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TRANSGENIC CROPS

hazards and uncertainties

More than 750 studies disregarded by the GMOs regulatory bodies

Special Secretariat for Family Farming
and Agrarian Development

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Transgenic Crops hazards and uncertainties

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Special Secretariat for Family Farming and Agrarian Development
Brasília, 2017

Dedication

to teacher Magda Zanoni

This publication represents the continuity of the work started by Magda Zanoni, in the Ministry of Agricultural Development (MDA), in order to discuss the risk of the transgenic plants to the health and the environment. In her work at the MDA and as a representative of the Ministry at CTNBio, Magda made efforts to call the attention to the importance of this debate.

Magda worked for many years as a researcher from the Center for Agricultural Studies and Rural Development (NEAD/MDA), and published a number of books. Among them, in 2007 she co-organized "Plantas Geneticamente Modificadas: riscos e incertezas" [Genetically Modified Plants: hazards and uncertaities], which was the embryo of this work.

In addition to her direct involvement with the biotechnologies subject, Magda always defended the need for a "good use" of science, with transdisciplinary vision and proposing the fair re-distribution of its benefits, especially for the rural populations, who develop the current agriculture as a perennial service for mankind.

Her academic background was always accompanied by a tireless social activism, writing up and promoting discussions about a "Citizen Science". She dedicated herself for many years to deepening the dialogue and consolidate the cooperation between Brazil and France, between academic institutions and, also, between farmers organizations. Her work was based on innovative actions and on the search for constructing new approaches about science, particularly in the articulations between research and qualification.

Graduated in Natural Sciences and Biological and Geological Sciences from the Federal University of Rio Grande do Sul (UFRGS), Magda continued her academic studies in France, as a result of an

enforced exile due to her resistance activism against the military dictatorship and in defend of Brazil's re-democratization. There, she was awarded a number of titles, among them the Doctorate in Development Sociology one, at University Paris I – Sorbonne. She retired as a *Maître de Conférence* in University Paris VII. She worked for many years as a researcher from LADYS - Laboratory for Social Dynamics and Recomposition of Spaces, of the University of Paris *Ouest Nanterre La Defense*.

Magda died on March 10th, 2015 and this work is dedicated to her.

Preface

For the fifth consecutive year, Brazil is considered as being the second largest producer of transgenic plants of the world, after the United States. The area for this type of culture already exceeded 40 million of hectares in our country.

The continuous increase of the area planted with transgenic soy, maize and cotton varieties, and the successive commercial release of new genetically modified organisms (GMOs), with combined alterated genes now, indicate the importance of monitoring their possible impacts to the environment, in general, and the human health, in particular.

With this respect, the Ministry of Agrarian Development (MDA) bring to the public this work which provides subsides to the qualification of risk evaluation processes associated to the growth of transgenic crops and to the consumption of their products and derivatives. The publication, intended to risk managers, researches, professionals from the biological, legal, economic and other areas, as well as to the other individuals interested on the subject, results from the Group of Agrobiodiversity Studies (GEA).

The extensive systematization work contained in this book gather references of scientific articles that are available for consultation via Internet, scientific publications or databank websites. The presented references correspond to studies published by independent researchers in magazines and indexed journals. Most of the articles can be accessed for free reading and download.

In more than 750 indications of texts from renowned research institutes established in several regions of the planet, scientists warn about the hazards and uncertainties involved in the massive environmental release of transgenic plants. By evidencing and documenting the absence of scientific consensus with respect to the transgenics impacts to the people's health and to the social-ecological

biodiversity, thise book brings vast elements for the evaluation of problems resulting from the adoption of this type of biotechnology. The information here gathered reinforce the urgent need for critical analyses of the current agricultural and agrarian development model, opening the discussion for the search for alternatives for the rural environment and livelihood.

With the book "Transgenic Crops – hazards and uncertainties: More than 750 studies disregarded by the GMOs regulatory bodies", the MDA's intention is, therefore, to promote the thinking and contribute to the debate about the release and use of transgenic plants, focused on the importance of maintaining the biodiversity and agrobiodiversity, the safety and food sovereignty and the sustainable rural development. Have a good reading!

Patrus Ananias

Minister of State of Agrarian Development
December 2014 until April 2016

Presentation

This book has a different format from the conventional ones that propose to present a bibliographic review of scientific publications related to a certain subject. It innovates by placing questions about aspects of the scientific debate on the genetic modification of living organisms for, subsequently, presenting a list of bibliographic references opposed to the versions adopted by regulatory agencies and disclosed in marketing campaigns of transgenics producer companies.

All the references presented here correspond to studies published by independent researchers in magazines and indexed journals. These articles enable to problematize those examined subjects, taking into consideration arguments sometimes radically opposed to those produced by the agrochemical and biotechnological companies or at their service.

Researchers, students, opinion makers and those in line with the groups which believe or are interested in defending the hypothesis of the absence of environmental and health risks in the commercial use of transgenic plants, will have here the opportunity to review their opinions . To the others, this document provides supportive elements to guide the debate with the first ones, when it comes to defending the interests of society, nature preservation and a more sustainable agricultural model.

The present bibliographic review includes hazards to the human and animal health, risks to the environment, as well as agronomic or socioeconomic problems. Examples include evidences of increased Cry[1] toxin-resistant insect attacks and multiplication of herbicide-tolerant weed[2] populations. The impacts of toxins and pesticides associated to genetically modified plants are also discussed.

1 Proteins with insecticidal properties that result in the death of certain insects when consumed.
2 Also mistakenly called "weeds".

The book evaluates biosafety issues with focus on aspects that have been neglected by most of transgenic risk evaluation bodies, such as the National Technical Biosafety Commission (CTNBio) and registration and inspections agencies like Anvisa and Ibama in Brazil. The elements described in about 750 studies validated by scientific magazines with editorial board clearly show that there is no consensus in the scientific community about the genetic modification subject and its impacts.

Such lack of consensus, object of many warnings from the Group of Agrobiodiversity Studies (GEA), linked to the Ministry of Agrarian Development (MDA), in its several publications, seminars and debates over the last ten years, is now supported by the extensive bibliographic documentation gathered here.

The initiative of this book, for its uniqueness has, therefore, a public interest role by calling the attention to the diffuse responsibilities in the society, concentrated at CTNBio and at the National Biosafety Council (CNBS), which would be responsible for reviewing the scientific base adopted by that Commission, the opinions of which have been repeatedly favorable to the transgenics developing companies' claims. By adopting a consensus inexistent in the scientific community, these opinions amplify the magnitude of the risks the brazilian population and biomes are subject to, disconsidering the Precautionary Principle and affecting the credibility of the government and its institutions. Thus, unfortunately, it is not only Science that is impaired by the scientific obscurantism.

Group of Agrobiodiversity Studies (GEA/NEAD/MDA)

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Introduction

The book "Transgenic Crops – Risks to the Health and the Environment – More than 750 studies disregarded by the GMOs regulatory bodies" incorporates results of an extensive bibliographic research covering the subject of the hazards and uncertainties associated to the use of transgenic plants.

The main scientific publications referenced by specialized editorial boards related to the growth or transgenic plants (in commercial or experimental scale[3]), to the management of those crops and to the animal and human consumption of their products were examined.

Being highlighted the issues related to biosafety, prioritizing environmental, human and animal aspects associated to the use of the concerned technologies, we accounted around 750 referenced articles, published between 1980 and 2015. Maintained in invisibility because their findings are opposite to the numerous pro-mgo marketing campaigns, such studies launch new perspectives about the subject. The abundance and the importance of such documents, as well as the invisibility imposed to them in the discussions conducted by the regulatory agencies, justify this publication, the objective of which is to enhance the scientific controversy concerning the biosafety of the transgenic plants. It is, as the documents gathered here evidence, a strong and active debate, weighing the attempts to impose false consensuses by the biotechnology industry and their associated lobbies. Opposite to what has been stated by foundations, institutes, associations and NGOs which incorporate the ideology of the biotechnology companies upon budget supports and members in their administration boards[4], the scientific community remains deeply divided.

3 In general called deliberated releases into the environment [field trials].

4 In Brazil one can cite the Council for Biotechnology Information (CIB). At the international level apperas the International Life Science Institute (ILSI).

The controversy, involving all the subjects related to the transgenic plants biosafety, can be summarized in terms of risks to the human and animal health and risks to the environment, being subdivided into sub-subjects and particularities associated to the involved transgenes, to the host organisms, to the technological packages and associated pesticides, among others. As it will be shown over this book, hundreds of studies evidence risks and weaknesses of affirmatives stating absence of toxicity of Bt proteins for human beings and farm animals. The same can be verified concerning the impact of underdosages and the results of sublethal effects of Cry toxins on non-target organisms or concerning the damages causes by crops that are tolerant to herbicides on the soil microbiota and the agricultural-ecological systems they are inserted to.

The statements about the impossibility to restrain the gene flow between GM species and these with native or agricultural species are also questionable. The impacts to the agrobiodiversity are greater as more extensive are the possibilities, the mechanisms and pollination vectors, but are not limited to this. The studies also reveal possibilities of horizontal transfers of genes and their fragments, increasing the risks resulting from overcoming the natural barriers between the species.

Highlight must further be given to the expansion in the use of pesticides associated to transgenic crops, to the hazards of glyphosate-, ammonium glufosinate-based herbicides and other pesticides succeeding the first ones in growing spiral of toxicity, as well as the risk evaluation processes practiced by the regulatory bodies. We call the attention to the fact that a similar research work - and not less wide - would be required to approach the set or socioeconomic risks represented by such plants. Effective agronomic performance analyses, such as the balance of costs and benefits taking into consideration medium term perspectives, were never conducted on an impartial and independent way, taking into consideration the

possibility to reject such technologies. The objective of establishing a systemic balance, analyzing the risk/benefit of the biotechnology applied to the agriculture domain, what should guide the National Biosafety Council decisions – in Brazil – has never been among CTNBio's concerns. If sought, this objective would certainly be supported not only in the studies gathered here, but also add those generated in another universe of specialized scientific magazines to these so as to cover the economy and sociology subjects.

The form this book is presented also differs from the usual format of books on which the bibliographic references used are indicated in the texts. In this case, an explanation of the approached subject is conducted and the quotes are listed with references so that the reader can access the original publications in full and use them as desired and draw their own conclusions about the several approached subjects.

We hope this effort reaches the purpose of challenging the scholars on such fields to complete this research, so as to consolidate the range of knowledge required for the National Biosafety Council[5] (CNBS) to better comply with its duty to evaluate the opportunity and convenience to support CTNBio's supposedly scientific decisions, which, ignoring this collection, invariably recommends unrestricted authorization for planting and consumption (human and animal) of transgenics in Brazil.

The first part of this publication, entitled **Unpredictable and non-intentional genetic modification effects**, focuses in studies pointing to the absence of control by the biotechnologist over the actions and the mechanisms he/she uses by imposing the transformed organism a new biological function. Despite of being random in terms of construction process, this function and others engaged by it will

5 Higher level policy instance regarding biosafety decisions in Brazil. The body is legally competent review CTNBio decisions and to analyze the socioeconomic risk of transgenic plants and to assess the opportunity and the convenience of technology. Composed of eleven ministers, met only twice after complaints issued by IBAMA and ANVISA against technical decision of CTNBio about the first GM corn commercially released in 2008/2009. In such opportunities, strengthened the latter part of the CTNBio and gave up their other responsibilities, being silent since.

be fixed on a perennial and stable way in its role over the future generations, of that being and by others affected by it.

The difficulties involved in voluntarily (and correctly) inserting certain gene sequences in other organisms by means of classic genetic modification techniques appear right in the beginning of the gene transformation process. These consolidate themselves, among other examples, by inserting a number of copies of the interest transgene in the transformed organism, which fix, in full or in part, in random places of the new transgenic being's genome.

In fact, without conducting long lasting and expensive tests and studies allowing to characterize the obtained transformation, the researchers will not know, strictly speaking, what they have performed. The basic fact is that the insertion mechanisms does not allow to anticipate the site where the desired genomic sequences will be incorporated, or how this will be completed, or even how many of them (and on which state, if as a whole or by parts) will be incorporated to the receiving DNA, or even what the transformed genome reaction will be in face of such transgenic invasion. Also, there is no way to anticipate if the plant will accept the transgenes and their functions, repairing, as possible, the damages caused by the method in the DNA, or if, to the contrary, it will silence the incorporated transgene, blocking its expression.

In parallel, the researchers also state that there are no mechanisms allowing to follow all the genetic and/or metabolic functions of the genomic sequences to be inserted. Indeed, it is possible that there is no scientific structure allowing at least to estimate, or know, all the metabolic relationships involved in the relationships triggered by a single gene, taking into consideration the environmental changes and the horizon of life of any individual.

The central dogma the genetic modification is supported by, frequently reiterated in the regulatory agencies and expanded with the assumption of the substantial equivalence, more than 20 years

ago revealed itself as failed and without scientific support. A gene does not encode a single protein which will play a clear and defined role. A gene, under the influence of the environment, will make possible the expression of a wide set of proteins which, in their turn will interact of different ways with the macro set of proteins of the transformed organism and its relationships with the environment.

Finally, the genetic modification technique itself operates on an unrelated way to the set of involved relationships, abstracting or ignoring the epigenetic phenomena which regulates a number of biological mechanisms of the organisms, including the hereditary ones. By concentrating in less than a single support of the biological information, a DNA fragment, assumes that the organisms result from the simple addition of their parts, as if, knowing the genome, we had a map that is able to organize life creation. In other words, the genetic modification success in transposing a new function to an organism which naturally did not have it, rests above everything on the chance, where the success probability is substantially lower than that of the lotteries.

In a second part, entitled **Agronomic issues related to the growth of transgenic plants**, the selected articles focus on biosafety studies noting agronomic problems related to planting and handling of commercially released transgenic plants.

Such problems refer to the development of insect populations and ruderal plants genetically resistant to transgenic technology – sensitivity to Cry proteins and herbicides, respectively, to existing biological interactions, potentially causers of the inefficacy of the virus-resistance technology, as well as to the occurrence of ecological disturbs in the agricultural systems. All these aspects result in a number of agronomic problems such as attacks of secondary pests and transgenic dissemination in genetically related organisms, with economic, social and ecological impacts which far extrapolate the areas cultivated by those farmers who decide to adopt such technologies.

It is worth emphasizing that some articles related to productivity, to the use of pesticides and to the economic profit of the transgenic plants producers were included, despite of being slightly out of the scope of this publication. Likewise, the coexistence subject is particularly approached with reference to some articles about the gene flow, horizontal and vertical, adding cases of contamination between transgenic and non-transgenic commercial crops. It is worth emphasizing that the "coexistence" subject, in addition to the consideration of biological studies and arguments found in the scientific literature, requires an approach adding social, economic and cultural elements appropriate to each administrative region (country, region, etc.) and their different agricultural-ecological systems and biomes.

The third part, entitled **Risks to the environment associated to the growth and/or use of transgenic plants**, covers a set of hazards and uncertainties associated to the growth and dissemination of genetically modified plants in the environment.

The systemization of such articles is particularly structured concerning the risks involving Bt plants. At this point, the controversy about the Bt proteins specificity theory, their persistence in the environment and in the trophic chains and the negative impacts – direct and indirect - of such proteins about non-target macro-organisms (NTOs) and the soil microbiota communities is included.

In a second time, studies addressing plants tolerant to herbicides (TH), specially the glyphosate-based one, were systematized. Here, articles related to the negative impact of the herbicides associated to HT plants on the environment, are included. In fact, the risk evaluation of the herbicides associated to the use of transgenic plants provides important biosafety information about the subject of the environmental hazards resulting from HT crops, even when the metabolism of the substances occur on a different way for the two types of plants (natural and GM).

Finally, references about the transgenic dissemination/contamination risks in non-agricultural species come to complement the issues related to the transgene dissemination in agricultural systems, analyzed in the second part of this publication.

The fourth part of this work is entitled **Risks to the health associated to the growth and/or use of transgenic plants**. Here, around 200 references are joined about this subject - from articles pointing out to the insufficiency of scientific data to conclude for the absence of risks to the health, when genetically modified plants or their parts are consumed, to details about problems related to Bt and HT plants, separately addressed, on a similar way to that conducted in parts two and three of this book.

Concerning plants synthesizing Bt toxins, the interactions of such proteins with mammalian cells, as well as their potentially toxic and allergenic effects (in vivo and in vitro) are highlighted.

In case of HT plant, following a brief review or articles indicating negative effects on the human and animal health of herbicides associates to these crops, especially the glyphosate-based ones, studies pointing out hazards and uncertainties connected to the consumption of such plants and their parts will be systematized. In addition, some references about the hazards and uncertainties to the health from non-Bt transgenic plants and tolerant to other herbicides are supplied, in addition the glyphosate-based ones.

Finally, this chapter joins studies pointing out hazards related to the horizontal gene transfer in mammalian cells and their symbiontic organisms, as well as uncertainties associated to epigenetic mechanisms (especially RNAi).

In the fifth and last part of this book, entitled **Scientific controversies and criticisms to the transgenic plants risk analysis process**, the growing academic and scientific clash, conducted in international scale is analyzed concerning the supposed absence of risks related

to the planting and consumption of transgenic plants. For that purposes, more than 90 articles which criticize from the risk evaluation process indicating the safety of such plants under the toxicological, allergenic, nutritional and environmental perspective, to those pointing out evidences of problems with this respect, such as toxicological, allergenic, nutritional and environmental hazards and damages will be systematized.

Credibility damaging campaigns for researches and authors mentioning such hazards and uncertainties are also described. The chapter further includes a number or reports about subjects suggesting explanations for the absence of consensus between the scientists, covering from conflicts of interest and methodological weaknesses to the commitment with possible research results and their eventual economic deployment.

Methodological Aspects

Selected articles

As mentioned before, the first criterion for the inclusion of approximately 750 articles referenced in this publication is their publication in a national and/or international renowned scientific magazine – the majority of them have a peer review committee (*peer-reviewed journals*). The publication of a study in such magazines in fact allows its access by the scientific community – which will have at their disposal the materials and methodology used for their conduction, as well as a significant part of the gross data, which does not result from the authors analysis and which are subject to a number of interpretations. With this respect, in case other researchers do not agree with the interpretation of the study authors, the latest express their divergent opinions, in general by means of a communication or another publication in the same magazine or even in another magazine specialized in the same subject. Such counter-argumenting

possibility for the scientific community is not possible in case of internal reports from institutes, NGOs or companies, on which only the authors had access to the data.

It is worth emphasizing that the fact that a study is published in one of such magazines does not mean *a priori* that the presented arguments are the most exact ones, and much less that the authors conclusions mean some type of absolute truth. However, in the absence of opposite manifestation by the scientific community members, such study can be considered as being the closest to a truthful description of a certain situation, at a certain moment. In fact, a scientific hypothesis can be considered as robust until the science itself proves the opposite: this is the way science progresses.

In addition, the fact that the studies are peer-reviewed before they are published minimizes the probability to make results incorporating methodological bias and conclusions not supported by the data and their implications/interpretations available to the society.

With this respect, the option to select only studies and works published in scientific magazines provided with a specialized editorial board and which practices the blind review (the reviewers do not know who the authors are and vice-versa) must be considered as the best alternative to collect independent opinions about any scientific subject.

Article categories

In order the assist the reader in using this publication, we adopted a classification typology in the document.

The references are preceded by acronyms indicating their nature. Thus, when the mentioned article is clearly intended for a bibliographic review of a certain subject, the reference is preceded by the acronym REV-; the acronym COM- refers to “communications” or “comments” (generally characterized by a rapid working time and which occupies a maximum of one or two pages in a

scientific magazine); the acronym MOD- indicates a study about modeling or experimental model and the acronym EPI- refers to an epidemiological study. The absence of the acronyms indicates articles supported by an experiment, with explicit material and method, regardless of being conducted in the field or in laboratory. Such classification typology is intended to clarify the reader about the type of mentioned reference, being that in general a direct comparison between two studies belonging to distinct categories is inappropriate.

Finally, in order to provide a historical overview of the subject, the referenced articles were chronologically ordered for each addressed problem. In addition to facilitate the reading, such ordering emphasizes the historical backgrounds of the debate and illustrates the continuity in time of the controversies about the several addressed subjects.

Access to information and target public

It is worth mentioning that all these articles are available in digital media through the internet - significant part of them for free (especially through Open Access) -, depending on the magazines' authors intention and the editors option, and the rest only upon payment or access through pre-paid servers in universities and research institutions.

In order to facilitate the access to the articles, the internet links directly taking to the online publication were associated to each free access reference. When the article is not in a free access system, the associated link will direct the reader to the abstract, always accessible online.

In general, the National Center for Biotechnology Information (NCBI – USA) internet links were prioritized. A number of reasons guided such option, such as the extensive databank about biosafety studies on transgenic plants, the presence of the *cross ref* tool – allowing to identify related studies, *corrigendum* and answers to a certain article

-, in addition to the public character of the body. When an article is not found in such databank, another internet site is indicated. In all of the cases it was prevented to prioritize, promote or support certain websites or institutions. Those specialized in the trade of scientific publication of participants of Civil Society Organizations were treated on a similar and secondary way, as mentioned above. In practical terms, the facility to locate online publications by means of a search tool in the basic internet (Google) consisted, somehow, in a criterion which significantly guided the indication of the internet links used in this publication. Evidently, the articles as do not depend on such indications and can be accessed through other means.

All the links found in this publication were accessed between 11/01/2014 and 03/30/2015.

The summaries and references[6] transcribed here maintain the language of the original publication. Because the English language is widely dominant in the scientific literature, this situations repeats in this book. We understand that the option can be justified by the technical difficulties involved in the translation of an abstract of an already peer-validated scientific study, without risk of changes – even if slight – in the meaning provided by the authors. As an example, consider that, even the interpretation of simples words, such as "might" or "may" (generally translated to Portuguese as "pode") are subject to imply relevant changes to the meaning of the original sentence.

Although considering the importance of the familiarity with the technical English for the appropriate use of the contents exposed here, we emphasize that this book is not exclusively intended for biotechnologists, molecular biologists or geneticists. In contrast, it is necessary to widen the debate concerning the hazards and uncertainties

6 For the option adopted in this publication to keep references in English, the ABNT standards system could not be used.

about the environment, health and agricultural production of transgenic plants. And this book is intended to contribute for so, by making scientific studies about these subjects available to other spheres of the scientific community. With this respect, the target public includes ecologists, statisticians, microbiologists, nutritionists, toxicologists, veterinarians, physicians, agronomic, agricultural and forest engineers, among others. We understand that such professional categories are poorly represented in the scientific debate and, consequently, in the decision making associated to such subjects, decisively affecting the exercise of their professions.

This book will contribute for such. It will be noted on it that, excluding few summaries singularly associated to certain specialties in the biology field, the studies referenced here, as well as the texts introducing them, provide robust support for opinion leaders connected to a number of already referred professional categories, as well as to public managers of the human area and environmental health areas. The content will also be very useful to legislators, law inspectors and the organized civil society representatives - even without academic qualification in "hard/exact sciences". It is worth mentioning that significant part of the foreign technical terms to non-specialists will be defined in footnotes, over the material. As a complement, at the end of the book we provide a small explanatory glossary which will help to clarify doubts concerning expressions that, even known by the general public, can have distinct meanings in the biology context.

Scholars connected to social sciences and the like are also included among the target public of this book. We understand that they can expand their technical knowledge about the subject and enrich them with expertise from their area. We consider as being relevant the approach of such professionals of the debate about the GMOs and with the practice of the analysis and decision making conducted in the regulatory instances, before the several interpretation of the

Precaution Principle. We suppose that this document may contribute for the acquisition of knowledge about biology and genetics and, consequently, encourage its incorporation to the debates of such scientific areas.

We also hope that this book is adopted as an information source for professionals and social actors who work in the subject of pesticides and their impact on the environment, the public health and the agricultural production.

Finally, this work incorporates, by means of the referred articles, great international effort applied to the construction of scientific knowledge, comprising a number of biology domains and representing an innovative challenge to the common citizen (potential involuntary consumer of GMOs!), who will find information here allowing him/her to better understand the technology associated to the GMOs and of some mechanisms inherent to the life, under their most different forms and complexities.

Have a good reading!

Gilles Ferment, Leonardo Melgarejo, Gabriel Fernandes and José Maria Ferraz

Contextualization

1 Transgenics and basic notions of biology

The genetic modification process changes the living being, allowing the fusion of genetic material from completely different species, whether bacteria, fungi, plants or animals. In order to better understand how such technology works and what risks it can bring to the environment and to the consumers, some basic notions of biology are indispensable.

1.1 Cells, proteins, genome and gene regulation

Every living being is constituted by one or several cells (unicellular or pluricellular organism). Depending on the species, the number of cells may largely range: while the human being has approximately $10E^{15}$ cells, *Caenorhabditis elegans* (a worm that is frequently used in biological research) has approximately 1,031 cells, and the bacteria have only one. In general, the number of cells of an organism depends on its size/volume.

These cells comprise a number of components performing several essential functions to those "life units", such as the reproduction (cell multiplication), communication (with other cells) and breathing (cellular) ones, among others.

In pluricellular organisms the cells do not have the same functions, performing distinct "tasks". The liver cells will have an important function in the detoxification of harmful molecules and the immune system cells (white cells, for example) will have wider biological roles, related to the protection of the organism against viral or bacterial infections, for example. In case of plants, the root cells will have different functions from those of the grains or the leaves, which will further present differentiations associated to the systems they participate in.

But, regardless of the involved functions (whether common or specific to certain cells), almost all the cell actions are conducted by means of proteins. The proteins correspond, with this respect, to the main functional molecules of the cells. Some proteins will carry materials out or to the cell, others will help to destroy or cut out old or dangerous elements, others will act in the production of energy or in cell multiplication, and so on.

1.2 Where does the protein diversity come from and how are they produced to develop certain and so distinct functions?

Part of the answers is located in the core of each cell. In such compartment there are macromolecules containing the information required for the cell (or the unicellular organism) operation: these are the main DNA strands[7].

Specifically, a DNA strand can be composed by billions of Deoxyribonucleic Acid (DNA) molecules. The variation will depend on the species of organism carrying that DNA. There are four types of nitrogenous bases (Adenine, Thymine, Guanine and Cytosine), designated by letters A, T, G and C.

The DNA complexity can be illustrated by the following estimate: the simple transcription of the information "written" in the core of a single human cell, using those four letters, would required the publication of 10 books, in A4 format, with 1,000 pages each.

Certain Deoxyribonucleic Acid sequences have particular significations. As in the case of the several letters of our alphabet, which when associated in a certain order generate sentences with their own meaning, the order on which those four bases occur defines what we can call biological messages.

7 During the process of cell division, especially, the DNA strands are clustered and compressed and can be viewed in the form of chromosomes.

The science designated such biological messages (codes) of genes. The current knowledge progressed and it is currently know that the genes alone are not able to command certain expressions, once their relationship with the environment, in addition to the existence of nitrogen bases sequences which do not form genes, but act as essential regulation elements so that those messages are issued, read or understood. In other words, less than half of the genetic basis is formed by genes. The rest, for not determining the protein formation, was initially interpreted as "waste DNA", has regulatory functions that are essential to the genes operation. It is in this sense that the initial interpretation that the genes define the life as it is, has been proven to be incorrect, being admitted in the present that it is not exactly known what a gene is.

But it is because of that reductionist interpretation that it is used to say that the DNA, with its set of genes, constitutes a kind of "book of life", where the support of the genetic information and all its possibilities are hidden.

There are usually various thousands of genes in each DNA molecule. Some organisms are more complex than others and the man, who has approximately 22 thousand genes, is not in the top of the list. Rice, for example, has around 50 thousand genes. In all the organisms, it is the message coded in the DNA, supported by the regulatory sequences and submitted to pressures and particularities of the cell medium, which guides the elaboration of proteins, encouraging the wide variety of functions and characteristics of all the live organisms. Thus, those nitrogen bases combinations are crucial for the formation of a wide range of molecules which gives the basis to all the known and imaginable biological processes.

On a simplified way, it is assumed that a gene is always translated into protein(s). But it is worth mentioning that the genetic information contained in the DNA strand cannot be directly translated into proteins. It needs to pass through a transition process, "rewriting or

recoding". This process allows the DNA to be copied (transcribed) in another molecule – the Ribonucleic Acid strand qualified as messenger (RNAm) – before it serves as the basis for the elaboration of proteins. In other words, it is in the process called transcription that the RNAm will be translated into amino acids, which in their turn will form one or more types of proteins.

1.3 Gene regulation

In summary, the DNA strand is comprised by genes containing the genetic information required for the manufacture of proteins responsible for almost all the biological processes performed by the cells (except viruses and other exceptions). In addition, all the cells of a certain organism will have the same DNA molecule and, therefore, the same genetic information. But, it is not guaranteed that such information will be expressed in the same way in all the cells. Such finding introduces the gene regulation or the gene expression regulation concept.

In fact, at any time over the history of a cell life, only a small part of the RNA and proteins coded in its genome will be expressed. At different moments, the profile of expressed gene products may markedly differ in qualitative and quantitative terms with respect to which proteins will be expressed and in what level or expression.

Any step of a gene expression can be modulated, from the DNA accessibility[8] for its transcription to the post-translational modification[9] of a protein, passing through the amount of synthesized RNA. The gene regulation guarantees the cell a significant part of the control over its structure and function, being the basis of extremely varied biological mechanisms such as the cell differentiation, morphogenesis or the adaptability of any organism to the environment.

8 In general, DNA is accessible to transcriptional machinery only when uncompressed, a known form of euchromatin.

9 Biological processes to complete the synthesis of a protein after translation process of RNA strands to chains of amino acids, by adding, for example, certain molecules (sugars, phosphorus) or giving it a specific three-dimensional spatial conformation (via folding and biochemical fixation).

Another regulatory part of the gene expression is controlled by the environment where the organism or cell is developed. Epigenetics relates to the study of the environmental influences, which modify the expression of the genetic code and the mechanisms involved therein[10]. The epigenetic mechanisms, briefly resumed in this publication (especially in part 1), will be diverse and may also act at various moments of the protein elaboration. In addition, the epigenetic mechanisms effects can be transmitted from generation to generation, although most of them appears to be reversible in time, while the RNA changes (such as mutations) are definite.

The noted characters (such as the physical structures which differentiate the several types of cells) are called phenotypic. In the scale of an organism, the color of the eyes, the spike insertion height, the size of capacity to resist to pathogenic agents would be examples of phenotypic expressions. Thus, the phenotype of an organisms (or of a cell) will result from a certain type of expression of the genotype, which will emerge from a wide set of possibilities, being partially controlled by the environment.

2 Genetically modified organisms and transgenic food plants: distinct debates

By means of molecular biology tools, research are able to transfer certain DNA sequences from an organism to the genome of another one, in order to provide the latest one with a new biological function, supposedly in the society's interest. Theoretically, any living being can be transformed in laboratory into a transgenic organism. The current examples include plants, bacteria, yeast, mice, fishes, sheeps and goats, among others. Some of these have revealed themselves as very pertinent and interesting research tools for the understanding

10 The field of epigenetics has been widely disregarded (when not simply ignored) in the risk assessment of GMOs. Possibly because it confronts outdated paradigms and dogmas but also underlies significant part of molecular biology and genetics, historical controversies reactivating such as the transmission of acquired characteristics (Lamarck's theory) or, more recently, the race in search for technologies set to the financially promising field of gene therapy. Disregard the knowledge of epigenetics clearly reduces the effectiveness of risk assessments and slow scientific progress.

and construction of the scientific knowledge about genetic and gene regulation concepts and mechanisms. Others, not so much.

In fact, the biotechnologies are used, for many decades, both in the research field and in the support/assistance to chemical-industrial processes in commercial scale. This is what happens with the production of insulin in GMO, of antiviral proteins (for the manufacture of vaccines) of of enzymes with detergent properties, among others. However, the trade of such products – produces in GMOs – did not close the debate about biotechnologies with the same intensity as the transgenic plants intended for feeding. A number of factors, social and biological, allow us to explain such differentiation. On a summarized way, the following arguments must be highlighted:

a) in case of transgenic food plants, the consumers' health is directly involved, affecting all the people. In case of medicinal products such as insulin or recombinant vaccines, only certain populations are involved, having the opportunity to choose, in general, consciously for that. In addition, these populations are protected by social and institutional mechanisms which allow a relatively efficient monitoring of the potential problems, because of the medical prescription and the case-by-case follow-up, for example;

b) when parts of a transgenic plant are consumed, it is the genetically modified organism itself that is being ingested, and not only a GMO expression product – such as an enzyme produced in transgenic yeast and subsequently purified before being integrated in the food chain. Both from the biological point of view and relatively to the risks to the health, this implies significant differences. The use, after the purification process of only one protein produced in GMO, does not extend to an entire set of possibilities of damages associated to eventual genetic modifications and/or metabolic changes which may occur in the scale of the whole organism. Such set of possibilities, in contrast, is present in the situation on which the GMO itself, or part of it, is consumed.

c) The commercial transgenic plants are grown in non-controlled environments, i.e., in the field. The GMOs, in their turn, developed with experimental objectives and/or intended for the production or recombinant molecules for various industrial uses are confined in "closed" environments, even if not necessarily tight, such as laboratory, incubators, vegetation chamber, etc. This fact substantially limits the risks associated to the consumption of such transgenic products by non-target organisms (NTOs), as well as the risks associated to the dissemination of transgenes in the environment and their social-environmental impact.

Such observations must not be interpreted as an argument which exempts the non-agricultural/food biotechnologies from any biological risk. However, they allow us to clearly delimit the picture of the scientific controversy addressed in this publication.

This book is clear in its effort of discrimination among the scientific debates related to the biotechnology risks in general and the risks associated to the commercial use of transgenic food plants. "Arguments" like "transgenics are good for our societies because they have already saved millions of lives of insulin-dependent people[11]" – in addition to not having scientific basis – do not fit into a serious reflection about the problems discussed here.

3 Transgenic plants and risk evaluation: international and Brazilian context

Currently, in the world scenario, more than 98% of the transgenic plants were genetically modified in order to express only two types of characteristics.

11 This type of fallacious argument, which aims to silence the debate about the actual risks to public health, environment and agriculture, associated with the commercial use of transgenic food plants, has been unfortunately repeated by significant part of the academic world and scientific societies of Brazil and other countries major producers of transgenic plants.

a) <u>the synthesis of an insecticide toxin in their tissues:</u> these are the so-called Bt plants, which theoretically are "resistant" to insects, because the target-insect, in general a caterpillar or a coleoptera larva, when feeding itself with the maize, soy or cotton leaf or the root (particularly of Bt maize), will ingests those toxins and will die.

b) <u>the modification of metabolic pathways in plants allowing them to be insensitive to the lethal action of certain herbicides:</u> These plants are called by the biotechnology industry as Herbicide-Tolerant (HT) plants. The best known example is represented by RR soy, which by receiving a bath of glyphosate does not die, in contrast to all the other plants of the crop treated with the herbicide, thus facilitating the management of ruderal plants in such crops. In addition to the tolerance to glyphosate, plants with a function of tolerance to ammonium glufosinate-based, 2,4-D herbicides, or those belonging to the chemical group of the imidazolinones are currently grown in commercial scale.

In the latest years, more en more areas are planted with transgenic plants combining[12] these two functions, i.e., they are toxic plants to certain insects and also insensitive to certain herbicides.

The transgenic plants are basically produced in only five countries of the world (USA, Brazil, Argentina, India[13] and Canada), totalizing around 95% of the 180 million cultivated hectares in the planet.

It is worth mentioning that basically six companies hold the world market of transgenic varieties and perticides associated to them[14]. This applies both to the national and the international scenario.

12 This combination of functions can be obtained directly by genetic engineering, moving in the same plant several transgenes responsible for different functions or by conventional crossing of two or more transgenic plants that have these functions alone. In this case, it is pyramided / staked events.

13 In India only transgenic cotton is allowed on a commercial scale.

14 For example, Monsanto is responsible for marketing the main varieties of soybeans, corn and cotton tolerant to glyphosate and also holds the main patent on the glyphosate-based herbicide; Bayer is responsible for marketing of the main varieties of soybeans, corn and cotton tolerant to gluphosinate ammonium and also holds key patents on herbicides based on gluphosinate ammonium; DowAgroscience is responsible for the marketing of the main varieties of soybeans and corn tolerant to 2,4-D and also holds the main patent based herbicide 2,4-D, etc.

Two main types of risks can be associated to the use of GMOs:

a) risks associated to the new function provided by means of genetic modification, the insecticide protein synthesized in Bt plants, for example, and to the presence of the associated transgene(s)

b) risks associated to undesirable effects resulting from the genetic modification process itself, such as the change to the metabolic pathways which may result in the synthesis of new proteins, potentially toxic or allergenic.

Such distinction between the risk types is noted, in most of the book, thanks to the elaboration of items related to specific risks. All the biological risks associated to the commercial use of transgenic plants basically result from these two types of risks. Included here are the risks associated to the transgenic dissemination in the environment or to the consumption of such plants by non-target organisms, animals and human beings.

Although this publication does not cover only risks specific to the Brazilian context, almost all the referenced articles warn to the hazards and uncertainties relevant to Brazil.

In fact, Brazil is the second largest producer of transgenic food plants in the world, with approximately 30 million hectares planted with transgenic soy, maize, and cotton. We further have a bean and an eucalyptus, not cultivated yet, but which have already been approved by CTNBio. For this reason, the entire area planted with transgenic plants in the country is covered by these two types of transgenic plants, Bt or HT varieties or which combine these two functions.

The risk evaluation in Brazil is coordinated[15] by National Technical Biosafety Commission (CTNBio), MCTI commision composed by

15 Theoretically subordinated to the socio-economic analysis of the National Biosafety Council (CNBS, composed of eleven ministers of state) - the technical opinions issued by CTNBio are actually transformed into policy decisions that result in marketing authorization of GM crops in record time. In the absence of manifestation of CNBS - as is the case for 95% of transgenic plants authorized today - is the CTNBio that is morally (but not legally) responsible for a significant part of the country's agricultural policy, strongly oriented to the use of transgenics.

27 doctors, including the government, the academy and the civil society representatives. In 10 years of existence, CTNBio approved around 60 transgenic events (45 plants). There has never been rejection to the company's requests. Well, all the CTNBio decisions were controversial since its establishment, in 2005. As it appears throughout this publication, CTNBio's technical opinion does not reflect the scientific community's opinion.

Part 1
Unpredictable and non-intentional genetic modification effects

1. Genome, epigenome and gene expression[16]

The genetic modification is a molecular biology tools intended to transfer certain genes from an organism to another, in order to also transfer characteristics that are supposedly dependent on such genes. However, in case of products which are released in the environment and consumed by human being, extremely caution is required concerning the total of implications and their possible consequences.

These gene transfers obviously incorporate potentially undesirable changes, able to result in dangerous effects to the health and the environment. The controls which would allow to operate with the required care evidently require the appropriate understanding of the implications associated to the genome and their relationship with the environment. The most essential point, in this discussion lies in the difficulty to, with the current knowledge, correctly understand the functioning of the genome and the changes imposed to it.

1.1 The gene concept in continuous evolution

The transgenic organisms were developed based on exaggeratedly simplified premises, of molecular biology and genetics. According to these, a gene codifies for a certain protein which will perform a certain function. This and nothing else. With this respect, it was believed that simply transferring a gene – with interesting function – from an organism (donor) to another organism (receptor) would be enough for the latest to be obliged to express the desired function, through the synthesis of the recombinant protein.

Well, the reasoning became complex when the gene concept progressed, being that current the name "genomic sequence of determined size"

16 Although most of the references mentioned in this item relates to biology in mammalian cells (or model organisms, such as certain yeasts), it is noteworthy that incorporates doubt valid for supposedly simpler organisms, including plants (focus of this publication).

or "DNA elements" is currently better accepted, operating on an articulated way, conditioned by the environment (they affect while they are affected by it).

In case of the human being, such simplified interpretation allowed that most part of the genome was considered, until some years ago, as "waste DNA", once it would not be a protein encoder. Subsequently, the Encode project concluded (in 2012) that 80% of the genome would have, in fact, several biochemical functions regulators of unpredictable complex systems based on the knowledge dominated by science.

The interpretation is maintained. Strictly speaking, we do not know what a gene is, although we know it is not what we supposed. With this respect, it is possible to state that the understanding of the basic biological mechanisms inherent to the life requires more studies in order to clarify its modes of action and provide safety in its manipulations. The studies below discuss such issue.

REV – El-Hani, C. 2007. Between the cross and the sword: the crisis of the gene concept. *Genet. Mol. Biol.,* vol.30, nº 2. São Paulo.

Challenges to the gene concept have shown the difficulty of preserving the classical molecular concept, according to which a gene is a stretch of DNA encoding a functional product (polypeptide or RNA). The main difficulties are related to the overlaying of the Mendelian idea of the gene as a 'unit': the interpretation of genes as structural and/or functional units in the genome is challenged by evidence showing the complexity and diversity of genomic organization. This paper discusses the difficulties faced by the classical molecular concept and addresses alternatives to it. Among the alternatives, it considers distinctions between different gene concepts, such as that between the 'molecular' and the 'evolutionary' gene, or between 'gene-P' (the gene as determinant of phenotypic differences) and 'gene-D' (the gene as developmental resource). It also addresses the process molecular gene concept, according to which genes are understood as the whole molecular process underlying the capacity to express a particular product, rather than as entities in 'bare' DNA; a treatment of genes as sets of domains (exons, introns, promoters, enhancers, etc.) in DNA; and a systemic understanding of genes as combinations of nucleic acid sequences corresponding to a product specified or demarcated by the cellular system. In all these cases, possible contributions to the advancement of our understanding of the architecture and dynamics of the genetic material are emphasized.

Full article available at http://www.scielo.br/scielo.php?pid=S1415-47572007000300001&script=sci_arttext

Encode Project Consortium. 2007. Identification and analysis of functional elements in 1% of the human genome by the Encode pilot project. *Nature*, v.447, p. 799-816.

We report the generation and analysis of functional data from multiple, diverse experiments performed on a targeted 1% of the human genome as part of the pilot phase of the ENCODE Project. These data have been further integrated and augmented by a number of evolutionary and computational analyses. Together, our results advance the collective knowledge about human genome function in several major areas. First, our studies provide convincing evidence that the genome is pervasively transcribed, such that the majority of its bases can be found in primary transcripts, including non-protein-coding transcripts, and those that extensively overlap one another. Second, systematic examination of transcriptional regulation has yielded new understanding about transcription start sites, including their relationship to specific regulatory sequences and features of chromatin accessibility and histone modification. Third, a more sophisticated view of chromatin structure has emerged, including its inter-relationship with DNA replication and transcriptional regulation. Finally, integration of these new sources of information, in particular with respect to mammalian evolution based on inter- and intra-species sequence comparisons, has yielded new mechanistic and evolutionary insights concerning the functional landscape of the human genome. Together, these studies are defining a path for pursuit of a more comprehensive characterization of human genome function.

http://www.ncbi.nlm.nih.gov/pubmed/17571346

Encode Project Consortium. 2012. An integrated encyclopedia of DNA elements in the human genome. *Nature*, Vol 489, 57-74.

The human genome encodes the blueprint of life, but the function of the vast majority of its nearly three billion bases is unknown. The Encyclopedia of DNA Elements (ENCODE) project has systematically mapped regions of transcription, transcription factor association, chromatin structure and histone modification. These data enabled us to assign biochemical functions for 80% of the genome, in particular outside of the well-studied protein-coding regions. Many discovered candidate regulatory elements are physically associated with one another and with expressed genes, providing new insights into the mechanisms of gene regulation. The newly identified elements also show a statistical correspondence to sequence variants linked to human disease, and can thereby guide interpretation of this variation. Overall, the project provides new insights into the organization and regulation of our genes and genome, and is an expansive resource of functional annotations for biomedical research.

http://www.ncbi.nlm.nih.gov/pubmed/22955616

COM – Pennisi, E. 2012. Encode Project Writes Eulogy for Junk DNA. *Science*, 1159-1161.

Without summary available.

http://www.ncbi.nlm.nih.gov/pubmed/22955811

Ibarra-Laclette, E.; Lyons, E.; *et al*. 2013. Architecture and evolution of a minute plant genome. *Nature,* 498, 94-98 doi:10.1038/nature12132.

It has been argued that the evolution of plant genome size is principally unidirectional and increasing owing to the varied action of whole-genome duplications (WGDs) and mobile element proliferation. However, extreme genome size reductions have been reported in the angiosperm family tree. Here

we report the sequence of the 82-megabase genome of the carnivorous bladderwort plant Utricularia gibba. Despite its tiny size, the U. gibba genome accommodates a typical number of genes for a plant, with the main difference from other plant genomes arising from a drastic reduction in non-genic DNA. Unexpectedly, we identified at least three rounds of WGD in U. gibba since common ancestry with tomato (Solanum) and grape (Vitis). The compressed architecture of the U. gibba genome indicates that a small fraction of intergenic DNA, with few or no active retrotransposons, is sufficient to regulate and integrate all the processes required for the development and reproduction of a complex organism.

http://www.ncbi.nlm.nih.gov/pubmed/23665961

Even today, a number of issues are still open, related to the biological roles played by most of the DNA elements, to the form how these rearrange themselves in the genome, how they are accepted or rejected when changed/broken by natural or artificial processes.

Sargent, R.; Brenneman, M.; Wilson, J. 1997. Repair of Site-Specific Double-Strand Breaks in a Mammalian Chromosome by Homologous and Illegitimate Recombination. *Molecular and Cellular Biology*, p. 267–277.

In mammalian cells, chromosomal double-strand breaks are efficiently repaired, yet little is known about the relative contributions of homologous recombination and illegitimate recombination in the repair process. In this study, we used a loss-of-function assay to assess the repair of double-strand breaks by homologous and illegitimate recombination. We have used a hamster cell line engineered by gene targeting to contain a tandem duplication of the native adenine phosphoribosyltransferase (APRT) gene with an I-SceI recognition site in the otherwise wild-type APRT+ copy of the gene. Site-specific double-strand breaks were induced by intracellular expression of I-SceI, a rare-cutting endonuclease from the yeast Saccharomyces cerevisiae. I-SceI cleavage stimulated homologous recombination about 100-fold; however, illegitimate recombination was stimulated more than 1,000-fold. These results suggest that illegitimate recombination is an important competing pathway with homologous recombination for chromosomal double-strand break repair in mammalian cells.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC231751/

Haber, J. 2006. Transpositions and translocations induced by site-specific double-strand breaks in budding yeast. *DNA Repair (Amst)*, 5(9-10):998-1009.

Much of what we know about the molecular mechanisms of repairing a broken chromosome has come from the analysis of site-specific double-strand breaks (DSBs). Such DSBs can be generated by conditional expression of meganucleases such as HO or I-SceI or by the excision of a DNA transposable element. The synchronous creation of DSBs in nearly all cells of the population has made it possible to observe the progress of recombination by monitoring both the DNA itself and proteins that become associated with the recombining DNA. Both homologous recombination mechanisms and non-homologous end-joining (NHEJ) mechanisms of recombination have been defined by using these approaches. Here I focus on recombination events that lead to alterations of chromosome structure: transpositions, translocations, deletions, DNA fragment capture and other small insertions. These rearrangements can occur from ectopic gene conversions accompanied by crossing-over, break-induced replication, single-strand annealing or non-homologous end-joining.

http://www.ncbi.nlm.nih.gov/pubmed/16807137

Katz, S.; Gimble, F.; Storici, F. 2014. To nick or not to nick: comparison of I-SceI single- and double-strand break-induced recombination in yeast and human cells. *PLOS ONE*, 9(2):e88840. doi: 10.1371/journal.pone.0088840.

Genetic modification of a chromosomal locus to replace an existing dysfunctional allele with a corrected sequence can be accomplished through targeted gene correction using the cell's homologous recombination (HR) machinery. Gene targeting is stimulated by generation of a DNA double-strand break (DSB) at or near the site of correction, but repair of the break via non-homologous end-joining without using the homologous template can lead to deleterious genomic changes such as in/del mutations, or chromosomal rearrangements. By contrast, generation of a DNA single-strand break (SSB), or nick, can stimulate gene correction without the problems of DSB repair because the uncut DNA strand acts as a template to permit healing without alteration of genetic material. Here, we examine the ability of a nicking variant of the I-SceI endonuclease (K223I I-SceI) to stimulate gene targeting in yeast Saccharomyces cerevisiae and in human embryonic kidney (HEK-293) cells. K223I I-SceI is proficient in both yeast and human cells and promotes gene correction up to 12-fold. We show that K223I I-SceI-driven recombination follows a different mechanism than wild-type I-SceI-driven recombination, thus indicating that the initial DNA break that stimulates recombination is not a low-level DSB but a nick. We also demonstrate that K223I I-SceI efficiently elevates gene targeting at loci distant from the break site in yeast cells. These findings establish the capability of the I-SceI nickase to enhance recombination in yeast and human cells, strengthening the notion that nicking enzymes could be effective tools in gene correction strategies for applications in molecular biology, biotechnology, and gene therapy.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3928301/

Thus, as the gene concept remains in full progress, also the scientific knowledge concerning the set of biological mechanisms allowing the regulation of such genes increases year after year. In fact, in addition to the promoters and the encoder DNA regulatory sequences, there is a number of other molecules with an important role in the gene regulation and expression and which are not covered by the risk evaluations in transgenic plants. Concentrated in the strict space of the promoters, the regulators and terminators, such analyses, as currently conducted, put aside wide field for the expression of risk possibility.

Since 20 years ago, new molecules have been identified and characterized as gene expression regulator elements. They are RNA molecules which are not translated into proteins, currently called small RNA species (or *non-coding* RNA/ncRNA[17] or functional RNA funcional/fRNA).

17 This terminology may prove misplaced, considering it was recently discovered the possibility of certain small RNA species encode for peptides. See, for example, Lauressergues et al., 2015 (Primary transcripts of microRNAs encode regulatory peptides, Nature, 520,90–93) and Waterhouse & Hellens, 2015 (Plant biology: Coding in non-coding RNAs Nature, 520,41–42).

The sRNAs group a number of RNA types[18], with several actions in the gene regulation, but also in the DNA replication or in post-translational modifications. In this context, related to possible sRNAs (and their consequences) changes when submitting and organism/cell to the genetic modification process, the miRNA and siRNA deserve attention. Both have an essential role in the mechanism currently called RNA interference (RNAi), which determines the gene regulation inhibition (in general destroying specific messenger RNA – preventing the formation of proteins from these).

The biological potential of this mechanisms, as well as the associated molecules, is immense, although full of uncertainties. In fact, a single miRNA may decrease the expression level of hundreds of genes. These molecules also have ability to interact in the form of the cell chromatin, leaving the DNA in a physical state that is incompatible with its transcription in RNAm.

Parallely, chemical changes – particularly consolidated by the addition of methyl groups to histones and/or in the DNA itself, by means of methylases – also change the gene availability for its transcription or directly regulate the methylated gene expression, respectively.

These gene regulation forms basically independent from the organism's DNA sequences are largely associated to epigenetic mechanisms, constituting a recent accumulation field which has not deserved attention compatible with its relevance, concerning the risk analysis of the transgenic plants. In fact, such analysis is currently focused only on possible changes to the DNA of the transformed organism.

Fire A.; Xu S.; *et al.* 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans. Nature,* 391(6669):806-11.

Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene. Such effects have been proposed to result from a simple antisense mechanism that depends on hybridization between the injected RNA and endogenous messenger RNA transcripts. RNA interference has been used in the nematode Caenorhabditis elegans to manipulate gene expression. Here we investigate the requirements for structure and delivery of the interfering RNA. To our surprise, we found that double-stranded RNA was substantially more effective at producing interference than was either strand individually. After injection into adult animals, purified single strands had at most a modest effect, whereas double-stranded mixtures caused potent and specific interference. The effects of this interference were evident in both the injected animals and their progeny. Only a few molecules of injected double-stranded RNA were required per affected cell, arguing against stochiometric interference with endogenous mRNA and suggesting that there could be a catalytic or amplification component in the interference process.

Full article available at http://www.nature.com/nature/journal/v391/n6669/full/391806a0.html

18 See http://en.wikipedia.org/wiki/List_of_RNAs for more detals

REV – Mello C.; Conte Jr D. 2004. Revealing the world of RNA interference. *Nature*, 432:338–42.

The recent discoveries of RNA interference and related RNA silencing pathways have revolutionized our understanding of gene regulation. RNA interference has been used as a research tool to control the expression of specific genes in numerous experimental organisms and has potential as a therapeutic strategy to reduce the expression of problem genes. At the heart of RNA interference lies a remarkable RNA processing mechanism that is now known to underlie many distinct biological phenomena.

http://www.ncbi.nlm.nih.gov/pubmed/15372040

Grewal, S.; Elgin, S. 2007. Transcription and RNA interference in the formation of heterochromatin. *Nature*, 447:399–406.

Recent findings have challenged the longstanding belief that heterochromatin is an inert and transcriptionally inactive structure. Studies in organisms ranging from fission yeast to animals have found that noncoding RNAs transcribed from heterochromatic DNA repeats function in the assembly and function of heterochromatin. In this review, we discuss the roles of RNA and RNA turnover in mechanisms that mediate heterochromatin assembly and keep heterochromatic domains silent.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2950806/

Washietl, S., Pedersen, J.; Korbel, Jan; *et al.* 2007. Structured RNAs in the Encode selected regions of the human genome. *Genome Research*, 17(6): 852–864.

Functional RNA structures play an important role both in the context of noncoding RNA transcripts as well as regulatory elements in mRNAs. Here we present a computational study to detect functional RNA structures within the ENCODE regions of the human genome. Since structural RNAs in general lack characteristic signals in primary sequence, comparative approaches evaluating evolutionary conservation of structures are most promising. We have used three recently introduced programs based on either phylogenetic–stochastic context-free grammar (EvoFold) or energy directed folding (RNAz and AlifoldZ), yielding several thousand candidate structures (corresponding to ~2.7% of the ENCODE regions). EvoFold has its highest sensitivity in highly conserved and relatively AU-rich regions, while RNAz favors slightly GC-rich regions, resulting in a relatively small overlap between methods. Comparison with the GENCODE annotation points to functional RNAs in all genomic contexts, with a slightly increased density in 3'-UTRs.

While we estimate a significant false discovery rate of ~50%–70% many of the predictions can be further substantiated by additional criteria: 248 loci are predicted by both RNAz and EvoFold, and an additional 239 RNAz or EvoFold predictions are supported by the (more stringent) AlifoldZ algorithm. Five hundred seventy RNAz structure predictions fall into regions that show signs of selection pressure also on the sequence level (i.e., conserved elements). More than 700 predictions overlap with noncoding transcripts detected by oligonucleotide tiling arrays. One hundred seventy-five selected candidates were tested by RT-PCR in six tissues, and expression could be verified in 43 cases (24.6%).

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1891344/

Reyes-Turcu, F.; Grewal S. 2012. Different means, same end — heterochromatin formation by RNAi and RNAi-independent RNA processing factors in fission yeast. *Curr Opin Genet* Dev, 22:156–63.

The assembly of heterochromatin in eukaryotic genomes is critical for diverse chromosomal events including regulation of gene expression, silencing of repetitive DNA elements, proper segregation of chromosomes and maintenance of genomic integrity. Previous studies have shown that noncoding RNAs and the RNA interference (RNAi) machinery promote the assembly of heterochromatin that serves as a multipurpose platform for targeting effectors involved in various chromosomal processes. Recent work has revealed that RNAi-independent mechanisms, involving RNA processing activities that utilize both noncoding and coding RNAs, operate in the assembly of heterochromatin. These findings have established that, in addition to coding for proteins, mRNAs also function as signaling molecules that modify chromatin structure by targeting heterochromatin assembly factors.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3331891/

Ahlenstiel C.; Lim, H.; Cooper, D; Ishida, T.; Kelleher, A.; Suzuki, K. 2012. Direct evidence of nuclear Argonaute distribution during transcriptional silencing links the actin cytoskeleton to nuclear RNAi machinery in human cells. *Nucl Acids Res,* 40: 1579–95.

Mammalian RNAi machinery facilitating transcriptional gene silencing (TGS) is the RNA-induced transcriptional gene silencing-like (RITS-like) complex, comprising of Argonaute (Ago) and small interfering RNA (siRNA) components. We have previously demonstrated promoter-targeted siRNA induce TGS in human immunodeficiency virus type-1 (HIV-1) and simian immunodeficiency virus (SIV), which profoundly suppresses retrovirus replication via heterochromatin formation and histone methylation. Here, we examine subcellular co-localization of Ago proteins with promoter-targeted siRNAs during TGS of SIV and HIV-1 infection. Analysis of retrovirus-infected cells revealed Ago1 co-localized with siRNA in the nucleus, while Ago2 co-localized with siRNA in the inner nuclear envelope. Mismatched and scrambled siRNAs were observed in the cytoplasm, indicating sequence specificity. This is the first report directly visualizing nuclear compartment distribution of Ago-associated siRNA and further reveals a novel nuclear trafficking mechanism for RITS-like components involving the actin cytoskeleton. These results establish a model for elucidating mammalian TGS and suggest a fundamental mechanism underlying nuclear delivery of RITS-like components.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3287199/

1.2 Omission of epigenetics in genetic modification concept

Genome studies in model organisms and the conduction of the Human Genome Sequencing Project evidenced that significant part of the physical and chemical characteristics of the living beings (the phenotype) was not only controlled by the DNA and, consequently, by the so-called genes. Currently, there is consensus that a number of such characteristics is controlled by epigenetic mechanisms, established in hierarchic levels which extrapolate the functions attributed to the genes, the definition of which is shown to be insufficient.

The epigenetics can be presented, on a simplified way, as an articulated governance means, defining the form through which the genotype may (or not) be used to generate the expression of on or another phenotype. With this respect, in the words of biologist Michel Morange, epigenetics "is a concept that partially denies the 'fatality' of the genes".

The epigenetic mechanisms, which involve molecules such as non-coding RNA/ncRNA (or small RNAs/sRNAs), are generally triggered/guided by environmental factors, by the diet or by the microbione (set of the symbiontic organisms of the living beings, such as the skin or the digestive system bacteria). Under the current knowledge, both in plants and arthropods such as in mammalians[19] certain epigenetic mechanisms seem to be universal, with much to be discovered about its implications. There are still many doubts concerning the operation of the epigenome, its several biological roles in the organisms and the potentially undesirable effects which may trigger disorders posed to them, by the abusive use of technologies affecting it (especially transgenic/exogenous dsRNA synthesis).

19 The epigenetic mechanisms control key biological functions. The permanent silencing of one of the X chromosomes in female mammals, stands out among its most symbolic examples.

COM – Bass BL. 2001. RNA interference: The short answer. *Nature*, 411: 428–9.

Without summary.

http://www.nature.com/nature/journal/v411/n6836/full/411428a0.html

Siomi, H.; Siomi, M. 2009. On the road to reading the RNA-interference code. *Nature*, 457: 396–404.

The finding that sequence-specific gene silencing occurs in response to the presence of double-stranded RNAs has had an enormous impact on biology, uncovering an unsuspected level of regulation of gene expression. This process, known as RNA interference (RNAi) or RNA silencing, involves small non-coding RNAs, which associate with nuclease-containing regulatory complexes and then pair with complementary messenger RNA targets, thereby preventing the expression of these mRNAs. Remarkable progress has been made towards understanding the underlying mechanisms of RNAi, raising the prospect of deciphering the 'RNAi code' that, like transcription factors, allows the fine-tuning and networking of complex suites of gene activity, thereby specifying cellular physiology and development.

http://www.ncbi.nlm.nih.gov/pubmed/19158785

Khan, A.; Betel, D.; Miller, M., Sander, C., Leslie, C., Marks, D. 2009. Transfection of small RNAs globally perturbs gene regulation by endogenous microRNAs. *Nat. Biotechnol.*, 27(6):549-55. doi: 10.1038/nbt.1543. Erratum in *Nat. Biotechnol.*, 27(7):671.

Transfection of small RNAs (such as small interfering RNAs (siRNAs) and microRNAs (miRNAs)) into cells typically lowers expression of many genes. Unexpectedly, increased expression of genes also occurs. We investigated whether this upregulation results from a saturation effect--that is, competition among the transfected small RNAs and the endogenous pool of miRNAs for the intracellular machinery that processes small RNAs. To test this hypothesis, we analyzed genome-wide transcript responses from 151 published transfection experiments in seven different human cell types. We show that targets of endogenous miRNAs are expressed at significantly higher levels after transfection, consistent with impaired effectiveness of endogenous miRNA repression. This effect exhibited concentration and temporal dependence. Notably, the profile of endogenous miRNAs can be largely inferred by correlating miRNA sites with gene expression changes after transfections. The competition and saturation effects have practical implications for miRNA target prediction, the design of siRNA and short hairpin RNA (shRNA) genomic screens and siRNA therapeutics.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2782465/

Jagtap, U.; Gurav, R.; Bapat, V. 2011. Role of RNA interference in plant improvement. *Naturwissenschaften*, 98(6):473-92. doi: 10.1007/s00114-011-0798-8.

Research to alter crops for their better performance involving modern technology is underway in numerous plants, and achievements in transgenic plants are impacting crop improvements in unparalleled ways. Striking progress has been made using genetic engineering technology over the past two decades in manipulating genes from diverse and exotic sources, and inserting them into crop plants for inducing desirable characteristics. RNA interference (RNAi) has recently been identified as a natural mechanism for regulation of gene expression in all higher organisms from plants to humans and promises greater accuracy and precision to plant improvement. The

expression of any gene can be down-regulated in a highly explicit manner exclusive of affecting the expression of any other gene by using RNAi technologies. Additional research in this field has been focused on a number of other areas including microRNAs, hairpin RNA, and promoter methylation. Manipulating new RNAi pathways, which generate small RNA molecules to amend gene expression in crops, can produce new quality traits and having better potentiality of protection against abiotic and biotic stresses. Nutritional improvement, change in morphology, or enhanced secondary metabolite synthesis are some of the other advantages of RNAi technology. In addition to its roles in regulating gene expression, RNAi is also used as a natural defense mechanism against molecular parasites such as jumping genes and viral genetic elements that affect genome stability. Even though much advancement has been made on the field of RNAi over the preceding few years, the full prospective of RNAi for crop improvement remains to be fully realized. The intricacy of RNAi pathway, the molecular machineries, and how it relates to plant development are still to be explained.

http://www.ncbi.nlm.nih.gov/pubmed/21503773

REV – Lejeune, E. ; Allshire, R. 2011. Common ground: small RNA programming and chromatin modifications. *Curr. Opin. Cell. Biol.*, 23:258–65.

Epigenetic mechanisms regulate genome structure and expression profiles in eukaryotes. RNA interference (RNAi) and other small RNA-based chromatin-modifying activities can act to reset the epigenetic landscape at defined chromatin domains. Centromeric heterochromatin assembly is a RNAi-dependent process in the fission yeast Schizosaccharomyces pombe, and provides a paradigm for detailed examination of such epigenetic processes. Here we review recent progress in understanding the mechanisms that underpin RNAi-mediated heterochromatin formation in S. pombe. We discuss recent analyses of the events that trigger RNAi and manipulations which uncouple RNAi and chromatin modification. Finally we provide an overview of similar molecular machineries across species where related small RNA pathways appear to drive the epigenetic reprogramming in germ cells and/or during early development in metazoans.

http://www.ncbi.nlm.nih.gov/pubmed/21478005

REV – Zhang H.; Zhu J-K. Seeing the forest for the trees: a wide perspective on RNA-directed DNA methylation. *Genes Dev.*, 26: 1769–73.

In this issue of Genes & Development, Wierzbicki and colleagues (pp. 1825-1836) examine the current model of RNA-directed DNA methylation (RdDM) by determining genome-wide distributions of RNA polymerase V (Pol V) occupancy, siRNAs, and DNA methylation. Their data support the key role of base-pairing between Pol V transcripts and siRNAs in targeting de novo DNA methylation. Importantly, the study also reveals unexpected complexity and provides a global view of the RdDM pathway.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3426756/

Cortessis, V.; Thomas D.; Levine A.; *et al.* 2012. Environmental epigenetics: prospects for studying epigenetic mediation of exposure–response relationships. *Hum Genet.,* 131: 1565–89.

Changes in epigenetic marks such as DNA methylation and histone acetylation are associated with a broad range of disease traits, including cancer, asthma, metabolic disorders, and various reproductive conditions. It seems plausible that changes in epigenetic state may be induced by environmental

exposures such as malnutrition, tobacco smoke, air pollutants, metals, organic chemicals, other sources of oxidative stress, and the microbiome, particularly if the exposure occurs during key periods of development. Thus, epigenetic changes could represent an important pathway by which environmental factors influence disease risks, both within individuals and across generations. We discuss some of the challenges in studying epigenetic mediation of pathogenesis and describe some unique opportunities for exploring these phenomena.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3432200/

Shenderov, B.; Midtvedt, T. 2014. Epigenomic programing: a future way to health? *Microb. Ecol. Health Dis.,* 8;25. doi: 10.3402/mehd.v25.24145.

It is now generally accepted that the 'central genome dogma' (i.e. a causal chain going from DNA to RNA to proteins and downstream to biological functions) should be replaced by the 'fluid genome dogma', that is, complex feed-forward and feed-back cycles that interconnect organism and environment by epigenomic programing - and reprograming - throughout life and at all levels, sometimes also down the generations. The epigenomic programing is the net sum of interactions derived from own metabolism and microbiota as well as external factors such as diet, pharmaceuticals, environmental compounds, and so on. It is a growing body of results indicating that many chronic metabolic and degenerative disorders and diseases - often called 'civilization diseases' - are initiated and/or influenced upon by non-optimal epigenomic programing, often taking place early in life. In this context, the first 1,000 days of life - from conception into early infancy - is often called the most important period of life. The following sections present some major mechanisms for epigenomic programing as well as some factors assumed to be of importance. The need for more information about own genome and metagenome, as well as a substantial lack of adequate information regarding dietary and environmental databases are also commented upon. However, the mere fact that we can influence epigenomic health programing opens up the way for prophylactic and therapeutic interventions. The authors underline the importance of creating a 'Human Gut Microbiota and Epigenomic Platform' in order to facilitate interdisciplinary collaborations among scientists and clinicians engaged in host microbial ecology, nutrition, metagenomics, epigenomics and metabolomics as well as in disease epidemiology, prevention and treatment.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4016746/

Zhang, L.; Hou, D.; Chen, X.; *et al.* 2012. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. *Cell Research* 22:107–126. doi:10.1038/cr.2011.158.

Our previous studies have demonstrated that stable microRNAs (miRNAs) in mammalian serum and plasma are actively secreted from tissues and cells and can serve as a novel class of biomarkers for diseases, and act as signaling molecules in intercellular communication. Here, we report the surprising finding that exogenous plant miRNAs are present in the sera and tissues of various animals and that these exogenous plant miRNAs are primarily acquired orally, through food intake. MIR168a is abundant in rice and is one of the most highly enriched exogenous plant miRNAs in the sera of Chinese subjects. Functional studies in vitro and in vivo demonstrated that MIR168a could bind to the human/mouse low-density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA, inhibit LDLRAP1 expression in liver, and consequently decrease LDL removal from mouse plasma. These findings demonstrate that exogenous plant miRNAs in food can regulate the expression of target genes in mammals.

Full article available at http://www.nature.com/cr/journal/v22/n1/full/cr2011158a.html

An excellent example of the industry precipitation in its search for rapidly placing in the market transgenic products which are not fully understood by the science relates to Flav/Svr[20] tomato. The first transgenic plant traded in the world, it was distributed for consumption even before the subjacent mechanisms to the effects obtained by the genetic manipulation have been known. Only 12 years after the launching of that tomato in the market, the researches reached clearer interpretations concerning the biological mechanisms responsible for the controlled ripening by a chemical inducer, at the desired moment. By the time of launching, it was supposed that the effect obtained in the Flav/svr tomato resulted from the direct mediation the anti-sense mRNA. Well, it is currently recognized that such technology truly resorted to an epigenetic mechanism of interference RNA (RNAi).

Sanders, R.; Hiatt, W. 2005. Tomato transgene structure and silencing. *Nat. Biotechnol.,* 23: 287–9.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/15765076

Krieger, E.; Allen, E.; Gilbertson, L.; Roberts, J.; Hiatt, W.; Sanders, R. 2008. The Flavr Savr Tomato, an early example of RNAi technology. *HortScience*, 43, 962–964.

The Flavr Savr tomato was introduced as the first genetically engineered whole food in 1994. The commercial event, resulting from transformation with an antisense expression cassette of the endogenous *polygalacturonase* gene, was sequenced and found to contain two contiguous, linked, transfer DNA insertions. We found polygalacturonase suppression correlates with accumulation of ≈21-nt small interfering RNAs, the hallmark of an RNA interference-mediated suppression mechanism.

Full article available at http://hortsci.ashspublications.org/content/43/3/962.full

20 A similar interpretation error was observed in case of papaya genetically modified to resist the ringspot virus (Chiang et al., 2001. Comparative reactions of recombinant papaya ringspot viruses with chimeric coat protein (CP) genes and wild-type viruses on CP-transgenic papaya. *J Gen Virol* 82 (Pt 11): 2827–36).

2 Genetically modified organism response to genetic modification

2.1 The transgene insertion technology inaccuracy results in important modifications in the transformed organism's genome

In contrast to what has been historically defended by the biotechnology companies, the development or transgenic plants results from a highly invasive and random technological process, responsible for damages to the transformed organism's DNA and in the configuration of the transgene itself (mutations, deletions, rearrays, etc.).

Insertions have also been noted on which the trangenes are attached to unstable sites of the genome, such as in retrotransposons[21] (mobile DNA elements).

The number of copies of the cassette/transgene which will be integrated in the target genome is also highly variable and depends on the case. It must be emphasized, at this point, that each inserted copy will correspond to the aggregation of new and additional uncertainties concerning the final effects of that change through genetic modification.

REV – Pawlowski, W.; Somers, D. 1996. Transgene inheritance in plants genetically engineered by microprojectile bombardment. *Mol. Biotechnol.*, 6 (1): 17-30.

Microprojectile bombardment to deliver DNA into plant cells represents a major breakthrough in the development of plant transformation technologies and accordingly has resulted in transformation of numerous species considered recalcitrant to Agrobacterium- or protoplast-mediated transformation methods. This article attempts to review the current understanding of the molecular and genetic behavior of transgenes introduced by microprojectile bombardment. The characteristic features of the transgene integration pattern resulting from DNA delivery via microprojectile bombardment include integration of the full length transgene as well as rearranged copies of the introduced DNA. Copy number of both the transgene and rearranged fragments is often highly variable. Most frequently the multiple transgene copies and rearranged fragments are inherited as a single locus.

21 In an evolutionary view, it is possible to illustrate the biological importance of retrotransposons into a plant genome, based on corn. For details, see SANMIGUEL, P. & BENNETZEN, J., L. 1998 (Evidence that a Recent Increase in Maize Genome Size was Caused by the Massive Amplification of Intergene Retrotransposons. *Annals of Botany* 82 (Supplement A): 37-44, 1998), available at http://aob.oxfordjournals.org/content/82/suppl_1/37.full.pdf.

However, a variable proportion of transgenic events produced by microprojectile bombardment exhibit Mendelian ratios for monogenic and digenic segregation vs events exhibiting segregation distortion. The potential mechanisms underlying these observations are discussed.

http://www.ncbi.nlm.nih.gov/pubmed/8887358

Srivastava V.; Anderson, O.; Ow, D. 1996. Single copy transgenic wheat generated through the resolution of complex integration patterns. *PNAS*, 96: 1117-21.

Genetic transformation of plants often results in multiple copies of the introduced DNA at a single locus. To ensure that only a single copy of a foreign gene resides in the plant genome, we used a strategy based on site-specific recombination. The transformation vector consists of a transgene flanked by recombination sites in an inverted orientation. Regardless of the number of copies integrated between the outermost transgenes, recombination between the outermost sites resolves the integrated molecules into a single copy. An example of this strategy has been demonstrated with wheat transformation, where four of four multiple-copy loci were resolved successfully into single-copy transgenes.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC17996/

Sawasaki, T.; Takahashi, M.; Goshima, N.; Morikawa, H. 1998. Structures of transgene loci in transgenic Arabidopsis plants obtained by particle bombardment: junction regions can bind to nuclear matrices. *Gene*, 218, 24-35.

To clarify the molecular structure of the integration sites of transgenes, we used particle bombardment to examine the DNA sequences of transgene loci. Three transgenic Arabidopsis lines gave a single Southern hybridization band with a selectable gene as the probe. Junction regions flanked by the transgenes were cloned by the inverse polymerase chain reaction method, and the characteristics of the DNA sequences of the 10 junction regions were investigated. All but two of these were AT-rich sequences bearing motifs characteristic of a scaffold/matrix-attachment region (S/MAR). Calculations showed that seven of them should have a propensity for curvature. An assay of in-vitro binding to tobacco nuclear matrices showed that all the junction regions bound to nuclear matrices and that the two input DNAs did not bind. The 12 chromosome/transgene (CT) junctions in these three transgene loci were investigated. Cleavage sites for topoisomerase I were found at 10 of the 12, near the junction point. The other two junctions had sites within 6bp of the junction point. The sequence near one terminal of the transgene in the transgene loci was compared with that near the other terminal. Short, direct repeats consisting of 4-6bp were present within 10bp of the junction points in the sequence. We speculate that the transgene introduced by particle bombardment is delivered on AT-rich S/MAR that has a propensity for curvature, and then a nucleotide near the short, direct repeat on the transgene is joined near the cleavage sites on the genome for topoisomerase I.

http://www.ncbi.nlm.nih.gov/pubmed/9751799

COM – Jank, B.; Haslberger, A. 2000. Recombinant DNA insertion into plant retrotransposons. *Tibtech,* Vol. 18.

Without summary.

Full article available at https://goo.gl/nllbiC

Svitashev, S.; Ananiev E.; Pawlowski, W.; Somers, D. 2000. Association of transgene integration sites with chromosome rearrangements in hexaploid oat. *Theor. Appl. Genet.* 100, 872-880.

Transgene loci in 16 transgenic oat (*Avena sativa* L.) lines produced by microprojectile bombardment were characterized using phenotypic and genotypic segregation, Southern blot analysis, and fluorescence in situ hybridization (FISH). Twenty-five transgene loci were detected; 8 lines exhibited single transgene loci and 8 lines had 2 or 3 loci. Double FISH of the transgene and oat C- and A/D-genome-specific dispersed and clustered repeats showed no preferences in the distribution of transgene loci among the highly heterochromatic C genome and the A/D genomes of hexaploid oat, nor among chromosomes within the genomes. Transgene integration sites were detected at different locations along individual chromosomes, although the majority of transformants had transgenes integrated into subtelomeric and telomeric regions. Transgene integration sites exhibited different levels of structural complexity, ranging from simple integration structures of two apparently contiguous transgene copies to tightly linked clusters of multiple copies of transgenes interspersed with oat DNA. The size of the genomic interspersions observed in these transgene clusters was estimated from FISH results on prometaphase chromosomes to be megabases long, indicating that some transgene loci were significantly larger than previously determined by Southern blot analysis. Overall, 6 of the 25 transgene loci were associated with rearranged chromosomes. These results suggest that particle bombardment-mediated transgene integration may result from and cause chromosomal breakage and rearrangements.

Full article available at http://pawlowski.cit.cornell.edu/2000.pdf

Svitashevy S.; Pawlowskiy, W.; Makarevitch I.; Plank, D.; Somers D. 2002. Complex transgene locus structures implicate multiple mechanisms for plant transgene rearrangement. *The Plant Journal*. 32, 433–445. **(1)**

To more fully characterize the internal structure of transgene loci and to gain further understanding of mechanisms of transgene locus formation, we sequenced more than 160 kb of complex transgene loci in two unrelated transgenic oat (Avena sativa L.) lines transformed using microprojectile bombardment. The transgene locus sequences from both lines exhibited extreme scrambling of non-contiguous transgene and genomic fragments recombined via illegitimate recombination. A perfect direct repeat of the delivered DNA, and inverted and imperfect direct repeats were detected in the same transgene locus indicating that homologous recombination and synthesis-dependent mechanism(s), respectively, were also involved in transgene locus rearrangement. The most unexpected result was the small size of the fragments of delivered and genomic DNA incorporated into the transgene loci via illegitimate recombination; 50 of the 82 delivered DNA fragments were shorter than 200 bp. Eleven transgene and genomic fragments were shorter than the DNA lengths required for Ku-mediated non-homologous end joining. Detection of these small fragments provided evidence that illegitimate recombination was most likely mediated by a synthesis-dependent strand-annealing mechanism that resulted in transgene scrambling. Taken together, these results indicate that transgene locus formation involves the concerted action of several DNA break-repair mechanisms.

http://www.ncbi.nlm.nih.gov/pubmed/12445116

Wu H.; Sparks, C.; Jones, H. 2006. Characterization of T-DNA loci and vector backbone sequences in transgenic wheat produced by Agrobacterium-mediated transformation. *Mol. Breeding* 18: 195-208.

Detailed molecular characterisation of transgene loci is a requirement for gaining regulatory approval for environmental release of genetically modified crops. In cereals, it is generally

accepted that *Agrobacterium*-mediated transformation generates cleaner transgene loci with lower copy number and fewer rearrangements than those generated by biolistics. However, in wheat there has been little detailed analysis of T-DNA insertions at genetic and molecular level. Wheat lines transformed using*Agrobacterium tumefaciens* with *bar* and *gus*A (GUS) genes were subjected to genetic and molecular analysis. Unlike previous studies of transgene loci in wheat, we used functional assays for PAT and GUS proteins, combined with PCR and Southern analysis to detect the presence, copy number, linkage and transmission of two transgenes inserted in the same T-DNA. Thirty-four independent transgenic lines were categorised into three types: type I events (38% of total) where the *gus*A and *bar* genes displayed complete genetic linkage, segregating together as a single functional locus at the expected ratio of 3:1; type II events (18%), which possessed two or more transgene loci each containing *gus*A and *bar*; and type III events (44%), containing an incomplete T-DNA in which either the *gus*A or *bar* gene was lost. Most lines in this last category had lost the *bar*gene situated near the left T-DNA border. Southern analysis indicated that 30% of all lines possessed a single T-DNA copy containing *gus*A and *bar*. However, when data on expression and molecular analysis are combined, only 23% of all lines have single copy T-DNAs in which both gene cassettes are functioning. We also report on the presence of plasmid backbone DNA sequence in transgene loci detected using primer pairs outside the left and right T-DNA borders and within the plasmid selectable marker (*Npt*I) gene. Approximately two thirds of the lines contained some vector backbone DNA, more frequently adjacent to the left border. Taken together, these data imply unstable left border function causing premature T-strand termination or read-through into vector backbone. As far as we are aware, this is the first report revealing near border T-DNA truncation and vector backbone integration in wheat transgenic lines produced by *Agrobacterium*-mediated transformation.

http://link.springer.com/article/10.1007%2Fs11032-006-9027-0#close

REV – Wilson, A.; Latham, J.; Steinbrecher, R. 2006. Transformation-induced mutations in transgenic plants: analysis and biosafety implications. *Biotechnol. Genet. Eng.* 23:209-37.

Without summary.

Full article available at http://www.econexus.info/publication/transformation-induced-mutations-transgenic-plants

REV – Latham, J.; Wilson, A.; Steinbrecher, R. 2006. The Mutational Consequences of Plant Transformation. *Journal of Biomedicine and Biotechnology*, Article ID 25376, Pages 1–7.

Plant transformation is a genetic engineering tool for introducing transgenes into plant genomes. It is now being used for the breeding of commercial crops. A central feature of transformation is insertion of the transgene into plant chromosomal DNA. Transgene insertion is infrequently, if ever, a precise event. Mutations found at transgene insertion sites include deletions and rearrangements of host chromosomal DNA and introduction of superfluous DNA. Insertion sites introduced using Agrobacterium tumefaciens tend to have simpler structures but can be associated with extensive chromosomal rearrangements, while those of particle bombardment appear invariably to be associated with deletion and extensive scrambling of inserted and chromosomal DNA. Ancillary procedures associated with plant transformation, including tissue culture and infection with A tumefaciens, can also introduce mutations. These genome-wide mutations can number from hundreds to many thousands per diploid genome. Despite the fact that confidence in the safety and dependability of crop species rests significantly on their genetic integrity, the frequency of transformation-induced mutations and their importance as potential biosafety hazards are poorly understood.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1559911/

In this context, it is necessary to highlight the fact that most of the transgenic events so far commercially released presents transgene configurations that are physically distinct from the predicted and expected ones as a result of the insertion process.

Windels, P.; Taverniers, I.; Depicker, A.; Van Bockstaele, E.; De Loose, M. 2001. Characterisation of the Roundup Ready soybean insert. *Eur. Food Res. Technol.,* 213, 107-112.

In this article we describe the isolation and characterisation of the junction between insert DNA and plant DNA in the transgenic Roundup Ready soybean line event 40-3-2. Our results establish that during integration of the insert DNA several rearrangements occurred at the 3' NOS junction and that the genomic plant DNA at the pre-integration site may have been rearranged. These findings highlight the utility of characterising junction regions to fulfil the request for information regarding which DNA sequences have been incorporated in commercialised transgenic lines. Furthermore, the characterisation of junction regions is, in our opinion, the method of choice to support method development for detection and identification of plant biotechnology-derived products.

Full article available at http://cera-gmc.org/docs/articles/09-090-008.pdf

Hernández, M.; Pla, M.; Esteve, T.; Prat, S.; Puigdomènech, P.; Ferrando, A. 2003. A specific real-time quantitative PCR detection system for event MON810 in maize YieldGard® based on the 3´-transgene integration sequence. *Transgenic Research,* 12: 179–189.

The increasing presence of transgenic plant derivatives in a wide range of animal and human consumables has provoked in western Europe a strong demand for appropriate detection methods to evaluate the existence of transgenic elements. Among the different techniques currently used, the real-time quantitative PCR is a powerful technology well adapted to the mandatory labeling requirements in the European Union (EU). The use of transgene flanking genomic sequences has recently been suggested as a means to avoid ambiguous results both in qualitative and quantitative PCR-based technologies. In this study we report the identification of genomic sequences adjacent to the 3'-integration site of event MON810 in transgenic maize. This genetically modified crop contains transgene sequences leading to ectopic expression of a synthetic CryIA(b) endotoxin which confers resistance to lepidopteran insects especially against the European corn borer. The characterization of the genome-transgene junction sequences by means of TAIL-PCR has facilitated the design of a specific, sensitive and accurate quantification method based on TaqMan chemistry. Cloning of event MON810 3'-junction region has also allowed to compare the suitability of plasmid target sequences versus genomic DNA obtained from certified reference materials (CRMs), to prepare standard calibration curves for quantification.

http://www.ncbi.nlm.nih.gov/pubmed/12739886

Rang, A.; Linke, B.; Jansen, B. 2005. Detection of RNA variants transcribed from the transgene in Roundup Ready soybean. *Eur. Food Res. Technol.,* 220, 438-43.

The acreage for genetically modified crops (GMOs)—particularly soybean—has steadily increased since 1996, when the first crop of Roundup Ready soybean (intended for food production) was grown. The Roundup Ready soybean varieties derive from a soybean line into which a glyphosate-resistant enolpyruvylshikimate- 3-phosphate-synthase (EPSPS) gene was introduced. The inserted

and the flanking regions in Roundup Ready soybean have recently been characterized. It was shown that a further 250-bp fragment of the epsps gene is localized downstream of the introduced nos terminator of transcription, derived from the nopaline synthase gene from Agrobacterium tumefaciens. We examined whether this 250-bp fragment could be of functional importance. Our data demonstrate that at least 150 bp of this DNA region are transcribed in Roundup Ready soybean. Transcription of the fragment depends on whether readthrough events ignore the nos terminator signal located upstream. Our data also indicate that the read-through product is further processed, resulting in four different RNA variants from which the transcribed region of the nos terminator is completely deleted. Deletion results in the generation of open reading frames which might code for (as yet unknown) EPSPS fusion proteins. The nos terminator is used as a regulatory element in several other GMOs used for food production. This implies that read through products and transcription of RNA variants might be a common feature in these GMOs.

Full article available at http://link.springer.com/article/10.1007%2Fs00217-004-1064-5#page-1

Collonnier, C.; Schattner, A.; Berthier, G.; *et al.* 2005. Characterization and event specific-detection by quantitative real-time PCR of T25 maize insert. *Journal AOAC International*, vol. 88, no. 2, p. 536-546.

T25 is one of the 4 maize transformation events from which commercial lines have so far been authorized in Europe. It was created by polyethylene glycol-mediated transformation using a construct bearing one copy of the synthetic pat gene associated with both promoter and terminator of the 35S ribosomal gene from cauliflower mosaic virus. In this article, we report the sequencing of the whole T25 insert and the characterization of its integration site by using a genome walking strategy. Our results confirmed that one intact copy of the initial construct had been integrated in the plant genome. They also revealed, at the 5' junction of the insert, the presence of a second truncated 35S promoter, probably resulting from rearrangements which may have occurred before or during integration of the plasmid DNA. The analysis of the junction fragments showed that the integration site of the insert presented high homologies with the Huck retrotransposon family. By using one primer annealing in the maize genome and the other in the 5' end of the integrated DNA, we developed a reliable event-specific detection system for T25 maize. To provide means to comply with the European regulation, a real-time PCR test was designed for specific quantitation of T25 event by using Taqman chemistry.

http://www.ncbi.nlm.nih.gov/pubmed/15859082

Rosati, A.; Bogani, P.; Santarlasci, A.; Bulatti, M. 2008. Characterisation of 3' transgene insertion site and derived mRNAs in MON810 YieldGard maize. *Plant Mol. Biol.*, 67 (3): 271-81.

The construct inserted in YieldGard MON810 maize, produced by Monsanto, contains the CaMV 35S promoter, the hsp70 intron of maize, the cryI(A)b gene for resistance to lepidopterans and the NOS terminator. In a previous work a truncation event at the 3' end of the cryI(A)b gene leading to the complete loss of the NOS terminator was demonstrated. The 3' maize genome junction region was isolated in the same experiment not showing any homology with known sequences. The aim of the experiments here reported was therefore to isolate and characterize a larger portion of the 3' integration junction from genomic DNA of two commercial MON810 maize lines. Specific primers were designed on the 3' integration junction sequence for the amplification of a 476 bp fragment downstream of the sequence previously detected. In silico analysis identified the whole isolated 3' genomic region as a gene putatively coding for the HECT E3 ubiquitin ligase. RT-PCR performed in this region produced cDNA variants of different length. In silico translation of these transcripts identified 2 and 18 putative additional aminoacids in different variants, all derived from the adjacent host genomic sequences, added to the truncated CRY1A protein. These putative

recombinant proteins did not show homology with any known protein domains. Our data gave new insights on the genomic organization of MON810 in the YieldGard maize and confirmed the previous suggestion that the integration in the genome of maize caused a complex recombination event without, apparently, interfering with the activity of the partial CRY1A endotoxin and both the vigor and yield of the YieldGard maize.

Full article available at http://www.salmone.org/wp-content/uploads/2009/05/buiatti2008.pdf

La Paz, J.; Vicient, C.; Puigdomènech, P.; Pla, M. 2010. Characterization of polyadenylated*cryIA(b)* transcripts in maize MON810 commercial varieties. *Analytical and Bioanalytical Chemistry*, Volume 396, Issue 6, pp 2125-2133.

The Zea mays L. event MON810 is one of the major commercialized genetically modified crops. The inserted expression cassette has a 3' truncation partially affecting the cryIA(b) coding sequence, resulting in the lack of the NOS terminator, with transcription of the transgene reported to read-through 3'-past the truncation site. Here, we demonstrate that the cryIA(b) transgene gives rise to a variety of polyadenylated transcripts of different sizes that extend to around 1 kbp downstream the truncation site. A Stop codon at position +7 downstream the truncation site indicates the production of a transgenic protein with two additional amino acids; which is compatible with the reported size of the CryIA(b) protein in MON810. There is no evidence of the existence of other translated products. Several main 3' transcription termination regions were detected close to the truncation site and in the transgene 3' flanking sequence. Next to these main termination sites, we identified some sequence motifs that could potentially act as 3'-end-processing elements and drive termination of the transgene transcripts. The MON810 transgene has been introduced into different commercial varieties through breeding programs. Here, we demonstrate that there are no significant differences among the levels of transgene mRNA accumulation, major transcript sizes and 3' termini profiles comparing a number of MON810 commercial varieties grown under similar environmental conditions. Commercial varieties of this event appear to be stable in terms of transgene expression.

http://www.ncbi.nlm.nih.gov/pubmed/19841912

Waminal, N.; Ryu, K.; Choi S-H.; Kim H. 2013. Randomly detected genetically modified (GM) maize *(Zea mays* L.) near a transport route revealed a fragile 45S rDNA phenotype. *PLOS ONE*. 9;8(9):e74060. doi: 10.1371/journal.pone.0074060.

Monitoring of genetically modified (GM) crops has been emphasized to prevent their potential effects on the environment and human health. Monitoring of the inadvertent dispersal of transgenic maize in several fields and transport routes in Korea was carried out by qualitative multiplex PCR, and molecular analyses were conducted to identify the events of the collected GM maize. Cytogenetic investigations through fluorescence in situ hybridization (FISH) of the GM maize were performed to check for possible changes in the 45S rDNA cluster because this cluster was reported to be sensitive to replication and transcription stress. Three GM maize kernels were collected from a transport route near Incheon port, Korea, and each was found to contain NK603, stacked MON863 x NK603, and stacked NK603 x MON810 inserts, respectively. Cytogenetic analysis of the GM maize containing the stacked NK603 x MON810 insert revealed two normal compact 5S rDNA signals, but the 45S rDNA showed a fragile phenotype, demonstrating a "beads-on-a-string" fragmentation pattern, which seems to be a consequence of genetic modification. Implications of the 45S rDNA cluster fragility in GM maize are also discussed.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3767626/

Ben Ali, S-E.; Madi, Z.; Hochegger, R.; Quist, D.; Prewein, B.; Haslberger, A.; Brandes, C. 2014. Mutation Scanning in a Single and a Stacked Genetically Modified (GM) Event by Real-Time PCR and High Resolution Melting (HRM) Analysis. *Int. J. Mol. Sci., 15*, 19898-19923; doi:10.3390/ijms151119898.

Genetic mutations must be avoided during the production and use of seeds. In the European Union (EU), Directive 2001/18/EC requires any DNA construct introduced via transformation to be stable. Establishing genetic stability is critical for the approval of genetically modified organisms (GMOs). In this study, genetic stability of two GMOs was examined using high resolution melting (HRM) analysis and real-time polymerase chain reaction (PCR) employing Scorpion primers for amplification. The genetic variability of the transgenic insert and that of the flanking regions in a single oilseed rape variety (GT73) and a stacked maize (MON88017×MON810) was studied. The GT73 and the 5' region of MON810 showed no instabilities in the examined regions. However; two out of 100 analyzed samples carried a heterozygous point mutation in the 3' region of MON810 in the stacked variety. These results were verified by direct sequencing of the amplified PCR products as well as by sequencing of cloned PCR fragments. The occurrence of the mutation suggests that the 5' region is more suitable than the 3' region for the quantification of MON810. The identification of the single nucleotide polymorphism (SNP) in a stacked event is in contrast to the results of earlier studies of the same MON810 region in a single event where no DNA polymorphism was found.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4264145/

Even in cases on which the DNA sequence mapped in the host plant appeared to be identical to the predicted one, this would not guarantee that its operation would produce the expected effects, and only these. The similarity in terms of identity of that genetic information does not guarantee by itself that the protein synthesis will be identical in the original being and in the transformed one. In fact, post-translational mechanisms – consolidated by the folding in the three-dimensional space of the amino acids sequence -, dependent of the cellular medium and the influenced the cell is submitted to, will define essential pathways for the configuration of the definite biological functions of that protein. The adoption of a spatial pattern distinct from the expected one – regardless of the DNA which generated a certain protein – may change its functionality, transforming macromolecules that are harmless to the organisms into harmful toxins[22] or allergens.

22 It should be noted that the difference between a normal type of prion and prion responsible for mad cow disease lies only in the spatial conformation of the protein, not the DNA sequence that generated. Check for example, Abid & Soto, 2006 (The intriguing prion disorders. *Cell Mol Life Sci,* 63, 2342–2351).

Prescott, V.; Campbell, P.; Moore, A.; Mattes, J.; Rothenberg, M.; Foster, P.; Higgins, T.; Hogan, S. 2005. Transgenic expression of bean alpha-amylase inhibitor in peas results in altered structure and immunogenicity. *Journal of Agricultural and Food Chemistry*, 53.

The development of modern gene technologies allows for the expression of recombinant proteins in non-native hosts. Diversity in translational and post-translational modification pathways between species could potentially lead to discrete changes in the molecular architecture of the expressed protein and subsequent cellular function and antigenicity. Here, we show that transgenic expression of a plant protein (alpha-amylase inhibitor-1 from the common bean (Phaseolus vulgaris L. cv. Tendergreen)) in a non-native host (transgenic pea (Pisum sativum L.)) led to the synthesis of a structurally modified form of this inhibitor. Employing models of inflammation, we demonstrated in mice that consumption of the modified alphaAI and not the native form predisposed to antigen-specific CD4+ Th2-type inflammation. Furthermore, consumption of the modified alphaAI concurrently with other heterogeneous proteins promoted immunological cross priming, which then elicited specific immunoreactivity of these proteins. Thus, transgenic expression of non-native proteins in plants may lead to the synthesis of structural variants possessing altered immunogenicity.

http://www.ncbi.nlm.nih.gov/pubmed/16277398

2.2 Instability of the transgene - and of its expression - after insertion

The forced insertion of transgenic material, which generally involves DNA elements of a number of phylogenetically distant species, tend to cause changes to the transformed organism's genome, generating favorable conditions to the transgene instability. Such instability – in the expression of the recombinant proteins (or in other expression products) can be expressed during the vegetative cycle of the plant and/or by the time the transgene is transmitted from generation to generation.

Considering the various internal and external factors to the organism which influence such instabilities, it is evident that its set generates very complex anticipation hazards and uncertainties, in a biosafety perspective.

Sometimes, the transgene is simply silenced[23], as it has been noted in commercial transgenic varieties.

23 Gene silencing of transgenes is also dealt with in item 2.3 of Part 2 of this book, where we discuss potential ineffectiveness of virus resistance technologies in genetically modified plants.

Meza, T.; Kamfjord, D.; Håkelien, A-M.; Evans, I.; Godager, L.; Mandal, A.; Jakobsen, K.; Aalen, R. 2001. The frequency of silencing in *Arabidopsis thaliana* varies highly between progeny of siblings and can be influenced by environmental factors. *Transgenic Research,* 10: 53–67.

In a collection of 111 transgenic Arabidopsis thaliana lines, silencing of the nptII gene was observed in 62 (56%) of the lines and three distinct nptII-silencing phenotypes were identified. Two T-DNA constructs were used, which differed in distance and orientation of the marker gene relative to the border sequences. Comparison of the sets of lines generated with each vector, indicate that the T-DNA construct configuration influence the incidence of lines displaying silencing, as well as the distribution of silencing phenotypes. Twenty lines were investigated more thoroughly. The frequency of silencing varied between siblings in 19 lines, including three lines containing a single T-DNA copy. The last line showed 100% silencing. The gus gene present in both constructs could be expressed in the presence of a silenced nptII gene. Investigation of methylation at a single site in the pnos promoter revealed partial methylation in multi-copy lines, but no methylation in single-copy lines. For 16 lines, the overall frequencies of silencing differed significantly between control plants and plants exposed to temperature stress; in 11 of these lines at the 0.1% level. In several cases, the frequency of silencing in progeny of stress-treated plants was higher than for the control group, while other lines showed higher frequencies of kanamycin-resistant progeny for the stress-treated sibling plants.

Full article available at https://goo.gl/hVmf48

Aguilera, M.; Querci, M.; Balla, B.; Prospero, A.; Ermolli, M.; Van den Eede, G. 2008. A Qualitative Approach for the Assessment of the Genetic Stability of the MON 810 Trait in Commercial Seed Maize Varieties. *Food Anal. Methods,* 1: 252-258.

Maize MON 810 is one of the European Union's (EU) authorized genetically modified organisms (GMO) for placing on the food and feed market. The total number of MON 810 varieties registered in the European Common Catalogue of varieties of agricultural plant species has almost tripled since 2005. One of the requirements described in EU legislation, namely the genetic stability of GM seed varieties, was thus assessed by analyzing the intactness of the entire MON 810 integration and its genotypic stability in commercial varieties available on the market for at least the last 2 years. A combined strategy using qualitative analytical methods made possible to determine the presence/absence of the individual genetic elements and of the whole GM construct. The restriction fragment length polymorphism patterns obtained from amplified whole constructs by long polymerase chain reaction (PCR) were compared side by side. CryIA(b) protein expression levels were determined by enzyme-linked immunosorbent assay. Twenty-four out of the 26 analyzed varieties met the expected stability features. One variety gave negative results in all assays, and one variety contained the necessary genetic elements for expressing CryIA(b) protein although giving negative results for the long PCR product. To our knowledge, this study is the first post-marketing stability analysis performed on GM commercial seed varieties.

http://link.springer.com/article/10.1007%2Fs12161-008-9035-2

2.2.1 The transgene instability due to internal factors

As previously mention, in addition to the transformation method used, also the organizational structure resulting from the insertion of the transgene influences its stability, the stability of its expression

and, also, the heritage standard of such DNA elements over the generations.

Kohli, A.; Twyman, R.; Abranches, R.; Wegel, E.; Stoger, E.; Christou, P. 2003. Transgene integration, organization and interaction in plants. *Plant Molecular Biology* 52: 247–258.

It has been appreciated for many years that the structure of a transgene locus can have a major influence on the level and stability of transgene expression. Until recently, however, it has been common practice to discard plant lines with poor or unstable expression levels in favor of those with practical uses. In the last few years, an increasing number of experiments have been carried out with the primary aim of characterizing transgene loci and studying the fundamental links between locus structure and expression. Cereals have been at the forefront of this research because molecular, genetic and cytogenetic analysis can be carried out in parallel to examine transgene loci in detail. This review discusses what is known about the structure and organization of transgene loci in cereals, both at the molecular and cytogenetic levels. In the latter case, important links are beginning to be revealed between higher order locus organization, nuclear architecture, chromatin structure and transgene expression.

http://www.ncbi.nlm.nih.gov/pubmed/12856933

Yin, Z; Plader, W.; Malepszy, S. 2004. Transgene inheritance in plants. *J Appl Genet.*, 45(2):127-44.

The patterns of transgene inheritance in plants and the possible explanations for non-Mendelian transmission are reviewed. The non-Mendelian inheritance of a transgene has been recorded with a frequency between 10% and 50% in transgenic plants produced either by Agrobacterium-mediated transformation or through particle bombardment. Different effects such as deletion, duplication, rearrangement, repeated sequence recombination as well as gene interaction have been observed for transgenic loci. The nature of the recipient genome, nature of the transgene and the interactions between them seem to contribute to the non-Mendelian segregation of transgenes.

Full article available at https://goo.gl/IZjczz

Epigenetic mechanisms may also influence - and even prevent - the transgene expression. One of the ways would be by means of the system called PTGS (Post Transcriptional Gene Silencing). Such mode of action – intended for the destruction of all the RNAm synthesized by one transgene – is indicated as a type of "genetic defense system" which recognizes and fights against the introduction of certain endogenous and/or exogenous DNA sequences. Under an evolutionary point of view, such mechanism would have progressed in order to protect the organisms from viruses and transposons.

Other information about the PTGS mechanism involved in the

inhibition of the transgene expression in case or tolerance to virus can be found in item 2.3 of Part 2 of this book.

Dougherty, W.; Parks, T.1995. Transgenes and gene suppression: telling us something new? *Curr Opin Cell Biol.*, Vol. 7(3): 399-405.

Transgenes provide unique opportunities to assess the relationship between genotype and phenotype in an organism. In most cases, introduction and subsequent expression of a transgene will increase (with a sense RNA) or decrease (with an antisense RNA) the steady-state level of a specific gene product. However, a number of surprising observations have been made in the course of many transgenic studies. We develop a hypothesis that suggests that many examples of endogenous gene suppression by either antisense or sense transcripts are mediated by the same cellular mechanism.

http://www.ncbi.nlm.nih.gov/pubmed/7662371

Kumpatla, S.; Chandrasekharan, M.; Iyer, M.; Li, G.; Hall, T. 1998. Genome intruder scanning and modulation systems and transgene silencing. *Trends in Plant Science*, Vol 3, Issue 3. 97-104.

The widespread occurrence of transgene inactivation in plants and classical cases of silencing of duplicated sequences in fungi suggest that all genomes contain defense systems that are capable of monitoring and manipulating intrusive DNA. Such DNA might be recognized by its structure, its sequence composition relative to that of its genomic environment and possibly by its disruption of normal biochemical functions. Although methylation, especially of repeated sequences, is widely associated with gene inactivation, other attributes, including chromatin modification, may be involved. Elimination of inactivated intrusive DNA (presently best documented for filamentous fungi) may also contribute to genomic defense mechanisms in plants. Stable integration and expression of introduced genes are essential for genetically engineered crops, and thus transformation constructs must be designed to avoid host surveillance processes.

Full article available at https://goo.gl/5Xwfqh

Kumpatla, S.; Hall; T. 1999. Organizational complexity of a rice transgene locus susceptible to methylation-based silencing. *Life*, 48, 459-467.

Molecular analyses of a rice (Oryza sativa L.) transgene locus introduced using biolistic techniques revealed the presence of multiple copies of rearranged fragments, as well as an intact copy of the supplied constructs. Both the gene of interest (35S-Btt cryIIIA) and the selectable marker used (Ubi1-bar) were methylated and silenced. Additionally, vector sequences were present in great abundance and were also highly methylated, indicating that the entire transgene insert was marked for methylation. The rearrangement of input DNA resulted in interspersion of plasmid backbone regions with the gene of interest. Permutation of segments encoding the gene of interest and the selectable marker was also detected, perhaps explaining why sequences introduced on separate plasmids are frequently found to be inserted at the same locus. The 35S promoter contained several hotspots for fragmentation. These observations strongly support the concept that intrusive DNA is recognized by host surveillance systems and that transgene loci with anomalous structural organization are subjected to inactivation by processes such as methylation.

http://www.ncbi.nlm.nih.gov/pubmed/10632579

Cogoni, C.; Macino, G. 2000. Post-transcriptional gene silencing across kingdoms. *Curr Opinion Genet Dev*, 10, 638-43.

Post-transcriptional gene silencing (PTGS) as a consequence of the introduction of either transgenes or double-stranded RNA molecules has been found to occur in a number of species. In the past year, studies in different systems have greatly enhanced our understanding of the molecular mechanisms of these phenomena. The ubiquitous presence of PTGS in both the plant and animal kingdoms and the finding of common genetic mechanisms suggest that PTGS is a universal gene-regulation system fundamental in biological processes such as protection against viruses and transposons.

Full article available at https://goo.gl/7zI52A

Hirai, S.; Takahashi, K.; Abiko, T.; Kodama, H. 2010. Loss of sense transgene-induced post-transcriptional gene silencing by sequential introduction of the same transgene sequences in tobacco. *The FEBS Journal*, 277(7):1695-703. doi: 10.1111/j.1742-4658.2010.07591.x.

RNA silencing is an epigenetic inhibition of gene expression and is guided by small interfering RNAs. Sense transgene-induced post-transcriptional gene silencing (S-PTGS) occurs in a portion of a transgenic plant population. When a sense transgene encoding a tobacco endoplasmic reticulum omega-3 fatty acid desaturase (NtFAD3) was introduced into tobacco plants, an S-PTGS line, S44, was obtained. Introduction of another copy of the NtFAD3 transgene into S44 plants caused a phenotypic change from S-PTGS to overexpression. Because this change was associated with the methylation of the promoter sequences of the transgene, reduced transcriptional activity may abolish S-PTGS and residual transcription of the sense transgene may account for the overexpression. To clarify whether RNA-directed DNA methylation (RdDM) can repress the transcriptional activity of the S44 transgene locus, we introduced several RdDM constructs targeting the transgene promoter. An RdDM construct harboring a 200-bp-long fragment of promoter sequences efficiently abrogated the generation of NtFAD3 small interfering RNAs in S44 plants. Transcription of the transgene was partially repressed, but the resulting NtFAD3 mRNAs successfully accumulated and an overexpressed phenotype was established. Our results indicate an example in which overexpression of the transgene is established by complex epigenetic interactions among the transgenic loci.

http://www.ncbi.nlm.nih.gov/pubmed/20180844

It is worth reminding that situations on which the integration of a transgene occurs on a physical basis, but without being expressed in the organism, have already been identified for more than 30 years. However, only recently the knowledge allowing to interpret some of the mechanisms involved in this process were accessed.

Barton, K.; Binns, A.; Matzke, A. Chilton, M-D. 1983. Regeneration of Intact Tobacco Plants Containing Full Length Copies of Genetically Engineered T-DNA, and Transmission of T-DNA to RI Progeny. *Cell*, Vol. 32, 1033-l 043.

Cloned DNA sequences encoding yeast alcohol dehydrogenase and a bacterial neomycin phosphotransferase have been inserted into the T-DNA of Agrobacterium tumefaciens plasmid pTiT37 at the "rooty" locus. Transformation of tobacco stem segments with the engineered bacterial strains produced attenuated crown gall tumors that were capable of regeneration into intact, normal

tobacco plants. The yeast gene and entire transferred DNA (T-DNA) were present in the regenerated plants in multiple copies, and nopaline was found in all tissues. The plants were fertile, and seedlings resulting from self-pollination also contained intact and multiple copies of the engineered T-DNA. Expression of nopaline in the germinated seedlings derived from one regenerated plant was variable and did not correlate with the levels of T-DNA present in the seedlings. Preliminary evidence indicates that nopaline in progeny of other similarly engineered plants is more uniform. The disarming of pTiT37 by insertions at the "rooty" locus thus appears to produce a useful gene vector for higher plants.

Full article available at https://goo.gl/xlp2fM

2.2.2 The transgene instability due to interactions with environmental factors

In addition to the internal factors, there is a number of external environmental factors which interact with the transgene, affecting its stability. In many circumstances, the local environmental conditions and the stress factors, in the field, seem to affect the transgene expression way and characteristics.

Olsen, K.; Daly, J.; Finnegan, E.; Mahon, R. 2005. Changes in Cry1Ac Bt Transgenic Cotton in Response to Two Environmental Factors: Temperature and Insect Damage. *J. Econ. Entomol.* 98(4): 1382Ð1390.

The efficacy of Cry1Ac Bacillus thuringiensis (Bt) cotton plants against field populations of Helicoverpa armigera (Hübner) has been inconsistent over the growing season. Any reduction in efficacy (where efficacy is the capacity of the plant to affect the survival of the insect) increases the opportunities for H. armigera to evolve resistance to Bt toxin. Changes in efficacy could be due to changes at the level of gene expression and/or in the physiological makeup of the plant and may be induced by environmental conditions. Two environmental factors, temperature and insect damage, were investigated. Temperature was found to affect efficacy, whether plants were grown at different temperatures continuously or were exposed to a change in temperature for a short period. Damage caused by chewing insects (H. armigera larvae) produced a dramatic increase in the efficacy of presquare Bt cotton. In contrast, damage by sucking insects (aphids) did not induce changes in efficacy. Changes in efficacy seemed to be mediated through modification of the physiological background of the plant rather than changes in the level of Cry1Ac expression or in the concentration of the Bt toxin. The impact of the non-Bt responses of plants on strains of H. armigera should be evaluated. It is possible that by enhancing existing defensive mechanisms of plants, the rate of evolution of resistance to Bt toxins could be retarded by increasing the plants overall toxicity through the additive effects of the toxins and plant defenses.

http://www.ncbi.nlm.nih.gov/pubmed/16156594

Bruns, H.; Abel, C. 2007. Effects of nitrogen fertility on Bt endotoxin levels in maize. *Journal of Entomological Science*, 42: 35-43.

A study was conducted to determine if Bt endotoxin concentrations during reproductive growth of Bt maize hybrids are affected by different N-fertility rates used to grow the crop. Previous research has shown N-fertility rates positively affect Bt concentrations of young Bt maize plants grown

in a glasshouse. Three Bt hybrids, two with Bt event MON-810 (AgriGold brand cv. A 6729Bt and Pioneer brand cv. 33V08Bt) one with Bt event DBT 418 (DeKalb 626Bt) and one non-Bt maize hybrid (DeKalb brand cv. 626) were grown at Stoneville, MS, USA in 2002 and 2003 with N-fertility rates of 0, 112, 224, and 336 kg N/ha. Tissue samples of the outer ear husks and primary ear leaf sheaths were collect at growth stage R3 and analyzed for Bt concentration. Agronomic data were collected at maturity. The concentrations of Bt endotoxin in both sets of tissue were positively correlated with N-fertility rate for the MON-810 Bt hybrids but not the DBT 418 Bt hybrid. Increases in N-fertility increased grain yields. The Bt hybrids had less lodging (0.7%-1.0%) than the non-Bt hybrid (5.1%). Adequate levels of N-fertility are important to MON-810 Bt hybrids not only for yield, but also to ensure sufficient levels of Bt endotoxin for maximum protection from susceptible insect pests.

Full article available at http://www.regional.org.au/au/asa/2004/poster/3/8/453_brunsha.htm

Griffiths, B.; Caul, S.; Thompson, J.; Birch, A.; Scrimgeour, C.; Cortet, J.; Foggo, A.; Hacket, C.; Krogh, P.; 2006. Soil microbial and faunal community responses to Bt maize and insecticide in two soils. *Journal of Environmental Quality*, 35: 734-741.

The effects of maize (Zea mays L.), genetically modified to express the Cry1Ab protein (Bt), and an insecticide on soil microbial and faunal communities were assessed in a glasshouse experiment. Soil for the experiment was taken from field sites where the same maize cultivars were grown to allow comparison between results under glasshouse conditions with those from field trials. Plants were grown in contrasting sandy loam and clay loam soils, half were sprayed with a pyrethroid insecticide (deltamethrin) and soil samples taken at the five-leaf stage, flowering, and maturity. The main effect on all measured parameters was that of soil type and there were no effects of Bt trait or insecticide on plant growth. The Bt trait resulted in more soil nematodes and protozoa (amoebae), whereas insecticide application increased plant Bt concentration and altered nematode community structure. The only significant effects on soil microbial community structure, microarthropods, and larvae of a nontarget root-feeding Dipteran, were due to soil type and plant growth stage. The results indicate that, although there were statistically significant effects of the Bt trait on soil populations, they were small. The relative magnitude of the effect could best be judged by comparison with the insecticide treatment, which was representative of current best practice. The Bt trait had no greater effect than the insecticide treatment. Results from this glasshouse experiment were in broad agreement with conclusions from field experiments using the same plant material grown in the same soils.

Full article available at https://goo.gl/yanQnO

Zeller, S.; Kalinina, O.; Brunner, S.; Keller, B.; Schmid, B. 2010. Transgene X environment interactions in genetically modified wheat. *PLOS One*, 12; 5(7):e11405.

Background: The introduction of transgenes into plants may cause unintended phenotypic effects which could have an impact on the plant itself and the environment. Little is published in the scientific literature about the interrelation of environmental factors and possible unintended effects in genetically modified (GM) plants.
Methods and Findings: We studied transgenic bread wheat *Triticum aestivum* lines expressing the wheat *Pm3b* gene against the fungus powdery mildew *Blumeria graminis* f.sp. *tritici*. Four independent offspring pairs, each consisting of a GM line and its corresponding non-GM control line, were grown under different soil nutrient conditions and with and without fungicide treatment in the glasshouse. Furthermore, we performed a field experiment with a similar design to validate our glasshouse results. The transgene increased the resistance to powdery mildew in all environments. However, GM plants reacted sensitive to fungicide spraying in the glasshouse. Without fungicide treatment, in the glasshouse GM lines had increased vegetative biomass and seed number and a twofold yield compared with control lines. In the field these results were reversed. Fertilization generally increased GM/control differences in the glasshouse but not in the field. Two of four GM

lines showed up to 56% yield reduction and a 40-fold increase of infection with ergot disease *Claviceps purpurea* compared with their control lines in the field experiment; one GM line was very similar to its control.
Conclusions: Our results demonstrate that, depending on the insertion event, a particular transgene can have large effects on the entire phenotype of a plant and that these effects can sometimes be reversed when plants are moved from the glasshouse to the field. However, it remains unclear which mechanisms underlie these effects and how they may affect concepts in molecular plant breeding and plant evolutionary ecology.

Full article available at https://goo.gl/zhXuoz

Blaise, D.; Kranthi, K. 2011. Cry1Ac expression in transgenic Bt cotton hybrids is influenced by soil moisture and depth. *Current Science*, Vol 101 (6).

Cry1Ac toxin concentration was assessed in leaves of *Bt* transgenic cotton hybrid grown on shallow (<60 cm) and deep (>90 cm) black soils of Nagpur, Maharashtra, India. Cry toxin concentration increased up to 80 days after sowing followed by a steep decline. In general, toxin concentration was greater on the deep black soils than the shallow soil. This was because of greater water-holding capacity of the deep soils. Cry toxin concentration was closely related to the soil water content. Beyond (excess moisture) and below (moisture deficit) field capacity, toxin concentration declined. A cubic polynomial best described the relationship between Cry toxin concentration and soil moisture content ($R2$ = 0.95).

Full article available at http://www.currentscience.ac.in/Volumes/101/06/0783.pdf

In case of Bt plants, the instability in the toxin synthesis (from a quantitative point of view) impairs the pest control strategy based on high doses, used in most recent events with views to limit the development of genetically resistant inset populations. Evidently such instability also sets a precedent for other implications and risks, such as agronomic, socioeconomic and environmental perspectives.

Coviella, C.; Morgan, D.; Trumble, J. 2000. Interactions of Elevated CO2 and Nitrogen Fertilization: Effects on Production of Bacillus thuringiensis Toxins in Transgenic Plants. *Environ. Entomol.*, 29(4): 781-787.

Elevated atmospheric CO_2 concentrations will cause plants to grow faster, lower nitrogen content per unit of plant tissue, and generate higher carbon to nitrogen (C/N) ratios. We hypothesize that production of transgenic proteins will be reduced, thus reducing the efficiency of *Bacillus thuringiensis* (Bt) transgenes against insect populations. Commercially available transgenic cotton plants expressing the *Cry 1Ac* gene from Bt were compared with a near isogenic non-Bt cotton line in a split-plot design with two levels of atmospheric CO_2 (ambient, 370 ppm and elevated, 900 ppm) incorporating a 2 × 2 factorial design with two nitrogen (N) fertilization regimes (low, 30 mg N/kg soil/wk and high, 130 mg N/kg soil/wk), and two levels of Bt (presence or absence). Bioassays using *Spodoptera exigua* (Hübner) and quantitative enzyme-linked immunosorbent assays for toxin content indicated reduced Bt protein production in elevated CO_2. The tendency for test insects to consume more foliage from plants with lower N, caused by the elevated CO_2, did not compensate for the reduction in toxin production. N fertilization regime interacted

with CO_2 concentration, showing that plants growing in N limited systems would produce substantially less toxin. The use of transgenic plants is becoming increasingly important and will continue to be so in the next decades. At the same time, atmospheric CO_2 increase will affect the effectiveness of this strategy. These observations have implications not only for agricultural use of transgenic plants, but also for the ecological consequences of transfer of Bt toxins to closely related wild plant genotypes.

http://www.bioone.org/doi/abs/10.1603/0046-225X-29.4.781?journalCode=enve

Abel, C.; Adamczyk, J. 2004. Relative concentration of Cry1A in maize leaves and cotton bolls with diverse chlorophyll content and corresponding larval development of fall armyworm (*Lepidoptera: Noctuldae*) and southwestern corn borer (*Lepidoptera: Crambidae*) on maize whorle leaf profiles. *Journal of Economic Entomology,* 97: 1737-1744.

To manage insect resistance to transgenic crops that express insecticidal proteins from Bacillus thuringiensis (Bt) Berliner, the U.S. Environmental Protection Agency recommends a refuge-based insect resistance management strategy where a percentage of non-Bt (refuge) crop is grown in proximity to a Bt-expressing crop. An important requirement for this strategy is that the toxin exists at a high effective dose for control of the target pest(s), so that heterozygous individuals in the population do not reach adulthood. Factors that cause reduced levels of toxin in the plant are a threat to this strategy. We quantified Cry1Ab from different areas of the maize, Zea mays L., leaf. In general, the distal tip of the V7 maize leaf had a higher concentration of Cry1Ab compared with the middle section of the V7 leaf, and the middle section of the developing V9 leaf had the lowest concentration of Cry1Ab. When these sections of maize tissue were fed to fall armyworm, Spodoptera frugiperda (J.E. Smith), and southwestern corn borer, Diatraea grandiosella Dyar, there was not a reduction in development or an increase in mortality with tissue that had higher concentrations of toxin. Another study tested the relative concentration of Cry1Ab between the white-yellow, yellow-green, and green portions of the developing ninth leaf within the maize whorl. There were differences in Cry1Ab concentration among these leaf areas. The green tissue had the highest concentration of toxin followed by the yellow-green and white-yellow tissues. Correlations between concentration of Cry1Ab and 5-d fall armyworm larval weights among the three leaf color profiles were all significant and negative, i.e., decreased concentration of Cry1Ab in the leaf tissue resulted in increased 5-d larval weights. There was 100% mortality to the southwestern corn borer larvae fed Cry1Ab maize leaf tissue. Differences in the amount of Cry1Ab in the developing V9 leaf profiles did not alter the absolute susceptibility of the southwestern corn borer to the toxin. In cotton, Gossypium hirsutum L., the amount of Cry1Ac was significantly lower in boll tips where flowers had remained attached compared with normal boll tips. Boll tips where the flowers remained attached are often the site where corn earworms, Helicoverpa zea (Boddie), penetrate Bt cotton bolls. This study demonstrated that, in two diverse plant species, tissue that has low chlorophyll content does not fully express Cry1A. Photosynthesis regulating factors related to mRNA transcription and translation should be studied for their effect on Cry1A production and insect control.

http://www.ncbi.nlm.nih.gov/pubmed/15568367

Kranthi, K.; Naidu, S.; Dhawad, C; Tatwawadi, A.; Mate, K.; Patil, E.; Bharose, A.; Behere, G.; Wadaskar, R.; Kranthi, S. 2005. Temporal and intra-plant variability of Cry1Ac expression in Bt-cotton and its influence on the survival of the cotton bollworm, *Helicoverpa armigera* (Hübner) (*Noctuidae: Lepidoptera*). *Current Science*, Vol. 89 (2), 291.

The quantitative levels of Cry1Ac and the seasonal decline in expression differed significantly among the eight commercial Bollgard hybrids tested. The Cry1Ac expression was found to be variable among the hybrids and also between different plant parts. The leaves of *Bt*-cotton plants were found

to have the highest levels of Cry1Ac expression followed by squares, bolls and flowers. The toxin expression in the boll-rind, square bud and ovary of flowers was clearly inadequate to confer full protection to the fruiting parts. Increasing levels of *Helicoverpa armigera* survival were correlated with the toxin levels decreasing below 1.8 mg/g in the plant parts. Genotype-independent seasonal decline of the Cry1Ac toxin levels was observed in all the hybrids. Cry1Ac expression decreased consistently as the plant aged. The decline in Cry1Ac was more rapid in some hybrids compared to others. The choice of parental background appeared to be crucial for sustainable expression of the *cry1Ac* transgene. The implications of variability in Cry1Ac expression and the seasonal decline on bollworm management are discussed.

Full article available at https://goo.gl/CpHSLd

Dong, H.; Li, W. 2007. Variability of endotoxin expression in Bt transgenic cotton. *J. Agron. Crop Sci.,* 193 (1), 21-29.

Transgenic cotton expressing Bt (Bacillus thuringiensis) *toxins* is currently cultivated on a large commercial scale in many *countries*, but observations have shown that it behaves variably in toxin efficacy against target insects under field conditions. Understanding of the temporal and spatial variation in efficacy and the resulting mechanisms is essential for cotton protection and production. In this review, we summarize current knowledge on variability in Bt cotton efficacy, in particular on the induced variability by environmental stresses. We also discuss the resulting mechanisms and the countermeasures for the inconsistence in efficacy in Bt cotton. It is indicated that insecticidal *protein* content in Bt cotton is variable with plant age, plant structure or under certain environmental stresses. Variability in Bt cotton efficacy against target insect pests is mainly attributed to the changes in Bt protein content, but physiological changes associated with the production of secondary compounds in plant tissues may also play an important role. Reduction of Bt protein content in late-season cotton could be due to the overexpression of Bt gene at earlier stages, which leads to gene regulation at post-transcription *levels* and consequently results in gene silencing at a later stage. Methylation of the promotor may be also involved in the declined expression of endotoxin *proteins*. As a part of total protein, the insecticidal protein in plant tissues changes its level through inhibited synthesis, degradation or translocation to developing plant parts, particularly under environmental stresses, thus being closely correlated to N metabolism. It can be concluded that developing new cotton varieties with more powerful resistance, applying certain plant growth regulators, enhancing intra-plant defensive capability, and maintenance of general health of the transgenic crop are important in realizing the full transgenic potential in Bt cotton.

http://onlinelibrary.wiley.com/doi/10.1111/j.1439-037X.2006.00240.x/abstract

Nguyen, H.; Jehle, J.; 2007. Quantitative analysis of the seasonal and tissue-specific expression of Cry1Ab in transgenic maize Mon810. *Journal of Plant Diseases and Protection*, 114 (2), 82–87.

The tissue-specific expression and seasonal abundance of Cry1Ab *protein* were determined in transgenic maize plants (Mon810, variety 'Novelis') from two field trials located near Bonn and Halle, Germany. *A total* of 1085 *samples* were analysed by using Double Antiserum-Enzyme Linked Immunosorbent Assay (DAS-ELISA). The Cry1Ab contents of various plant tissues (root, stem, upper leaf, lower leaf, anther, pollen and kernel) were determined at four different growth stages (BBCH19, BBCH30, BBCH61 and BBCH83) collected in 2001, 2002 and 2003. Mon810 showed the highest Cry1Ab contents in the leaves (5.5 - 6.4 mu g g(-1) fresh weight [fw]) at BBCH83, whereas the lowest Cry1Ab contents were detected in the pollen (1 - 97 ng g(-1) fw). Cry1Ab *content of* residual root stocks collected in the field nine months after harvest was 15 - 17 ng g(-1) fw. This demonstrated that the Cry1Ab concentration in residual root stocks was reduced to about one-hundredth of the fresh roots. The monitoring of Cry1Ab expression showed that the

Cry1Ab contents varied strongly between different plant individuals.

https://www.jstor.org/stable/43228900

Bakhsh, A.; Shahzad, K.; Husnain, T. 2011. Variation in the spatio-temporal expression of insecticidal genes in cotton. *Czech J. Genet. Plant Breed.*, 47 (2011): 1-9

The most significant breakthrough in plant biotechnology is the development of the techniques to transform genes from unrelated sources into commercially important crop plants to develop resistance against targeted insect pests. The spatio-temporal expression of insecticidal genes in transgenic cotton varies with plant age, plant parts and environmental conditions. The understanding of this temporal and spatial variation in efficacy and the resulting mechanisms is essential for cotton protection and production. This review summarizes variations in the efficacy of introduced insecticidal genes in cotton crop. The factors contributing to the variability of endotoxins have also been highlighted. The reduction in *Bt* protein biosynthesis in late-season cotton tissues could be attributed to the overexpression of the Bt gene at earlier stages, which leads to gene regulation at post-transcription levels and consequently results in gene silencing at a later stage. Methylation of the promoter may also play a role in the declined expression of endotoxin proteins. In genetically modified crops several environmental factors have been reported to affect the expression of transgenes. Among environmental factors nitrogen metabolism, inhibition of synthesis, degradation, remobilization and high temperature are attributable to the quantitative reduction in Bt proteins. Applying plant growth regulators or protein enhancers such as Chaperone™ may improve Bt cotton efficacy through enhancing the synthesis of proteins. Also some agronomic practices such as nitrogen fertilization and timely irrigation favour the endotoxin expression. Thus, variations in the efficacy of insecticidal genes in transgenic cotton and the involved mechanisms need to be understood fully so as to plan rational resistance management strategies to retard the rate of resistance development and to control target pests effectively by enhancing the endotoxin expression through genetic or agronomic management.

Full article available at http://www.agriculturejournals.cz/publicFiles/36993.pdf

Mejia, R.; Polanía, I.; 2012. Expression of the Cry1Ab Toxin in Transgenic Corn Yieldgard® in the Eastern Plains of Colombia. *Southwestern Entomologist,* 37(2):209-223.

The knowledge of the Bt toxin concentracion within a transgenic plant is basic for the estimation of the performance of the insect pest aimed to be managed with this technology. In a *commercial* lot at the National Cereal Federation (FENALCE) in the municipality of Granada (Meta), *Colombia*, *samples* from plants of transgenic maize YieldGard® were taken to determine the average concentracion of the Cry1Ab toxin. In the laboratory by means of the sandwich ELISA assay, the*protein* was quantified in vegetative and reproductive tissues with the kit "for Cry1Ab/Cry1Ac Qualiplate Envirologix." For this purpose, leaves, stem, ear, ear styles, husk, cob, and corn kernels were lyophilized. The analyses indicated that expression fluctuates between tissues from the same plant and between plants in the same plot. The average concentracion of Cry1Ab was: 8.97 µg/g fresh weight in leaves (V7), 8.96 in stems, 2.3 in male inflorescence (VT), 9.57 in unfertilized styles, 7.27 in fertilized styles (R1), 8.39 in cob (R3), 12.19 in husks, and 1.85 µg/g fresh weight in grains (R3). concentracions in seeds and leaves were similar to those obtained in the U.S.A. and Europe. This marked difference in the toxin expression makes Bt corn a plant susceptible to some pests, because there is no uniformity in the Cry1Ab concentracion in the tissues consumed.

http://www.bioone.org/doi/abs/10.3958/059.037.0214

2.3 Diversity of unexpected effects in the gene expression, in metabolome or in the proteome of the transformed organism

The insertion/transfer of a certain genetic sequence to another organism may induce/activate new and unexpected effects on the expression of physiological/biological characteristics of such transformed organism. Such changes can be associated or not to the physical changes resulting from the genetic transformation process or even from the transgene instabilities, as mentioned in the previous items (2.1 and 2.2).

Some of these effects can be noted by means of evaluation of the set of proteins and/or metabolites synthesized by the transformed organism. The theory which supports the genetic modification – based in the weak hypothesis that the transfer of a single "gene" would exclusively result in its full expression and similar to its origin organism – would imply that the transgene expression products would represent the only differences between the GMO and its isogenic (organism with the genome identical to the transgene exception). As a result, the identification of metabolic differences distinct from the predicted ones, after the insertions, would be sufficient to characterize the fragility of that hypothesis and of the interpretations resulting from it.

Well, a number of studies verify situations on which dozens of proteins, the expressions of which were modified by the insertion of a transgene, can be mapped. The fact is so relevant that, from the knowledge and Metabolome studies, evidencing the possibility of changing the metabolic profile of the GMOs, the European Economic Community became mandatory (as of 2004) the labeling - as "transgenic" - of food containing more than 0.9% of component from ingredients extracted from GMOs[24].

24 In Brazil, Decree No. 4680 of April 24, 2003, which regulates the labeling of products containing GMOs, is also based in part on that basis.

Villas-Boas, S.; Gombert, A. 2006. Análise do Metaboloma. *Biotecnologia, Ciência e Desenvolvimento,* nº 36 p.58-69.

Without summary.

Full article available at https://pt.scribd.com/document/223431273/Analise-de-Metaboloma

Portuguese

Batista, R.; Saibo, N.; Lourenço, T.; Oliveira, M. 2008. Microarray analyses reveal that plant mutagenesis may induce more transcriptomic changes than transgene insertion. 3640–3645, PNAS, vol. 105, nº. 9.

Controversy regarding genetically modified (GM) plants and their potential impact on human health contrasts with the tacit acceptance of other plants that were also modified, but not considered as GM products (e.g., varieties raised through conventional breeding such as mutagenesis). What is beyond the phenotype of these improved plants? Should mutagenized plants be treated differently from transgenics? We have evaluated the extent of transcriptome modification occurring during rice improvement through transgenesis versus mutation breeding. We used oligonucleotide microarrays to analyze gene expression in four different pools of four types of rice plants and respective controls: (*i*) a γ-irradiated stable mutant, (*ii*) the M1 generation of a 100-Gy γ-irradiated plant, (*iii*) a stable transgenic plant obtained for production of an anticancer antibody, and (*iv*) the T1 generation of a transgenic plant produced aiming for abiotic stress improvement, and all of the unmodified original genotypes as controls. We found that the improvement of a plant variety through the acquisition of a new desired trait, using either mutagenesis or transgenesis, may cause stress and thus lead to an altered expression of untargeted genes. In all of the cases studied, the observed alteration was more extensive in mutagenized than in transgenic plants. We propose that the safety assessment of improved plant varieties should be carried out on a case-by-case basis and not simply restricted to foods obtained through genetic engineering.

Full article available at http://www.pnas.org/content/105/9/3640.full

Liu, Z.; Li, Y.; Zhao, J.; Chen, X.; Jian, G.; Peng, Y.; Qi , F. 2012. Differentially Expressed Genes Distributed Over Chromosomes and Implicated in Certain Biological Processes for Site Insertion Genetically Modified Rice Kemingdao. *Int J Biol Sci.*, 8(7): 953-963.

Release of genetically modified (GM) plants has sparked off intensive debates worldwide partly because of concerns about potential adverse unintended effects of GM plants to the agro system and the safety of foods. In this study, with the aim of revealing the molecular basis for unintended effects of a single site insertion GM Kemingdao (KMD) rice transformed with a synthetic cry1Ab gene, and bridging unintended effects of KMD rice through clues of differentially expressed genes, comparative transcriptome analyses were performed for GM KMD rice and its parent rice of Xiushui11 (XS11). The results showed that 680 differentially expressed transcripts were identified from 30-day old seedlings of GM KMD rice. The absolute majority of these changed expression transcripts dispersed and located over all rice chromosomes, and existed physical distance on chromosome from the insertion site, while only two transcripts were found to be differentially expressed within the 21 genes located within 100 kb up and down-stream of the insertion site. Pathway and biology function analyses further revealed that differentially expressed transcripts of KMD rice were involved in certain biological processes, and mainly implicated in two types of pathways. One type was pathways implicated in plant stress/defense responses, which were considerably in coordination with the reported unintended effects of KMD rice, which were more susceptible to rice diseases compared to its parent rice XS11; the other type was pathways associated with amino acids metabolism. With this clue, new unintended effects for changes in amino acids

synthesis of KMD rice leaves were successfully revealed. Such that an actual case was firstly provided for identification of unintended effects in GM plants by comparative transciptome analysis.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3399318/

Barbosa, H., S., Arruda, S., Azevedo, R., Arruda, M., 2012. New insights on proteomics of transgenic soybean seeds: evaluation of differential expressions of enzymes and proteins**.** *Analytical and Bioanalytical Chemistry*, January 2012, Volume 402, Issue 1, pp 299-314.

This work reports the evaluation of differentially expressed enzymes and proteins from transgenic and nontransgenic soybean seeds. Analysis of malondialdehyde, ascorbate peroxidase (EC 1.11.1.11), glutathione reductase (EC 1.6.4.2), and catalase (EC 1.11.1.6) revealed higher levels (29.8, 30.6, 71.4, and 35.3%, respectively) in transgenic seeds than in nontransgenic seeds. Separation of soybean seed proteins was done by two-dimensional polyacrylamide gel electrophoresis, and 192 proteins were identified by matrix-assisted laser desorption/ionization (MALDI) quadrupole time-of-flight (QTOF) mass spectrometry (MS) and electrospray ionization (ESI) QTOF MS. Additionally, the enzyme CP4 EPSPS, involved in the genetic modification, was identified by enzymatic digestions using either trypsin or chymotrypsin and ESI-QTOF MS/MS for identification. From the proteins identified, actin fragment, cytosolic glutamine synthetase, glycinin subunit G1, and glycine-rich RNA-binding protein were shown to be differentially expressed after analysis using the two-dimensional difference gel electrophoresis technique, and applying a regulator factor of 1.5 or greater.

http://www.ncbi.nlm.nih.gov/pubmed/21947011

Agapito-Tenfen, S.; Guerra, M.; Wikmark , O-G; Nodari, R. 2013. Comparative proteomic analysis of genetically modified maize grown under different agroecosystems conditions in Brazil. *Proteome Science*, 11:46.

Bakground: Profiling technologies allow the simultaneous measurement and comparison of thousands of cell components without prior knowledge of their identity. In the present study, we used two-dimensional gel electrophoresis combined with mass spectrometry to evaluate protein expression of Brazilian genetically modified maize hybrid grown under different agroecosystems conditions. To this effect, leaf samples were subjected to comparative analysis using the near-isogenic non-GM hybrid as the comparator.
Results: In the first stage of the analysis, the main sources of variation in the dataset were identified by using Principal Components Analysis which correlated most of the variation to the different agroecosystems conditions. Comparative analysis within each field revealed a total of thirty two differentially expressed proteins between GM and non-GM samples that were identified and their molecular functions were mainly assigned to carbohydrate and energy metabolism, genetic information processing and stress response.
Conclusions: To the best of our knowledge this study represents the first evidence of protein identities with differentially expressed isoforms in Brazilian MON810 genetic background hybrid grown under field conditions. As global databases on outputs from "omics" analysis become available, these could provide a highly desirable benchmark for safety assessments.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4176129/

A significant part of the undesirable effect notes following genetic

modification occurs through pleiotropy[25] (situation on which a certain gene conditions or influences the expression of more than one characteristic) or through epistasis/gene interaction (situation that is opposite to the preceding one, on which a number of genes interact among each other in order to influence a single characteristic). Some of these pleiotropic and/or epistatic effects, which tend to be hidden in optimized research conditions, are noted only when the plant is submitted to specific environmental conditions, determined in the field by a wide variety and combination of biotic and abiotic stress factors.

In this context, a number of studies point out side effects of genetic modification, expressed by the change to several metabolic and biochemical pathways, in transformed plants. As an example, relationships involved in the lignin and nitrogen metabolism must be highlighted.

Escher, N.; Käch, B.; Nentwig, W. 2000. Decomposition of transgenic *Bacillus thuringiensis* maize by microorganisms and woodlice *Porcellio scaber (Crustacea: Isopoda)*. *Basic Appl. Ecol.* 1, 161–169.

Foliage of transgenic maize *Zea mays* L., expressing a Cry1Ab protein derived from *Bacillus thuringiensis* (Berliner) subsp. *kurstaki*, was compared with foliage of the corresponding non-transgenic maize variety in laboratory feeding and decomposition experiments to study the effects of the *B. thuringiensis* protein on the chemical composition of the maize leaves, on the decomposer *Porcellio scaber* (Crustacea: Isopoda), and on leaf-litter-colonising microorganisms. Initial contents of fructose and soluble carbohydrates were significantly higher in non-transgenic maize. Lignin was decomposed more quickly in transgenic maize. Starch, cellulose, hemicellulose and ash content did not differ. Bacterial growth on faeces of *P. scaber* fed on non-transgenic maize was up to 60% higher than on faeces of the transgenic-fed woodlice, but bacterial growth on leaves and fungal growth on faeces were equal on both maize varieties. *P. scaber* showed no significant difference in its consumption rate of transgenic and non-transgenic maize. The number of offspring did not differ between the two treatment groups, but the mortality of juveniles reared on non-transgenic maize leaves was significantly higher. During the first 131 days weight increase of the offspring was significantly higher in the non-transgenic group, but weight increase of adult *P. scaber* was higher in the transgenic group. Due to a slightly lower C:N ratio, a lower lignin content, and a higher content of soluble carbohydrates, the nutritional quality of transgenic maize leaves was better than that of the non-transgenic variety. This explains the lower mortality of *P. scaber* offspring and the faster weight gain of adult *P. scaber*on the transgenic diet.

http://www.sciencedirect.com/science/article/pii/S1439179104700213

25 As for the biological importance of pleiotropy, we recommend working Wanga et al., 2010 (Genomic patterns of pleiotropy and the evolution of complexity. www.pnas.org/cgi/doi/10.1073/pnas.1004666107), available at http://www.pnas.org/content/107/42/18034.full

Saxena, D.; Stotzky, G. 2001. Bt corn has a higher lignin content than non-Bt corn. *American Journal of Botany,* 88: 1704-1706.

Bt corn has been genetically modified to express the Cry1Ab protein of Bacillus thuringiensis to kill lepidopteran pests. Fluorescence microscopy and staining with toluidine blue indicated a higher content of lignin in the vascular bundle sheaths and in the sclerenchyma cells surrounding the vascular bundle in all ten Bt corn hybrids, representing three different transformation events, studied than of their respective non-Bt isolines. Chemical analysis confirmed that the lignin content of all hybrids of Bt corn, whether grown in a plant growth room or in the field, was significantly higher (33-97% higher) than that of their respective non-Bt isolines. As lignin is a major structural component of plant cells, modifications in lignin content may have ecological implications.

Full article available at http://www.amjbot.org/content/88/9/1704.long

Stotzky, G. 2004. Persistence and biological activity in soil of the insecticidal proteins from *Bacillus thuringiensis*, especially from transgenic plants. *Plant and Soil,* 266: 77–89.

Insecticidal proteins produced by various subspecies (*kurstaki, tenebrionis*, and *israelensis*) of*Bacillus thuringiensis* (*Bt*) bound rapidly and tightly on clays, both pure mined clay minerals and soil clays, on humic acids extracted from soil, and on complexes of clay and humic acids. Binding reduced susceptibility of the proteins to microbial degradation. However, bound proteins retained biological activity. Purified Cry1Ab protein and protein released from biomass of transgenic *Bt* corn and in root exudates of growing *Bt* corn (13 hybrids representing three transformation events) exhibited binding and persistence in soil. Insecticidal protein was also released in root exudates of*Bt* potato (Cry3A protein) and rice (Cry1Ab protein) but not in root exudates of *Bt* canola, cotton, and tobacco (Cry1Ac protein). Vertical movement of Cry1Ab protein, either purified or in root exudates or biomass of *Bt* corn, decreased as the concentration of the clay minerals, kaolinite or montmorillonite, in soil increased. Biomass of transgenic *Bt* corn decomposed less in soil than biomass of near-isogenic non-*Bt* corn, possibly because biomass of *Bt* corn had a significantly higher content of lignin than biomass of non-*Bt* corn. Biomass of *Bt* canola, cotton, potato, rice, and tobacco also decomposed less than biomass of the respective near-isogenic non-*Bt* plants. However, the lignin content of these *Bt* plants, which was significantly less than that of *Bt* corn, was not significantly different from that of their near-isogenic non-*Bt* counterparts, although it was consistently higher. The Cry1Ab protein had no consistent effects on organisms (earthworms, nematodes, protozoa, bacteria, fungi) in soil or *in vitro*. The Cry1Ab protein was not taken up from soil by non-*Bt* corn, carrot, radish, or turnip grown in soil in which *Bt* corn had been grown or into which biomass of *Bt* corn had been incorporated.

http://link.springer.com/article/10.1007%2Fs11104-005-5945-6

Flores, S.; Saxena, D.; Stotzky, G. 2005. Transgenic Bt plants decompose less in soil than non-Bt plants. *Soil Biology & Biochemistry*, 37: 1073-1082.

Bt plants are plants that have been genetically modified to express the insecticidal proteins (e.g. Cry1Ab, Cry1Ac, Cry3A) from subspecies of the bacterium, *Bacillus thuringiensis* (*Bt*), to kill lepidopteran pests that feed on corn, rice, tobacco, canola, and cotton and coleopteran pests that feed on potato. The biomass of these transgenic *Bt* plants (*Bt*+) was decomposed less in soil than the biomass of their near-isogenic non-*Bt*plant counterparts (*Bt*–). Soil was amended with 0.5, 1, or 2% (wt wt^{-1}) ground, dried (50 °C) leaves or stems of *Bt* corn plants; with 0.5% (wt wt^{-1}) ground, dried biomass of *Bt* rice, tobacco, canola, cotton, and potato plants; with biomass of the near-isogenic plants without the respective *cry* genes; or not amended. The gross metabolic activity of the soil was determined by CO_2 evolution. The amounts of C evolved as CO_2 were significantly lower from soil microcosms amended with biomass of *Bt* plants than of non-*Bt* plants. This difference occurred with stems and leaves from two hybrids of *Bt* corn, one of which had a higher C:N ratio

than its near-isogenic non-*Bt* counterpart and the other which had essentially the same C:N ratio, even when glucose, nitrogen (NH_4NO_3), or glucose plus nitrogen were added with the biomass. The C:N ratios of the other *Bt* plants (including two other hybrids of *Bt* corn) and their near-isogenic non-*Bt* counterparts were also not related to their relative biodegradation. *Bt* corn had a significantly higher lignin content than near-isogenic non-*Bt* corn. However, the lignin content of the other *Bt* plants, which was significantly lower than that of both*Bt* and non-*Bt* corn, was generally not statistically significantly different, although 10–66% higher, from that of their respective non-*Bt* near-isolines. The numbers of culturable bacteria and fungi and the activity of representative enzymes involved in the degradation of plant biomass were not significantly different between soil amended with biomass of *Bt* or non-*Bt* corn. The degradation of the biomass of all *Bt* plants in the absence of soil but inoculated with a microbial suspension from the same soil was also significantly less than that of their respective inoculated non-*Bt* plants. The addition of streptomycin, cycloheximide, or both to the soil suspension did not alter the relative degradation of *Bt*+ and *Bt*– biomass, suggesting that differences in the soil microbiota were not responsible for the differential decomposition of *Bt*+ and *Bt*– biomass. All samples of soil amended with biomass of *Bt* plants were immunologically positive for the respective Cry proteins and toxic to the larvae of the tobacco hornworm (*Manduca sexta*), which was used as a representative lepidopteran in insect bioassays (no insecticidal assay was done for the Cry3A protein from potato). The ecological and environmental relevance of these findings is not clear.

http://www.sciencedirect.com/science/article/pii/S0038071704004201

Poerschmann, J.; Gathmann, A.; Augustin, J.; Langer, U.; Górecki, T. 2005. Molecular composition of leaves and stems of genetically modified Bt and near-isogenic non-Bt maize - Characterization of lignin patterns. *Journal of Environmental Quality,* 34: 1508-1518.

Transformation of crops, including maize (Zea mays L.), with the cry1Ab gene from Bacillus thuringiensis to combat lepidopteran pests results in pleiotropic effects regarding lignin biosynthesis. Lignin patterns in stems and leaves of two genetically modified Bt-maize varieties (Novelis T and Valmont T) were studied along with their non-Bt near-isolines (Nobilis and Prelude, respectively). Molecular-level based thermochemolysis using tetramethylammonium hydroxide (TMAH) in combination with gas chromatography-mass spectrometry (GC-MS) was used to quantitate the total lignin contents and to identify monomeric lignin subunits including p-hydroxyphenyl (P), guaiacyl (G), and syringyl (S) moieties. The results were supplemented and confirmed by cupric oxide oxidation. The stems of the transgenic lines had higher concentrations of total lignin than the respective isogenic lines: Valmont T/Prelude by 18% and Novelis T/Nobilis by 28%. In contrast, differences in the total lignin concentration of leaves between the transgenic and the respective near-isogenic lines were marginal. There were significant modifications in the ratio of p-hydroxyphenyl/guaiacyl/syringyl molecular marker units of stem lignin between transgenic and isogenic lines. The guaiacyl units (in particular the G18 marker) accounted chiefly for the higher total lignin contents in the transgenic lines. The leaf lignin patterns did not show significant differences in molecular markers between isogenic and transgenic lines. TMAH-induced thermochemolysis--conducted in both the on-line and off-line modes--provided detailed information on the molecular composition of lignin, thus proving superior to the established "wet chemistry" methods of lignin determination.

http://www.ncbi.nlm.nih.gov/pubmed/16091603

Manetti, C.; Bianchetti, C.; Casciani, L.; Castro, C.; Di Cocco, M.; Miccheli, A.; Motto, M.; Conti, F. 2006. A metabonomic study of transgenic maize (*Zea mays*) seeds revealed variations in osmolytes and branched amino acids. *Journal of Experimental Botany*, 57 (11):2613-2625.

The aim of the research was to investigate metabolic variations associated with genetic modifications in the grains of Zea mays using metabonomic techniques. With this in mind, the non-targeted

characteristic of the technique is useful to identify metabolites peculiar to the genetic modification and initially undefined. The results obtained showed that the genetic modification, introducing Cry1Ab gene expression, induces metabolic variations involving the primary nitrogen pathway. Concerning the methodological aspects, the experimental protocol used has been applied in this field for the first time. It consists of a combination of partial least square-discriminant analysis and principal component analysis. The most important metabolites for discrimination were selected and the metabolic correlations linking them are identified. Principal component analysis on selected signals confirms metabolic variations, highlighting important details about the changes induced on the metabolic network by the presence of a Bt transgene in the maize genome.

Full article available at http://jxb.oxfordjournals.org/content/57/11/2613.long

Relevant compositional and nutritional changes have been registered – in genetically modified plants and, comparatively, in their isogenics -, which can be interpreted as a consequence of disturbs in the metabolic and biochemical pathways, resulting from the insertions.

Masoero, F.; Moschini, M.; Rossi, F.; Prandini, A.; Pietri, A. 1999. Nutritive value, mycotoxin contamination and in vitro rumen fermentation of normal and genetically modified corn (cry1A(b)) grown in northern Italy. *Journal Maydica,* Volume: 44 Issue: 3 Pages: 205-209.

An assessment was made on the effect of inserting the cry1A(b) gene of *Bacillus thuringiensis* (Bt) into the genome of two maize hybrids (the newly-developed hybrid from Cargill Semences identified as CR and the traditional hybrid B73 × Mo17) on the analytical composition, the *in vitro* rumen degradability and the mycotoxin contamination of the plant. Transgenicity changed the plant chemical composition as a function of the recipient genotype: starch was increased in the CR-Bt$^+$ plant (73.3 vs. 70.4%, P<0.10), whereas higher lignin content (7.3 vs. 6.3%, P<0.05) and lower protein (7.1 vs. 7.7%, P<0.10) and soluble nitrogen (26.9 vs. 34.8%, P<0.10) contents were observed in the B73 × Mo17-Bt$^+$ plants in comparison with CR-Bt$^+$ plants. When not considering the hybrid pedigree, there was a tendency (P<0.1) towards a lower protein content in the Bt$^+$ maize seeds (8.2 vs. 9.2%) and a higher sugar content in stalk and leaves (5.7 vs. 2.9%). The stover degradation increased in the CR-Bt$^+$variety, probably as the consequence of the higher content of lower structured carbohydrates. Transgenic plants had less ergosterol and fumonisin content than standard maize, suggesting a reduced susceptibility to mould attack.

https://www.researchgate.net/publication/282498875_Nutritive_value_mycotoxin_contamination_and_in_vitro_rumen_fermentation_of_normal_and_genetically_modified_corn_CRY-1AB_grown_in_Northern_Italy

Lappe, M.; Bailey, E.; Childress, C.; Setchell, K. 1999. Alterations in clinically important phytoestrogens in genetically modified, herbicide-tolerant soybeans. *Journal of Medicinal Food,* 1, 241-245.

The growing clinical interest and use of soybean-based food products or extracts to increase dietary phytoestrogen intake makes the precise composition of the key biologically active ingredients of soybeans, notably genistin and daidzin of substantial medical interest. Conventional soybeans are increasingly being replaced by genetically modified varieties. We analyzed the phytoestrogen concentrations in two varieties of genetically modified herbicide tolerant soybeans and their isogenic conventional counterparts grown under similar conditions. An overall reduction in phytoestrogen

levels of 12-14 percent was observed in the genetically altered soybean strains. Most of this reduction was attributable to reductions in genistin and to a lesser extent daidzin levels, which were significantly lower in modified compared to conventional soybeans in both strains. Significant sample to sample variability in these two phytoestrogens, but not glycitin, was evident in different batches of genetically altered soybeans. Given the high biological potency of isoflavones and their metabolic conversion products, these data suggest genetically modified soybeans may be less potent sources of clinically relevant phytoestrogens than their conventional precursors. These observations, if confirmed in other soybean varieties, heighten the importance of establishing baselines of expected isoflavone levels in transgenic and conventional soy products to ensure uniformity of clinical results. Disclosure of the origins and isoflavone composition of soy food products would be a valuable adjunct to clinical decision-making.

Full article available at https://goo.gl/OMJUdM

Shewmaker, C.; Sheehy, J.; Daley, M.; Colburn, S.; Ke, D. 1999. Seed-specific overexpression of phytoene synthase: Increase in carotenoids and other metabolic effects. *The Plant Journal*, 20(4): 401–412X.

A bacterial phytoene synthase (crtB) gene was overexpressed in a seed-specific manner and the protein product targeted to the plastid in Brassica napus (canola). The resultant embryos from these transgenic plants were visibly orange and the mature seed contained up to a 50-fold increase in carotenoids. The predominant carotenoids accumulating in the seeds of the transgenic plants were alpha and beta-carotene. Other precursors such as phytoene were also detected. Lutein, the predominant carotenoid in control seeds, was not substantially increased in the transgenics. The total amount of carotenoids in these seeds is now equivalent to or greater than those seen in the mesocarp of oil palm. Other metabolites in the isoprenoid pathway were examined in these seeds. Sterol levels remained essentially the same, while tocopherol levels decreased significantly as compared to non-transgenic controls. Chlorophyll levels were also reduced in developing transgenic seed. Additionally, the fatty acyl composition was altered with the transgenic seeds having a relatively higher percentage of the 18 : 1 (oleic acid) component and a decreased percentage of the 18 : 2 (linoleic acid) and 18 : 3 (linolenic acid) components. This dramatic increase in flux through the carotenoid pathway and the other metabolic effects are discussed.

http://www.ncbi.nlm.nih.gov/pubmed/10607293

Zhou, J. ; Ma, C. ; Xu, H. ; *et al*. 2009. Metabolic profiling of transgenic rice with cryIAc and sck genes: an evaluation of unintended effects at metabolic level by using GC-FID and GC-MS. *J Chromatogr B Analyt Technol Biomed Life Sci*, 877(8-9): 725-732.

The cryIAc and sck genes were introduced to the rice for the purpose of improving the insect resistance. Metabolic profiles of wild and transgenic rice were compared to assess the unintended effects related to gene modification. Wild samples with different sowing dates or sites were also examined to determine the environmental effects on metabolites. The polar compounds of grains were extracted, trimethylsilylated and analyzed by gas chromatography-flame ionization detection (GC-FID). Partial least squares-discriminant analysis (PLS-DA) and principal component analysis (PCA) were applied to differentiate transgenic and wild rice grains. The significantly distinguishable metabolites were picked out, and then identified by gas chromatography-mass spectrometry (GC-MS). It was found that both the environment and gene manipulation had remarkable impacts on the contents of glycerol-3-phosphate, citric acid, linoleic acid, oleic acid, hexadecanoic acid, 2,3-dihydroxypropyl ester, sucrose, 9-octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester and so on. Sucrose, mannitol and glutamic acid had a significant increase in transgenic grains in contrast to those in non-genetically modified (GM) rice.

http://www.ncbi.nlm.nih.gov/pubmed/19233746

Jiao, Z.; Si, X.; Li, G.; Zhang, Z.; Xu, X. 2010. Unintended compositional changes in transgenic rice seeds (*Oryza sativa L.*) studied by spectral and chromatographic analysis coupled with chemometrics methods. *J. Agric. Food Chem.*, 58, 1746-1754.

Unintended compositional changes in transgenic rice seeds were studied by near-infrared reflectance, GC-MS, HPLC, and ICP-AES coupled with chemometrics strategies. Three kinds of transgenic rice with resistance to fungal diseases or insect pests were comparatively studied with the nontransgenic counterparts in terms of key nutrients such as protein, amino acids, fatty acids, vitamins, elements, and antinutrient phytic acid recommended by the Organization for Economic Co-operation and Development (OECD). The compositional profiles were discriminated by chemometrics methods, and the discriminatory compounds were protein, three amino acids, two fatty acids, two vitamins, and several elements. Significance of differences for these compounds was proved by analysis of variance, and the variation extent ranged from 20 to 74% for amino acids, from 19 to 38% for fatty acids, from 25 to 57% for vitamins, from 20 to 50% for elements, and 25% for protein, whereas phytic acid content did not change significantly. The unintended compositional alterations as well as unintended change of physical characteristic in transgenic rice compared with nontransgenic rice might be related to the genetic transformation, the effect of which needs to be elucidated by additional studies.

http://www.ncbi.nlm.nih.gov/pubmed/20050687

Abdo, E. ; Barbary O.; Shaltout O. 2013. Chemical Analysis of Bt corn "Monsanto810: Ajeeb-YG" and its counterpart non-Bt corn "Ajeeb". *IOSR Journal of Applied Chemistry*, Vol 4 Issue 1, pp55-60.

Commercialization of biotech crops has started since 1996, where the cultivated area of these crops was increased from 1.7 million hectares in 1996 to 170.3 million hectares in 2012 according to the latest statistics in 2012. Bt corn "MON810: Ajeeb YG®" is one of these crops that express endotoxin from Bacillus thuringiensis (Bt) throughout the whole plant. This study was designed to assess the safety of Bt corn by comparing its compositional chemical analysis with its conventional counterpart "Ajeeb". Moisture content, crude fat, total saccharides, starch, & crude fiber were determined; sodium, potassium, magnesium, calcium and phosphorous content were measured, tannins & phytic acid were determined as anti-nutrients. Amino acids and fatty acids profiles were also evaluated. Results indicated the presence of significant differences between both of Bt corn and its counterpart.

Full article available at https://goo.gl/VJdZAu

Li, S.; Chen, L.; Zheng, F.; Li, Y. 2013. Effect of the cp4-epsps gene on metal bioavailability in maize and soybean using bionic gastrointestinal tracts and ICP-MS determination. *J. Agric. Food Chem.,* 20;61(7):1579-84. doi: 10.1021/jf303962a.

The transformation and metabolism of dietary compounds are affected significantly by gut microbiota. Hence, gut microbiota are used to improve bionic gastrointestinal tracts. The effect of the cp4-epsps gene on metal bioavailability was proved by the comparison of the affinity-liposome metal content ratio (AMCR) in transgenic and conventional crops. The bioavailability of V, Mn, Co, Ga, Ag, Ba, and Pb in roundup ready soybean decreased significantly because the ratio of AMCR (R(AMCR)) in the transgenic crop and its corresponding conventional

type ranged from 0.36 to 0.69. In roundup ready maize, metal bioavailability decreased for Li and Cr (i.e., R(AMCR) was 0.26 and 0.39, respectively) but increased for V, Co, and Pb (i.e., R(AMCR) was 1.48, 2.07, and 2.12, respectively). Compared with conventional crops, safe dosage and maximum consumption of roundup ready crops were 1.59 times for soybean and 0.78 times for maize.

http://pubs.acs.org/doi/abs/10.1021/jf303962a

The consequences of the metabolic and biochemical disturbs noted in transgenic plants cannot always be fully characterized. Changes to metabolic pathways are difficult to be predicted and may affect important agronomic characteristics, associated to productivity.

Preeti, R.; Singh, A.; Ray, K.; Chaudhary, B.; et al. 2011. Detrimental effect of expression of Bt endotoxin Cry1Ac on in vitro regeneration, in vivo growth and development of tobacco and cotton transgenics. *Journal of Biosciences*, 36(2), 363-376.

High levels of expression of the cry1Ac gene from Bacillus thuringiensis cannot be routinely achieved in transgenic plants despite modifications made in the gene to improve its expression. This has been attributed to the instability of the transcript in a few reports. In the present study, based on the genetic transformation of cotton and tobacco, we show that the expression of the Cry1Ac endotoxin has detrimental effects on both the in vitro and in vivo growth and development of transgenic plants. A number of experiments on developing transgenics in cotton with different versions of cry1Ac gene showed that the majority of the plants did not express any Cry1Ac protein. Based on Southern blot analysis, it was also observed that a substantial number of lines did not contain the cry1Ac gene cassette although they contained the marker gene nptII. More significantly, all the lines that showed appreciable levels of expression were found to be phenotypically abnormal. Experiments on transformation of tobacco with different constructs expressing the cry1Ac gene showed that in vitro regeneration was inhibited by the encoded protein. Further, out of a total of 145 independent events generated with the different cry1Ac gene constructs in tobacco, only 21 showed expression of the Cry1Ac protein, confirming observations made in cotton that regenerants that express high levels of the Cry1Ac protein are selected against during regeneration of transformed events. This problem was circumvented by targeting the Cry1Ac protein to the chloroplast, which also significantly improved the expression of the protein.

Full article available at

https://www.ncbi.nlm.nih.gov/pubmed/21654089

Powell, A.; Nguyen, C.; Hill, T.; Cheng, K.; et al. 2012. Uniform ripening encodes a Golden 2-like transcription factor regulating tomato fruit chloroplast development. *Science*, vol 336 29.

Modern tomato (Solanum lycopersicum) varieties are bred for uniform ripening (u) light green fruit phenotypes to facilitate harvests of evenly ripened fruit. U encodes a Golden 2-like (GLK) transcription factor, SlGLK2, which determines chlorophyll accumulation and distribution in developing fruit. In tomato, two GLKs--SlGLK1 and SlGLK2--are expressed in leaves, but only SlGLK2 is expressed in fruit. Expressing GLKs increased the chlorophyll content of fruit, whereas

SlGLK2 suppression recapitulated the u mutant phenotype. GLK overexpression enhanced fruit photosynthesis gene expression and chloroplast development, leading to elevated carbohydrates and carotenoids in ripe fruit. SlGLK2 influences photosynthesis in developing fruit, contributing to mature fruit characteristics and suggesting that selection of u inadvertently compromised ripe fruit quality in exchange for desirable production traits.

http://www.ncbi.nlm.nih.gov/pubmed/22745430

Also, the activation of new metabolic pathways or the synthesis of new byproducts upon transgenic organisms may represent new potential biological hazard spectra, both to the human and animal health such as for non-target organisms.

Donegan, K.K., Palm, C.J., Fieland, V.J.,et al. Changes in levels, species and DNA fingerprints of soil microorganisms associated with cotton expressing the Bacillus thuringiensis var. kurstaki endotoxin. *Applied soil ecology*., 1995;2(2):111-124.

An important aspect of the risk assessment of pesticidal transgenic plants is the potential for detrimental effects on the soil ecosystem from residual plant material following harvesting and tillage. We evaluated this concern by placing leaves of three different lines of cotton genetically engineered to produce the *Bacillus thuringiensis* var. *kurstaki* (B.t.k.) endotoxin in soil and monitoring numbers and species of indigenous soil bacteria and fungi. Four experiments, lasting 28 or 56 days, were performed using combinations of the following treatments: (1) soil only; (2) soil +purified B.t.k. toxin; (3) soil +parental cotton; (4) soil +purified B.t.k. toxin +parental cotton; (5) soil + B.t.k. toxin-producing cotton.
Two of the three transgenic cotton lines caused a transient increase in total bacterial and fungal population levels that was significantly higher on several sample days in the experiments than the levels in the other treatments. In contrast, neither the third transgenic cotton line nor the purified B.t.k. toxins had any significant effects on the total numbers of bacteria and fungi.
Transient changes in bacterial species composition, measured by biochemical tests of individual cultures, community substrate utilization and DNA fingerprinting, were also observed in treatments with the two transgenic plant lines.
The plant line specificity of the response, and the lack of effects from the purified B.t.k. toxins, suggest that the observed effects of the two transgenic plant lines on soil microorganisms may not have resulted from the plants' production of B.t.k. toxin. We suggest that genetic manipulation or tissue culturing of the plants may have produced a change in plant characteristics, aside from B.t.k. toxin production, that can influence growth and species composition of soil microorganisms.

http://www.sciencedirect.com/science/article/pii/0929139394000437

Schrøder, M.; Poulsen, M.; Wilcks, A.; et al. 2007. A 90-day safety study of genetically modified rice expressing Cry1Ab protein (*Bacillus thuringiensis* toxin) in Wistar rats. *Food Chem Toxicol.,* 45(3): 339-349.

An animal model for safety assessment of genetically modified foods was tested as part of the SAFOTEST project. In a 90-day feeding study on Wistar rats, the transgenic KMD1 rice expressing Cry1Ab protein was compared to its non-transgenic parental wild type, Xiushui 11. The KMD1 rice contained 15mg Bt toxin/kg and based on the average feed consumption the

daily intake was 0.54mg Bt toxin/kg body weight. No adverse effects on animal behaviour or weight gain were observed during the study. Blood samples collected one week prior to sacrifice were analyzed and compared for standard haematological and biochemical parameters. A few parameters were significantly different, but all within the normal reference intervals for rats of this breed and age and not in relation to any other findings, thus not considered treatment related. Upon sacrifice a large number of organs were weighed, macroscopic and histopathological examinations were performed with only minor changes to report. The aim of the study was to use a known animal model in performance of safety assessment of a GM crop, in this case KMD1 rice. The results show no adverse or toxic effects of KMD1 rice when tested in the design used in this 90-day study. Nevertheless the experiences from this study lead to the overall conclusion that safety assessment for unintended effects of a GM crop cannot be done without additional test group(s).

http://www.ncbi.nlm.nih.gov/pubmed/17050059

Zolla, L.; Rinalducci, S.; Antonioli, P.; Righetti, P. 2008. Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of genetic modifications. *Journal of Proteome Research,* 7, 1850-1861.

To improve the probability of detecting unintended side effects during maize gene manipulations by bombardment, proteomics was used as an analytical tool complementary to the existing safety assessment techniques. Since seed proteome is highly dynamic, depending on the species variability and environmental influence, we analyzed the proteomic profiles of one transgenic maize variety (event MON 810) in two subsequent generations (T05 and T06) with their respective isogenic controls (WT05 and WT06). Thus, by comparing the proteomic profiles of WT05 with WT06 we could determine the environmental effects, while the comparison between WT06 and T06 seeds from plants grown under controlled conditions enabled us to investigate the effects of DNA manipulation. Finally, by comparison of T05 with T06 seed proteomes, it was possible to get some indications about similarities and differences between the adaptations of transgenic and isogenic plants to the same strictly controlled growth environment. Approximately 100 total proteins resulted differentially modulated in the expression level as a consequence of the environmental influence (WT06 vs WT05), whereas 43 proteins resulted up- or down-regulated in transgenic seeds with respect to their controls (T06 vs WT06), which could be specifically related to the insertion of a single gene into a maize genome by particle bombardment. Transgenic seeds responded differentially to the same environment as compared to their respective isogenic controls, as a result of the genome rearrangement derived from gene insertion. To conclude, an exhaustive differential proteomic analysis allows to determine similarities and differences between traditional food and new products (substantial equivalence), and a case-by-case assessment of the new food should be carried out in order to have a wide knowledge of its features.

Full article available at https://goo.gl/INb6TE

Bortolotto, O.; Silva G.; Bueno, A.; Pomari, A.; Martinelli, S.; Head, G.; Carvalho, R.; Barbosa, G. 2014. Development and reproduction of *Spodoptera eridania* (*Lepidoptera: Noctuidae*) and its egg parasitoid *Telenomus remus* (*Hymenoptera: Platygastridae*) on the genetically modified soybean (Bt) MON 87701 × MON 89788. *Bulletin of Entomological Research,* vol 104, Issue 06, pp 724-730.

Genetically modified crops with insect resistance genes from Bacillus thuringiensis Berliner (Bt-plants) are increasingly being cultivated worldwide. Therefore, it is critical to improve our knowledge of their direct or indirect impact not only on target pests but also on non-target arthropods. Hence,

this study evaluates comparative leaf consumption and performance of Spodoptera eridania (Cramer), a species that is tolerant of the Cry1Ac protein, fed with Bt soybean, MON 87701×MON 89788 or its non-Bt isoline. We also assessed the comparative performance of the egg parasitoid Telenomus remus Nixon on eggs of S. eridania produced from individuals that fed on these two soybean isolines as larvae. Results showed that Bt soybean reduced by 2 days larval development and increased by 3 days adult male longevity. Therefore, we conclude that the effect of Bt soybean MON 87701×MON 89788 on S. eridania development and reproduction is small, and favorable to pest development. These differences are less likely to directly result from the toxin presence but indirectly from unintended changes in plant characteristics caused by the insertion of the transgene. Our results should be viewed as an alert that S. eridania populations may increase in Bt soybeans, but on the other hand, no adverse effects of this technology were observed for the egg parasitoid T. remus which can help to prevent S. eridania outbreaks on these crops.

http://www.ncbi.nlm.nih.gov/pubmed/25248849

2.4 Pyramided events: towards the scientific reductionism

The designation "pyramided event" (also called "stacked event" or "piled") started to be used in order to characterize transgenic plants obtained through conventional breeding involving two or more simple transgenic events (with a transgene). Double, triple or n-pyramided (with n transgenics) are called the same way. Since the last half decade, almost all the "new" transgenic plants, commercially released correspond to pyramided events which, in some cases, support more than five transgenes.

Evidently, the complexity of the interactions is growing, with the multiplication of risk factors. For this reason, in order to prevent difficult and expensive evaluation processes – and consequently facilitate the commercial release of transgenic plants – the regulatory bodies, supported by the industry, started to adopt evaluation alternatives which deserve detailed discussion. Prioritizing practicality aspects and by default of conduct adjustment to scientific basis principles, they started to use the approach based on the assumption that phenomena perceived on an isolated way would not be changed when obliged to joint expression. In other words, the effects noted in each individual event would present merely additive expression in the pyramided events, without possibility of

interaction which could take to unexpected expressions, inexistent in simple events. I.e.: if the transgenic events A and B alone do not present risks, then, the new AxB event, a priori, would not present risks.

Thus, and in the interest of the companies, studies applied to the isolated cases, which deny the possibility for damages, start to serve as the basis to certify the safety of the multiple events. Thus, despite of the risks to the society, more complex events, instead of complete evaluations, start to be judged based on expedite analyses.

Well, this decreased the possibilities to understand the pyramided events, increasing the risk ranges associated to the use of the technology. Even so, despite of the extremely decreased number of researches applied to the pyramided events, the scientific literature registers studies pointing out to unexpected effects, resulting from the stacking of transgenes.

More severe than this is the fact that, at the same time on which the gene interactions – among transgenes – are poorly known, the knowledge about their potential consequences in the epigenome of the new organisms is practically null.

Londo, J.; Bollman, M.; Sagers, C.; Lee, E.; Watrud, L. 2011. Changes in fitness-associated traits due to the stacking of transgenic glyphosate resistance and insect resistance in *Brassica napus* L. *Heredity*, 107, 328–337; doi:10.1038/hdy.2011.19.

Increasingly, genetically modified crops are being developed to express multiple 'stacked' traits for different types of transgenes, for example, herbicide resistance, insect resistance, crop quality and tolerance to environmental stresses. The release of crops that express multiple traits could result in ecological changes in weedy environments if feral crop plants or hybrids formed with compatible weeds results in more competitive plants outside of agriculture. To examine the effects of combining transgenes, we developed a stacked line of canola (Brassica napus L.) from a segregating F(2) population that expresses both transgenic glyphosate resistance (CP4 EPSPS) and lepidopteran insect resistance (Cry1Ac). Fitness-associated traits were evaluated between this stacked genotype and five other Brassica genotypes in constructed mesocosm plant communities exposed to insect herbivores (Plutella xylostella L.) or glyphosate-drift. Vegetative biomass, seed production and relative fecundity were all reduced in stacked trait plants when compared with non-transgenic plants in control treatments, indicating potential costs of expressing multiple transgenes without selection pressure. Although costs of the transgenes were offset by selective treatment, the stacked genotype continued to produce fewer seeds than either single transgenic line. However, the increase

in fitness of the stacked genotype under selective pressure contributed to an increased number of seeds within the mesocosm community carrying unselected, hitchhiking transgenes. These results demonstrate that the stacking of these transgenes in canola results in fitness costs and benefits that are dependent on the type and strength of selection pressure, and could also contribute to changes in plant communities through hitchhiking of unselected traits.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3182500/

Shi, G.; Chavas, J-P.; Lauer, J. 2013. Commercialized transgenic traits, maize productivity and yield risk. *Nature Biotechnology,* vol 31 number 2.

Without summary.

Full article available at http://www.nature.com/nbt/journal/v31/n2/full/nbt.2496.html

Agapito-Tenfen, S.; Vilperte, V.; Benevenuto, R.; Rover, C.; Traavik, T.; Nodari, R. 2014. Effect of stacking insecticidal cry and herbicide tolerance *epsps t*ransgenes on transgenic maize proteome. *Plant Biology*, 14:346 Open access.

Background: The safe use of stacked transgenic crops in agriculture requires their environmental and health risk assessment, through which unintended adverse effects are examined prior to their release in the environment. Molecular profiling techniques can be considered useful tools to address emerging biosafety gaps. Here we report the first results of a proteomic profiling coupled to transgene transcript expression analysis of a stacked commercial maize hybrid containing insecticidal and herbicide tolerant traits in comparison to the single event hybrids in the same genetic background.
Results: Our results show that stacked genetically modified (GM) genotypes were clustered together and distant from other genotypes analyzed by PCA. Twenty-two proteins were shown to be differentially modulated in stacked and single GM events versus non-GM isogenic maize and a landrace variety with Brazilian genetic background. Enrichment analysis of these proteins provided insight into two major metabolic pathway alterations: energy/carbohydrate and detoxification metabolism. Furthermore, stacked transgene transcript levels had a significant reduction of about 34% when compared to single event hybrid varieties.
Conclusions: Stacking two transgenic inserts into the genome of one GM maize hybrid variety may impact the overall expression of endogenous genes. Observed protein changes differ significantly from those of single event lines and a conventional counterpart. Some of the protein modulation did not fall within the range of the natural variability for the landrace used in this study. Higher expression levels of proteins related to the energy/carbohydrate metabolism suggest that the energetic homeostasis in stacked versus single event hybrid varieties also differ. Upcoming global databases on outputs from ¿omics¿ analyses could provide a highly desirable benchmark for the safety assessment of stacked transgenic crop events. Accordingly, further studies should be conducted in order to address the biological relevance and implications of such changes.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4273480/

It is worth emphasizing that the risk evaluation associated to stacked events must consider both the possibilities of interactions between the transgene expression products and between the pesticides systematically associated to them (herbicides, for example). Discussions restricted to the examination of direct connections and interactions, between the transgenes themselves,

besides insufficient, are very limited in terms of coverage to provide minimum safety to the consumption and to the environment.

Admittedly, the genetic modification expression products interact with environmental factors, being likely to result in effects that are different to the expected ones or even noted on an isolate way. In addition, difficulties for simulation and forecasting prevent – based on information collected in the absence of those combinations – the conduction of appropriate inferences about possible impacts related to the adoption level and the synergy of products from such technologies.

Accinelli, C.; Screpanti, C; Vicari, A.; Catizone, P. 2004. Influence of insecticidal toxins from *Bacillus thuringiensis* subsp. *kurstaki* on the degradation of glyphosate and glufosinate-ammonium in soil samples. *Agriculture, Ecosystems and Environment,* 103 497–507.

Investigations dealing with the persistence in soil of glyphosate [*N*-(phosphonomethyl) glycine] (GLYP) and glufosinate-ammonium [the ammonium salt of DL-homoalanin-4-yl(methyl)phosphinic acid] (GLUF) herbicides and of insecticidal toxins produced by *Bacillus thuringiensis* subsp. *kurstaki* (Berliner) are largely reported in the literature. However, no information on the influence of these insecticidal toxins on the persistence in soil of herbicides is available. Preliminary results regarding the influence of insecticidal toxins extracted from a commercial formulation of *B. thuringiensis* subsp. *kurstaki* (Berliner) (Btk) on the degradation of the herbicides glyphosate and glufosinate-ammonium in a loam and a sandy loam soil, under laboratory conditions, were obtained. Soil microbial carbon (SMC) and insecticidal activity of incubated soil samples were also estimated. In both soil types, persistence of GLYP was significantly higher with respect to GLUF. Average GLYP and GLUF half-life was 14.4 and 8.0 days, respectively. Addition of Btk toxins lead to a significant increase of GLYP and GLUP persistence in both soil types. More specifically, average GLYP and GLUF half-life in soil samples receiving the Btk treatment was 24.3 and 14.2 days, respectively. In contrast to herbicide persistence in soil, Btk toxins did not influence microbial carbon content of incubated soil samples. The insecticidal activity of Btk toxins in soil rapidly decreased during the 28-day incubation time. Considering that degradation of GLYP and GLUF was mainly a microbial process, the absence of effects of Btk toxins on the soil microbial carbon and the rapid decrease of insecticidal activity of Btk toxins in the soil suggest a possible effect of the Btk toxins on other soil properties and/or mechanisms influencing herbicide degradation. The present preliminary investigation permitted to highlight the possibility of the Btk toxins to enhance the persistence of GLYP and GLUF in soil, under laboratory conditions. However, further studies are necessary to investigate whether or not the effects observed in this study under artificial and controlled conditions can be extrapolated to field conditions.

Full article available at http://stopogm.net/sites/stopogm.net/files/IITBT.pdf

Mesnage, R.; Clair, E.; Gress, S.; Then, C.; Székács, A.; Séralini, G-H. 2012. Cytotoxicity on human cells of Cry1Ab and Cry1Ac Bt insecticidal toxins alone or with a glyphosate-based herbicide. *Journal of Applied Toxicology,* DOI 10.1002/jat.2712

The study of combined effects of pesticides represents a challenge for toxicology. In the case of the new growing generation of genetically modified (GM) plants with stacked traits, glyphosate-based

herbicides (like Roundup) residues are present in the Roundup-tolerant edible plants (especially corns) and mixed with modified *Bt* insecticidal toxins that are produced by the GM plants themselves. The potential side effects of these combined pesticides on human cells are investigated in this work. Here we have tested for the very first time Cry1Ab and Cry1Ac *Bt* toxins (10 ppb to 100 ppm) on the human embryonic kidney cell line 293, as well as their combined actions with Roundup, within 24 h, on three biomarkers of cell death: measurements of mitochondrial succinate dehydrogenase, adenylate kinase release by membrane alterations and caspase 3/7 inductions. Cry1Ab caused cell death from 100 ppm. For Cry1Ac, under such conditions, no effects were detected. The Roundup tested alone from 1 to 20 000 ppm is necrotic and apoptotic from 50 ppm, far below agricultural dilutions (50% lethal concentration 57.5 ppm). The only measured significant combined effect was that Cry1Ab and Cry1Ac reduced caspases 3/7 activations induced by Roundup; this could delay the activation of apoptosis. There was the same tendency for the other markers. In these results, we argue that modified *Bt* toxins are not inert on nontarget human cells, and that they can present combined side-effects with other residues of pesticides specific to GM plants.

Full article available at https://goo.gl/iLjcJX

3. Interspecific genetic recombinations and transgene escape

The possibility/ability for the transgene inserted into a certain organism comes to escape from that genome, moving towards the environment and affecting other organisms established therein, is an important source of uncertainties and hazards.

Such escape is possible by means of Vertical (VGT) or Horizontal (HGT) Gene Transfer. In the first case, the escape occurs through the sexual reproduction between genetically related organisms (in general, of the same species). This transfer modality will be especially discussed in Part 2 (item 4), where we will address agronomic problems associated to genetic modification.

In case of Horizontal Gene Transfer (HGT), the transgene may escape from the organism to the environment through direct interaction involving the DNA of the transgenic organism and genetic transportation agents, such as viruses, plasmids and transposons.

3.1 Horizontal gene transfer (HGT): revolution of the scientific knowledge

The Horizontal Gene Transfer consists in the modality of DNA

exchanges between non-related organisms, without the fertilization resource. The HGT allows the genetic information exchange and operates in a so important and frequent scale that it seems to constitute one of the forces of the species evolution. The HGT occurs on a continuous and permanent way in the living world, without distinction between the biological species.

REV – Koonin, E. 2003. Horizontal gene transfer: the path to maturity. *Molecular Microbiology* 50 (3): 725-27.

Without summary.

Full article available at http://onlinelibrary.wiley.com/doi/10.1046/j.1365-2958.2003.03808.x/full

Woloszynska, M.; Bocer, T.; Mackiewicz, P.; Janska, H. 2004. A fragment of chloroplast DNA was transferred horizontally, probably from non-eudicots, to mitrochondrial genome of Phaseolus. *Plant Mol Biol.*, 56 (5): 811-20.

The mitochondrial genomes of some Phaseolus species contain a fragment of chloroplast trnA gene intron, named pvs-trnA for its location within the Phaseolus vulgaris sterility sequence (pvs). The purpose of this study was to determine the type of transfer (intracellular or horizontal) that gave rise to pvs-trnA. Using a PCR approach we could not find the respective portion of the trnA gene as a part of pvs outside the Phaseolus genus. However, a BLAST search revealed longer fragments of trnA present in the mitochondrial genomes of some Citrus species, Helianthus annuus and Zea mays. Basing on the identity or near-identity between these mitochondrial sequences and their chloroplast counterparts we concluded that they had relocated from chloroplasts to mitochondria via recent, independent, intracellular DNA transfers. In contrast, pvs-trnA displayed a relatively higher sequence divergence when compared with its chloroplast counterpart from Phaseolus vulgaris. Alignment of pvs-trnA with corresponding trnA fragments from 35 plant species as well as phylogenetic analysis revealed that pvs-trnA grouped with non-eudicot sequences and was well separated from all Fabales sequences. In conclusion, we propose that pvs-trnA arose via horizontal transfer of a trnA intron fragment from chloroplast of a non-eudicot plant to Phaseolus mitochondria. This is the first example of horizontal transfer of a chloroplast sequence to the mitochondrial genome in higher plants.

http://www.ncbi.nlm.nih.gov/pubmed/15803417

Daubin, V.; Ochman, H. 2004. Quartet mapping and the extent of lateral transfer in bacterial genomes. *Mol Biol Evol,* 21 (1): 86-89.

Several recent analyses have used quartet-based methods to assess the congruence among phylogenies derived for large sets of genes from prokaryotic genomes. The principal conclusion from these studies is that lateral gene transfer (LGT) has blurred prokaryotic phylogenies to such a degree that the darwinian scheme of treelike evolution might be abandoned in favor of a net or web. Here, we focus on one of these methods, quartet mapping, and show that its application can lead to overestimation of the extent of inferred LGT in prokaryotes, particularly when applied to distantly related taxa.

Full article available at http://mbe.oxfordjournals.org/content/21/1/86.long

Steale, R.; Hampson, S.; Stover, N.; Kibler, D.; Bode, H.; 2004. Probable horizontal transfer between a protist and a cnidarian. *Current Biology,* 14(8): 298-9.

Without summary.

Full article available at https://goo.gl/VfklsE

Davis, C.; Wurdack, K. 2004. Host-to-parasite gene transfer in flowering plants: Phylogenetic evidence from Malpighiales. *Science*, 305: 676-78.

Horizontal gene transfer (HGT) between sexually unrelated species has recently been documented for higher plants, but mechanistic explanations for HGTs have remained speculative. We show that a parasitic relationship may facilitate HGT between flowering plants. The endophytic parasites Rafflesiaceae are placed in the diverse order Malpighiales. Our multigene phylogenetic analyses of Malpighiales show that mitochondrial (matR) and nuclear loci (18S ribosomal DNA and PHYC) place Rafflesiaceae in Malpighiales, perhaps near Ochnaceae/Clusiaceae. Mitochondrial nad1B-C, however, groups them within Vitaceae, near their obligate host Tetrastigma. These discordant phylogenetic hypotheses strongly suggest that part of the mitochondrial genome in Rafflesiaceae was acquired via HGT from their hosts.

http://www.ncbi.nlm.nih.gov/pubmed/15256617

REV – Andersson, J. 2005. Lateral gene transfer in eukaryotes. *Cell Mol Life Sci,* 62: 1182–1197.

Lateral gene transfer -- the transfer of genetic material between species -- has been acknowledged as a major mechanism in prokaryotic genome evolution for some time. Recently accumulating data indicate that the process also occurs in the evolution of eukaryotic genomes. However, there are large rate variations between groups of eukaryotes; animals and fungi seem to be largely unaffected, with a few exceptions, while lateral gene transfer frequently occurs in protists with phagotrophic lifestyles, possibly with rates comparable to prokaryotic organisms. Gene transfers often facilitate the acquisition of functions encoded in prokaryotic genomes by eukaryotic organisms, which may enable them to colonize new environments. Transfers between eukaryotes also occur, mainly into larger phagotrophic eukaryotes that ingest eukaryotic cells, but also between plant lineages. These findings have implications for eukaryotic genomic research in general, and studies of the origin and phylogeny of eukaryotes in particular.

http://www.ncbi.nlm.nih.gov/pubmed/15761667

Babić, A.; Lindner, A.; Vulić, M.; Stewart, E.; Radman, M. 2008. Direct Visualization of Horizontal Gene Transfer. *Science*, vol 319, no. 5869 pp. 1533-1536 DOI:10.1126/science.1153498.

Conjugation allows bacteria to acquire genes for antibiotic resistance, novel virulence attributes, and alternative metabolic pathways. Using a fluorescent protein fusion, SeqA-YFP, we have visualized this process in real time and in single cells of Escherichia coli. We found that the F pilus mediates DNA transfer at considerable cell-to-cell distances. Integration of transferred DNA by recombination occurred in up to 96% of recipients; in the remaining cells, the transferred DNA was fully degraded by the RecBCD helicase/nuclease. The acquired integrated DNA was tracked through successive replication rounds and was found to occasionally split and segregate with different chromosomes, leading to the inheritance of different gene clusters within the cell lineage. The incidence of DNA splitting corresponds to about one crossover per cell generation.

Full article available at http://www.sciencemag.org/content/319/5869/1533.long

Dana, C.; Glauber, K.; Chan, T.; Bridge, D.; Steele, R. 2012. Incorporation of a Horizontally Transferred Gene into an Operon during Cnidarian Evolution. *PLOS ONE,* 7(2): e31643.

Genome sequencing has revealed examples of horizontally transferred genes, but we still know little about how such genes are incorporated into their host genomes. We have previously reported the identification of a gene (*flp*) that appears to have entered the *Hydra* genome through horizontal transfer. Here we provide additional evidence in support of our original hypothesis that the transfer was from a unicellular organism, and we show that the transfer occurred in an ancestor of two medusozoan cnidarian species. In addition we show that the gene is part of a bicistronic operon in the *Hydra* genome. These findings identify a new animal phylum in which trans-spliced leader addition has led to the formation of operons, and define the requirements for evolution of an operon in *Hydra*. The identification of operons in *Hydra* also provides a tool that can be exploited in the construction of transgenic *Hydra* strains.

Full article available at https://goo.gl/8VufJ1

Domingues, S.; Harms, K.; Fricke, W.; Johnsen, P.; Silva, G.; *et al.* 2012. Natural Transformation Facilitates Transfer of Transposons, Integrons and Gene Cassettes between Bacterial Species. *PLoS Pathog,* 8(8): e1002837. doi:10.1371/journal.ppat.1002837.

We have investigated to what extent natural transformation acting on free DNA substrates can facilitate transfer of mobile elements including transposons, integrons and/or gene cassettes between bacterial species. Naturally transformable cells of Acinetobacter baylyi were exposed to DNA from integron-carrying strains of the genera Acinetobacter, Citrobacter, Enterobacter, Escherichia, Pseudomonas, and Salmonella to determine the nature and frequency of transfer. Exposure to the various DNA sources resulted in acquisition of antibiotic resistance traits as well as entire integrons and transposons, over a 24 h exposure period. DNA incorporation was not solely dependent on integrase functions or the genetic relatedness between species. DNA sequence analyses revealed that several mechanisms facilitated stable integration in the recipient genome depending on the nature of the donor DNA; homologous or heterologous recombination and various types of transposition (Tn21-like and IS26-like). Both donor strains and transformed isolates were extensively characterized by antimicrobial susceptibility testing, integron- and cassette-specific PCRs, DNA sequencing, pulsed field gel electrophoreses (PFGE), Southern blot hybridizations, and by re-transformation assays. Two transformant strains were also genome-sequenced. Our data demonstrate that natural transformation facilitates interspecies transfer of genetic elements, suggesting that the transient presence of DNA in the cytoplasm may be sufficient for genomic integration to occur. Our study provides a plausible explanation for why sequence-conserved transposons, IS elements and integrons can be found disseminated among bacterial species. Moreover, natural transformation of integron harboring populations of competent bacteria revealed that interspecies exchange of gene cassettes can be highly efficient, and independent on genetic relatedness between donor and recipient. In conclusion, natural transformation provides a much broader capacity for horizontal acquisitions of genetic elements and hence, resistance traits from divergent species than previously assumed.

Full article available at https://goo.gl/po4V4b

This subject will be discussed again in item 4.2. of Part 4, with articles which examine the possibility of horizontal (trans)gene transfer in mammalian cells and/or mammalian symbiontic microorganisms (bacteria from the digestive system and/or specially from the oral cavity), and in item 3.3 of Part 3, where articles addressing the possibility of HGT between organisms from the environment in

general and transgenic organisms are joined.

3.2 Specific concerns with the CaMv promoter

There are not very well worked biosafety aspects in the conventional risk analyses, related to genetic construction elements which – in addition to the transgene of interest – contain information required for the transgene expression in the transformed organisms. One of these elements is the originating promoter of the cauliflower mosaic virus (CaMV) and is associated to almost all the genetic constructions inserted into traded GM plants. Called CaMv PS35S, this element carries the possibility to interact with other natural vegetable viruses and, thus, generate new viruses. In addition, it may activate the CaMV in plant species normally not susceptible to that disease.

These risks, the agronomic consequences of which must be considered as relevant, have been a reason for warning for almost two decades by independent researchers.

Gal, S.; Pisan, B.; Hohn, T.; Grimsley, N; Hohn, B. 1992. Agroinfection of transgenic plants leads to viable cauliflower mosaic virus by intermolecular recombination. *Virology,* 187, 525–533.

Intermolecular reconstitution of a plant virus has been detected in whole plants in a system using a defective cauliflower mosaic virus genome and transgenic host plants containing the missing viral gene. The information for the gene VI protein of the virus was integrated into the chromosome of host Brassica napus plants and leaves of these plants were inoculated with Agrobacterium tumefaciens containing the complementing viral sequences. In several cases, upper leaves contained replicating viral DNA which was able to incite CaMV symptoms on turnip plants. The sequence of the resultant recombinant viral molecules suggested that both DNA and RNA recombination events may have been involved in the production of functional virus, one event being gene targeting of the T-DNA.

http://www.ncbi.nlm.nih.gov/pubmed/1546451

Wintermantel, W.; Schoelz, J. 1996. Isolation of Recombinant Viruses between Cauliflower Mosaic Virus and a Viral Gene in Transgenic Plants under Conditions of Moderate Selection Pressure. *Virology*, 223, 156–164.

We demonstrate that recombinant viruses formed between a wild-type virus and a viral transgene can be isolated from transgenic plants under conditions of moderate to weak selection pressure. We inoculated cauliflower mosaic virus (CaMV) strain W260 to transgenic Nicotiana bigelovii

plants that expressed a copy of CaMV gene VI derived from CaMV strain D4, a gene that determines systemic infection of solanaceous species, including N. bigelovii. Because W260 infects nontransformed N. bigelovii systemically, a recombinant virus formed between W260 and the D4 transgene would be expected to have little selective advantage over the wild-type W260 virus W260 was inoculated to approximately 100 plants each of nontransformed and transgenic N. bigelovii and it systemically infected nearly all of the plants. An analysis of viral DNA recovered from 23 transgenic plants infected with W260 revealed that 20 infections resulted from the systemic movement of the wild-type W260 virus, while a recombinant between W260 and the D4 transgene was detected in three of the infections. To determine the percentage of recovery of recombinant viruses under strong selection pressure, we inoculated approximately 100 nontransformed and 100 D4 gene VI transgenic plants with CaMV strain CM1841, a virus that is unable to infect nontransformed N. bigelovii. CM1841 infected 36% of the transgenic plants systemically, but none of the nontransformed controls. An analysis of 24 infected plants showed that a recombination event occurred in every plant, demonstrating that under strong selection conditions, the recovery of CaMV recombinants from transgenic plants can be very high.

Full article available at http://www.sciencedirect.com/science/article/pii/S004268229690464X

The physical structure of the CaMV 35S promoter – which has palindromic nucleotid sequences[26] – tends to favor illegitimate genetic recombinations, potentially generators of unexpected genomic modifications.

Muller, A.; Kamisugi, Y.; Gruneberg, R.; Niedenhof, I.; Horold, R.; Meyer, P. 1999. Palindromic sequences and A+T-rich DNA elements promote illegitimate recombination in *Nicotiana tabacum*. *J. Mol. Biol.,* 291, 29-46.

Illegitimate recombination is the prevailing molecular mechanism for the integration of recombinant DNA into the genome of most eukaryotic systems and the generation of deletions by intrachromosomal recombination. We developed a selectable marker system to screen for intrachromosomal illegitimate recombination events in order to assess the sequence and structure-specific requirements for illegitimate recombination in tobacco. In 12 illegitimate recombination products analysed, we found that all deletion termini localise to sites of palindromic structures or to A+T-rich DNA elements. All deletion termini showed microhomologies of two to six nucleotides. In three plants, the recombination products contained filler-DNA or an inversion of an endogenous segment. Our data strongly suggest that illegitimate recombination in plants is mediated by a DNA synthesis-dependent process, and that this mechanism is promoted by DNA regions that can form palindromic structures or facilitate DNA unwinding.

http://www.ncbi.nlm.nih.gov/pubmed/10438604

Kohli, A.; Griffiths, S.; Palacios, N.; Twyman, R.; Vain, P.; Laurie, D.; Christou, P. 1999. Molecular characterization of transforming plasmid rearrangments in transgenic rice reveals a recombinaison hotspot in the CaMV 35S promoter and confirms the predominance of microhomologie mediated recombination. *The Plant Journal*, 17, 6, p. 591-601.

26 It is of opposite polarity DNA strands, but with the same nucleotide sequence. Consider as an example the palindromic sequence 5'-GAATTC-3' and 3'-CTTAAG-5'.

The characterization of plasmid-genomic DNA junctions following plant transformation has established links between DNA double-strand break repair (DSBR), illegitimate recombination and plasmid DNA integration. The limited information on plasmid-plasmid junctions in plants comes from the dicot species tobacco and Arabidopsis. We analyzed 12 representative transgenic rice lines, carrying a range of transforming plasmid rearrangements, which predominantly reflected microhomology mediated illegitimate recombination involving short complementary patches at the recombining ends. Direct end-ligation, in the absence of homology between the recombining molecules, occurred only rarely. Filler DNA was found at some of the junctions. Short, purine-rich tracts were present, either at the junction site or in the immediate flanking regions. Putative DNA topoisomerase I binding sites were clustered around the junctions. Although different regions of the transforming plasmid were involved in plasmid-plasmid recombination, we showed that a 19 bp palindromic sequence, including the TATA box of the CaMV 35S promoter, acted as a recombination hotspot. The purine-rich half of the palindromic sequence was specifically involved at the recombination junctions. This recombination hotspot is located within the 'highly recombinogenic' region of the full-length CaMV RNA that has been shown to promote viral recombination in dicot plants. Clustering of plasmid recombination events in this highly recombinogenic region, even in the absence of viral enzymes and other cis-acting elements proves that the plant cellular machinery alone is sufficient to recognize and act on these viral sequences. Our data also show the similarity between mechanisms underlying junction formation in dicot and monocot plants transformed using different procedures.

Full article available at http://onlinelibrary.wiley.com/doi/10.1046/j.1365-313X.1999.00399.x/pdf

REV – Ho, M.; Ryan, A.; Cummins, J. 2000. Hazards of transgenic plants containing the cauliflower mosaic viral promoter. *Microbial Ecology in Health and Disease,* 12, 6-11.

Without summary.

Full article available at http://www.i-sis.org.uk/camv-mehd.php

COM – Cummins, J.; Ho, M.; Ryan, A. 2000. Hazardous CaMV promoter? *Nature Biotechnology,* 18, 363.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/10748474

Podevin, N.; du Jardin, P. 2012. Possible consequences of the overlap between the CaMV 35S promoter regions in plant transformation vectors used and the viral gene VI in transgenic plants.*GM Crops and Food*, 3 (4): 296-300.

Multiple variants of the Cauliflower mosaic virus 35S promoter (P35S) are used to drive the expression of transgenes in genetically modified plants, for both research purposes and commercial applications. The genetic organization of the densely packed genome of this virus results in sequence overlap between P35S and viral gene VI, encoding the multifunctional P6 protein. The present paper investigates whether introduction of P35S variants by genetic transformation is likely to result in the expression of functional domains of the P6 protein and in potential impacts in transgenic plants. A bioinformatic analysis was performed to assess the safety for human and animal health of putative translation products of gene VI overlapping P35S. No relevant similarity was identified between the putative peptides and known allergens and toxins, using different databases. From a literature study it became clear that long variants of the P35S do contain an open reading frame, when expressed, might

result in unintended phenotypic changes. A flowchart is proposed to evaluate possible unintended effects in plant transformants, based on the DNA sequence actually introduced and on the plant phenotype, taking into account the known effects of ectopically expressed P6 domains in model plants.

http://www.ncbi.nlm.nih.gov/pubmed/22892689

Considering that the CaMv P35S can be active in a great set of living organisms (and not only in plants), biosafety aspects associated to the possibility that this promoter allows the expression of new DNA elements – whether recombined or not – under its control, once ingested, integrated by competent organisms via HGT[27] (symbiontic bacteria of the human being, for example) or disseminated in the environment, must be considered.

Ballas, N.; Broido, S.; Soreq, H.; Loyter, A. 1989. Efficient functioning of plant promoters and poly(A) sites in *Xenopus* oocytes. *Nucleic Acids Res.,* 17: 7891–7903.

Mature Xenopus oocytes were challenged with DNA constructs including plant regulatory elements, namely, the Cauliflower mosaic virus (CaMV) 35S promoter as well as the nopaline synthase (NOS) promoter and polyadenylation signal. The bacterial chloramphenicol acetyl transferase (CAT) was used as a reporter gene. When microinjected into these cells, the plant-derived DNA constructs effectively promoted CAT synthesis in a manner dependent on the presence of the plant promoters and probably also on the polyadenylation signals. Structural studies revealed that the supercoiled structures of the above DNA plasmids were much more active in supporting CAT synthesis in microinjected oocytes than their linear forms, with clear correlation between efficient gene expression and DNA topology. In contrast, the linear forms of these plasmids were considerably more active than the supercoiled ones in transfected plant protoplasts. These findings demonstrate, for the first time, the activity of regulatory elements from plant genes in Xenopus oocytes and shed new light on the specific rules applicable for gene expression in plant and animal cells.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC334895/

Gmunder, H.; Kohli, J. 1989. Cauliflower mosaic virus promoters direct efficient expression of a bacterial G418 resistance gene in Schizosaccharomyces pombe. *Mol. Gen. Genet.*, 220: 95-101.

A system is presented for transformation of the fission yeast Schizosaccharomyces pombe to resistance against the antibiotic G418. The bacterial resistance gene of the transposon Tn5 is expressed under the control of promoters and transcription terminators from cauliflower mosaic virus (CaMV). The promoter of the S. pombe alcohol dehydrogenase gene has also been used. Transformants can be selected directly on medium containing G418 (up to 1 mg/ml) due to inactivation of G418 by the Tn5 gene product, the aminoglycoside 3'-phosphotransferase (II). The plant viral promoter 35S confers higher resistance to G418 than the 19S promoter. This corresponds to the relative strengths of these promoters in plant cells. The strong plant promoter 35S yields resistance comparable to that obtained

27 See section 4.2 of part 4, which presents articles dealing with the risks associated to horizontal transfer involving the CaMV 35S promoter in mammalian cells.

with the strong S. pombe promoter from the alcohol dehydrogenase gene. The constructions with the two plant promoters have been used on multicopy shuttle plasmids that replicate autonomously in S. pombe and Escherichia coli. In addition the 35S and the 19S constructions have been inserted into the S. pombe genome where they confer G418 resistance as single copy genes. Since vector sequences are excluded in this case, all the necessary signals for expression of G418 resistance are contained within the DNA fragments containing the plant promoters, the resistance gene and the plant terminators. This transformation system is independent of S. pombe mutants. It may be useful for the transformation of other lower eukaryotes. The activity of the CaMV promoters in S. pombe may be exploited for the expression of plant genes in fission yeast.

http://www.ncbi.nlm.nih.gov/pubmed/2558289

Hirt, H.; Kogl, M.; Murbacher, T.; Heberlebors, E. 1990. Evolutionary conservation of transcriptional machinery between yeast and plants as shown by the efficient expression from the CaMV 35S promoter and 35S terminator. *Curr. Genet.,* 17: 473–479.

Complementation of fission yeast mutants by plant genomic libraries could be a promising method for the isolation of novel plant genes. One important prerequisite is the functioning of plant promoters and terminators in Schizosaccharomyces pombe and Saccharomyces cerevisiae. Therefore, we studied the expression of the bacterial beta-glucuronidase (GUS) reporter gene under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter and 35S terminator. We show here that S. pombe initiates transcription at exactly the same start site as was reported for tobacco. The 35S CaMV terminator is appropriately recognized leading to a polyadenylated mRNA of the same size as obtained in plant cells transformed with the same construct. Furthermore, the GUS-mRNA is translated into fully functional GUS protein, as determined by an enzymatic assay. Interestingly, expression of the 35S promoter in the budding yeast S. cerevisiae was found to be only moderate and about hundredfold lower than in S. pombe. To investigate whether different transcript stabilities are responsible for this enormous expression difference in the two yeasts, the 35S promoter was substituted by the ADH (alcohol dehydrogenase) promoter from fission yeast. In contrast to the differential expression pattern of the 35S promoter, the ADH promoter resulted in equally high expression rates in both fission and budding yeast, comparable to the 35S promoter in S. pombe. Since the copy number of the 35S-GUS constructs differs only by a factor of two in the two yeasts, it appears that differential recognition of the 35S promoter is responsible for the different transcription rates.

http://www.ncbi.nlm.nih.gov/pubmed/2202523

Assaad, F.; Signer, E. 1990. Cauliflower mosaic-virus p35S promoter activity in *Escherichia-coli*. *Molecular and General Genetics,* 223(3): 517-520.

We present evidence that the cauliflower mosaic virus promoter P35S can direct expression of the bacterial neomycin phosphotransferase II (NPTII) gene in Escherichia coli. Transcription is initiated at several sites, the major one being located approximately 315 bases upstream of the plant start site. The nucleotide sequence directly preceding this start site is strongly homologous to the prokaryotic promoter consensus sequence. Thus constructs designed for introduction into plants can be expressed in E. coli.

http://www.ncbi.nlm.nih.gov/pubmed/2176717

Burke, C.; Yu X.; Marchitelli, L.; Davis, E.; Ackerman, S. 1990. Transcription Factor IIA of wheat and human function similarly with plant and animal viral promoters. *Nucleic Acid Research,* 18(12):3611-3620.

Eucaryotic transcription initiation by RNA polymerase II involves protein:DNA interactions during the formation of a transcription complex. In addition to RNA polymerase II there are at least five other general transcription factors necessary for initiation with the adenovirus major late promoter. One of these, TFIIA, is involved in the earliest events during transcription complex assembly. We have purified TFIIA from wheat germ and characterized it in an in vitro transcription system. Wheat TFIIA is a single polypeptide of Mr approximately 35 kd which functionally replaces human (HeLa) TFIIA to form a wheat/HeLa transcription system. [This polypeptide can be eluted from a SDS-polyacrylamide gel, refolded to a native conformation, and will function as wheat TFIIA in the heterologous system.] The heterologous system requires a lower optimal incubation temperature than the HeLa system. Biochemical characterization, using the adenovirus major late promoter, indicates that transcription reaction parameters for both wheat and HeLa TFIIA are similar but the kinetics of transcription for both TFIIAs are somewhat dissimilar. A plant viral promoter, the cauliflower mosaic virus 35S promoter, accurately and efficiently directs in vitro transcription in both the wheat/HeLa and HeLa systems with identical transcription kinetics. We conclude that TFIIA function has been conserved during evolution.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC331017/

Pobjecky, N.; Rosenberg, G.; Dintergottlieb, G.; Kaufer, N. 1990. Expression of the betaglucuronidase gene under the control of the CaMV-35S promoter in Schizosaccharomyces-pombe. *Molecular & General Genetics,* 220 (2): 314-316.

We have transformed Schizosaccharomyces pombe with the beta-glucuronidase (GUS) gene from Escherichia coli under the control of the plant cauliflower mosaic virus (CaMV) 35S promoter element. Efficient expression of GUS enzyme was observed. Moreover, transcription initiated at a unique site identical to that used in plant cells.

http://www.ncbi.nlm.nih.gov/pubmed/2325625

Ruth, J.; Hirt, H.; Schweyen, R. 1992. The cauliflower mosaic virus 35S promoter is regulated by cAMP in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.,* 235:365–372.

The cauliflower mosaic virus 35S promoter confers strong gene expression in plants, animals and fission yeast, but not in budding yeast. On investigating this paradox, we found that in budding yeast the promoter acts through two domains. Whereas the upstream domain acts as a silencer, the downstream domain couples expression to the nutritional state of the cells via the RAS/cAMP pathway. Point mutations indicate that two boxes with similarity to the cAMP regulated element (CRE) of mammalian cells mediate this response. Gel retardation assays show that, in both yeast and plant protein extracts, factors bind to this promoter element. Therefore, transcriptional activation appears to be highly conserved at the level of transcription factors and specific DNA target elements in eukaryotes. This offers new ways to investigate gene regulation mechanisms of higher eukaryotes, which are not as amenable to genetic analysis as yeast.

http://www.ncbi.nlm.nih.gov/pubmed/1334531

Lewin, A.; Jacob, D.; Freytag, B.; Appel, B. 1998. Gene expression in bacteria directed by plant-specific regulatory sequences. *Transgenic Research*, 7:403-411.

The regulation of gene expression represents a specific process which has different structural and functional requirements in different groups of organisms. It is thus assumed that regulatory sequences of eucaryotes cannot be recognized in procaryotes. This assumption is of interest for risk assessments of the environmental impact of deliberate release experiments with genetically modified organisms. In order to analyse the extent of heterologous gene expression caused by the transfer of plant-specific regulatory sequences into bacteria, we constructed fusions between plant-specific regulatory sequences and the coding regions of the luxAB genes for the luciferase of the bioluminescent bacterium Vibrio harveyi, transferred the fusions into different bacterial species and measured the luminescence to quantify the expression of the luciferase genes. The regulatory sequences investigated included (a) the 35S promoter of the Cauliflower mosaic virus, (b) the B33 promoter of a class I patatin gene of potatoes, (c) the promoter of the ST-LS1 gene of potatoes and (d) the promoter of the rolC gene of Agrobacterium rhizogenes. We could show that in addition to the 35S promoter, which has already been described as being recognized in Escherichia coli, the sequences containing the B33 and the ST-LS1 promoters are recognized in bacteria. Luciferase gene expression promoted by the sequence with the ST-LS1 promoter could be observed in E. coli, Yersinia enterocolitica and Agrobacterium tumefaciens. Comparison of the luminescence caused by fusions between luxAB and different promoters on the chromosome and on an endogenous plasmid of Y. enterocolitica demonstrated that the level of the heterologous gene expression caused by the fragment with the ST-LS1 promoter was within the range of gene expression levels caused by endogenous promoters of Y. enterocolitica.

http://link.springer.com/article/10.1023/A%3A1008876826415

Ryabova, L.; Hohn, T. 2000. Ribosome shunting in cauliflower mosaic virus 35S RNA leader is a special case of reinitiation of translation functioning in plant and animal systems. *Genes Dev.,* 14: 817-829.

The shunt model predicts that small ORFs (sORFs) within the cauliflower mosaic virus (CaMV) 35S RNA leader and downstream ORF VII are translated by different mechanisms, that is, scanning-reinitiation and shunting, respectively. Wheat germ extract (WGE) and rabbit reticulocyte lysate (RRL) in vitro translation systems were used to discriminate between these two processes and to study the mechanism of ribosomal shunt. In both systems, expression downstream of the leader occurred via ribosomal shunt under the control of a stable stem and a small ORF preceding it. Shunting ribosomes were also able to initiate quite efficiently at non-AUG start codons just downstream of the shunt landing site in WGE but not in RRL. The short sORF MAGDIS from the mammalian AdoMetDC RNA, which conditionally suppresses reinitiation at a downstream ORF, prevented shunting if placed at the position of sORF A, the 5'-proximal ORF of the CaMV leader. We have demonstrated directly that sORF A is translated and that proper termination of translation at the 5'-proximal ORF is absolutely required for both shunting and linear ribosome migration. These findings strongly indicate that shunting is a special case of reinitiation.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC316492/

COM – Ho, M.; Ryan, A.; Cummins, J. 2000. CaMv 35S Promoter Fragmentation Hotspot Confirmed, and it is Active in Animals. *Microbial Ecology in Health and Disease,* 12, 189.

Without summary.

Full article available at http://www.i-sis.org.uk/pdf/CAMV_35s_promo_hotspot_confirmed.pdf

Jacob, D.; Lewin, A.; Meister, B.; Appel, B. 2002. Plant-specific promoter sequences carry

elements that are recognised by the eubacterial transcription machinery. *Trans. Research,* 11, 291-303.

During evolution the promoter elements from prokaryotes and eukaryotes have developed differently with regard to their sequence and structure, implying that in general a transfer of eukaryotic promoter sequences into prokaryotes will not cause an efficient gene expression. However, there have been reports on the functionality of the 35S promoter from cauliflower mosaic virus (CaMV) in bacteria. We therefore decided to experimentally investigate the capability of plant promoter sequences to direct gene expression in various bacteria. Accordingly, we tested ten different plant-specific promoters from Solanum tuberosum, Nicotiana tabacum, CaMV, Agrobacterium tumefaciens, and A. rhizogenes for their ability to initiate transcription in five different eubacterial species (Escherichia coli, Yersinia enterocolitica, A. tumefaciens, Pseudomonas putida, and Acinetobacter sp. BD413). To monitor the strength of the plant-specific promoters in bacteria we created fusions between these promoters and the coding region of the luciferase genes from Vibrio harveyi and measured the luminescence in the bacteria. Heterologous gene expression was observed in 50% of the combinations analysed. We then mapped the transcription start site caused by one of the plant-specific promoters, the ST-LS1 promoter from S. tuberosum, in these bacterial species. The location of the mapped transcription start site indicated that the sequences of the plant promoter themselves were recognised by the bacterial transcription apparatus. The recognition of plant-specific promoter sequences by the bacterial RNA polymerase was further confirmed by site-directed mutagenesis of the ST-LS1 promoter and the analysis of the effects of the mutations on the strength of gene expression in E. coli. Using these mutants in our reporter assays we could localise the sequences of the ST-LS1 promoter serving as -10 region in E. coli. The results of our study show that promoter sequences are much less specific than is generally assumed. This is of great importance for our knowledge about the evolution of gene expression systems and for the construction of optimised expression vectors.

http://www.ncbi.nlm.nih.gov/pubmed/12113461

Vlasak, J.; Smahel, M.; Pavlik, A.; Pavingerova, D.; Briza, J. 2003. Comparison of hCMV immediate early and CaMV 35S promoters in both plant and human cells. *J. Biotechnol.*, 103, 197-202.

Cauliflower mosaic virus 35S promoter, widely used in transgenic crop plants, is known to be recognized in widely differing kinds of cells. Its activity in human cells may have impact on the risk assessment for the environmental release of genetically modified plants. In this study, transient expression of several constructs containing beta-glucuronidase (GUS) gene driven by cauliflower mosaic virus 35S promoter or by immediate early promoter of human cytomegalovirus (pCMV) was tested in both potato leaf protoplasts and cultured human cells. The results showed very low but measurable activity of 35S promoter in human 293T-cells (0.01% of that revealed when using pCMV) and in 293 cells that do not produce SV40 T antigen this activity was even lower. On the other hand, in potato protoplasts, pCMV displayed nearly 1% activity seen with p35S.

http://www.ncbi.nlm.nih.gov/pubmed/12890606

Tepfer, M. ; Gaubert, S. ; Leroux-Coyau, M. ; Prince, S. ; Houdebine, L. 2004. Transient expression in mammalian cells of transgenes transcribed from the Cauliflower mosaic virus 35S promoter. *Environ. Biosafety Res.,* 3, 91-97.

Gene constructs containing the Cauliflower mosaic virus (CaMV) 35S promoter and a sequence coding either for a green fluorescent protein (GFP) or for firefly luciferase were transfected into Chinese hamster ovary (CHO) cells. Both reporter genes were expressed to significant levels. The 35S promoter was 40 times less active than the human eF1 alpha promoter, which is known to be one of the most potent promoters in mammalian cells. The 35S promoter must therefore be considered to be

a promoter of significant potency in mammalian cells. RT-PCR analysis suggested that transcription initiation in CHO cells occurred between the TATA box and the transcription start site of the 35S promoter that function in plant cells. Further analysis by 5'RACE confirmed that transcription was initiated in CHO cells at different sites located essentially between the TATA box and the plant transcription start site, showing that 35S promoter activity in animal cells is due to the presence of promoter elements that are functional in mammalian cells, but that are not those used in plants. The data reported here raise the possibility that genes controlled by the 35S promoter, which is commonly used in transgenic plants, have the potential for expression in animal cells.

http://www.ncbi.nlm.nih.gov/pubmed/15612506

Myhre, M.; Fenton, K.; Eggert, J.; Nielsen, K.; Traavik, T. 2006. The 35S CaMV plant virus promoter is active in human enterocyte-like cells. *Eur Food Res Technol,* 222: 185–193.

The 35S cauliflower mosaic virus (CaMV) promoter is commonly used to drive transgene expression in the genetically engineered (GE) crop plants that have been commercialized so far. Whether, and how far, the 35S promoter might be active in mammalian cells has been scientifically unsettled and controversial. Very recently it was established that the 35S promoter is transcriptionally active following transient reporter gene transfections in continuous cell lines of human [J Biotechnol 103:197–202, 2003] and hamster ovary [Environ Biosafety Res 3:41–47, 2004] fibroblasts. The initial exposure of a human organism to DNA from GE food takes place in the gastrointestinal tract (GIT). Hence, we have now investigated the promoter capacity of 35S in human enterocyte-like cells. We constructed expression vectors with 35S promoter inserted in front of two reporter genes encoding firefly luciferase and green fluorescent protein (GFP), respectively, and performed transient transfection experiments in the human enterocyte-like cell line Caco-2. It was demonstrated that the 35S CaMV promoter was able to drive the expression of both reporter genes to significant levels, although the protein expression levels might seem modest compared to those obtained with the strong promoters derived from human cytomegalo virus (hCMV) and simian virus 40 (SV40). Furthermore, computer-based searches of the 35S CaMV DNA sequence for putative mammalian transcription factor binding motifs gave a high number of hits. Some of the identified motifs indicate that transcriptional activation by the 35S CaMV promoter may be stronger in other human and animal cell types than in those investigated so far.

Full article available at https://goo.gl/aH2zhf

3.3 Risks associated to the use of *Agrobacterium tumefaciens* in transgenic plants

Agrobacterium tumefaciens is naturally competent to perform the horizontal gene transfer. For this reason, the bacterium has been used to perform the insertion of desirable genes into cultivated plants, whether transgenic or not. It has recently been noted that it is also able to transfer genetic material between certain fungi species and into human cells, under semi-natural conditions. The implications are relevant and suggest that the use of such bacterium for the transformation of plants may result in the insertion of undesirable

genes, appropriate to the *A. tumefaciens* genome, such as those responsible for the tumors in infected plants. The possibility that the recombinant DNA is exsudated by the transformed plants' roots and transferred to *A. tumefaciens* strains natural from the soil (or to other competent bacteria) – event favored by the homologies of the genomic sequences – suggests potential important and irreversible risks, associated to the dissemination of transgenes in the environment.

With this respect, the use of *A. tumefaciens* in the transformation of GMP increases risk factors associated to HGT between organisms present in cultivated areas with those transgenic plants, organisms which consume[28] them or which interact with them.

Knight, C.; Bailey, A.; Foster, G. 2010. Investigating Agrobacterium-mediated transformation of *Verticillium* albo-atrum on plant surfaces. *PLOS ONE*, 5(10): 13684.

Background: *Agrobacterium tumefaciens* has long been known to transform plant tissue in nature as part of its infection process. This natural mechanism has been utilized over the last few decades in laboratories world wide to genetically manipulate many species of plants. More recently this technology has been successfully applied to non-plant organisms in the laboratory, including fungi, where the plant wound hormone acetosyringone, an inducer of transformation, is supplied exogenously. In the natural environment it is possible that *Agrobacterium* and fungi may encounter each other at plant wound sites, where acetosyringone would be present, raising the possibility of natural gene transfer from bacterium to fungus.
Methodology/Principal Findings: We investigate this hypothesis through the development of experiments designed to replicate such a situation at a plant wound site. *A. tumefaciens* harbouring the plasmid pCAMDsRed was co-cultivated with the common plant pathogenic fungus *Verticillium albo-atrum* on a range of wounded plant tissues. Fungal transformants were obtained from co-cultivation on a range of plant tissue types, demonstrating that plant tissue provides sufficient *vir* gene inducers to allow *A. tumefaciens* to transform fungi *in planta*.
Conclusions/Significance: This work raises interesting questions about whether *A. tumefaciens* may be able to transform organisms other than plants in nature, or indeed should be considered during GM risk assessments, with further investigations required to determine whether this phenomenon has already occurred in nature.

Full article available at https://goo.gl/voGY13

Kunik, T.; Tzfira, T.; Kapulnik, Y.; Gafni, Y.; Dingwall, C.; Citovsky, V. 2001. Genetic

28 Risks associated with the HGT are treated in items 3.3 and 4.2 of Parts 3 and 4, respectively.

transformation of HeLa cells by Agrobacterium. *Proc Natl Acad Sci USA*, 98(4): 1871-1876.

Agrobacterium tumefaciens is a soil phytopathogen that elicits neoplastic growths on the host plant species. In nature, however, *Agrobacterium* also may encounter organisms belonging to other kingdoms such as insects and animals that feed on the infected plants. Can *Agrobacterium*, then, also infect animal cells? Here, we report that *Agrobacterium* attaches to and genetically transforms several types of human cells. In stably transformed HeLa cells, the integration event occurred at the right border of the tumor-inducing plasmid's transferred-DNA (T-DNA), suggesting bona fide T-DNA transfer and lending support to the notion that *Agrobacterium* transforms human cells by a mechanism similar to that which it uses for transformation of plants cells. Collectively, our results suggest that *Agrobacterium* can transport its T-DNA to human cells and integrate it into their genome.

Full article available at http://www.pnas.org/content/98/4/1871.full

Barrett, C.; Cobb, E.; McNicol, R.; Lyon, G. 1997. A risk assessment study of plant genetic transformation using Agrobacterium and implications for analysis of transgenic plants. *Plant Cell, Tissue and Organ Culture*, 47: 135–144.

Agrobacterium transformation systems for *Brassica, Solanum* and *Rubus*, using carbenicillin, cefotaxime and ticaracillin respectively to eliminate contamination, were examined for the presence of residual *Agrobacterium*. The results indicated that none of the antibiotics in question, succeeded in eliminating *Agrobacterium* and the contamination levels increased in explants from 12 to 16 weeks to such an extent that *Solanum* cultures senesced and died. This may be due to the fact that four times the Minimum bactericidal concentration values (concentration to be used for elimination of contaminants in culture), for the three antibiotics, were higher than the concentrations employed in the culture medium. Contamination in shoot material decreased over 16 to 24 weeks possibly due to bacteriostatis and the use only of the apical node for further culture. The presence of the binary vector was also noted under non-selective conditions, even up to 6 months after transformation, where approx. 50% of contaminated material still harboured bacterial cells with the binary vector at levels of approx. 10_7 Colony forming units per gram.

http://link.springer.com/article/10.1007/BF02318949

Ulker, B.; Li, Y.; Rosso, M.; Logemann, E.; Somssich, I.; Weisshaar, B. 2008. T-DNA–mediated transfer of Agrobacterium tumefaciens chromosomal DNA into plants. *Nature Biotechnology*, volume 26, number 9.

Besides the well-documented integration of DNA flanked by the transfer DNA borders, occasional insertion of fragments from the tumor-inducing plasmid into plant genomes has also been reported during Agrobacterium tumefaciens-mediated transformation. We demonstrate that large (up to approximately 18 kb) gene-bearing fragments of Agrobacterium chromosomal DNA (AchrDNA) can be integrated into Arabidopsis thaliana genomic DNA during transformation. One in every 250 transgenic plants may carry AchrDNA fragments. This has implications for horizontal gene transfer and indicates a need for greater scrutiny of transgenic plants for undesired bacterial DNA.

http://www.ncbi.nlm.nih.gov/pubmed/18758448

Part 2
Agronomic issues related to the transgenic plants growth

1 Productivity, production cost and use of pesticides in commercial transgenic plants crops

Although this work focuses biological aspects related to hazards and uncertainties associated to the commercial use of transgenic plants, it is necessary to approach some issues related to their socioeconomic deployment.

Here, studies questioning suppositions reiterated in marketing campaigns and endorsed by studies of questionable quality are presented. Such campaigns inform increased productivity for transformed plants intended only to support herbicide baths, or decreased production costs of crops cultivates with seeds costing 4 times more, or even the decrease of the use of pesticides, when an exponential growth is noted in their sales. It is an important discussion because such statements are still repeated, although different from reality, and have an important role in the decision making which directly contribute for the authorizations for growing and trading transgenic plants.

With this objective, in the first item of this chapter we presented some studies which are opposite to the arguments produced by the biotechnologies industry or at their service (frequently reiterated by certain authors in some scientific publications). In this case, the objective is to highlight that also in these points the controversy is intensive and the divergences are far from being resolved.

The conditions allowing oscillations in the most relevant indictors for promotional campaigns of the transgenics range a lot. Basically, the answers noted in each case and for each parameter will depend on interactions between the particularities of the genome and the environmental conditions, being obvious the fact that, in the absence of changes which directly affect the productivity

construction factors (absorption and metabolism of water and nutrients, weight and number of grains, use of solar energy, etc.), any result in this sense will be the result of random factors (of genetic, environmental or agronomic order).

Thus, once the generations of transgenic plants available in the market had no changes allowing gain in productivity, and taking into consideration the frequency with which drops in production and/or increased use of pesticides are noted, and, consequently, lower economic benefit for the producer (especially in case of small producers and familiar farmers who were originally provided with their own seeds), it is important to examine studies indicating technical reasons allowing to doubt of myths associated to the benefits of such cultures.

Elmore, R.; Roeth, F.; Nelson, L.; Shapiro, C.; Klein, R.; Knezevic, S.; Martin, A. 2001. Glyphosate resistant soyabean cultivar yields compared with sister lines. *Agronomy Journal*, 93, 408-412.

Herbicide-resistant crops like glyphosate resistant (GR) soybean [Glycine max (L.) Merr.] are gaining acceptance in U.S. cropping systems. Comparisons from cultivar performance trials suggest a yield suppression may exist with GR soybean. Yield suppressions may result from either cultivar genetic differentials, the GR gene/gene insertion process, or glyphosate. Grain yield of GR is probably not affected by glyphosate. Yield suppression due to the GR gene or its insertion process (GR effect) has not been reported. We conducted a field experiment at four Nebraska locations in 2 yr to evaluate the GR effect on soybean yield. Five backcross-derived pairs of GR and non- GR soybean sister lines were compared along with three high-yield, nonherbicide-resistant cultivars and five other herbicide-resistant cultivars. Glyphosate resistant sister lines yielded 5% (200 kg ha21) less than the non-GR sisters (GR effect). Seed weight of the non-GR sisters was greater than that of the GR sisters (in 1999) and the non-GR sister lines were 20 mm shorter than the GR sisters. Other variables monitored were similar between the two cultivar groups. The high-yield, nonherbicide-resistant cultivars included for comparison - yielded 5% more than the non-GR sisters and 10% more than the GR sisters.

Full article available at https://goo.gl/j89jus

Men, X.; Ge, F.; Edwards, C.; Yardim, E. 2004. Influence of Pesticide Applications on Pest and Predatory Arthropods Associated with Transgenic Bt Cotton and Nontransgenic Cotton Plants. *Phytoparasitica,* 32(3):246-254.

The effects of pesticide applications on pests (aphids and acarid mites) and predators (ladybeetles and spiders) were investigated in transgenic *Bt* cotton and nontransgenic cotton agroecosystems in 1999, 2000 and 2001. Transgenic cotton did not cause changes in populations of acarids and did

not reduce numbers of predators considerably; its effects on aphids were inconsistent. Although insecticides were not applied against the main pest . cotton bollworm . on transgenic cotton, the total number of insecticide applications in 3 years was no less than the total applied on nontransgenic cotton, because additional applications were required against sucking pests on transgenic *Bt* cotton. Pesticide applications decreased numbers of aphids, acarids and predatory spiders significantly on both transgenic and nontransgenic cottons. The results suggest that the use of *Bt* cotton should be evaluated carefully in China.

https://goo.gl/O8X6bZ

Ma, B.; Subedi, K. 2005. Development, yield, grain moisture and nitrogen uptake of Bt corn hybrids and their conventional near-isolines. *Field Crops Research*, 93: 199-211.

There are concerns over the economic benefits of corn (Zea mays L.) hybrids with the Bt trait transferred from Bacillus thuringiensis. A field experiment including three to seven pairs of commercial hybrids and their transgenic Bt near-isolines were grown side-by-side for three consecutive years in Ottawa, Canada (458170N, 758450W; 93 m above sea level) to determine (i) which hybrid had the highest yielding potential, (ii) if there was a differential response of Bt and non-Bt hybrids to N application, and (iii) under natural infestation of European corn borer (ECB), whether there was a yield advantage of Bt over non-Bt hybrids to justify their cost.We found that some of the Bt hybrids took 2–3 additional days to reach silking and maturity, and produced a similar or up to 12% lower grain yields with 3–5% higher grain moisture at maturity, in comparison with their non-Bt counterpart. Although N application increased grain yield and N uptake in 2 of the 3 years, there was no N-by-hybrid interaction on yield or other agronomic traits. Most Bt hybrids had similar to or lower total N content in grain with higher N in stover than their respective non-Bt near-isolines. Under extreme weather conditions (e.g. cool air temperature at planting and severe drought during the development), some of the hybrids (both Bt and non-Bt) required up to 400 additional crop heat units (CHU) to reach physiological maturity than indicated by the supplying companies. Our data suggest that within the same maturity group, it was the superior hybrids (non-Bt trait) that led to the greatest N accumulation, and the highest grain yield. Under the conditions tested, there was no yield advantage of Bt hybrids in comparison with their conventional counterparts when stalk lodging and breakage of the non-Bt counterpart by ECB was low to moderate.

http://www.sciencedirect.com/science/article/pii/S0378429004002564

Hofs, J.; Fok, M.; Vaissayre, M. 2006. Impact of Bt cotton adoption on pesticide use by smallholders: A 2-year survey in Makhatini Flats (South Africa). *Crop Protection*, 25(9): 984–988.

This paper explores insecticide use in fields cropped with conventional or Bt cotton varieties in a smallholder farming area (Makhathini Flats, KwaZulu Natal, South Africa). The study was carried out during the 2002-2003 and 2003-2004 growing seasons as part of a broader survey based on daily monitoring of a sample of smallholdings. The adoption of Bt cotton led to a decrease in pyrethroid use, but the level of insect resistance of this cultivar was not sufficient to completely drop this pesticide from the spraying programme. On the other hand, organophosphates were still being applied in substantial amounts, thus raising questions as to the impact of Bt cotton adoption on farmers' health. The overall economic results obtained with Bt cotton were slightly positive despite the low cotton yields obtained in the Flats during our survey. Bt cotton adoption did lead to labour savings, but the extent of this gain was not as high as expected. In conclusion, cropping Bt cotton in Makhathini Flats did not generate sufficient income to expect a tangible and sustainable socioeconomic improvement due to the way the crop is currently managed. Adoption of an innovation like Bt cotton seems to pay only in an agro-system with a sufficient level of

intensification.

http://publications.cirad.fr/une_notice.php?dk=533741

Gordon, B. 2007. Manganese nutrition of glyphosate resistant and conventional soybeans. *Better Crops,* 91 (4): 12-13.

This study was conducted to determine if glyphosate-resistant (GR) soybeans respond differently to Mn fertilizer than conventional soybean varieties in an irrigated high-yield environment, and if so to develop fertilization strategies that will prevent or correct deficiencies. Yield of the GR variety was less than the conventional variety without Mn fertilizer. However, Mn application (banded at planting) to the GR variety closed the yield gap. The conventional soybean variety was not responsive to Mn fertilization. Conversely, yield was reduced at the highest rate of Mn. A second phase of the study showed that a combination of Mn applied as starter and foliar application provided maximum yield response.

Full article available at https://goo.gl/JpXnWe

Swaminathan, M.; Rawal, V. 2011. Are there Benefits from the Cultivation of Bt cotton? *Review of Agrarian Studies,* Vol 1(1).

This note examines costs and returns from the cultivation of different types of cotton in a rainfed village in the Vidarbha region of Maharashtra, India. While the pros and cons of GM cotton are extensively debated, there are only a few empirical studies on the economic performance of Bt cotton, particularly under rainfed conditions. The results from a detailed survey of farm business incomes show that Bt cotton was a clear leader in terms of production and gross value of output when grown as a stand-alone crop. However, on the fields of small and marginal farmers, where cotton was usually intercropped with sorghum (or other cereals and pulses), the relative income advantage of Bt cotton declined. Further, expenditure on chemical pesticides was higher for Bt cotton than for other varieties of cotton. Variability in production was also higher for Bt cotton than for other types of cotton.

Full article available at https://goo.gl/608ejN

Benbrook, C. 2012. Impacts of genetically engineered crops on pesticide use in the U.S. the first sixteen years. *Environmental Sciences Europe,* 24:24.

Background: Genetically engineered, herbicide-resistant and insect-resistant crops have been remarkable commercial successes in the United States. Few independent studies have calculated their impacts on pesticide use per hectare or overall pesticide use, or taken into account the impact of rapidly spreading glyphosate-resistant weeds. A model was developed to quantify by crop and year the impacts of six major transgenic pest-management traits on pesticide use in the U.S. over the 16-year period, 1996–2011: herbicide-resistant corn, soybeans, and cotton; Bacillus thuringiensis (Bt) corn targeting the European corn borer; Bt corn for corn rootworms; and Bt cotton for Lepidopteron insects.
Results: Herbicide-resistant crop technology has led to a 239 million kilogram (527 million pound) increase in herbicide use in the United States between 1996 and 2011, while Bt crops have reduced insecticide applications by 56 million kilograms (123 million pounds). Overall, pesticide use increased by an estimated 183 million kgs (404 million pounds), or about 7%.
Conclusions: Contrary to often-repeated claims that today's genetically-engineered crops have, and are reducing pesticide use, the spread of glyphosate-resistant weeds in herbicide-resistant

weed management systems has brought about substantial increases in the number and volume of herbicides applied. If new genetically engineered forms of corn and soybeans tolerant of 2,4-D are approved, the volume of 2,4-D sprayed could drive herbicide usage upward by another approximate 50%. The magnitude of increases in herbicide use on herbicide-resistant hectares has dwarfed the reduction in insecticide use on Bt crops over the past 16years, and will continue to do so for the foreseeable future.

Full article available at http://www.enveurope.com/content/24/1/24

Heinemann, J.; Massaro, M.; Coray, D.; Agapito-Tenfen, S.; Wen, J. 2013. Sustainability and innovation in staple crop production in the US Midwest. *International Journal of Agricultural Sustainability*, http://dx.doi.org/10.1080/14735903.2013.806408.

An agroecosystem is constrained by environmental possibility and social choices, mainly in the form of government policies. To be sustainable, an agroecosystem requires production systems that are resilient to natural stressors such as disease, pests, drought, wind and salinity, and to human constructed stressors such as economic cycles and trade barriers. The world is becoming increasingly reliant on concentrated exporting agroecosystems for staple crops, and vulnerable to national and local decisions that affect resilience of these production systems. We chronicle the history of the United States staple crop agroecosystem of the Midwest region to determine whether sustainability is part of its design, or could be a likely outcome of existing policies particularly on innovation and intellectual property. Relative to other food secure and exporting countries (e.g. Western Europe), the US agroecosystem is not exceptional in yields or conservative on environmental impact. This has not been a trade-off for sustainability, as annual fluctuations in maize yield alone dwarf the loss of caloric energy from extreme historic blights. We suggest strategies for innovation that are responsive to more stakeholders and build resilience into industrialized staple crop production.

Full article available at http://sustainablepulse.com/wp-content/uploads/Jack.pdf

2 Low efficiency of the transgenic plants in pests and diseases management

Few years after the biotechnologies involving plants-insecticides (Bt) and herbicide-tolerant plants (TH) were made available in commercial scale, the transgenic plant producers started to face difficulties in the management and control of certain species of insects considered as pests and ruderal plants (wrongly called "invasive" or "weeds"[29]). Effectively, the continuous and massive synthesis of Bt proteins, as well as the systematic and continuous use of the same principles

29 The ruderal expression employed throughout this publication is to the effect proposed by Schneider (2007) (Schneider, A. A. *A flora naturalizada no estado do Rio Grande do Sul, Brasil: herbáceas subespontâneas. Biociências*, Porto Alegre, v. 15, nº 2, p. 257-268, jul. 2007) and relates to plant species that grow without cultivation and without human care, encompassing both native and naturalized species. Unlike the term "harmful", ruderal has no value judgment and rejects the false premise that any plant other than the object culture would be harmful, which is not true of the natural systems that have diversity as an essential inherent element to the homeostasis.

and ingredients with herbicide activities, generates significant selective pressure over the organisms of the involved agricultural-ecological systems.

Well, in the intraspecific diversity of insects and ruderal plants, individuals naturally resistant to Bt toxins and to the active substances of herbicides can be found. These will survive in those agricultural systems of transgenic plants, in contrast to most of the other organisms, naturally sensitive to the referred technologies. Over the generations (and in some cases, depending on the ecology of the involved beings, this happens in few agriculture harvests), the few individuals initially selected due to their resistance generate important progenies to the point to form full populations provided with such evolutionarily advantageous genetic profile – in the context transformed by the presence of those toxins.

Thus, the emergence and the development of populations resistant to the technological packages associated to BT- and HT-type GM plants has conformed in general condition species. Observe in several sites of the planet, with emphasis for those regions where the transgenics expanded on and earlier and massive way, such condition already reaches various species, affecting the efficacy of those technologies. The socioeconomic impact resulting from that are expressive and aggravated by the fact that the resistance character, once incorporated to the genome of the so-called "pests" and "invasive plants", is irreversible, making the control ways complex and preventing the adoption of strategies which were efficient before the expansion of the Bt and HT technologies.

2.1 Development of populations of insects resistant to the main Bt proteins

Even before the wide scale commercial use of transgenic Bt plants, the adaptation of insect populations to Cry toxins generated

concerns in the scientific community. The hypothesis of rapid loss of toxicological/agronomic efficacy of such insecticide proteins indicated the need for caution in their use, which should be topic, concentrated in focuses of incidence of insects and used only upon expectations of economic damages. With plants which permanently produce such proteins in all their cells, implying a massive use regardless of the presence of pests, it would be expected a rapid emergence of resistant insects. This fact has been noted in times ranging around five years after the commercial growth, in large scale, of Bt maize, soy and cotton in several countries and continents.

REV – McGaughey, W.; Whalon, M. 1992. Managing insect resistance to *Bacillus thuringiensis* toxins. *Science,* 258: 1451-1455.

Bacillus thuringiensis (B.t.) δ-endotoxins provide an alternative to chemical insecticides for controlling many species of pest insects. Recent biotechnological developments offer the promise of even greater use of B.t. toxins in genetically transformed pest-resistant crops. However, the discovery that insects can adapt to these toxins raises concerns about the long-term usefulness of B.t. toxins. Several methods for managing the development of resistance to B.t. toxins have been suggested, but none of these approaches offer clear advantages in all situations.

http://www.ncbi.nlm.nih.gov/pubmed/17755107

COM – Kaiser, J. 1996. Pests Overwhelm Bt Cotton Crop. *Science*, vol. 273

Thousands of acres of cotton bioengineered to make its own insecticide have fallen victim in the southern United States to cotton bollworms, one of three pests that the crops were supposed to kill. The result has heightened the fears of environmental activists that the insects will eventually develop resistance to the toxin, known as Bt, and that fear has revived calls for tougher federal biosafety regulations. The reasons behind the disappointing results—involving one of the first large-scale plantings of a transgenic crop—also serve as a reminder to researchers that Mother Nature still has a few tricks up her sleeve.

http://www.sciencemag.org/content/273/5274/423.summary

2.1.1 High development potential of Bt toxin-resistant populations (notes in bioassays)

Cases of resistance in populations of insects obtained in laboratories are known for more than two decades. Studies of bioassays allow

to evaluate the resistance potential of species with a certain genetic profile for various Bt proteins. It is currently known that such populations may become insensitive to the toxins in just one or two dozens of generations – some years for some species of insects of short biological cycle.

Ali, M.; Luttrell, R.; Young, S. 2006. Susceptibilities of *Helicoverpa zea* and *Heliothis virescens* (*Lepidoptera: Noctuidae*) Populations to Cry1Ac Insecticidal Protein. *J. Econ. Entomol.,* 99(1): 164-175.

Susceptibilities of bollworm, *Helicoverpa zea* (Boddie) and tobacco budworm, *Heliothis virescens* (F.) to Cry1Ac were measured via a diet-incorporated assay with MPV II at the University of Arkansas during 2002–2004. Lethal concentration–mortality (LC_{50}) estimates of five laboratory, seven laboratory-cross, and 10 field populations of *H. virescens* varied 12-fold. Pooled susceptibilities of *H. virescens* across all laboratory and field populations varied five-fold. The LC_{50} estimates for *H. virescens* were higher than those reported by previous research before the introduction of transgenic crops. However, the ratio of susceptibility of laboratory and field populations was similar, suggesting no change in overall species susceptibility. Individual LC_{50} estimates of five laboratory, nine laboratory-cross, and 57 field populations of *H. zea* varied over 130-fold. Pooled susceptibilities across laboratory and field populations varied widely. Among the field populations, colonies from non-*Bacillus thuringiensis* (Bt) crops were generally more susceptible than those from Bt crops. Across the Bt crops expressing Cry protein, colonies from Bollgard (Monsanto Company) cotton had lower susceptibility to Cry1Ac than those from Bt corn and those from non-Bt crops.

http://www.ncbi.nlm.nih.gov/pubmed/16573337

Huang, F.; Leonard, B.; Wu, X. 2007. Resistance of sugarcane borer to *Bacillus thuringiensis* Cry1Ab toxin. *Entomologia Experimentalis et Applicata*,124: 117-123.

The sugarcane borer, *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae), strain (F52-3-R) was developed from F_3 survivors of a single-pair mating on commercial Cry1Ab *Bacillus thuringiensis* (Bt) corn plants in the greenhouse. The susceptibility of a Bt-susceptible and the F52-3-R strain of *D. saccharalis* to trypsin-activated Cry1Ab toxin was determined in a laboratory bioassay. Neonatestage larvae were fed a meridic diet incorporating Cry1Ab toxin at a concentration range of 0.0625 to 32mg g^{-1}. Larval mortality, larval weight, and number of surviving larvae that did not gain significant weight (<0.1 mg per larva) were recorded on the 7th day after inoculation. The F52-3-R strain demonstrated a significant level of resistance to the activated Cry1Ab toxin. Larval mortality of the Bt-susceptible strain increased in response to higher concentrations of Cry1Ab toxin, exceeding 75% at 32mg g^{-1}, whereas mortality of the F52-3-R strain was below 8% across all Cry1Ab concentrations. Using a measure of practical mortality (larvae either died or gained no weight), the median lethal concentration (LC50) of the F52-3-R strain was 102-fold greater than that of the Bt-susceptible insects. Larval growth of both Bt-susceptible and F52-3-R strains was inhibited on Cry1Ab-treated diet, but the inhibition of the F52-3-R strain was significantly less than that of the Bt-susceptible insects. These results confirm that the survival of the F52-3-R strain on commercial Bt corn plants was related to Cry1Ab protein resistance and suggest that this strain may have considerable value in studying resistance management strategies for Bt corn.

https://goo.gl/i8p7x9

Ali, M. ; Luttrell, R. 2007. Susceptibility of Bollworm and Tobacco Budworm (*Lepidoptera: Noctuidae*) to Cry2Ab2 Insecticidal Protein. *J. Econ. Entomol.,* 100(3): 921-931.

Susceptibilities of 82 bollworm, Helicoverpa zea (Boddie), and 44 tobacco budworm, Heliothis virescens (F.) (Lepidoptera: Noctuidae), populations to Cry2Ab2 protein were measured in diet incorporated assays at the University of Arkansas from 2002 to 2005. Resulting data were used to calculate overall (pooled data) estimates of species susceptibility for future benchmarks of resistance. Variabilities among populations also were studied by comparing regressions for individual populations and calculating mean susceptibilities for different subgroups of the colonies studied. Individual lethal concentration (LC50) estimates for nine laboratory, seven laboratory-cross, and 28 field populations of H. virescens varied up to 48-fold when adjusted for the response of the most susceptible laboratory colony studied. Mean susceptibilities of all laboratory, laboratory-cross, or field colonies varied only two-fold. When grouped by host plants, populations collected on tobacco, Nicotiana tabacum (L.), seemed to be less susceptible than those collected on other host plants. Individual LC50 values for 82 laboratory, laboratory-cross and field populations of H. zea varied up to 37-fold. Mean LC50 values of all laboratory, laboratory-cross, or field populations varied only three-fold. Susceptibilities of populations from Bollgard cotton were up to four-fold less than those from Bacillus thuringiensis corn, Zea mays L. Field populations collected during late season were generally less susceptible than those collected early in the season. Across the two species, H. zea was less sensitive to Cry2Ab2 than H. virescens. Both species seem to be less sensitive to Cry2Ab2 than to CrylAc.

http://www.ncbi.nlm.nih.gov/pubmed/17598557

Meihis, L.; Higdon, M.; Siegfried, B.; Miller, N.; Sappington, T.; Ellersieck, M.; Spencer, T.; Hibbard, B. 2008. Increased survival of western corn rootworm on transgenic corn within 3 generations of on-plant greenhouse selection. *Proc Natl Acad Sci USA*, 105(49): 19177-82.

To delay evolution of insect resistance to transgenic crops producing *Bacillus thuringiensis* (Bt) toxins, nearby "refuges" of host plants not producing Bt toxins are required in many regions. Such refuges are expected to be most effective in slowing resistance when the toxin concentration in Bt crops is high enough to kill all or nearly all insects heterozygous for resistance. However, Bt corn, *Zea mays*, introduced recently does not meet this "high-dose" criterion for control of western corn rootworm (WCR), *Diabrotica virgifera virgifera*. A greenhouse method of rearing WCR on transgenic corn expressing the Cry3Bb1 protein was used in which approximately 25% of previously unexposed larvae survived relative to isoline survival (compared to 1–4% in the field). After three generations of full larval rearing on Bt corn (Constant-exposure colony), WCR larval survival was equivalent on Bt corn and isoline corn in greenhouse trials, and the LC_{50} was 22-fold greater for the Constant-exposure colony than for the Control colony in diet bioassays with Cry3Bb1 protein on artificial diet. After six generations of greenhouse selection, the ratio of larval recovery on Bt corn to isoline corn in the field was 11.7-fold greater for the Constant-exposure colony than the Control colony. Removal from selection for six generations did not decrease survival on Bt corn in the greenhouse. The results suggest that rapid response to selection is possible in the absence of mating with unexposed beetles, emphasizing the importance of effective refuges for resistance management.

Full article available at http://www.pnas.org/content/105/49/19177.full

Tabashnik, B.; Unnithan, G.; Masson, L.; Crowder, D.; Li, X.; Carrière, Y. 2009. Asymmetrical cross-resistance between Bacillus thuringiensis toxins Cry1Ac and Cry2Ab in pink bollworm. *PNAS USA*, vol. 106, nº 29.

Transgenic crops producing *Bacillus thuringiensis* (Bt) toxins kill some key insect pests and can reduce reliance on insecticide sprays. Sustainable use of such crops requires methods for delaying evolution of resistance by pests. To thwart pest resistance, some transgenic crops produce 2 different

Bt toxins targeting the same pest. This "pyramid" strategy is expected to work best when selection for resistance to 1 toxin does not cause cross-resistance to the other toxin. The most widely used pyramid is transgenic cotton producing Bt toxins Cry1Ac and Cry2Ab. Cross-resistance between these toxins was presumed unlikely because they bind to different larval midgut target sites. Previous results showed that laboratory selection with Cry1Ac caused little or no cross-resistance to Cry2A toxins in pink bollworm (*Pectinophora gossypiella*), a major cotton pest. We show here, however, that laboratory selection of pink bollworm with Cry2Ab caused up to 420-fold crossresistance to Cry1Ac as well as 240-fold resistance to Cry2Ab. Inheritance of resistance to high concentrations of Cry2Ab was recessive. Larvae from a laboratory strain resistant to Cry1Ac and Cry2Ab in diet bioassays survived on cotton bolls producing only Cry1Ac, but not on cotton bolls producing both toxins. Thus, the asymmetrical cross-resistance seen here does not threaten the efficacy of pyramided Bt cotton against pink bollworm. Nonetheless, the results here and previous evidence indicate that crossresistance occurs between Cry1Ac and Cry2Ab in some key cotton pests. Incorporating the potential effects of such cross-resistance in resistance management plans may help to sustain the efficacy of pyramided Bt crops.

Full article available at http://www.pnas.org/content/106/29/11889.full.pdf+html

Oswald, K.; French, B.; Nielson C.; Bagley, M. 2011. Selection for Cry3Bb1 Resistance in a genetically diverse population of nondiapausing western corn rootworm (*Coleoptera: Chrysomelidae*). *Journal of Economic Entomology*, 104: 1038-1044.

Five short-diapause laboratory lines of western corn rootworm, Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae), were selected for resistance to MON863, a variety of corn genetically modified with the Bacillus thuringiensis Berliner (Bt) transgene that expresses the Cry3Bb1 delta-endotoxin. Three of the selected lines were developed by incremental increase in the duration of exposure to MON863 over 11 generations (moderate selected lines). Two selected lines were developed from a control group by constant exposure to MON863 for at least 14 d posthatch over seven generations (intense selected lines). At the end of the experiment, survivorship, as measured by adult emergence, was approximately 4 times higher in each of the selected lines reared on MON863 compared with control lines. Estimates of realized heritabilities (h2) were 0.16 and 0.15 for the moderate and intense selected lines, respectively, and are consistent with h2 estimates reported previously from a variety of pest insects. These lines provide data necessary for evaluating the potential for Bt resistance within diabroticite beetles and will be useful for developing improved insect resistance management strategies.

http://www.ncbi.nlm.nih.gov/pubmed/21735927

Frank, D.; Zukoff, A.; Barry, J.; Higdon, M.; Hibbard, B. 2013. Development of Resistance to eCry3.1Ab-Expressing Transgenic Maize in a Laboratory-Selected Population of Western Corn Rootworm (Coleoptera: Chrysomelidae*). Journal of Economic Entomology,* 106(6):2503-2513.

A laboratory colony of western corn rootworm, *Diabrotica virgifera virgifera* LeConte, was selected for resistance to transgenic maize expressing the eCry3.1Ab protein. The selected colony was developed by rearing larvae on nonelite noncommercial Bt maize expressing the eCry3.1Ab protein. After four generations, selected and control colonies were screened on eCry3.1Ab-expressing and isoline maize using greenhouse experiments. There was a significant colony × maize pedigree interaction in terms of the number of larvae recovered. There was no significant difference in the number of larvae recovered from eCry3.1Ab-expressing and isoline maize for the selected colony, whereas this difference was significant for the control colony. There was not a significant colony × maize pedigree interaction in terms of root damage, or the number of beetles recovered, but the effect of maize pedigree was significant. After four and eight generations of selection, seedling

bioassays were performed. Again, there was a significant colony × maize pedigree interaction in terms of the number of larvae recovered. After 11 generations of selection, larvae from the selected colony had higher LC_{50} values than the control colony when exposed to increasing concentrations of the eCry3.1Ab protein. The resistance ratio of the selected colony was 2.58. These data provide necessary information for understanding the potential for Bt resistance by western corn rootworm and underscores the need for insect resistance management plans for this pest.

http://www.bioone.org/doi/abs/10.1603/EC13148?af=R

2.1.2 Cases of Bt toxin-resistant insect populations noted in the field

Populations of insects totally insensitive to Bt toxins already exist in five of the largest species considered as pests (this number at least doubles when taking into consideration the less sensitive populations), considering the four main insecticide proteins (Cry1Ab, Cry1Ac, Cry1F and Cry3Bb1). While the first populations were confirmed in the USA in the beginning of the 2000s, resistant insects are currently noted in all the great countries producers of transgenics, including Brazil. This "break of resistance, which has been considered as responsible for suicide waves between cotton producers in India, has already taken associations of Brazilian producers to bring an action in order to denounce the misleading advertising of the biotechnology companies which continue to sell these transgenic varieties as being "resistant to insects".

It is worth mentioning that the biological mechanisms through with the insects considered as pests develop such resistance to Bt are various, involving a set of molecules and genetic mutations. Considering that new mechanisms appear over the time, the break of resistance is not something that can be resolve with the development of "differentiated" Bt toxins (modified to work around a type of resistance mechanism).

Shelton, A.; Robertson, J.; Tang, J. 1993. Resistance of diamondback moth (*Lepidoptera: Plutellidae*) to *Bacillus thuringiensis* subspecies in the field. *J. Econ. Entomol.*, 86, 697-705.

Eleven populations of diamondback moth, *Plutella xylostella* (L.), were collected in 1990 from

Brassica plants in six states of the United States and in Indonesia and tested for their responses to two formulations of *Bacillus thuringiensis* subsp. *Kurstaki* (Javelin we and DipeI2X), permethrin, and methomyI. Populations from Florida that had been treated extensively over several years with these insecticides displayed significantly higher LC50s. In 1992, field tests in geographically separate areas in Florida and laboratory assays of populations from those fields indicated control failures and resistance to products containing *B. thuringiensis* subsp. *kurstaki* and low levels of resistance to a product containing *B. thuringiensis* subsp. *aizawai* (XenTari). These *B. thuringiensis* subspp. differ in the number of toxins produced, but whether resistance to them is a result of cross-resistance or independent selection was not determined. We documented significant differences between the response of resistant and susceptible populations to two products containing *B. thuringiensis* subsp. *kurstaki,* thus suggesting that the products actually differed in the number or amounts of toxins. In laboratory bioassays of three products containing *B. thuringiensis* suhsp. *aizawai* and two products containing *B. thuringiensis* subsp. *kurstaki,* the variation in response (as determined by resistance ratios) varied by 321- to 461-fold for *B. thuringiensis* subsp. *kurstaki* and by 3- to 4.1-fold for *B. thuringiensis* subsp. *aizawai.* These studies indicate increasing resistance problems caused by intensive use of any *B. tlmringiensis* product. We conclude that if *B. thuringiensis* is to remain a durable insecticide in parts of the world where resistance does not already occur, other tactics such as biological control, host-free periods, plant resistance, and cultural controls must he incorporated into the management programs.

Full article available at https://goo.gl/RjGWex

Gunning, R.; Dang, H.; Kemp, F.; Nicholson, I.; Moores, G. 2005. New Resistance Mechanism in *Helicoverpa armigera* Threatens Transgenic Crops Expressing *Bacillus thuringiensis* Cry1Ac Toxin. *Applied and Environmental Microbiology*, p. 2558–2563 Vol. 71, No. 5.

In Australia, the cotton bollworm, *Helicoverpa armigera*, has a long history of resistance to conventional insecticides. Transgenic cotton (expressing the *Bacillus thuringiensis* toxin Cry1Ac) has been grown for *H. armigera* control since 1996. It is demonstrated here that a population of Australian *H. armigera* has developed resistance to Cry1Ac toxin (275-fold). Some 70% of resistant *H. armigera* larvae were able to survive on Cry1Ac transgenic cotton (Ingard) The resistance phenotype is inherited as an autosomal semidominant trait. Resistance was associated with elevated esterase levels, which cosegregated with resistance. In vitro studies employing surface plasmon resonance technology and other biochemical techniques demonstrated that resistant strain esterase could bind to Cry1Ac protoxin and activated toxin. In vivo studies showed that Cry1Ac-resistant larvae fed Cy1Ac transgenic cotton or Cry1Ac-treated artificial diet had lower esterase activity than non-Cry1Ac-fed larvae. A resistance mechanism in which esterase sequesters Cry1Ac is proposed.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1087549/

Van Rensburg, J. 2007. First report of field resistance by the stem borer, *Busseola fusca* (Fuller) to Bt transgenic maize. *South African Journal of Plant and Soil,* 24(3): 147-151.

Reports of severe damage caused by the African stem borer, *Busseola fusca* (Fuller) to Mon810-transgenic maize (Bt) prompted a study in which the survival of progenies of diapause larvae collected from both a Bt and non-Bt planting were compared when feeding on various Bt and non-Bt hybrids. Field and greenhouse grown plants were artificially infested with neonate larvae. Larval mass was recorded at two-day intervals for three weeks. Data were subjected to simple regression analyses followed by pair wise comparison of the slopes. The two borer populations showed similar larval mass gains on non-Bt hybrids but differed in the response to Bt-hybrids. Appreciable numbers of larvae from the non-Bt derived population survived only to the eighth day. In contrast, substantial numbers of larvae of the Bt-derived population survived over the entire trial period. The mean larval mass of the Bt-derived population at the conclusion of the experiment was less on Bt-hybrids than

on their susceptible counterparts. This indicates that the Bt-derived population has attained a level of resistance where some larvae are able to survive in the presence of the Bt-toxin but not without some detrimental effect on larval growth rate. Since producers are inclined to provide refugia under rain fed conditions in the immediate vicinity of irrigated plantings rather than as part of irrigated fields, the known preference of moths for high humidity could have contributed to increased selection pressure towards the development of resistance to the Bt-toxin.

Full article available at http://www.tandfonline.com/doi/abs/10.1080/02571862.2007.10634798

Liu, F.; Xu, Z.; Chang, J; Chen, J.; Meng, F.; Zhu, Y.; Shen, J. 2008. Resistance Allele Frequency to Bt Cotton in Field Populations of *Helicoverpa armigera* (Lepidoptera: Noctuidae) *China. J. Econ. Entomol.*, 101(3): 933-943.

Resistance evolution in target insects to Bacillus thurningiensis (Bt) cotton, Gossypium hirsutum L., is a main threat to Bt cotton technology. An increasing trend of population density of Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) has been observed since 2001 in Qiuxian County (Hebei, China), where Bt cotton has been planted dominantly since 1998. This region was selected in 2006 and 2007 for estimating frequency of gene alleles conferring resistance to Bt cotton by screening the F1 progeny from single-pair cross between field-collected male and laboratory female of the Bt-resistant strain of H. armigera (F1 screen). F1 offspring from each single-pair line were screened for resistance alleles based on larval growth, development, and survival on Bt cotton leaves for 5 d. Two-year results indicated that approximately equal to 20% of field-collected males carried resistance alleles. The conservative estimate of the resistance allele frequency was 0.094 (95% CI, 0.044-0.145) for 2006 and 0.107 (95% CI, 0.055-0.159) for 2007. This is the first report of resistance allele frequency increase to such a high level in the field in China. Long-term adoption of Bt sprays, dominant planting of single-toxin-producing Bt cotton, and lack of conventional cotton refuge system might accelerate the resistance evolution in the region.

http://www.ncbi.nlm.nih.gov/pubmed/18613597

REV – Tabashnik, B.; van Rensburg, J.; Carrière, Y. 2009. Field-Evolved Insect Resistance to Bt Crops: Definition, Theory, and Data. *J. Econ. Entomol.,* 102(6): 2011-2025.

Transgenic crops producing *Bacillus thuringiensis* (*Bt*) toxins for insect pest control have been successful, but their efÞcacy is reduced when pests evolve resistance. Here we review the deÞnition of Þeld-evolved resistance, the relationship between resistance and Þeld control problems, the theory underlying strategies for delaying resistance, and resistance monitoring methods. We also analyze resistance monitoring data from five continents reported in 41 studies that evaluate responses of field populations of 11 lepidopteran pests to four *Bt* toxins produced by *Bt* corn and cotton. After more than a decade since initial commercialization of *Bt* crops, most target pest populations remain susceptible, whereas field-evolved resistance has been documented in some populations of three noctuid moth species: *Spodoptera frugiperda* (J. E. Smith) to Cry1F in *Bt* corn in Puerto Rico, *Busseola fusca* (Fuller) to Cry1Ab in *Bt* corn in South Africa, and *Helicoverpa zea* (Boddie) to Cry1Ac and Cry2Ab in *Bt* cotton in the southeastern United States. Field outcomes are consistent with predictions from theory, suggesting that factors delaying resistance include recessive inheritance of resistance, abundant refuges of non-*Bt* host plants, and two-toxin *Bt* crops deployed separately from one-toxin *Bt* crops. The insights gained from systematic analyses of resistance monitoring data may help to enhance the durability of transgenic insecticidal crops. We recommend continued use of the longstanding definition of resistance cited here and encourage discussions about which regulatory actions, if any, should be triggered by specific data on the magnitude, distribution, and impact of field-evolved resistance.

http://www.ncbi.nlm.nih.gov/pubmed/20069826

COM – Bagla, P. 2010. Hardy Cotton-Munching Pests Are Latest Blow to GM Crops. *Science*, vol 327.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/20299559

Caccia, S.; Hernández-Rodríguez, C.; Mahon, R.; Downes, S.; James, W.; et al. 2010. Binding site alteration is responsible for field-isolated resistance to *Bacillus thuringiensis* Cry2A insecticidal proteins in two Helicoverpa species. *PLOS ONE,* 5(4): e9975.

Background: Evolution of resistance by target pests is the main threat to the long-term efficacy of crops expressing *Bacillus thuringiensis* (*Bt*) insecticidal proteins. Cry2 proteins play a pivotal role in current Bt spray formulations and transgenic crops and they complement Cry1A proteins because of their different mode of action. Their presence is critical in the control of those lepidopteran species, such as *Helicoverpa* spp., which are not highly susceptible to Cry1A proteins. In Australia, a transgenic variety of cotton expressing Cry1Ac and Cry2Ab (Bollgard II) comprises at least 80% of the total cotton area. Prior to the widespread adoption of Bollgard II, the frequency of alleles conferring resistance to Cry2Ab in field populations of *Helicoverpa armigera* and *Helicoverpa punctigera* was significantly higher than anticipated. Colonies established from survivors of F_2 screens against Cry2Ab are highly resistant to this toxin, but susceptible to Cry1Ac.
Methodology/Principal Findings: Bioassays performed with surface-treated artificial diet on neonates of *H. armigera* and *H. punctigera* showed that Cry2Ab resistant insects were cross-resistant to Cry2Ae while susceptible to Cry1Ab. Binding analyses with ^{125}I-labeled Cry2Ab were performed with brush border membrane vesicles from midguts of Cry2Ab susceptible and resistant insects. The results of the binding analyses correlated with bioassay data and demonstrated that resistant insects exhibited greatly reduced binding of Cry2Ab toxin to midgut receptors, whereas no change in ^{125}I-labeled-Cry1Ac binding was detected. As previously demonstrated for *H. armigera*, Cry2Ab binding sites in *H. punctigera* were shown to be shared by Cry2Ae, which explains why an alteration of the shared binding site would lead to cross-resistance between the two Cry2A toxins.
Conclusion/Significance: This is the first time that a mechanism of resistance to the Cry2 class of insecticidal proteins has been reported. Because we found the same mechanism of resistance in multiple strains representing several field populations, we conclude that target site alteration is the most likely means that field populations evolve resistance to Cry2 proteins in *Helicoverpa* spp. Our work also confirms the presence in the insect midgut of specific binding sites for this class of proteins. Characterizing the Cry2 receptors and their mutations that enable resistance could lead to the development of molecular tools to monitor resistance in the field.

Full article available at https://goo.gl/ubhXCI

Storer, N.; Babcock, J.; Schlenz, M.; Meade, T.; Thompson, G.; Bing, J.; Huckaba, R. 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (*Lepidoptera: Noctuidae*) in Puerto Rico. *J Econ Entomol.*, 103(4): 1031-8.

Transgenic maize, Zea mays L., event TC1507 produces the Cry1F protein to provide protection from feeding by several important lepidopteran pests, including Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae). Reports of reduced field performance against this species in Puerto Rico were investigated, and laboratory bioassays showed that S. frugiperda collected from the affected area exhibited lower sensitivity to the Cry1F protein compared with typical colonies from other regions. The resistance was shown to be autosomally inherited and highly recessive. The Puerto Rico colony was shown to be moderately less sensitive than susceptible laboratory strains to Cry1Ab and Cry1Ac, but the differences in sensitivity were dramatically smaller than for Cry1F. Potential contributory factors to the emergence of resistance to Cry1F in Puerto Rico populations of S. frugiperda include the tropical island geography, unusually large population sizes in 2006, and

drought conditions reducing the availability of alternative hosts. In response to this resistance incident, the technology providers have stopped commercial sales of TC1507 maize in Puerto Rico pending potential reversion to susceptibility.

http://www.ncbi.nlm.nih.gov/pubmed/20857709

Ranjith, M.; Prabhuraj, A.; Srinivasa, Y. 2010. Survival and reproduction of natural populations of Helicoverpa armigera on Bt-cotton hybrids in Raichur, India. *Current Science*, vol. 99 (11).

Transgenic *Bt*-cotton is commercially cultivated on the rationale that it produces toxins that defend the plants primarily from caterpillars damaging cotton bolls. From the context of crop protection, it is important that these bollworms remain susceptible to the toxins, so that their populations are under check. However, if certain individuals are able to survive and breed on the transgenics, they can build populations resistant to the toxins. In one such instance we discovered individuals of *Helicoverpa armigera*, the most prominent among bollworms in India, surviving on commercial *Bt*-cotton hybrids containing single (*Cry*1Ac) and double (*Cry*1Ac and *Cry*2Ab) genes in experimental plots of the University of Agricultural Sciences, Raichur campus, India. Analyses of various biological parameters measured through laboratory breeding on the respective hybrids revealed that these surviving individuals could not only complete their life cycle but also reproduce. A proportion of individuals of the succeeding generation were also able to complete their life cycle on the transgenic commercial hybrids. Interestingly, many of the biological parameters of the bollworm across *Bt* and non-*Bt* hybrids were mostly comparable. These results not only validate the occurrence of natural populations of *H. armigera* on *Bt* cotton hybrids, but also provide evidence for its survival and successful reproduction in India.

Full article available at https://www.researchgate.net/publication/291688988_Survival_and_reproduction_of_natural_populations_of_Helicoverpa_armigera_on_bt-cotton_hybrids_in_Raichur_India

Gassmann, A.; Petzold-Maxwell, J.; Keweshan, R.; Dunbar, M. 2011. Field evolved resistance to Bt maize by Western Corn Rootworm. *PLOS ONE,* 6(7): 222629.

The widespread planting of crops genetically engineered to produce insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt) places intense selective pressure on pest populations to evolve resistance. Western corn rootworm is a key pest of maize, and in continuous maize fields it is often managed through planting of Bt maize. During 2009 and 2010, fields were identified in Iowa in which western corn rootworm imposed severe injury to maize producing Bt toxin Cry3Bb1. Subsequent bioassays revealed Cry3Bb1 resistance in these populations. Here, we report that, during 2011, injury to Bt maize in the field expanded to include mCry3A maize in addition to Cry3Bb1 maize and that laboratory analysis of western corn rootworm from these fields found resistance to Cry3Bb1 and mCry3A and cross-resistance between these toxins. Resistance to Bt maize has persisted in Iowa, with both the number of Bt fields identified with severe root injury and the ability western corn rootworm populations to survive on Cry3Bb1 maize increasing between 2009 and 2011. Additionally, Bt maize targeting western corn rootworm does not produce a high dose of Bt toxin, and the magnitude of resistance associated with feeding injury was less than that seen in a high-dose Bt crop. These first cases of resistance by western corn rootworm highlight the vulnerability of Bt maize to further evolution of resistance from this pest and, more broadly, point to the potential of insects to develop resistance rapidly when Bt crops do not achieve a high dose of Bt toxin.

Full article available at http://www.pnas.org/content/111/14/5141.full

Dhurua, S.; Gujar, G. 2011. Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (*Lepidoptera: Gelechiidae*) from India. *Pest Management Science,*vol. 67 (8): 898-903.

Background: The pink bollworm is one of the most destructive pests of cotton. Transgenic cotton producing Bt toxin Cry1Ac or a combination of Cry1Ac and Cry2Ab2 has been used effectively against this pest. However, some other insects have evolved resistance to Bt toxins in the field. During the 2007-2008 and 2008-2009 seasons, pink bollworm populations in India were surveyed to evaluate their responses to Cry1Ac and seed powder containing Cry1Ac and Cry2Ab2.
Results: The results provide evidence that resistance to Cry1Ac had evolved by 2008 in a population sampled from non-Bt cotton in the Amreli district of Gujarat in western India. The median lethal concentration of Cry1Ac for five-day-old larvae (LC50) was significantly higher for insects derived in 2008 from Amreli than for any of the other field populations tested from four locations in India. For Cry1Ac, the mean LC50 for the strain derived from Amreli in 2008 was 44 times higher than for the most susceptible population. However, for seed powder of Bollgard II containing primarily Cry2Ab2, the 2008 Amreli population was only slightly less susceptible than the most susceptible population.
Conclusions: The data reported here constitute the first evidence of field-evolved resistance of pink bollworm to Cry1Ac. This initial evidence spurred more extensive evaluations during the 2009-2010 growing season, which confirmed field-evolved resistance to Cry1Ac in Amreli. The lack of cross-resistance to Cry2Ab2 suggests that plants producing this toxin are likely to be more effective against resistant populations than plants producing only Cry1Ac.

http://www.ncbi.nlm.nih.gov/pubmed/21438121

Tabashnik, B.; Wu, K.; Wu, Y. 2012. Early detection of field-evolved resistance to Bt cotton in China: cotton bollworm and pink bollworm. J Invertebr Pathol., 110(3):301-6. doi: 10.1016/j.jip.2012.04.008. Epub 2012 Apr 16.

Transgenic crops producing Bacillus thuringiensis (Bt) toxins kill some major insect pests, but pests can evolve resistance and thereby reduce the effectiveness of such Bt crops. The main approach for slowing pest adaptation to Bt crops uses non-Bt host plants as "refuges" to increase survival of susceptible pests. To delay evolution of pest resistance to cotton producing Bt toxin Cry1Ac, several countries have required refuges of non-Bt cotton, while farmers in China have relied on "natural" refuges of non-Bt host plants other than cotton. This strategy is designed for cotton bollworm (Helicoverpa armigera), which attacks many crops and is the primary target of Bt cotton in China, but it does not apply to pink bollworm (Pectinophora gossypiella), which feeds almost entirely on cotton in China. Here we review evidence of field-evolved resistance to Cry1Ac by cotton bollworm in northern China and by pink bollworm in the Yangtze River Valley of China. For both pests, results of laboratory diet bioassays reveal significantly decreased susceptibility of field populations to Cry1Ac, yet field control failures of Bt cotton have not been reported. The early detection of resistance summarized here may spur countermeasures such as planting Bt cotton that produces two or more distinct toxins, increased planting of non-Bt cotton, and integration of other management tactics together with Bt cotton.

http://www.ncbi.nlm.nih.gov/pubmed/22537835

REV – Tabashnik, B.; Brévault, T.; Carrière, Y. 2013. Insect resistance to Bt crops: lessons from the first billion acres. *Nature Biotechnology*, 31, 510–521 doi:10.1038/nbt.2597.

Evolution of resistance in pests can reduce the effectiveness of insecticidal proteins from *Bacillus thuringiensis* (Bt) produced by transgenic crops. We analyzed results of 77 studies from five continents reporting field monitoring data for resistance to Bt crops, empirical evaluation of factors affecting resistance or both. Although most pest populations remained susceptible, reduced efficacy of Bt

crops caused by field-evolved resistance has been reported now for some populations of 5 of 13 major pest species examined, compared with resistant populations of only one pest species in 2005. Field outcomes support theoretical predictions that factors delaying resistance include recessive inheritance of resistance, low initial frequency of resistance alleles, abundant refuges of non-Bt host plants and two-toxin Bt crops deployed separately from one-toxin Bt crops. The results imply that proactive evaluation of the inheritance and initial frequency of resistance are useful for predicting the risk of resistance and improving strategies to sustain the effectiveness of Bt crops.

http://www.ncbi.nlm.nih.gov/pubmed/23752438

Farias, J.; Andow, D.; Horikoshi, R.; Sorgatto, R.; Fresia, P; Santos, A.; Omoto, C. 2014. Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda (Lepidoptera: Noctuidae*) in Brazil. *Crop Protection*, volume 64, Pages 150–158.

The Cry1F protein from *Bacillus thuringiensis* Berliner expressed in event TC1507 maize (*Zea mays* L.) was one of the most effective ways to control *Spodoptera frugiperda* (J. E. Smith) in Brazil. After reports of reduced effectiveness of this Bt maize event in some areas of Brazil, research was undertaken to investigate if damage to Cry1F maize was caused by resistant *S. frugiperda*. Additional investigations were conducted to evaluate the genetic basis of the resistance and to test if Cry1F resistant *S. frugiperda* selected from populations of different regions of Brazil share the same resistance locus by using complementation tests. Neonate larvae of *S. frugiperda* collected from TC1507 maize fields with damage in Western Bahia region in 2011 were able to survive on Cry1F maize plants under laboratory conditions and subsequently produced normal adults. Survival of Cry1F-susceptible *S. frugiperda* on non-Bt maize was significantly higher in leaf than plant bioassays. Resistance ratio in diet overlay bioassays was >5000-fold. A discriminating concentration of 2000 ng cm^{-2} of Cry1F protein was defined for monitoring the frequency of resistance of *S. frugiperda* to Cry1F. Cry1F resistant *S. frugiperda* showed a recessive autosomal inheritance for alleles involved in resistance to Cry1F protein. In complementation tests, the resistant population from Western Bahia was crossed with the other seven resistant populations collected from different States of Brazil. F_1 larvae from each cross had the same survival at discriminating concentration of 2000 ng cm^{-2} of Cry1F protein, indicating that the resistance alleles in each population were likely at the same locus. Therefore, implementation of resistance management strategies is urgent to prolong the lifetime of Cry1F for controlling *S. frugiperda* in Brazil.

http://www.sciencedirect.com/science/article/pii/S026121941400204X

Gassmann, A.; Petzold-Maxwell, J.; Clifton, E.; Dunbar, M.; Hoffmann, A.; Ingber, D.; Keweshan, R. 2014. Field-evolved resistance by western corn rootworm to multiple *Bacillus thuringiensis* toxins in transgenic maize. *PNAS* USA, vol. 111, no. 14, 5141–5146.

The widespread planting of crops genetically engineered to produce insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt) places intense selective pressure on pest populations to evolve resistance. Western corn rootworm is a key pest of maize, and in continuous maize fields it is often managed through planting of Bt maize. During 2009 and 2010, fields were identified in Iowa in which western corn rootworm imposed severe injury to maize producing Bt toxin Cry3Bb1. Subsequent bioassays revealed Cry3Bb1 resistance in these populations. Here, we report that, during 2011, injury to Bt maize in the field expanded to include mCry3A maize in addition to Cry3Bb1 maize and that laboratory analysis of western corn rootworm from these fields found resistance to Cry3Bb1 and mCry3A and cross-resistance between these toxins. Resistance to Bt maize has persisted in Iowa, with both the number of Bt fields identified with severe root injury and the ability western corn rootworm populations to survive on Cry3Bb1 maize increasing between 2009 and 2011. Additionally, Bt maize targeting western corn rootworm does not produce a high dose of Bt toxin, and the magnitude of resistance associated with feeding injury was less than that seen in a high-dose Bt crop. These first cases

of resistance by western corn rootworm highlight the vulnerability of Bt maize to further evolution of resistance from this pest and, more broadly, point to the potential of insects to develop resistance rapidly when Bt crops do not achieve a high dose of Bt toxin.

Full article available at http://www.pnas.org/content/111/14/5141.full.pdf+html

Fabrick, J.; Ponnuraj, J.; Singh, A.; Tanwar, R.; Unnithan, G.; Yelich, A.; Li, X.; Carrière, Y.; Tabashnik, B. 2014. Alternative splicing and highly variable cadherin transcripts associated with field-evolved resistance of pink bollworm to Bt cotton in India. *PLOS ONE,* 19;9(5):e97900. doi: 10.1371/journal.pone.0097900. eCollection 2014.

Evolution of resistance by insect pests can reduce the benefits of insecticidal proteins from Bacillus thuringiensis (Bt) that are used extensively in sprays and transgenic crops. Despite considerable knowledge of the genes conferring insect resistance to Bt toxins in laboratory-selected strains and in field populations exposed to Bt sprays, understanding of the genetic basis of field-evolved resistance to Bt crops remains limited. In particular, previous work has not identified the genes conferring resistance in any cases where field-evolved resistance has reduced the efficacy of a Bt crop. Here we report that mutations in a gene encoding a cadherin protein that binds Bt toxin Cry1Ac are associated with field-evolved resistance of pink bollworm (Pectinophora gossypiella) in India to Cry1Ac produced by transgenic cotton. We conducted laboratory bioassays that confirmed previously reported resistance to Cry1Ac in pink bollworm from the state of Gujarat, where Bt cotton producing Cry1Ac has been grown extensively. Analysis of DNA from 436 pink bollworm from seven populations in India detected none of the four cadherin resistance alleles previously reported to be linked with resistance to Cry1Ac in laboratory-selected strains of pink bollworm from Arizona. However, DNA sequencing of pink bollworm derived from resistant and susceptible field populations in India revealed eight novel, severely disrupted cadherin alleles associated with resistance to Cry1Ac. For these eight alleles, analysis of complementary DNA (cDNA) revealed a total of 19 transcript isoforms, each containing a premature stop codon, a deletion of at least 99 base pairs, or both. Seven of the eight disrupted alleles each produced two or more different transcript isoforms, which implicates alternative splicing of messenger RNA (mRNA). This represents the first example of alternative splicing associated with field-evolved resistance that reduced the efficacy of a Bt crop.

Full article available at http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0097900

In addition to the growing presence of insects insensitive to Bt toxins, some researchers noted that such individuals may have greater fitness than their similar of sensitive populations. Thus, some insects which acquire resistance reproduce themselves on a more efficient way, rapidly increasing their populations and generating agronomic and socioeconomic damages potentially more relevant than those notes in their absence.

Oswald, K.; French, B.; Nielson, C.; Bagley, M. 2012. Assessment of fitness costs in Cry3Bb1-resistant and susceptible western corn rootworm (*Coleoptera: Chrysomelidae*) laboratory colonies. *Journal of Applied Entomology,* Doi: 10.1111/j.1439-0418.2012.01704.x

Maize production in the United States is dominated by plants genetically modified with transgenes

from *Bacillus thuringiensis* (Bt). Cry3Bb delta endotoxins expressed by Bt maize specifically target corn rootworms (genus *Diabrotica*) and have proven highly efficacious. However, development of resistance to Bt maize, especially among western corn rootworm (*Diabrotica virgifera virgifera*) populations, poses a significant threat to the future viability of this pest control biotechnology. The structured refuge insect resistance management (IRM) strategy implemented in the United States for Bt maize adopts a conservative approach to managing resistance by assuming no fitness costs of Bt resistance, even though these trade-offs strongly influence the dynamics of Bt resistance within numerous agricultural pest species. To investigate the effects of Bt resistance on fitness components of western corn rootworm, we compared survivorship, fecundity and viability of five Bt-resistant laboratory lines reared on MON863 (YieldGard Rootworm), a Bt maize product that expresses Cry3Bb1 delta endotoxin, and on its non-transgenic isoline. Analysis of performance on the isoline maize demonstrated no fitness costs associated with Bt resistance. In fact, resistant lines emerged approximately 2–3 days earlier than control lines when reared on both MON863 and the isoline, indicating that selection for Bt resistance resulted in a general increase in the rate of larval development. In addition, resistant lines reared on Bt maize displayed higher fecundity than those reared on the isoline, which may have significant management implications. These data will be valuable for formulating improved IRM strategies for a principal agricultural pest of maize.

https://goo.gl/etHlOf

2.1.3 Low efficacy of the Bt technology's useful life maintenance strategies

Soon after the recording of the first populations of genetically resistant insects to Bt toxins, handling strategies were developed in order to extend the efficacy time of Bt technology. With a concern directly related to the maintenance of the profit rates, with views to extend the useful life of the technology, mechanisms intended to transfer to the farmer the responsibility for natural condition, resulting from the oppressive presence of Cry proteins, were proposed. With this respect, two strategies have been recommended to the producers of Bt transgenic plants by the biotechnology sector.

The first strategy involves the companies which develop biotechnologies and the producers. The companies started to trade Bt plants which synthesize - supposedly[30] – high doses of toxins (with the objective to maximize the possibilities of death of the insects [31]), being he producers responsible for implementing the so-called refuge zones. Close to their crops, such refuges

30 Variations in protein synthesis Cry for Bt plants are exemplified in section 2.2.2 of Part 1.

31 One of the prerequisites for efficacy of high-dose / refuge strategy is that the genetic mutations responsible for insensitivity to the toxin is transmitted in recessive mode (insensitivity will be expressed in the next generation only if the insect mate with individual also endowed with the mutation). Now, certain agronomically important pests have not recessive transmission (or dominant) of this character, invalidating this management strategy.

correspond to parts of the cultivation areas seeded with non-genetically modified varieties. It would be a species of seedling "for the insects", so as to maintain the numerical prevalence of sensitive populations. Well, the resistance by the farmers was expected, with all of them waiting for their neighbors to perform anti-economic cultivation of the refuge areas, which would basically serve to preserve the companies' profits. However, increased loads of insecticide proteins did not prevent or even delayed the emergence of tolerant populations. In addition, the refuge strategy has shown to be fragile, once, in many cases - due to the species and the predispositions of certain populations to be selected -, more than half of the crop would need to be planted with conventional varieties in order to maintain the efficacy of the technology during some years.

MOD-REV – Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. *Annu Rev Entomol.*, 43: 701-26.

This review examines potential impacts of transgenic cultivars on insect population dynamics and evolution. Experience with classically bred, insecticidal cultivars has demonstrated that a solid understanding of both the target insect's ecology and the cultivar's performance under varied field conditions will be essential for predicting area-wide effects of transgenic cultivars on pest and natural enemy dynamics. This experience has also demonstrated the evolutionary capacity of pests for adaptive response to insecticidal traits in crops. Biochemical and genetic studies of insect adaptation to the *Bacillus thuringiensis* (Bt) toxins expressed by currently marketed transgenic cultivars indicate a high risk for rapid adaptation if these cultivars are misused. Theoretical and practical issues involved in implementing strategies to delay pest adaptation to insecticidal cultivars are reviewed. Emphasis is placed on examining the "high dose"/refuge strategy that has become the goal of industry and regulatory authorities.

http://www.ncbi.nlm.nih.gov/pubmed/15012402

Huang, F.; Buschman, L.; Higgins, R.; McGaughey, W. 1999. Inheritance of resistance to *Bacillus thuringiensis* toxin (Dipel ES) in the European corn borer. *Science,* 284: 965-966.

Resistance in the European corn borer, *Ostrinia nubilalis* (Hu¬bner), to a commercial formulation of *Bacillus thuringiensis* (*Bt*) Berliner toxin, Dipel ES, appears to be inherited as an incompletely dominant autosomal gene. This contrasts with the inheritance of resistance to *Bt* in other insects, where it has usually been characterized as a recessive trait. The proposed high-dose/refuge strategy for resistance management in *Bt* maize depends on resistance being recessive or partially recessive. If field resistance turns out to be similar to this laboratory resistance, the usefulness of the high-dose/refuge strategy for resistance management in *Bt* maize may be diminished.

http://www.ncbi.nlm.nih.gov/pubmed/10320377

Shelton, A.; Tang, J.; Roush, R.; Metz, T.; Earle, E. 2000. Field tests on managing resistance to Bt-engineered plants. *Nature Biotechnology*, vol 18.

Several important crops have been engineered to express toxins of *Bacillus thuringiensis* (*Bt*) for insect control. In 1999, US farmers planted nearly 8 million hectares (nearly 20 million acres) of transgenic *Bt* crops approved by the EPA. *Bt*-transgenic plants can greatly reduce the use of broader spectrum insecticides, but insect resistance may hinder this technology. Present resistance management strategies rely on a "refuge" composed of non-*Bt* plants to conserve susceptible alleles. We have used *Bt*-transgenic broccoli plants and the diamondback moth as a model system to examine resistance management strategies. The higher number of larvae on refuge plants in our field tests indicate that a "separate refuge" will be more effective at conserving susceptible larvae than a "mixed refuge" and would thereby reduce the number of homozygous resistant (RR) offspring. Our field tests also examined the strategy of spraying the refuge to prevent economic loss to the crop while maintaining susceptible alleles in the population. Results indicate that great care must be taken to ensure that refuges, particularly those sprayed with efficacious insecticides, produce adequate numbers of susceptible alleles. Each insect/*Bt* crop system may have unique management requirements because of the biology of the insect, but our studies validate the need for a refuge. As we learn more about how to refine our present resistance management strategies, it is important to also develop the next generation of technology and implementation strategies.

http://www.ncbi.nlm.nih.gov/pubmed/10700153

REV – Tabashnik, B.; Carrière, Y.; Dennehy, T.; Morin, S.; Sisterson, M.; Roush, R. Shelton, A.; Zhao, J-Z. 2003. Insect Resistance to Transgenic Bt Crops: Lessons from the Laboratory and Field. *J. Econ. Entomol.*, 96(4): 1031-1038.

Transgenic crops that produce insecticidal toxins from the bacterium Bacillus thuringiensis (Bt) grew on >62 million ha worldwide from 1996 to 2002. Despite expectations that pests would rapidly evolve resistance to such Bt crops, increases in the frequency of resistance caused by exposure to Bt crops in the field have not yet been documented. In laboratory and greenhouse tests, however, at least seven resistant laboratory strains of three pests (Plutella xylostella [L.], Pectinophora gossypiella [Saunders], and Helicoverpa armigera [Hübner]) have completed development on Bt crops. In contrast, several other laboratory strains with 70- to 10,100-fold resistance to Bt toxins in diet did not survive on Bt crops. Monitoring of field populations in regions with high adoption of Bt crops has not yet detected increases in resistance frequency. Resistance monitoring examples include Ostrinia nubilalis (Hübner) in the United States (6 yr), P. gossypiella in Arizona (5 yr), H. armigera in northern China (3 yr), and Helicoverpa zea (Boddie) in North Carolina (2 yr). Key factors delaying resistance to Bt crops are probably refuges of non-Bt host plants that enable survival of susceptible pests, low initial resistance allele frequencies, recessive inheritance of resistance to Bt crops, costs associated with resistance that reduce fitness of resistant individuals relative to susceptible individuals on non-Bt hosts ("fitness costs"), and disadvantages suffered by resistant strains on Bt hosts relative to their performance on non-Bt hosts ("incomplete resistance"). The relative importance of these factors varies among pest-Bt crop systems, and violations of key assumptions of the refuge strategy (low resistance allele frequency and recessive inheritance) may occur in some cases. The success of Bt crops exceeds expectations of many, but does not preclude resistance problems in the future.

http://www.ncbi.nlm.nih.gov/pubmed/14503572

Chilcutt, C.; Tabashnik, B. 2004. Contamination of refuges by *Bacillus thuringiensis* toxin

genes from transgenic maize. *Proceedings of the National Academy of Sciences,* 101:7526-7529.

Transgenic crops producing insecticidal toxins from *Bacillus thuringiensis* (Bt) are widely used to control pests, but their benefits will be lost if pests evolve resistance. The mandated high-dose/refuge strategy for delaying pest resistance requires planting refuges of toxin-free crops near Bt crops to promote survival of susceptible pests. We report that pollen-mediated gene flow up to 31 m from Bt maize caused low to moderate Bt toxin levels in kernels of non-Bt maize refuge plants. Immunoassays of non-Bt maize sampled from the field showed that the mean concentration of Bt toxin Cry1Ab in kernels and the percentage of kernels with Cry1Ab decreased with distance from Bt maize. The highest Bt toxin concentration in pooled kernels of non-Bt maize plants was 45% of the mean concentration in kernels from adjacent Bt maize plants. Most previous work on gene flow from transgenic crops has emphasized potential effects of transgene movement on wild relatives of crops, landraces, and organic plantings, whereas implications for pest resistance have been largely ignored. Variable Bt toxin production in seeds of refuge plants undermines the high dose/refuge strategy and could accelerate pest resistance to Bt crops. Thus, guidelines should be revised to reduce gene flow between Bt crops and refuge plants.

Full article available at https://goo.gl/VcjWTb

MOD – Kranthi, K.; Kranthi, N. 2004. Modelling adaptability of cotton bollworm, *Helicoverpa armigera* (Hübner) to Bt-cotton in India. *Current Science,* vol. 87, nº 8.

A stochastic model '*Bt*-Adapt' was developed to simulate the rate of resistance development of *Helicoverpa armigera* to Cry1Ac under Indian farming conditions. The model integrates genetic and ecological parameters of *H. armigera* in relation to its response to the Cry1Ac expressing *Bacillus thuringiensis* (*Bt*)-cotton. Simulation analysis showed that relative survival rate of the Cry1Ac-resistant homozygous (RR), heterozygous (RS) and homozygous susceptible (SS) *H. armigera* genotypes on *Bt*-cotton, was the most important factor influencing resistance development. In the order of significance, the other factors that had the greatest impact on resistance development were the relative proportion of area under *Bt*-cotton, dominance of the resistant allele and initial frequency of resistant alleles in field populations. The extent of population reduction in *Bt*-cotton and non-*Bt* crops due to pest control, was found to have a significant impact on the rate of resistance development. Simulation studies showed that cultivation of *Bt*-cotton in 10, 20, 30 and 40% of the total area under cotton, is likely to result in resistant allele frequency reaching 0.5, which would be adequate to cause crop failure, after 54, 25, 16 and 11 years respectively, if no pest control measures were adopted in both *Bt*-cotton and non-*Bt* crops. With a pest control efficacy of 0.9 in *Bt*-cotton and 0.5 in non- *Bt* crops, it would take 70 and 45 years for resistant allele frequency to reach 0.5 with the *Bt*-cotton area at 30 and 40% respectively. Based on the simulation analysis, resistance management strategies are proposed with emphasis on reducing populations of *H.armigera* that survive *Bt*-cotton and enhancement of area of alternate host crops that are as attractive as cotton to *H. armigera*, to be used as trap crop or intercrop refuges.

Full article available at http://www.iisc.ernet.in/currsci/oct252004/1096.pdf

REV – Tabashnik, B.; Gassmann, A.; Crowder, D.; Carrière, Y. 2008. Insect resistance to Bt crops: Evidence versus Theory. *Nature Biotechnology,* 26: 199-202.

Evolution of insect resistance threatens the continued success of transgenic crops producing *Bacillus thuringiensis* (*Bt*) toxins that kill pests. The approach used most widely to delay insect resistance to *Bt* crops is the refuge strategy, which requires refuges of host plants without *Bt* toxins near *Bt* crops to promote survival of susceptible pests. However, large-scale tests of the refuge strategy have been problematic. Analysis of more than a decade of global monitoring data reveals that the frequency of resistance alleles has increased substantially in some field populations of *Helicoverpa zea*, but not in

five other major pests in Australia, China, Spain and the United States. The resistance of *H. zea* to *Bt* toxin Cry1Ac in transgenic cotton has not caused widespread crop failures, in part because other tactics augment control of this pest. The field outcomes documented with monitoring data are consistent with the theory underlying the refuge strategy, suggesting that refuges have helped to delay resistance.

http://www.ncbi.nlm.nih.gov/pubmed/18259177

COM – Tabashnik, B. 2008. Delaying insect resistance to transgenic crops. *PNAS USA*, 105 (49) 19029-19030, vol. 105, nº 49 19029–19030 – 1.

Without summary.

Full article available at http://www.pnas.org/content/105/49/19029.full.pdf+html

Kruger, M.; van Rensburg, J.; Van den Berg, J. 2009. Perspective on the development of stem borer resistance to Bt maize and refuge compliance at the Vaalharts irrigation scheme in South Africa. *Crop Protection,* 28 (8): 684-9.

Bt maize has been grown at the Vaalharts irrigation scheme in South Africa since its first release during 1998. Interest in Bt maize refuge compliance, pest incidence and production practices at Vaalharts were recently stimulated by the first report of field resistance of Busseola fusca (Lepidoptera: Noctuidae) to Bt maize. Objectives of this study were to evaluate farmer's perceptions of the regulatory aspects guiding the planting of Bt maize and refugia and how the field situation developed between 1998 and 2008. A survey, using a self-administered questionnaire, was conducted amongst 80 farmers at the irrigation scheme. The questionnaire addressed signing of contracts upon purchasing genetically modified (GM) seed, refuge compliance, refuge design and general farming practices. Farmers were also questioned on the perceived benefits and disadvantages of Bt maize and their perceptions of the pest status of B. fusca. The two greatest advantages associated with Bt maize were indicated to be convenient management (88%) and increased productivity (61.3%) while 42.5% indicated that they perceived Bt-technology to be environmental friendly. Initial levels of refuge compliance were low, and even though farmers were obligated to plant a refuge area for each Bt maize field, only 7.7% of farmers planted refuges during 1998. This number increased to 100% during 2008. Eight percent of farmers, however, indicated that they did not plant a refuge field for each Bt maize field, which was justified on the basis of small farm sizes (25 ha). Nearly all farmers (99.8%) allow no spatial separation between the Bt maize field and adjacent refuge area. Farmers preferred to plant the refuge option where 5% of the field area is planted to conventional maize, which is not sprayed with insecticide instead of the 20% refuge area on which insecticide application against the target pest is allowed. In South Africa stewardship programs instituted during the 2008/2009 growing season, involve grower education programs as well as the compulsory signing of contracts between companies and farmers that contractually bind them to comply with refuge requirements accompanied by on-farm inspections. It appears that stem borer resistance to Bt maize in the Vaalharts area resulted from a combination of a late general planting date with consequent increased levels of infestation and variance in time of planting providing a continuous supply of moths.

http://www.sciencedirect.com/science/article/pii/S0261219409000921

Carrière, Y.; Crowder, D.; Tabashnik, B. 2010. Evolutionary ecology of insect adaptation to Bt crops. *Evolutionary Applications* (Special Issue: Evolution in Agro-Ecosystems) vol. 3, Issue 5-6: 561-73.

Transgenic crops producing *Bacillus thuringiensis* (Bt) toxins are used worldwide to control major pests of corn and cotton. Development of strategies to delay the evolution of pest resistance to Bt

crops requires an understanding of factors affecting responses to natural selection, which include variation in survival on Bt crops, heritability of resistance, and fitness advantages associated with resistance mutations. The two main strategies adopted for delaying resistance are the refuge and pyramid strategies. Both can reduce heritability of resistance, but pyramids can also delay resistance by reducing genetic variation for resistance. Seasonal declines in the concentration of Bt toxins in transgenic cultivars, however, can increase the heritability of resistance. The fitness advantages associated with resistance mutations can be reduced by agronomic practices, including increasing refuge size, manipulating refuges to increase fitness costs, and manipulating Bt cultivars to reduce fitness of resistant individuals. Manipulating costs and fitness of resistant individuals on transgenic insecticidal crops may be especially important for thwarting evolution of resistance in haplodiploid and parthenogenetic pests. Field-evolved resistance to Bt crops in only five pests during the last 14 years suggests that the refuge strategy has successfully delayed resistance, but the accumulation of resistant pests could accelerate.

Full article available at http://onlinelibrary.wiley.com/doi/10.1111/j.1752-4571.2010.00129.x/pdf

Kruger, M.; van Rensburg, J.; Van Den Berg, J. 2011. Resistance to Bt Maize in *Busseola fusca* (*Lepidoptera: Noctuidae*) from Vaalharts, South Africa. *Environmental Entomology,* 40(2):477-483.

The first report of resistance of the maize stem borer [*Busseola fusca* (Fuller)] to Bt maize (MON810) was made in the Christiana area of South Africa during 2007. The objective of this study was to evaluate the status of resistance of other populations of *B. fusca* to Bt maize. One greenhouse and two laboratory studies were conducted. *B. fusca* populations were collected on Bt maize as well as the adjacent refugia (conventional maize and non-Bt maize) in the Vaalharts area, 50 km from the Christiana site. Control populations were collected from sites where Bt maize was not planted. In the greenhouse study 720 potted plants were each artificially infested with 10 neonate larvae of the F1-generation after the field collected populations were reared through to adults. Numbers of live larvae and larval mass per plant were determined at regular intervals over a 35-d period. Larvae of the Christiana conventional population (Bt-susceptible) on Bt maize (CHR08ConBt) and Bethal conventional population (Bt-susceptible) on Bt maize (BET08Con-Bt) did not survive on Bt maize for longer than 12 d. The populations collected from both Bt (VAA08Bt-Bt) maize and refuges (VAA08Ref-Bt) at Vaalharts were resistant and the subsequent generation of larvae completed their life cycle on Bt maize. Similar results were observed in the laboratory experiments. This study confirmed resistance of *B. fusca* to the Cry1Ab toxin (MON810). The geographical distribution of resistance was shown to include at least the Vaalharts area, in addition to the original report for the Christiana area. These observations that larvae collected from refugia at Vaalharts was resistant, show that the efficacy of the refuge strategy is compromised in this area because the contribution of refugia did not produce large enough numbers of susceptible individuals to mate with moths of which larvae survived inside Bt maize fields.

Full article available at https://goo.gl/iVjAxD

REV – Zhang, H.; Tian, W.; Zhao, J.; Jun, L.; Chunhui, Y.; Yihua, L.; Shuwen, Y.; Kongming, W.; *et al.* 2012. Diverse genetic basis of field-evolved resistance to Bt cotton in cotton bollworm from China. *Proceedings of the National Academy of Sciences,* vol. 109, issue 26, pp. 10275-10280.

Evolution of pest resistance reduces the efficacy of insecticidal proteins from Bacillus thuringiensis (Bt) used in sprays or in transgenic crops. Although several pests have evolved resistance to Bt crops in the field, information about the genetic basis of fieldevolved resistance to Bt crops has been limited. In particular, laboratory-selected resistance to Bt toxin Cry1Ac based on recessive mutations

in a gene encoding a toxin-binding cadherin protein has been identified in three major cotton pests, but previous work has not determined if such mutations are associated with fieldselected resistance to Bt cotton. Here we show that the most common resistance alleles in field populations of cotton bollworm, Helicoverpa armigera, selected with Bt cotton in northern China, had recessive cadherin mutations, including the deletion mutation identified via laboratory selection. However, unlike all previously studied cadherin resistance alleles, one field-selected cadherin resistance allele conferred nonrecessive resistance. We also detected nonrecessive resistance that was not genetically linked with the cadherin locus. In field-selected populations, recessive cadherin alleles accounted for 75–84% of resistance alleles detected. However, most resistance alleles occurred in heterozygotes and 59–94% of resistant individuals carried at least one nonrecessive resistance allele. The results suggest that resistance management strategies must account for diverse resistance alleles in field-selected populations, including nonrecessive alleles.

Full article available at http://www.pnas.org/content/109/26/10275.full.pdf

Tabashnik, B.; Gould, F. 2012. Delaying Corn Rootworm Resistance to Bt Corn. *Journal of Economic Entomology,* 105(3):767-776.

Transgenic crops producing *Bacillus thuringiensis* (Bt) toxins for insect control have been successful, but their efÞcacy is reduced when pests evolve resistance. To delay pest resistance to Bt crops, the U.S. Environmental Protection Agency (EPA) has required refuges of host plants that do not produce Bt toxins to promote survival of susceptible pests. Such refuges are expected to be most effective if the Bt plants deliver a dose of toxin high enough to kill nearly all hybrid progeny producedby matings between resistant and susceptible pests. In 2003, the EPA Þrst registered corn, *Zea mays* L., producing a Bt toxin (Cry3Bb1) that kills western corn rootworm, *Diabrotica virgifera virgifera* LeConte, one of the most economically important crop pests in the United States. The EPA requires minimum refuges of 20% for Cry3Bb1 corn and 5% for corn producing two Bt toxins active against corn rootworms.We conclude that the current refuge requirements are not adequate, because Bt corn hybrids active against corn rootworms do not meet the high-dose standard, and western corn rootworm has rapidly evolved resistance to Cry3Bb1 corn in the laboratory, greenhouse, and Þeld. Accordingly, we recommend increasing the minimum refuge for Bt corn targeting corn rootworms to 50% for plants producing one toxin active against these pests and to 20% for plants producing two toxins active against these pests. Increasing the minimum refuge percentage can help to delay pest resistance, encourage integrated pest management, and promote more sustainable crop protection.

http://www.ncbi.nlm.nih.gov/pubmed/22812111

Campagne, P.; Kruger, M.; Pasquet, R.; Le Ru, B.; Van den Berg, J. 2013. Dominant Inheritance of Field-Evolved Resistance to Bt Corn in *Busseola fusca*. *PLOS ONE,* 8(7): e69675. doi:10.1371/journal.pone.0069675.

Transgenic crops expressing Bacillus thuringiensis (*Bt*) toxins have been adopted worldwide, notably in developing countries. In spite of their success in controlling target pests while allowing a substantial reduction of insecticide use, the sustainable control of these pest populations is threatened by the evolution of resistance. The implementation of the "high dose/refuge" strategy for managing insect resistance in transgenic crops aims at delaying the evolution of resistance to *Bt* crops in pest populations by promoting survival of susceptible insects. However, a crucial condition for the "high dose/refuge" strategy to be efficient is that the inheritance of resistance should be functionally recessive. *Busseola fusca* developed high levels of resistance to the *Bt* toxin *Cry 1Ab* expressed in *Bt* corn in South Africa. To test whether the inheritance of *B. fusca* resistance to the *Bt* toxin could be considered recessive we performed controlled crosses with this pest and evaluated its survival on *Bt* and non-*Bt* corn. Results show that resistance of *B. fusca* to *Bt* corn is dominant, which refutes the hypothesis

of recessive inheritance. Survival on *Bt* corn was not lower than on non-*Bt* corn for both resistant larvae and the F_1 progeny from resistant × susceptible parents. Hence, resistance management strategies of *B. fusca* to *Bt* corn must address non-recessive resistance.

Full article available at https://goo.gl/RF6AHH

Another strategy adopted by the biotechnology sector in order to maximize the useful life of the Bt technologies – obtained following investments of millions of dollar – refers to the development of "stacked" or "pyramided" transgenic varieties, or "piled", on which two or more transgenes are incorporated to the same plant, by means of conventional breeding involving simple transgenic varieties.

This strategy, which initially raised notable hopes, rapidly showed its limits. Generally speaking, the insect resistance to one of the toxins would be overcome/complemented by the lethal action of another toxin, with a different mode of action – preventing the survival and the reproduction of individuals holding genomes insensitive to Bt technology. Currently, the scientific community converges to consider that pyramided transgenic plants do not prevent the development of insensitive insect populations, especially when there are already populations resistant to one of the toxins synthesized in the pyramided event in the region.

Zhao, J-Z.; Cao, J.; Collins, H.; Bates, S.; Roush, R.; Earle, E.; Shelton, A. 2005. Concurrent use of transgenic plants expressing a single and two *Bacillus thuringiensis* genes speeds insect adaptation to pyramided plants. *PNAS*, 8426–8430, vol. 102, nº 24.

Transgenic plants expressing insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) were grown on over 13 million ha in the United States and 22.4 million ha worldwide in 2004. Preventing or slowing the evolution of resistance by insects ("resistance management") is critical for the sustainable use of Bt crops. Plants containing two dissimilar Bt toxin genes in the same plant ("pyramided") have the potential to delay insect resistance. However, the advantage of pyramided Bt plants for resistance management may be compromised if they share similar toxins with single-gene plants that are deployed simultaneously. We tested this hypothesis using a unique model system composed of broccoli plants transformed to express different Cry toxins (Cry1Ac, Cry1C, or both) and a synthetic population of the diamondback moth (*Plutella xylostella*) carrying genes for resistance to Cry1Ac and Cry1C at frequencies of _0.10 and 0.34, respectively. After 24–26 generations of selection in the greenhouse, the concurrent use of one- and two-gene plants resulted in control failure of both types of Bt plants. When only two-gene plants were used in the selection, no or few insects survived on one- or two-gene Bt plants, indicating that concurrent use of transgenic plants expressing a single and two Bt genes will select for resistance to two-gene plants more rapidly than the use of two-gene plants alone. The results of this experiment agree with the

predictions of a Mendelian deterministic simulation model and have important implications for the regulation and deployment of pyramided Bt plants.

Full article available at http://www.pnas.org/content/102/24/8426.full

Downes, S.; Parker, T.; Mahon, R. 2010. Incipient resistance of Helicoperva punctigera to the Cry2Ab Bt toxin in Bollgard II cotton. *PLOS ONE,* 5(9): e12567.

Combinations of dissimilar insecticidal proteins ("pyramids") within transgenic plants are predicted to delay the evolution of pest resistance for significantly longer than crops expressing a single transgene. Field-evolved resistance to Bacillus thuringiensis (Bt) transgenic crops has been reported for first generation, single-toxin varieties and the Cry1 class of proteins. Our five year data set shows a significant exponential increase in the frequency of alleles conferring Cry2Ab resistance in Australian field populations of Helicoverpa punctigera since the adoption of a second generation, two-toxin Bt cotton expressing this insecticidal protein. Furthermore, the frequency of cry2Ab resistance alleles in populations from cropping areas is 8-fold higher than that found for populations from non-cropping regions. This report of field evolved resistance to a protein in a dual-toxin Bt-crop has precisely fulfilled the intended function of monitoring for resistance; namely, to provide an early warning of increases in frequencies that may lead to potential failures of the transgenic technology. Furthermore, it demonstrates that pyramids are not 'bullet proof' and that rapid evolution to Bt toxins in the Cry2 class is possible.

Full article available at https://goo.gl/9oUOSp

Brévault, T.; Heuberger, S.; Zhang, M.; Ellers-Kirk, K.; Ni, X.; Masson, L.; Li, X.; Tabashnik, B.; Carrière, Y. 2013. Potential shortfall of pyramided transgenic cotton for insect resistance management. *PNAS*, 5806–5811, vol. 110, nº 1.

To delay evolution of pest resistance to transgenic crops producing insecticidal proteins from *Bacillus thuringiensis* (Bt), the "pyramid" strategy uses plants that produce two or more toxins that kill the same pest. In the United States, this strategy has been adopted widely, with two-toxin Bt cotton replacing one-toxin Bt cotton. Although two-toxin plants are likely to be more durable than one-toxin plants, the extent of this advantage depends on several conditions. One key assumption favoring success of two-toxin plants is that they kill insects selected for resistance to one toxin, which is called "redundant killing." Here we tested this assumption for a major pest, *Helicoverpa zea*, on transgenic cotton producing Bt toxins Cry1Ac and Cry2Ab. Selection with Cry1Ac increased survival on two-toxin cotton, which contradicts the assumption. The concentration of Cry1Ac and Cry2Ab declined during the growing season, which would tend to exacerbate this problem. Furthermore, analysis of results from 21 selection experiments with eight species of lepidopteran pests indicates that some cross-resistance typically occurs between Cry1A and Cry2A toxins. Incorporation of empirical data into simulation models shows that the observed deviations from ideal conditions could greatly reduce the benefits of the pyramid strategy for pests like *H. zea,* which have inherently low susceptibility to Bt toxins and have been exposed extensively to one of the toxins in the pyramid before two-toxin plants are adopted. For such pests, the pyramid strategy could be improved by incorporating empirical data on deviations from ideal assumptions about redundant killing and cross-resistance.

Full article available at http://www.pnas.org/content/110/15/5806.full.pdf+html

Van den Berg, J.; Hilbeck, A.; Bøhn, T. 2013. Pest resistance to Cry1Ab Bt maize:

Field resistance, contributing factors and lessons from South Africa. *Crop Protection,* 54, 154-160.

This paper documents the historical development of resistance of the African maize stem borer, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) to Bt maize (*Zea mays* L.). This pest was one of the first to evolve resistance to Bt maize expressing Cry1Ab protein. A time-line of events and contributing factors are presented, from the commencement of efficacy testing through to the present situation, where the Cry1Ab toxin has lost its efficacy against *B. fusca* at many localities throughout the maize producing region, and single-gene Bt maize events often require insecticide treatments for which farmers are compensated. Significant levels of pest survival on Bt maize was observed in the first season after commercial release in 1998 and confirmed seven years later. Reduced selection pressure on the target pest is the objective of insect resistance management (IRM), and strategies to accomplish this should receive highest priority. Where resistance is prevalent, the only viable options to reduce selection pressure are withdrawal of the product and/or enforcement of high-dose/refuge requirements. The latter action may however be of no value under conditions where resistance is prevalent, since the value of refugia to an IRM strategy may be compromised. Remedial actions taken in South Africa included the propagation and enforcement of refuge compliance followed by the release of pyramided maize hybrids in 2011. These pyramids combine Cry1A.105 and Cry2Ab2 toxin-producing transgenes, replacing the ineffective single-transgene. However, it remains uncertain if cross-resistance occurs between Cry1A.105/Cry2Ab2 and the closely related Cry1Ab toxin, and for how long this pyramided event will endure. Cultivation of Cry1Ab-expressing hybrids continues in areas where resistance levels have been confirmed to be high. In retrospect, this case provides lessons regarding IRM, not only in South Africa, but wherever Bt crops are being introduced.

http://www.sciencedirect.com/science/article/pii/S0261219413002093

In any case, after some years of commercial use of the Bt plants, there is consensus in the scientific community about the need to associated such technology to an entire set of pre-existing management alternatives, such as the use of natural predators, complementary use of agrochemicals, crop rotation, etc. The original objective of extending to the maximum the useful life of Bt technology, which for its use causes its own obsolescence, is maintained. Even so, by the fact that the Bt plants synthesize toxins on a continuous and permanent way over their biological cycle, in all their cells, some authors consider the Bt plants as being incompatible with the Integrated Pest Management (IPM).

Gray, M.; 2011. Relevance of Traditional Integrated Pest Management (IPM) Strategies for Commercial Corn Producers in a Transgenic Agroecosystem: A Bygone Era? *Journal of Agricultural and Food Chemistry,* 59 (11), pg. 5852-5858.

The use of transgenic Bt maize hybrids continues to increase significantly across the Corn Belt of the

United States. In 2009, 59% of all maize planted in Illinois was characterized as a "stacked" gene variety. This is a 40% increase since 2006. Stacked hybrids typically express one Cry protein for corn rootworm control and one Cry protein for control of several lepidopteran pests; they also feature herbicide tolerance (to either glyphosate or glufosinate). Slightly more than 50 years has passed since Vernon Stern and his University of California entomology colleagues published (1959) their seminal paper on the integrated control concept, laying the foundation for modern pest management (IPM) programs. To assess the relevance of traditional IPM concepts within a transgenic agroecosystem, commercial maize producers were surveyed at a series of meetings in 2009 and 2010 regarding their perceptions on their use of Bt hybrids and resistance management. Special attention was devoted to two insect pests of corn, the European corn borer and the western corn rootworm. A high percentage of producers who participated in these meetings planted Bt hybrids in 2008 and 2009, 97 and 96.7%, respectively. Refuge compliance in 2008 and 2009, as mandated by the U.S. Environmental Protection Agency (EPA), was 82 and 75.7%, respectively, for those producers surveyed. A large majority of producers (79 and 73.3% in 2009 and 2010, respectively) revealed that they would, or had, used a Bt hybrid for corn rootworm (Diabrotica virgifera virgifera LeConte) or European corn borer (Ostrinia nubilalis Hübner) control even when anticipated densities were low. Currently, the EPA is evaluating the long-term use of seed blends (Bt and non-Bt) as a resistance management strategy. In 2010, a large percentage of producers, 80.4%, indicated they would be willing to use this approach. The current lack of integration of management tactics for insect pests of maize in the U.S. Corn Belt, due primarily to the escalating use of transgenic Bt hybrids, may eventually result in resistance evolution and/or other unforeseen consequences.

http://pubs.acs.org/doi/abs/10.1021/jf102673s

Birch, A.; Begg, G.; Squire, G. 2011. How agro-ecological research helps to address food security issues under new IPM and pesticide reduction policies for global crop production systems. *Journal of Experimental Botany*, page 1 of 11. doi:10.1093/jxb/err064

Drivers behind food security and crop protection issues are discussed in relation to food losses caused by pests. Pests globally consume food estimated to feed an additional one billion people. Key drivers include rapid human population increase, climate change, loss of beneficial on-farm biodiversity, reduction in per capita cropped land, water shortages, and EU pesticide withdrawals under policies relating to 91/414 EEC. IPM (Integrated Pest Management) will be compulsory for all EU agriculture by 2014 and is also being widely adopted globally. IPM offers a 'toolbox' of complementary crop- and region-specific crop protection solutions to address these rising pressures. IPM aims for more sustainable solutions by using complementary technologies. The applied research challenge now is to reduce selection pressure on single solution strategies, by creating additive/synergistic interactions between IPM components. IPM is compatible with organic, conventional, and GM cropping systems and is flexible, allowing regional fine-tuning. It reduces pests below economic thresholds utilizing key 'ecological services', particularly biocontrol. A recent global review demonstrates that IPM can reduce pesticide use and increase yields of most of the major crops studied. Landscape scale 'ecological engineering', together with genetic improvement of new crop varieties, will enhance the durability of pest-resistant cultivars (conventional and GM). IPM will also promote compatibility with semiochemicals, biopesticides, precision pest monitoring tools, and rapid diagnostics. These combined strategies are urgently needed and are best achieved via multi-disciplinary research, including complex spatio-temporal modelling at farm and landscape scales. Integrative and synergistic use of existing and new IPM technologies will help meet future food production needs more sustainably in developed and developing countries, in an era of reduced pesticide availability. Current IPM research gaps are identified and discussed.

Full article available at http://jxb.oxfordjournals.org/content/early/2011/06/08/jxb.err064.full

2.2 Populations of ruderal herbs which develop genetic resistance to the main herbicides used in HT crops

For evolutionary reasons similar to the case of loss of efficacy of Bt technology before the development of populations of insects insensitive to the toxins, producers of HT plants also face difficulties in managing the ruderal plants in their crops.

In fact, the intensive use of some molecules of active products of herbicides systematically associated to the growth of HT plants, especially the glyphosate ones, generated a selection pressure which strengthened populations of ruderal plants naturally resistant to such herbicides.

The articles listed in this item illustrate the cases of populations resistant to the main herbicides systematically associated to the growth of HT plants (glyphosate, ammonium glufosinate and, in close trade phase, 2,4-D), emphasizing its implications in terms of difficulty of management and fight. Currently, there is a consensus in the scientific community in considering that these populations represent a severe threat to the efficacy of the herbicide-tolerance technology, notably in case of glyphosate, for its increased pressure of use. Even so, similar mechanisms of reaction to the selective pressures will fatally take to the emergence of plants tolerant to the other active substances, provided they are used in the same massive form, in terms of coverage and continuity.

Owen, M.; Zelaya, I. 2005. Herbicide-resistant crops and weed resistance to herbicides. *Pest Manag.*, Sci. 61: 301-311.

The adoption of genetically modified (GM) crops has increased dramatically during the last 3 years, and currently over 52 million hectares of GM crops are planted world-wide. Approximately 41 million hectares of GM crops planted are herbicide-resistant crops, which includes an estimated 33.3million hectares of herbicide-resistant soybean. Herbicide-resistant maize, canola, cotton and soybean accounted for 77% of the GM crop hectares in 2001. However, sugarbeet, wheat, and as many as 14 other crops have transgenic herbicide-resistant cultivars that may be commercially available in the near future. There are many risks associated with the production of GM and herbicide-resistant crops, including problems with grain contamination, segregation and introgression of herbicide-

resistant traits, marketplace acceptance and an increased reliance on herbicides for weed control. The latter issue is represented in the occurrence of weed population shifts, the evolution of herbicide-resistant weed populations and herbicide-resistant crops becoming volunteer weeds. Another issue is the ecological impact that simple weed management programs based on herbicide-resistant crops have on weed communities. Asiatic dayflower (*Commelina cumminus* L) common lambsquarters (*Chenopodium album* L) and wild buckwheat (*Polygonum convolvulus* L) are reported to be increasing in prominence in some agroecosystems due to the simple and significant selection pressure brought to bear by herbicideresistant crops and the concomitant use of the herbicide. Finally, evolution of herbicide-resistant weed populations attributable to the herbicide-resistant crop/herbicide program has been observed. Examples of herbicide-resistant weeds include populations of horseweed (*Conyza canadensis* (L) Cronq) resistant to *N*-(phosphonomethyl)glycine (glyphosate). An important question is whether or not these problems represent significant economic issues for future agriculture.

Full article available at https://goo.gl/oWB96f

Sandermann, H. 2006. Plant biotechnology: ecological case studies on herbicide resistance. *Trends in Plant Science,* 11 (7), 324–328.

The emerging field of molecular ecology aims to improve the ecological predictability of transgenic crop plants. The most widely cultivated lines are Roundup-Ready plants, which are genetically modified to be resistant to the broad-spectrum herbicide glyphosate. Recent publications demonstrate two ecological effects that were not anticipated: the widespread emergence of glyphosate-resistant weed biotypes and the formation of a metabolic herbicidal residue. Both effects appear to be due to the increased use of glyphosate rather than the genetic modification in the transgenic crop plant. With one prominent exception, opinions collected from the literature point towards a certain degree of resistance mismanagement and an inadequate testing of the ecological effects of extensive glyphosate use.

http://www.ncbi.nlm.nih.gov/pubmed/16781885

COM – Waltz, E. 2010. Glyphosate resistance threatens Roundup hegemony. *Nature Biotechnology,* 28, 537-538.

Without summary.

Full article available at http://www.emilywaltz.com/NAS_-_June_2010.pdf

Gaines, T.; Zhang, W.; Wang, D.; Bukun, B.; Chisholm, S.; Shaner, D.; *et al.* 2010. Gene Amplification confers glyphosate resistance in *Amaranthus palmeri*. *Proceedings of the National Academy of Sciences*, vol. 107 (3), 1029-1034.

The herbicide glyphosate became widely used in the United States and other parts of the world after the commercialization of glyphosate-resistant crops. These crops have constitutive overexpression of a glyphosate-insensitive form of the herbicide target site gene, 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*). Increased use of glyphosate over multiple years imposes selective genetic pressure on weed populations. We investigated recently discovered glyphosate-resistant *Amaranthus palmeri* populations from Georgia, in comparison with normally sensitive populations. EPSPS enzyme activity from resistant and susceptible plants was equally inhibited by glyphosate, which led us to use quantitative PCR to measure relative copy numbers of the *EPSPS* gene. Genomes of resistant plants contained from 5-fold to more than 160-fold more copies of the *EPSPS* gene than did genomes of susceptible plants. Quantitative RT-PCR on cDNA revealed that *EPSPS* expression was positively correlated with genomic *EPSPS* relative copy number. Immunoblot analyses showed that increased EPSPS protein level also correlated with *EPSPS* genomic copy number. *EPSPS* gene amplification was heritable, correlated with

resistance in pseudo-F_2 populations, and is proposed to be the molecular basis of glyphosate resistance. FISH revealed that *EPSPS* genes were present on every chromosome and, therefore, gene amplification was likely not caused by unequal chromosome crossing over. This occurrence of gene amplification as an herbicide resistance mechanism in a naturally occurring weed population is particularly significant because it could threaten the sustainable use of glyphosate-resistant crop technology.

Full article available at http://www.pnas.org/content/107/3/1029.full

Mortensen, D.; Egan, J.; Maxwell, B.; Ryan, M.; Smith, R. 2012. Navigating a Critical Juncture for Sustainable Weed Management. *BioScience,* vol. 62, nº 1, pp. 75-84.

Agricultural weed management has become entrenched in a single tactic—herbicide-resistant crops—and needs greater emphasis on integrated practices that are sustainable over the long term. In response to the outbreak of glyphosate-resistant weeds, the seed and agrichemical industries are developing crops that are genetically modified to have combined resistance to glyphosate and synthetic auxin herbicides. This technology will allow these herbicides to be used over vastly expanded areas and will likely create three interrelated challenges for sustainable weed management. First, crops with stacked herbicide resistance are likely to increase the severity of resistant weeds. Second, these crops will facilitate a significant increase in herbicide use, with potential negative consequences for environmental quality. Finally, the short-term fix provided by the new traits will encourage continued neglect of public research and extension in integrated weed management. Here, we discuss the risks to sustainable agriculture from the new resistant crops and present alternatives for research and policy.

Full article available at http://bioscience.oxfordjournals.org/content/62/1/75.full

REV – Ward, S.; Webster, T.; Stecke, L. 2013. Palmer Amaranth (*Amaranthus palmeri*): A Review. *Weed Technology,* 27(1): 12-27.

In little over 20 yr, Palmer amaranth has risen from relative obscurity to its current status as one of the most widespread, troublesome, and economically damaging agronomic weeds in the southeastern U.S. Numerous factors have enabled Palmer amaranth to become such a dominant and difficult-to-control weed, including its rapid growth rate, high fecundity, genetic diversity, ability to tolerate adverse conditions, and its facility for evolving herbicide resistance. It is both a serious threat to several U.S. cropping systems and a fascinating model weed. In this paper, we review the growing body of literature on Palmer amaranth to summarize the current state of knowledge on the biology, agricultural impacts, and management of this weed, and we suggest future directions for research.

Full article available at http://wssajournals.org/doi/full/10.1614/WT-D-12-00113.1

COM – Service, R. 2013. What Happens When Weed Killers Stop Killing? *Science,* 20, vol. 341 nº 6152 pp. 1329-1329.

Without summary.

http://www.sciencemag.org/content/341/6152/1329

COM - Nature Editorial, 2014. A growing problem. *Nature,* vol. 510, p. 187.

Without summary.

Full article available at http://www.nature.com/news/a-growing-problem-1.15382

2.2.1 Resistance to the glyphosate-based herbicides

REV – Nandula, V.; Reddy, K.; Duke, S.; Poston, D. 2005. Glyphosate-resistant weeds: Current status and future outlook. *Outlooks on Pest Management*, 16: 183–187.

Without summary.

Full article available at https://goo.gl/yF1RZr

Vila-Aiub, M.; Vidal, A.; Balbi, M.; Gundel P.; Trucco, F.; Ghersa, C. 2007. Glyphosate-resistant weeds of South American cropping systems: an overview. *Pest Management Science*, 64, 366-371.

Herbicide resistance is an evolutionary event resulting from intense herbicide selection over genetically diverse weed populations. In South America, orchard, cereal and legume cropping systems show a strong dependence on glyphosate to control weeds. The goal of this report is to review the current knowledge on cases of evolved glyphosate-resistant weeds in South American agriculture. The first reports of glyphosate resistance include populations of highly diverse taxa (Lolium multiflorum Lam., Conyza bonariensis L., C. canadensis L.). In all instances, resistance evolution followed intense glyphosate use in fruit fields of Chile and Brazil. In fruit orchards from Colombia, Parthenium hysterophorus L. has shown the ability to withstand high glyphosate rates. The recent appearance of glyphosate-resistant Sorghum halepense L. and Euphorbia heterophylla L. in glyphosate-resistant soybean fields of Argentina and Brazil, respectively, is of major concern. The evolution of glyphosate resistance has clearly taken place in those agroecosystems where glyphosate exerts a strong and continuous selection pressure on weeds. The massive adoption of no-till practices together with the utilization of glyphosate-resistant soybean crops are factors encouraging increase in glyphosate use. This phenomenon has been more evident in Argentina and Brazil. The exclusive reliance on glyphosate as the main tool for weed management results in agroecosystems biologically more prone to glyphosate resistance evolution.

http://www.ncbi.nlm.nih.gov/pubmed/18161884

Vidal, A.; Trezzi, M.; Prado, R; Ruiz-Santaella, J.; Vila-Aiub, M. 2007. Glyphosate resistant biotypes of wild poinsettia (*Euphorbia heterophylla* L.) and its risk analysis on glyphosate-tolerant soybeans. *Journal of Food, Agriculture & Environment*, 5: 265-269.

The continuous use of a single herbicide for weed control can result in selection of biotypes resistant to that compound. Greenhouse experiments were conducted to assess the occurrence of wild poinsettia (*Euphorbia heterophylla*, EPHHL) resistant biotypes to glyphosate. Two suspected glyphosate- resistant biotypes from the northern part of Rio Grande do Sul, Brazil, were compared to known glyphosate-susceptible biotypes. Dose-response curves were used to compare the biotypes, with rates ranging from 0 to 450 g ha^{-1} in one experiment, and from 0 to 1200 g ha^{-1} in another. The resistance factor, calculated with the I_{50} data, indicated the resistant biotypes were about three times less sensitive to glyphosate than the susceptible biotypes. This is the first report of a glyphosate-resistant biotype in a weed species of major importance and distribution in Brazil. A risk analysis is discussed for the occurrence of glyphosate-resistant wild poinsettia in glyphosate-tolerant soybeans.

Full article available at http://world-food.net/download/journals/2007-issue_2/e5.pdf

REV – Powles S. 2008. Evolved glyphosate-resistant weeds around the world: lesson to be learnt. *Pest Management Science,* 64, 360-365.

Glyphosate is the world's most important herbicide, with many uses that deliver effective and sustained control of a wide spectrum of unwanted (weedy) plant species. Until recently there were relatively few reports of weedy plant species evolving resistance to glyphosate. Since 1996, the advent and subsequent high adoption of tr-genic glyphosate-resistant crops in the Americas has meant unprecedented and often exclusive use of glyph,osate for weed controlover verylarge areas. Consequently, in regions oCthe USA where transgenic glyphosateresistant crops dominate, there are now evolved glyphosate-resistant populations of the economically damaging weed species *Ambrosia artemissifolia* L., *Atnbrosia trifida* L., *Atnaranthus paltneri* S Watson, *Amaranthus rudis'* JD Sauer, *Amaranthus tuberculatus* (Moq) JD Sauer and various *Conyza* and *Lolium* spp. Likewise, in areas' oftransgenic glyphosate-resistant crops in Argentina and Brazil, there are now evolved glyphosate-resistant populations of*Sorghum halepense* (L.) Pen and *Euphorbia hete-rophylla* L. respectively. As transgenic glyphosateresistant crops will remain very popular with producers, it is anticipated that glyphosate-resistant biotypes of other: prominent weed species will evolve over the next few years. Therefore, evolved glyphosate-resistant weeds are a major risk for the continued success of glyphosate and transgenic glyphosate-resistant crops. However, glyphosate-resistant weeds are not yet a problem in many parts ofthe world, and lessons can be learnt and actions taken, to achieve glyphosate sustainability. A major lesson is that maIntenance of diversity in weed management systems is crucial for glyphosate to be sustainable. Glyphosate is essential for present and future world food production, and action to secure its sustainability for future generations is a global imperative.

Full article available at https://www.ncbi.nlm.nih.gov/pubmed/18273881

REV - Binimelis, R.; Pengue, W.; Monterroso, I. 2009: Transgenic treadmill: Responses to the emergence and spread of glyphosate-resistant johnsongrass in Argentina. *Geoforum*, 40, 623-633.

The broad-spectrum herbicide glyphosate has become the largest-selling crop-protection product worldwide. The increased use of glyphosate is associated with the appearance of a growing number of tolerant or resistant weeds, with socio-environmental consequences apart from the loss of productivity. In 2002, a glyphosate-resistant biotype of johnsongrass (Sorghum halepense (L.)) appeared in Argentina and now covers at least 10,000 ha. This paper analyzes the driving forces behind the emergence and spread of this weed and also examines management responses and their implications. Preventive strategies against glyphosate-resistant johnsongrass fail because of the institutional setting. Reactive measures, however, transfer the risks to the society and the environment through the introduction of novel genetically modified crops that allow the use of yet more herbicide. This in turn reinforcesthe emergence of herbicide-resistant weeds, constituting a new phenomenon of intensification, the "transgenic treadmill".

http://stopogm.net/sites/stopogm.net/files/123456.pdf

Riar, D.; Norsworthy, J.; Johnson, D.; Scott, R.; Bagavathiannan, M. 2011. Glyphosate resistance in a Johnsongrass (*Sorghum halepense*) biotype from Arkansas. *Weed Science*, 59, 299-304. (1)

Johnsongrass is one of the most troublesome weeds of the world and is listed as a noxious weed in Arkansas. Reduced johnsongrass control with the recommended application rate of glyphosate (840 g ae ha21) was reported in a continuous soybean field near West Memphis, AR, in the fall of 2007. A greenhouse study was conducted (1) to confirm and characterize glyphosate resistance in the johnsongrass biotype from West Memphis and (2) to determine whether resistant and susceptible biotypes have differential glyphosate absorption or translocation. Dose–response studies revealed that the resistant biotype was five- to seven-fold less sensitive to glyphosate than the susceptible

biotype. Glyphosate absorption was similar in resistant and susceptible biotypes at 72 h after treatment (HAT). However, the treated leaf of the resistant biotype retained 28 percentage points more absorbed 14C glyphosate compared to the susceptible biotype at 72 HAT. Additionally, the resistant biotype had less 14C glyphosate translocated to the aboveground tissue below the treated leaf and to roots compared to the susceptible biotype at 24 and 72 HAT. Reduced translocation and increased retention of glyphosate in treated leaves is a probable mechanism of resistance in this glyphosate-resistant johnsongrass biotype.

Full article available at http://www.bioone.org/doi/pdf/10.1614/WS-D-10-00150.1

REV – Price, A.; Balkcom, K.; Culpepper, S.; Kelton, J.; Nichols, R.; Schomberg, H. 2011. Glyphosate-resistant Palmer amaranth: A threat to conservation tillage. *Journal of Soil and Water Conservation,* vol. 66(4) 265-275.

Conservation tillage reduces the physical movement of soil to the minimum required for crop establishment and production. When consistently practiced as a soil and crop management system, it greatly reduces soil erosion and is recognized for the potential to improve soil quality and water conservation and plant available water. Adoption of conservation tillage increased dramatically with the advent of transgenic, glyphosate-resistant crops that permitted in-season, over-the-top use of glyphosate (N-[phosphonomethyl] glycine), a broad-spectrum herbicide with very low mammalian toxicity and minimal potential for off-site movement in soil or water. Glyphosate-resistant crops are currently grown on approximately 70 million ha (173 million ac) worldwide. The United States has the most hectares (45 million ha [99 million ac]) of transgenic, glyphosate-resistant cultivars and the greatest number of hectares (46 million ha [114 million ac]) in conservation tillage. The practice of conservation tillage is now threatened by the emergence and rapid spread of glyphosate-resistant Palmer amaranth (*Amaranthus palmeri* [S.] Wats.), one of several amaranths commonly called pigweeds. First identified in Georgia, it now has been reported in Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Tennessee. Another closely related dioecious amaranth, or pigweed, common waterhemp (*Amaranthus rudis* Sauer), has also developed resistance to glyphosate in Illinois, Iowa, Minnesota, and Missouri. Hundreds of thousands of conservation tillage hectares, some currently under USDA Natural Resources Conservation Service conservation program contracts, are at risk of being converted to higher-intensity tillage systems due to the inability to control these glyphosate-resistant *Amaranthus* species in conservation tillage systems using traditional technologies. The decline of conservation tillage is inevitable without the development and rapid adoption of integrated, effective weed control strategies. Traditional and alternative weed control strategies, such as the utilization of crop and herbicide rotation and integration of high residue cereal cover crops, are necessary in order to sustain conservation tillage practices.

Full article available at https://goo.gl/K2F8A8

Gaines, T.; Cripps, A.; Powles, S. 2012. Evolved Resistance to Glyphosate in Junglerice (*Echinochloa colona*) from the Tropical Ord River Region in Australia. *Weed Technology* 26(3):480-484.

The objective of this study was to determine whether a junglerice population from the tropical Ord River region of northwest Australia was glyphosate resistant, and whether alternative herbicides labeled for junglerice control were still effective. Seed samples collected from the field site were initially screened with glyphosate in the glasshouse, and surviving individuals were self-pollinated for subsequent glyphosate dose-response studies. Glyphosate resistance was confirmed, as the suspected resistant population was found to be 8.6-fold more resistant to glyphosate than a susceptible population based on survival (LD_{50} of 3.72 kg ha^{-1}), and 5.6-fold more resistant based on biomass reduction (GR_{50} of 1.16 kg ha^{-1}). The glyphosate-

resistant population was susceptible to label-recommended doses of all other herbicides assessed, including three acetyl-CoA carboxylase (ACC) –inhibiting herbicides (fluazifop-P, haloxyfop, and sethoxydim), two acetolactate synthase (ALS) –inhibiting herbicides (imazamox and sulfometuron), paraquat, and glufosinate. Glyphosate resistance has previously evolved in numerous species found in glyphosate-resistant cropping systems, no-till chemical fallow, fence line, and perennial crop situations. Here we report the evolution of glyphosate resistance in a cropping system that included annual tillage. The evolution of glyphosate resistance in junglerice from a tropical cropping system further demonstrates the need for improved glyphosate stewardship practices globally.

http://www.bioone.org/doi/abs/10.1614/WT-D-12-00029.1

2.2.2 Resistance to the 2,4-D-based herbicides

COM – Egan, J.; Maxwell, B.; Mortensen, D.; Ryan, M.; Smith, R. 2011. 2,4-Dichlorophenoxyacetic acid (2,4-D)-resistant crops and the potential for evolution of 2,4-D-resistant weeds. *PNAS,* 108(11): E37.

Without summary.

Full article available at http://www.pnas.org/content/108/11/E37.full

Bernards, M.; Crespo, R.; Kruger, G.; Gaussoin, R.; Tranel, P. 2012. A Waterhemp (*Amaranthus tuberculatus*) Population Resistant to 2,4-D. *Weed Science*, 60 (3): 379 DOI: 10.1614/WS-D-11-00170.

A waterhemp population from a native-grass seed production field in Nebraska was no longer effectively controlled by 2,4-D. Seed was collected from the site, and dose-response studies were conducted to determine if this population was herbicide resistant. In the greenhouse, plants from the putative resistant and a susceptible waterhemp population were treated with 0, 18, 35, 70, 140, 280, 560, 1,120, or 2,240 g ae ha^{-1} 2,4-D. Visual injury estimates (I) were made 28 d after treatment (DAT), and plants were harvested and dry weights (GR) measured. The putative resistant population was approximately 10-fold more resistant to 2,4-D (RS ratio) than the susceptible population based on both I_{50} (50% visual injury) and GR_{50} (50% reduction in dry weight) values. The RS ratio increased to 19 and 111 as the data were extrapolated to I_{90} and GR_{90} estimates, respectively. GR_{50} doses of 995 g ha^{-1} for the resistant and 109 g ha^{-1} for the susceptible populations were estimated. A field dose-response study was conducted at the suspected resistant site with 2,4-D doses of 0, 140, 280, 560, 1,120, 2,240, 4,480, 8,960, 17,920, and 35,840 g ha^{-1}. At 28 DAT, visual injury estimates were 44% in plots treated with 35,840 g ha^{-1}. Some plants treated with the highest rate recovered and produced seed. Plants from the resistant and susceptible populations were also treated with 0, 9, 18, 35, 70, 140, 280, 560, or 1,120 g ae ha^{-1} dicamba in greenhouse bioassays. The 2,4-D resistant population was threefold less sensitive to dicamba based on I_{50} estimates but less than twofold less sensitive based on GR_{50} estimates. The synthetic auxins are the sixth mechanism-of-action herbicide group to which waterhemp has evolved resistance.

http://www.bioone.org/doi/abs/10.1614/WS-D-11-00170.1

2.2.3 Resistance to the Ammonium Glufosinate (AG)-based herbicides

Avila-Garcia, W.; Mallory-Smith, C. 2011. Glyphosate-resistant Italian ryegrass (*Lolium perenne*) populations also exhibit resistance to glufosinate. *Weed Science*, 59, 305-309.

Resistance to glufosinate has been confirmed in glyphosate-resistant Italian ryegrass populations collected in hazelnut orchards in Oregon. Dose–response, ammonia accumulation, and enzyme activity studies were conducted to test the sensitivity of three glyphosate-resistant and three susceptible Italian ryegrass populations to glufosinate. The glufosinate rates required to reduce the growth by 50% (GR_{50}) were 0.15, 0.18, and 0.21 for the control populations C1, C2, and C3, respectively, whereas for the resistant populations OR1, OR2, and OR3, the GR_{50} values were 0.49, 0.42, and 0.40 kg ai ha^{-1}, respectively, exhibiting an average resistance index of 2.4. The same trend was observed in ammonia accumulation studies between 48 and 96 h after glufosinate treatment where the susceptible populations accumulated on average two times more ammonia than the resistant populations. The glufosinate concentration required to reduce the glutamine synthetase enzyme activity by 50% (I_{50}) was not different for the resistant and susceptible populations. The I_{50}s ranged from 3.1 to 3.6 μM for the resistant populations and from 3.7 to 4.3 μM for the susceptible populations; therefore, an insensitive target site is not responsible for the glufosinate resistance.

Full article available at http://wssajournals.org/doi/full/10.1614/WS-D-11-00012.1

2.3 Inefficiency of the virus-resistance technologies

The transgenic technology of resistance to virus is still poorly developed and presents unsatisfactory results in terms of commercial scale[32]. One of the main reasons is associated to the fact that such technology uses biological mechanisms only partially known/understood, submitted to a number of genetic and epigenetic interactions susceptible to inactivate the transgene or prevent its full or partial expression, resulting in the inefficacy of its purposes.

In these latest years, one of the mechanisms responsible for the inefficacy of the virus resistance technology, obtained through genetic modification, is being better understood. It is the

32 In Brazil, this type of technology was commercially released in the case of beans 5.1, developed by Embrapa. With trade initially scheduled for 2013, the technology is not yet available for producers. According to the institution that developed the technology, this delay is due to supposed unexpected agronomic problems (greater sensitivity to other bean pathogens) and observed during the pre-marketing phase - but nevertheless released for commercial use with support from CTNBio.

PTG(*post-transducional gene silencing*[33]), epigenetic mechanism of gene silencing intended to the destruction of all the RNAm produced by certain transgenes, as well as all the homologous RNAm produced in the cell. Well, the permanent presence of these RNAm is essential for the transgenic plant to express the desired characteristic – the resistance to virus, in this case.

Researchers identified that, by "detecting" certain genomic sequences, a local response of the plant involving the PTGS extends to all the cells, inhibiting the operation of the technology.

Brault, V.; Pfeffer, S.; Erdinger, M.; Mutterer, J.; Ziegler-Graff, V. 2002. Virus-Induced Gene Silencing in Transgenic Plants Expressing the Minor Capsid Protein of *Beet western yellows virus. MPMI,* vol. 15, No. 8, pp. 799–807.

Transgenic *Nicotiana benthamiana* expressing the minor coat protein P74 of the phloem-limited *Beet western yellows virus* (BWYV) exhibited an unusual spatial pattern of posttranscriptional gene silencing (PTGS) when infected with BWYV or related viruses. Following infection, transgenic P74 and its mRNA accumulated to only low levels, 21 to 23 nucleotide RNAs homologous to the transgene appeared, and the transgene DNA underwent methylation. The infecting viral RNA, however, was not subject to significant silencing but multiplied readily and produced P74 in the phloem tissues, although the P74 encoded by the transgene disappeared from the phloem as well as the nonvascular tissues.

Full article available at http://apsjournals.apsnet.org/doi/abs/10.1094/MPMI.2002.15.8.799

Lucioli, A.; Noris, E.; Brunetti, A.; Tavazza, R.; Ruzza, V.; *et al.* 2003. *Tomato Yellow Leaf Curl Sardinia Virus* Rep-Derived Resistance to Homologous and Heterologous Geminiviruses Occurs by Different Mechanisms and Is Overcome if Virus-Mediated Transgene Silencing Is Activated. *J. Virol.,* 77, 6785–6798.

The replication-associated protein (Rep) of geminiviruses is involved in several biological processes brought about by the presence of distinct functional domains. Recently, we have exploited the multifunctional character of the *Tomato yellow leaf curl Sardinia virus* (TYLCSV) Rep to develop a molecular interference strategy to impair TYLCSV infection. We showed that transgenic expression of its N-terminal 210 amino acids (Rep-210) confers resistance to the homologous virus by inhibiting viral transcription and replication. We have now used biochemical and transgenic approaches to carry out a fuller investigation of the molecular resistance mechanisms in transgenic plants expressing Rep-210. We show that Rep-210 confers resistance through two distinct molecular mechanisms, depending on the challenging virus. Resistance to the homologous virus is achieved by the ability of Rep-210 to tightly inhibit C1 gene transcription, while that to heterologous virus is due to the interacting property of the Rep-210 oligomerization domain. Furthermore, we present evidence that in Rep-210-expressing plants, the duration of resistance is related to the ability of the

33 Neither the quality nor the quantity of mRNA produced are changed by the PTGS mechanism, indicating this mechanism is strictly post-transcriptional.

challenging virus to shut off transgene expression by a posttranscriptional homology-dependent gene silencing mechanism. A model of Rep-210-mediated geminivirus resistance that takes transgene- and virus-mediated mechanisms into account is proposed.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC156158/

Bian, X-Y.; Rasheed, M.; Seemanpillai, M.; Rezajan, M. 2006. Analysis of Silencing Escape of Tomato leaf curl virus: An Evaluation of the Role of DNA Methylation. *Mol. Plant Microbe Interact.,* 19, 614–624.

RNA silencing is a sequence-specific mechanism regulating gene expression and has been used successfully for antiviral defense against RNA viruses. Similar strategies to develop resistance against DNA containing *Tomato leaf curl virus* (TLCV) and some other geminiviruses have been unsuccessful. To analyze this silencing escape, we transformed tomato plants with a hairpin construct from the TLCV *C2* open reading frame (ORF). The transgenic plants showed a strong RNA silencing response, and following TLCV inoculation, their infection was delayed. However, the viral infection was not prevented and TLCV DNA accumulated to the levels found in nontransgenic plants. To determine the fate of a transgene carrying homology to the virus, we used transgenic plants carrying the TLCV *C4* gene, which induces a distinct phenotype. Upon TLCV infection, the phenotype was abolished and *C4* transcript disappeared. Concurrently, TLCV-specific small interfering RNAs were produced. In situ hybridization showed abundant levels of TLCV DNA in phloem cells of TLCV-infected *C4* transgenic plants. However, the *C4* transcripts were no longer detectable in nonvascular cells. Analysis of the transgene by methylation sequencing revealed a high level of de novo methylation of asymmetric cytosines in both the *C4* ORF and its 35S promoter. A high level of methylation also was found at both symmetric and asymmetric cytosines of the complementary-sense strand of TLCV double-stranded DNA. Given the previous finding that methylated geminiviral DNA is not competent for replication, we provide a model whereby TLCV evades host defense through a population of de novo synthesized unmethylated DNA.

Full article available at http://apsjournals.apsnet.org/doi/abs/10.1094/MPMI-19-0614

Ribeiro, S.; Lohuis, H.; Goldbach, R.; Prins, M. 2007. Tomato Chlorotic Mottle Virus Is a Target of RNA Silencing but the Presence of Specific Short Interfering RNAs Does Not Guarantee Resistance in Transgenic Plants. *J. Virol.,* 81, 1563–1573.

Tomato chlorotic mottle virus (ToCMoV) is a begomovirus found widespread in tomato fields in Brazil.ToCMoV isolate BA-Se1 (ToCMoV–[BA-Se1]) was shown to trigger the plant RNA silencing surveillancein different host plants and, coinciding with a decrease in viral DNA levels, small interfering RNAs(siRNAs) specific to ToCMoV-[BA-Se1] accumulated in infected plants. Although not homogeneouslydistributed, the siRNA population in both infected *Nicotiana benthamiana* and tomato plantsrepresented the entire DNA-A and DNA-B genomes. We determined that in *N. benthamiana*, the primary targetscorresponded to the 5_ end of AC1 and the embedded AC4, the intergenic region and 5_ end of AV1 andoverlapping central part of AC5. Subsequently, transgenic *N. benthamiana* plants were generated that were preprogrammed to express double-stranded RNA corresponding to this most targeted portion of the virusgenome by using an intron-hairpin construct. These plants were shown to indeed produce ToCMoVspecificsiRNAs. When challenge inoculated, most transgenic lines showed significant delays in symptomdevelopment, and two lines had immune plants. Interestingly, the levels of transgene-produced siRNAs were similar in resistant and susceptible siblings of the same line. This indicates that, in contrast to RNA viruses, the mere presence of transgene siRNAs corresponding to DNA virus sequences does not guarantee virus resistance and that other factors may play a role in determining RNA-mediated resistance to DNA viruses.

Full article available at http://jvi.asm.org/content/81/4/1563.full

COM – Lucioli, A.; Sallustio, D.; Barboni, D.; Berardi, A.; Papacchioli, V.; Tavazza, R.; Tavazza, M. 2008. A cautionary note on pathogen derived sequences. *Nat Biotechnol.,* v.26, p.617–619.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/18536679

Lafforgue, G.; Martinez, F.; Sardanyes, J.; Iglesia, F., Niu, Q-W.; Lin S-S., et al. 2011. Tempo and mode of plant RNA virus-escape from RNA interference-mediated resistance. *J Virol*, 85: 9686–95.

A biotechnological application of artificial microRNAs (amiRs) is the generation of plants that are resistant to virus infection. This resistance has proven to be highly effective and sequence specific. However, before these transgenic plants can be deployed in the field, it is important to evaluate the likelihood of the emergence of resistance-breaking mutants. Two issues are of particular interest: (i) whether such mutants can arise in nontransgenic plants that may act as reservoirs and (ii) whether a suboptimal expression level of the transgene, resulting in subinhibitory concentrations of the amiR, would favor the emergence of escape mutants. To address the first issue, we experimentally evolved independent lineages of *Turnip mosaic virus* (TuMV) (family Potyviridae) in fully susceptible wild-type Arabidopsis thaliana plants and then simulated the spillover of the evolving virus to fully resistant A. thaliana transgenic plants. To address the second issue, the evolution phase took place with transgenic plants that expressed the amiR at subinhibitory concentrations. Our results show that TuMV populations replicating in susceptible hosts accumulated resistance-breaking alleles that resulted in the overcoming of the resistance of fully resistant plants. The rate at which resistance was broken was 7 times higher for TuMV populations that experienced subinhibitory concentrations of the antiviral amiR. A molecular characterization of escape alleles showed that they all contained at least one nucleotide substitution in the target sequence, generally a transition of the G-to-A and C-to-U types, with many instances of convergent molecular evolution. To better understand the viral population dynamics taking place within each host, as well as to evaluate relevant population genetic parameters, we performed *in silico* simulations of the experiments. Together, our results contribute to the rational management of amiR-based antiviral resistance in plants.

Full article available at http://jvi.asm.org/content/85/19/9686.full.pdf+html

Other records applied to potential risks resulting from the use of transgenic technologies of resistance to virus indicate potential interactions between the recombinant viral protein synthesized in transgenic plants resistant to viruses and other pre-existing vegetable viruses. This could affect the efficiency of such biotechnologies, in addition to generating new types of risks, for which the evaluation reveals as being of great complexity.

REV – Latham, J.; Wilson, A. 2008. Trans-complementation and Synergism in Plants: Implications for Viral Transgenes? *Molecular Plant Pathology, 9* (1): 85-103.

In plants, viral synergisms occur when one virus enhances infection by a distinct or unrelated virus. Such synergisms may be unidirectional or mutualistic but, in either case, synergism implies that

protein(s) from one virus can enhance infection by another. A mechanistically related phenomenon is transcomplementation, in which a viral protein, usually expressed from a transgene, enhances or supports the infection of a virus from a distinct species. To gain an insight into the characteristics and limitations of these helper functions of individual viral genes, and to assess their effects on the plant–pathogen relationship, reports of successful synergism and transcomplementation were compiled from the peer-reviewed literature and combined with data from successful viral gene exchange experiments. Results from these experiments were tabulated to highlight the phylogenetic relationship between the helper and dependent viruses and, where possible, to identify the protein responsible for the altered infection process. The analysis of more than 150 publications, each containing one or more reports of successful exchanges, transcomplementation or synergism, revealed the following: (i) diverse viral traits can be enhanced by synergism and transcomplementation; these include the expansion of host range, acquisition of mechanical transmission, enhanced specific infectivity, enhanced cell-to-cell and long-distance movement, elevated or novel vector transmission, elevated viral titre and enhanced seed transmission; (ii) transcomplementation and synergism are mediated by many viral proteins, including inhibitors of gene silencing, replicases, coat proteins and movement proteins; (iii) although more frequent between closely related viruses, transcomplementation and synergism can occur between viruses that are phylogenetically highly divergent. As indicators of the interoperability of viral genes, these results are of general interest, but they can also be applied to the risk assessment of transgenic crops expressing viral proteins. In particular, they can contribute to the identification of potential hazards, and can be used to identify data gaps and limitations in predicting the likelihood of transgene-mediated transcomplementation.

http://www.ncbi.nlm.nih.gov/pubmed/18705887

Finally, as in the case of Bt and HT plants, it is possible that the genetic modification of resistance to virus generates ecological disturbs resulting in agronomic damages caused by insects or pathogens which, so far, had no expression taking them to be considered as crop pests[34]. For example, researchers noted that the presence of the virus resistance transgene in a transgenic cucumber fomented the aggressiveness of certain insect pests of such plant. The main "attraction" of the plant, for those herbivores, would have resulted in the crop contamination with another pathogen, a lethal bacterium for the cucumber and transmitted by that insect.

Sasu, M.; Ferrari, M.; Du, D.; Winsor, J.; Stephenson, A. 2009. Indirect costs of a nontarget pathogen mitigate the direct benefits of a virus-resistant transgene in wild Cucurbita. *Proc Natl Acad Sci USA,* 106(45): 19067-19071.

Virus-resistant transgenic squash are grown throughout the United States and much of Mexico and it is likely that the virus-resistant transgene (VRT) has been introduced to wild populations repeatedly. The evolutionary fate of any resistance gene in wild populations and its environmental impacts depend upon trade-offs between the costs and benefits of the resistance gene. In a 3-year field study using a wild gourd and transgenic and nontransgenic introgressives, we measured the effects of the transgene on fitness, on herbivory by cucumber beetles, on the incidence of mosaic viruses, and on the incidence of bacterial wilt disease (a fatal disease vectored by cucumber beetles). In each year, the first incidence of zucchini yellow

34 Such risks are illustrated in the following item 3.1.

mosaic virus occurred in mid-July and spread rapidly through the susceptible plants. We found that the transgenic plants had greater reproduction through both male and female function than the susceptible plants, indicating that the VRT has a direct fitness benefit for wild gourds under the conditions of our study. Moreover, the VRT had no effect on resistance to cucumber beetles or the incidence of wilt disease before the spread of the virus. However, as the virus spread through the fields, the cucumber beetles became increasingly concentrated upon the healthy (mostly transgenic) plants, which increased exposure to and the incidence of wilt disease on the transgenic plants. This indirect cost of the VRT (mediated by a nontarget herbivore and pathogen) mitigated the overall beneficial effect of the VRT on fitness.

Full article available at http://www.pnas.org/content/106/45/19067.full

3 Unexpected agronomic problems associated to the transgenic plants growth

3.1 Agronomic data per secondary and/or potential pests in Bt crops

In certain cases – and notably when there has been no development of populations of insects insensitive to Bt proteins – farmers who grow transgenic crops "protected" by Cry toxins may come to face significant damages due to the attack of other insects. The suppression of the traditional control forms, as well as the ecological gap resulting from the elimination of the target insects of Bt technology, has been pointed out as a stimulation element to the emergence of secondary pests. Under these conditions, insects which did not cause concerns assume such characteristic of intensity which may cause significant damages, with relevant economic impact.

This is particularly due to the selective emptying of certain ecological niches, which start to be occupied by competitor organisms of the affected species.

Ashouri, A.; Michaud, D.; Cloutier, C. 2001. Unexpected Effects of Different Potato Resistance Factors to the Colorado Potato Beetle (*Coleoptera: Chrysomelidae*) on the Potato Aphid (*Homoptera: Aphididae). Environmental Entomology*, vol. 30, no. 3, 524-532.

Improving the use of bioctechnological and classical plant resistance for herbivore pest control with less reliance on chemicals critically depends on predictable interactions with secondary pests. Performance

of the potato aphid *Macrosiphum euphorbiae* (Thomas), a secondary pest of potato in eastern North America, was studied on potato, *Solanum tuberosum* L., lines with traits of potential resistance to primary pests. The lines tested were 'Newleaf', a transgenic 'Superior' cultivar expressing the *Bacillus thuringiensis* Berliner CryIIIA toxin, which is highly resistant to the Colorado potato beetle, *Leptinotarsa decemlineata* (Say); a transgenic 'Kennebec' cultivar expressing rice cystatin I, a protease inhibitor previously shown to inhibit cathepsin like digestive enzymes in the Colorado potato beetle; NYL 235–4, a potato derived by selective breeding following hybridization with *S. berthaulthii*, with a moderate density of glandular trichomes providing resistance to small insects by contact; and the commercial cultivars Superior and Kennebec used as controls. Transgenic Superior potatoes negatively affected *M. euphorbiae*'s growth and fecundity, in contrast with the OCI potato, which improved aphid performance. The flight incidence of young alatae of *M. euphorbiae* that completed development on transgenic Superior was significantly higher than in aphids from other potato lines. Aphid resistance in the 'NYL 235–4' line was complex and depended on aphid access being limited to leaves, which reduced survival and fecundity. However, when aphids had access to whole NYL 235–4 plants, aphid performance was restored, as they preferentially fed and reproduced on NYL 235–4 stems and apical buds of unfolding leaflets. The results illustrate that the performance of a secondary pest of potato can vary unpredictably, depending on the nature of the resistance factors involved in developing specific resistance to a primary pest.

http://www.bioone.org/doi/abs/10.1603/0046-225X-30.3.524?journalCode=enve

Men, X.; Ge, F.; Edwards, C.; Yardim, E. 2004. Influence of Pesticide Applications on Pest and Predatory Arthropods Associated with Transgenic Bt Cotton and Nontransgenic Cotton Plants. *Phytoparasitica,* 32(3):246-254.

The effects of pesticide applications on pests (aphids and acarid mites) and predators (ladybeetles and spiders) were investigated in transgenic *Bt* cotton and nontransgenic cotton agroecosystems in 1999, 2000 and 2001. Transgenic cotton did not cause changes in populations of acarids and did not reduce numbers of predators considerably; its effects on aphids were inconsistent. Although insecticides were not applied against the main pest . cotton bollworm . on transgenic cotton, the total number of insecticide applications in 3 years was no less than the total applied on nontransgenic cotton, because additional applications were required against sucking pests on transgenic *Bt* cotton. Pesticide applications decreased numbers of aphids, acarids and predatory spiders significantly on both transgenic and nontransgenic cottons. The results suggest that the use of *Bt* cotton should be evaluated carefully in China.

Full article available at http://www.biosci.ohio-state.edu/~soilecol/Full%20articles/Ecology%20and%20Ecotoxicology/2004/Influence%20of%20pesticide%20applications.pdf

Men, X.; Ge, F.; Edwards, C.; Yardim, E. 2005. The influence of pesticide applications on Helicoverpa armigera Hübner and sucking pests in transgenic Bt cotton and non-transgenic cotton in China. *Crop Protection,* 24 (4): 319-324.

Effects of pesticide applications, based on an IPM program on cotton bollworm, *Helicoverpa armigera* Hübner, cotton mirids and cotton leafhoppers, were evaluated in transgenic *Bt*-cotton and non-transgenic cotton agroecosystems between 1999 and 2001 in China. Differences in pest populations between cotton varieties were also compared. In 1999 and 2000, bollworm populations on non-transgenic cotton were larger than those on transgenic *Bt*-cotton. In *Bt*-cotton fields, the numbers of fourth-generation bollworms were greater than those of in the second and the third generations over all 3 years of study. Leafhopper populations on *Bt*-cotton were consistently larger than those on non-transgenic cotton during the 3 years of study. Although the use of transgenic *Bt*-cotton decreased the need for insecticide applications against cotton bollworm, this relaxation from pesticide applications could cause increased populations of sucking insects, which could require additional insecticide applications.

http://www.sciencedirect.com/science/article/pii/S0261219404002182

COM – Pearson, H. 2006. Transgenic cotton drives insect boom. *Nature*. doi:10.1038/news060724-5.

Without summary.

Full article available at http://www.nature.com/news/2006/060724/full/news060724-5.html

Catangui, M.; Berg, R. 2006. Western bean cutworm, *Striacosta albicosta* (Smith) *(Lepidoptera: Noctuidae*), as a potential pest of transgenic Cry1Ab Bacillus thuringiensis corn hybrids in South Dakota. *Environmental Entomology,* 35: 1439-1452.

Injuries caused by the western bean cutworm, *Striacosta albicosta* (Smith), on transgenic Cry1Ab *Bacillus thuringiensis* (*Bt*) corn hybrids were documented and quantiÞed. The western bean cutworm is an emerging or potential pest of transgenic *Bt* corn in South Dakota. The proportion of ears infested with western bean cutworm larvae in the Cry1Ab *Bt* corn hybrids were 18-20, 38-70, and 0-34% in 2000, 2003, and 2004, respectively. The Cry1Ab *Bt* corn hybrids were almost completely free of European corn borer infestations. Untreated conventional corn hybrids were less infested with western bean cutworm larvae but more infested with European corn borer larvae. The proportion of ears infested with European corn borer larvae alone were 33, 58-80, and 8-25% in 2000, 2003, and 2004, respectively. Infestations with western bean cutworm alone were 28, 8-28, and 13-19%, respectively. Proportion of ears simultaneously infested with both western bean cutworm and European corn borer larvae were much lower than single infestations by either species alone, indicating niche overlap and competition. Simultaneous infestations by the two species on untreated conventional corn hybrids were only 8, 0-18, and 0-1% in 2000, 2003, and 2004. The corn grains harvested from injured ears were also analyzed for fumonisin and aßatoxin through quantitative enzyme-linked immunosorbent assays. More mycotoxins were found in 2003 when the levels of insect infestation in the corn ears were higher than in 2004. Results from this study underscore the need to investigate other emerging or potential arthropod pests of transgenic *Bt* corn hybrids in addition to the western bean cutworm.

Full article available at https://goo.gl/657KGJ

Wang, S.; Just, D. 2008. Pinstrup-Andersen P. Bt-cotton and secondary pests. *Int. J. Biotechnology,* 10(2/3): 113–121.

Bt-cotton seed has been effective to control the damage of bollworm in Chinese cotton production since 1999, reducing the need for pesticides and increasing incomes of Chinese farmers. Field data collected in 2004 indicates that these benefits have been eroded by increasing the use of pesticides aimed to control secondary pests. The combination of Bt-cotton seed and other forms of biological pest control may help farmers regain the economic and environmental benefits of previous years. Failure to find a solution, may lead to the discontinuation of the use of Bt-cotton seed in China and elsewhere.

http://www.inderscience.com/info/inarticle.php?artid=18348

Dorhout, D.; Rice, M. 2010. Intraguild competition and enhanced survival of western bean cutworm *(Lepidoptera: Noctuidae*) on transgenic Cry1Ab (MON810) *Bacillus thuringiensis* corn. *Journal of Economic Entomology,* 103: 54–62.

The effect of genetically modified corn (event MON810, YieldGard Corn Borer) expressing the Bacillus thuringiensis sp. kurstaki (Berliner) (Bt) endotoxin, Cry1Ab, on the survival of western bean cutworm, Striacosta albicosta (Smith), larvae was examined during intraguild competition studies with either European corn borer, Ostrinia nubilalis (Hübner), or corn earworm, Helicoverpa zea (Boddie), larvae. Competition scenarios were constructed by using either a laboratory or field competition arena containing one of five different diets and one of 13 different larval size-by-species scenarios. The survival of western bean cutworms competing with corn earworms in the laboratory arenas on either a meridic diet or isoline corn silk diet was significantly lower (P < or = 0.01) than the controls in 13 out of 14 competition scenarios and larval survival was frequently zero. In contrast, the survival of western bean cutworm competing with corn earworm on a Cry1Ab-MON810 corn silk diet was significant higher (P < or = 0.01) than the controls in four out of six competition scenarios. The results observed in the three way competitions involving the addition of European corn borers generally did not alter the outcomes observed in the western bean cutworm and corn earworm only two-way competitions. These data suggest that Cry1Ab-MON810 corn may confer a competitive advantage to western bean cutworm larvae during intraguild competition, particularly from corn earworms, and that western bean cutworms become equal competitors only when they are of equal or larger size and the diet is Cry1Ab-MON810 corn.

http://www.ncbi.nlm.nih.gov/pubmed/20214368

Lu, Y.; Wu, K.; Jiang, Y.; Xia, B.; *et al*. 2010. Mirid Bug Outbreaks in Multiple Crops Correlated with Wide-Scale Adoption of Bt Cotton in China. *Science,* vol. 328 nº 5982 pp. 1151-1154.

Long-term ecological effects of transgenic *Bacillus thuringiensis* (Bt) crops on nontarget pests have received limited attention, more so in diverse small holder–based cropping systems of the developing world. Field trials conducted over 10 years in northern China show that mirid bugs (Heteroptera: Miridae) have progressively increased population sizes and acquired pest status in cotton and multiple other crops, in association with a regional increase in Bt cotton adoption. More specifically, our analyses show that Bt cotton has become a source of mirid bugs and that their population increases are related to drops in insecticide use in this crop. Hence, alterations of pest management regimes in Bt cotton could be responsible for the appearance and subsequent spread of nontarget pests at an agro-landscape level.

Full article available at http://www.sciencemag.org/content/328/5982/1151.full

Zhao, J.; Ho, P.; Azadi, H. 2011. Benefits of Bt cotton counterbalanced by secondary pests? Perceptions of ecological change in China. *Environmental Monitoring and Assessment*, volume 173, Issue 1-4, pp 985-994.

In the past, scientific research has predicted a decrease in the effectiveness of Bt cotton due to the rise of secondary and other sucking pests. It is suspected that once the primary pest is brought under control, secondary pests have a chance to emerge due to the lower pesticide applications in Bt cotton cultivars. Studies on this phenomenon are scarce. This article furnishes empirical evidence that farmers in China perceive a substantial increase in secondary pests after the introduction of Bt cotton. The research is based on a survey of 1,000 randomly selected farm households in five provinces in China. We found that the reduction in pesticide use in Bt cotton cultivars is significantly lower than that reported in research elsewhere. This is consistent with the hypothesis suggested by recent studies that more pesticide sprayings are needed over time to control emerging secondary pests, such as aphids, spider mites, and lygus bugs. Apart from farmers' perceptions of secondary pests, we also assessed their basic knowledge of Bt cotton and their perceptions of Bt cotton in terms of its strengths and shortcomings (e.g., effectiveness, productivity, price, and pesticide use) in comparison with non-transgenic cotton.

http://www.ncbi.nlm.nih.gov/pubmed/20437270

Hagenbucher, S.; Wackers, F.; Wettstein, F.; Olson, D.; Ruberson, J.; Romeis J. 2013.

Pest tradeoffs in technology: reduced damage by caterpillars in Bt cotton benefits aphids. *Proceedings of the Royal Society B,* 20130042.

The rapid adoption of genetically engineered (GE) plants that express insecticidal Cry proteins derived from Bacillus thuringiensis (Bt) has raised concerns about their potential impact on non-target organisms. This includes the possibility that non-target herbivores develop into pests. Although studies have now reported increased populations of non-target herbivores in Bt cotton, the underlying mechanisms are not fully understood. We propose that lack of herbivore-induced secondary metabolites in Bt cotton represents a mechanism that benefits non-target herbivores.We showthat, because of effective suppression of Bt-sensitive lepidopteran herbivores, Bt cotton contains reduced levels of induced terpenoids.We also showthat changes in the overall level of these defensive secondary metabolites are associated with improved performance of a Bt-insensitive herbivore, the cotton aphid, under glasshouse conditions. These effects, however,were not as clearly evident under field conditions as aphid populations were not correlated with the amount of terpenoids measured in the plants. Nevertheless, increased aphid numbers were visible in Bt cotton compared with non-Bt cotton on some sampling dates. Identification of this mechanism increases our understanding of how insect-resistant crops impact herbivore communities and helps underpin the sustainable use of GE varieties.

Full article available at http://rspb.royalsocietypublishing.org/content/280/1758/20130042.full

Bortolotto, O.; Silva, G.; Bueno, A.; Pomari, A.; Martinelli, S.; Head, G.; Carvalho, R.; Barbosa, G. 2014.[35]Development and reproduction of Spodoptera eridania (Lepidoptera: Noctuidae) and its egg parasitoid Telenomus remus (Hymenoptera: Platygastridae) on the genetically modified soybean (Bt) MON 87701×MON 89788. *Bull Entomol Res,* 104(6):724-30. doi: 10.1017/S0007485314000546. Epub 2014 Sep 24.

Genetically modified crops with insect resistance genes from Bacillus thuringiensis Berliner (Bt-plants) are increasingly being cultivated worldwide. Therefore, it is critical to improve our knowledge of their direct or indirect impact not only on target pests but also on non-target arthropods. Hence, this study evaluates comparative leaf consumption and performance of Spodoptera eridania (Cramer), a species that is tolerant of the Cry1Ac protein, fed with Bt soybean, MON 87701×MON 89788 or its non-Bt isoline. We also assessed the comparative performance of the egg parasitoid Telenomus remus Nixon on eggs of S. eridania produced from individuals that fed on these two soybean isolines as larvae. Results showed that Bt soybean reduced by 2 days larval development and increased by 3 days adult male longevity. Therefore, we conclude that the effect of Bt soybean MON 87701×MON 89788 on S. eridania development and reproduction is small, and favorable to pest development. These differences are less likely to directly result from the toxin presence but indirectly from unintended changes in plant characteristics caused by the insertion of the transgene. Our results should be viewed as an alert that S. eridania populations may increase in Bt soybeans, but on the other hand, no adverse effects of this technology were observed for the egg parasitoid T. remus which can help to prevent S. eridania outbreaks on these crops.

http://www.ncbi.nlm.nih.gov/pubmed/25248849

In parallel, the use of the Bt technology in large scale may drastically decrease populations of certain target and non-target species,

35 A corrigendum, which comes to minimizing the risks associated with the use of this event (Bt soybeans) and to reduce the possibility of unintended effects of transgenic (explained in item 2 of Part 1 of this publication), was published by the same authors and is available at http://journals.cambridge.org/action/displayAbstract?fromPage=online&aid=9500235&fileId=S0007485314000881.

exacerbating the ecological imbalances existing in the agricultural-ecological systems. Well, some of these species are sometimes natural enemies (or competitors) of others, potentially pests of the same cultures. Under these circumstances, the elimination of some insects promotes population explosions of others - not sensitive to Bt. The scientific literature has recorded the emergence of new pests in Bt maize crops, supposedly stimulated by the population decrease of others, which controlled the first ones and which had their populations decreased for being a Bt target[36].

Tooker, J.; Fleischer, S. 2010. First report of western bean cutworm (*Striacosta albicosta*) in Pennsylvania. Online. *Crop Management*, doi:10.1094/CM-2010-0616-01-RS.

These results demonstrate that western bean cutworm moths were present in Pennsylvania in 2009, and the distribution and quality of the specimens suggest that populations have established. The data also suggest that universal traps work well for detection of low-level populations at least as well as milk jug traps.

Full article available at http://ento.psu.edu/publications/wbc-pa

Michel, A.; Krupke, C.; Baute, T.; Difonzo, C. 2010. Ecology and Management of the Western Bean Cutworm (Lepidoptera:Noctuidae) in Cornand Dry Beans. *Journal of Integrated Pest Management*, 1(1): 2010; DOI:10.1603/IPM10003.

The western bean cutworm, *Striacosta albicosta* (Smith) (Lepidoptera:Noctuidae),is a native North American pest that feeds mainly on corn and dry beans. The historical geographic range of the western bean cutworm coveredthe western Great Plains states, including Colorado, Nebraska, and Wyoming. Since 1999, the geographic range of the western bean cutworm hasrapidly expanded east ward across the United States Corn Belt, causing significant and economic damage to corn and dry beans in parts of this region. This expansion has led to a resurgence of interesting this pest, particularly in areas where it has most recently caused damage. We summarize the ecology and biology of western bean cutworm and discuss options for scouting and management, with an emphasis in the expanded geographical range.

Full article available at https://goo.gl/tNRzOe

36 In Brazil this chain of ecological effects is seen as responsible for major economic losses faced by producers of soy and cotton (measured in millions of *reais* to the state of Bahia, years 2012/2013). Indeed, the elimination of large populations of *Spodoptera sp.* (natural predators of the pest *Helicoverpa armigera*) would have led to a population explosion of *H. armigera*, new species of pest of soybeans and cotton, among other crops. No articles on the topic were found in scientific journals.

3.2 Agronomic damages by the intensive use of the herbicides associated to HT plants

The herbicide-tolerance (HT) technology depend on the use of a certain herbicide – generally systemic – which will be applied in several moments of the production cycle, on an isolate way or combined with other herbicide(s) which also do not cause lethal damages to that plant. Such facility to operate applications regardless of the culture cycle deeply simplified the agriculture management of the main crops, at the same time on which it has drastically expanded the sales of agrochemicals associated to the HT-type transgenic varieties.

In addition to the impacts to the health and the environment, resulting from the increased use of the same poisons, the technology showed ability to generate disturbs in the soil microbiota and in the exchanges of soil-plant nutrients, decreasing the crop's productive capacity, further causing agronomic damages to crops located in adjacent areas or in the same sites, in subsequent harvests.

3.2.1 Negative impacts of glyphosate on the productivity of HT plants

Many documents available in the scientific literature point out that glyphosate-based herbicides physiologically weaken the plants (particularly soy), affect the soil microbiota communities which contribute to the good agronomic performance of the crops and create imbalances in the seed banks and population relationship associated to them.

Ismail, B.; Kader, A.; Omar, O. 1995. Effects of Glyphosate on Cellulose Decomposition in Two Soils. *Folia Microbiol.*, 40 (5), 499-502.

Glyphosate with an equivalent concentration of either 0, 2.16 or 8.64 kg/hm² was sprayed on to cellulosic materials before burying in two soil types; peat (soil I) and sandy clay loam (soil II). Alternatively the soils were sprayed with 0, 20 or 150 ppm of the herbicide before burying the cellulosic material either

immediately or after preincubation for 4 weeks. In soil I, the increase in glyphosate concentrations substantially reduced the decomposition of cellulosic material regardless of the method of application employed. Glyphosate at 8.64 kg/hm² reduced the mass loss of the treated substrate by 83 %. However, cellulose decomposition in soil preincubated for 4 weeks before burying was affected almost to the same extent as the untreated control. Glyphosate stimulated cellulose decomposition when substrates were buried in soil II. Mass loss in soil treated with 150 ppm increased by about 100 % while when glyphosate was sprayed directly to the substrate (at 8.64 kg/hm²), the loss was about 25 %.

http://link.springer.com/article/10.1007/BF02814731

King, C.; Purcell, L.; Vories, E. 2001. Plant growth and nitrogenase activity of glyphosate-tolerant soybean in response to foliar glyphosate applications. *Agronomy Journal,* 93 (1), 179-186.

Glyphosate [*N*-(phosphonomethyl)glycine] inhibits 5-enolpyruvylshikimate-3-phosphate synthase, EC 2.5.1.19 (EPSPS), thereby blocking aromatic amino acid synthesis. While glyphosate-tolerant (GT) soybean [*Glycine max* (L.) Merr.] contains resistant EPSPS, the N_2-fixing symbiont in soybean root nodules, *Bradyrhizobium japonicum*, does not contain a resistant enzyme, and glyphosate spray to GT soybean may interfere with the symbiotic relationship. Glyphosate-tolerant soybean was treated with glyphosate at several different stages of development to evaluate N_2 fixation, growth, and yield in a series of greenhouse, growth chamber, and field experiments. Early applications of glyphosate generally delayed N_2 fixation and decreased biomass and N accumulation in the cultivar Terral TV5866RR (TV5866RR) harvested at 19 d after emergence (DAE), but plants had recovered by 40 DAE. The biomass and N content of GT soybean were also decreased by glyphosate in plants that were grown with available soil N. There were differences in sensitivity to glyphosate among GT cultivars, with biomass decreases in response to glyphosate ranging from 0 to 30% at 40 DAE for the most tolerant and sensitive cultivars that were evaluated. In growth chamber studies, N_2 fixation was more sensitive to water deficits in glyphosate-treated plants. In field studies, there was no measured effect of glyphosate on GT soybean at Fayetteville, AR where there was adequate soil water throughout the growing season. However, glyphosate tended to decrease biomass and seed yields under conditions of limited soil water at Keiser, AR.

https://www.agronomy.org/publications/aj/abstracts/93/1/179

Reddy, K.; Zablotowicz, R. 2003. Glyphosate resistant soybean response to various salts of glyphosate and glyphosate accumulation in soybean nodules. *Weed Science,* 51 (4): 496-502.

A field study was conducted during 2000 and 2001 at Stoneville, MS, to determine the effects of isopropylamine, trimethylsulfonium (Tms), diammonium, and aminomethanamide dihydrogen tetraoxosulfate (Adt) salt formulations of glyphosate on weed control, growth, chlorophyll content, nodulation, nitrogen content, and grain yield in glyphosate-resistant soybean and to assess potential glyphosate accumulation in soybean nodules. Glyphosate-Tms and glyphosate-Adt injured soybean, and visible injury ranged from 29 to 38% 2 d after late postemergence (LPOST) application; however, soybean recovered by 14 d. Glyphosate formulations had no effect on chlorophyll content, root and shoot dry weight, or nodule number but reduced nodule biomass by 21 to 28% 14 d LPOST. Glyphosate levels in nodules from treated plants ranged from 39 to 147 ng g21 (dry weight), and leghemoglobin content was reduced by as much as 10%. Control of five predominant weed species 14d after LPOST was . 83% with one application and . 96% with two applications regardless of the glyphosate salts used. Soybean yields were generally higher with two applications than with one application regardless of glyphosate formulation. These results indicate that soybean injury and inhibition of nodule development with certain glyphosate formulations can occur, but soybean has the potential to recover from glyphosate stress.

Full article available at http://naldc.nal.usda.gov/download/48637/PDF

Kremer, R.; Means, N.; Kim, S. 2005. Glyphosate affects soybean root exudation and rhizosphere microorganisms. *Int. J. of Environmental Analytical Chemistry,* 85 (15): 1165-1174.

Glyphosate is a nonselective, broad-spectrum herbicide that kills plants by inhibiting the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS), which is necessary for synthesis of aromatic amino acids. A secondary mode of action involves infection of roots by soilborne microorganisms due to decreased production of plant protection compounds known as phytoalexins. Varieties of several crops, including glyphosate-resistant (GR) or Roundup Ready® soybean, are genetically modified to resist the herbicidal effects of glyphosate and provide farmers with an effective weed management tool. After glyphosate is applied to GR soybean, glyphosate that is not bound to glyphosate-resistant EPSPS is translocated throughout the plant and accumulates primarily in meristematic tissues. We previously reported that fungal colonization of GR soybean roots increased significantly after application of glyphosate but not after conventional postemergence herbicides. Because glyphosate may be released into soil from GR roots, we characterized the response of rhizosphere fungi and bacteria to root exudates from GR and non-GR cultivars treated with and without glyphosate at field application rates. Using an immunoassay technique, the flux of glyphosate detected in exudates of hydroponically-grown GR soybean was > 1000 ng plant-1 over the 16-d post-glyphosate application period. Glyphosate also increased carbohydrate and amino acid contents in root exudates in both soybean cultivars. However, GR soybean released higher carbohydrate and amino acid contents in root exudates than non-GR soybean without glyphosate treatment. In vitro bioassays showed that glyphosate in the exudates stimulated growth of selected rhizosphere fungi, possibly by providing a selective C and N source combined with the high levels of soluble carbohydrates and amino acids associated with glyphosate treatment of the soybean plants. Increased fungal populations that develop under glyphosate treatment of GR soybean may adversely affect plant growth and biological processes in the soil and rhizosphere.

Full article available at https://goo.gl/uyJaCw

Neumann, G.; Kohls, S.; Landsberg, E.; Souza, K.; Yamada, T.; Romheld, V. 2006. Relevance of glyphosate transfer to non-target plants via the rhizosphere. *Journal of Plant Diseases and Protection,* 20: 963–969.

There is a common understanding that the widely used herbicide glyphosate is easily degraded and adsorbed in soils and thus, harmless for use in agriculture. We can demonstrate, however, that this conclusion is wrong and dangerous for farmers because in former risk assessments the behaviour of glyphosate in the rhizosphere was not properly considered. In nutrient solution, rhizobox and pot experiments we can show that foliar applied glyphosate to target plants is released into the rhizosphere after a fast translocation from shoots to roots. In the rhizosphere glyphosate can obviously be stabilized long enough to achieve negative effects on non-target plants. Such a negative side effect is for example inhibited acquisition of micronutrients such as Mn, but also Zn, Fe and B, which are involved in plant own disease resistance mechanisms. From this glyphosate transfer from target to non-target plants (e.g. from weed to trees in orchards) we predict an increase in disease problems, particularly on soils with low micronutrient availability as already reported in the USA. In view of plant and soil health, we urgently call for a re-assessment of glyphosate as herbicide.

Full article available at https://www.researchgate.net/publication/228489677_Relevance_of_glyphosate_transfer_to_non-target_plants_via_the_rhizosphere

Zablotowicz, R.; Reddy, K. 2007. Nitrogenase activity, nitrogen content, and yield responses to glyphosate in glyphosate-resistant soybean. *Crop Prot,* 26:370-6.

Transgenic glyphosate-resistant (GR) soybean [*Glycine max* (L.) Merr.] expressing a glyphosate-insensitive 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme has provided new opportunities for weed control in soybean production. However, glyphosate is toxic to the soybean nitrogen-fixing symbiont, *Bradyrhizobium japonicum,* as its EPSPS enzyme is sensitive to glyphosate. The effects of glyphosate on symbiotic parameters, nitrogen accumulation, and yield in GR soybean under weed-free conditions were determined in a 3-yr field study during 2002–2004. Four glyphosate (0.84, 1.68, 2.52+2.52, and 0.84+0.84 kg ae/ha) treatments applied at 4 and 6 weeks after planting (WAP) soybean were compared to a no glyphosate, hand weeded (weed-free) control. In 2002 and 2003, soybean plants were harvested at 5, 6, 7, and 8 WAP, and roots assessed for nitrogenase activity (acetylene reduction assay, ARA), root respiration, nodulation, and root biomass. Soybean seed yield, leaf and seed nitrogen content were determined in all three years. No consistent effect of glyphosate was observed on either ARA or root respiration. In 2002, both ARA and respiration were about a third of that in 2003, attributed to early-season drought in 2002. All glyphosate treatments reduced foliar nitrogen content (26–42%) in 2002. In 2003 and 2004, three and two glyphosate treatments, respectively, reduced foliar nitrogen content (8–13%), with the greatest reduction when glyphosate was applied at the highest rate. Soybean yield was reduced by 11% with two applications of 2.52 kg ae/ha glyphosate compared to hand weeded control in 2002, but seed yield was not affected in 2003 and 2004. Total seed nitrogen harvested in 2002 and 2003 was reduced by 32% and 17%, respectively, when two applications of 2.52 kg ae/ha glyphosate were applied compared to hand weeded soybean. These studies indicate that nitrogen fixation and/or assimilation in GR soybean was only slightly affected at label use rate, but was consistently reduced at above label use rates of glyphosate and the greatest reductions occurred with soil moisture stress following glyphosate application.

http://www.sciencedirect.com/science/article/pii/S0261219406002584

Bott, S.; Tesfamariam, T.; Candan, H.; Cakmak, I.; Römheld, V.; Neumann, G. 2008. Glyphosate-induced impairment of plant growth and micronutrient status in glyphosate-resistant soybean (*Glycine max* L.). *Plant Soil,* 312:185–194.

This investigation demonstrated potential detrimental side effects of glyphosate on plant growth and micronutrient (Mn, Zn) status of a glyphosate-resistant (GR) soybean variety (Glycine max cv. Valiosa), which were found to be highly dependent on the selected growth conditions. In hydroponic experiments with sufficient Mn supply [0.5 µM], the GR cv. Valiosa produced similar plant biomass, root length and number of lateral roots in the control treatment without glyphosate as compared to its non-GR parental line cv. Conquista. However, this was associated with 50% lower Mn shoot concentrations in cv. Conquista, suggesting a higher Mn demand of the transgenic cv. Valiosa under the selected growth conditions. Glyphosate application significantly inhibited root biomass production, root elongation, and lateral root formation of the GR line, associated with a 50% reduction of Mn shoot concentrations. Interestingly, no comparable effects were detectable at low Mn supply [0.1 µM]. This may indicate Mn-dependent differences in the intracellular transformation of glyphosate to the toxic metabolite aminomethylphosphonic acid (AMPA) in the two isolines. In soil culture experiments conducted on a calcareous loess sub-soil of a Luvisol (pH 7.6) and a highly weathered Arenosol (pH 4.5), shoot biomass production and Zn leaf concentrations of the GR-variety were affected by glyphosate applications on the Arenosol but not on the calcareous Loess sub-soil. Analysis of micronutrient levels in high and low molecular weight (LMW) fractions (80% ethanol extracts) of young leaves revealed no indications for internal immobilization of micronutrients (Mn, Zn, Fe) by excessive complexation with glyphosate in the LMW phase.

Full article available at http://stopogm.net/sites/stopogm.net/files/GlyphosateBott.pdf

Johal, G.; Huber, D. 2009. Glyphosate effects on diseases of plants. *Europ. J. Agronomy,* 31, 144-152.

Glyphosate, N-(phosphonomethyl) glycine, is the most extensively used herbicide in the history of agriculture. Weed management programs in glyphosate resistant (GR) field crops have provided highly effective weed control, simplified management decisions, and given cleaner harvested products. However, this relatively simple, broad-spectrum, systemic herbicide can have extensive unintended effects on nutrient efficiency and disease severity, thereby threatening its agricultural sustainability. A significant increase in disease severity associated with the wide spread application of the glyphosate herbicide can be the result of direct glyphosate-induced weakening of plant defenses and increased pathogen population and virulence. Indirect effects of glyphosate on disease predisposition result from immobilization of specific micronutrients involved in disease resistance, reduced growth and vigor of the plant from accumulation of glyphosate in meristematic root, shoot, and reproductive tissues, altered physiological efficiency, or modification of the soil microflora affecting the availability of nutrients involved in physiological disease resistance. Strategies to ameliorate the predisposing effects of glyphosate on disease include judicious selection of herbicide application rates, micronutrient amendment, glyphosate detoxification in meristematic tissues and soil, changes in cultural practices to enhance micronutrient availability for plant uptake, and biological amendment with glyphosate-resistant microbes for nitrogen fixation and nutrient availability. Given that recommended doses of glyphosate are often many times higher than needed to control weeds, we believe the most prudent method to reduce the detrimental effects of glyphosate on GR crops will be to use this herbicide in as small a dose as practically needed. Such a frugal approach will not only curtail disease predisposition of GR crops, but will also benefit the grower and the environment.

Full article available at https://goo.gl/fDLrwb

Fernandez, M.; Zentner, R.; Basnyat, P.; Gehl, D.; Selles, F.; Huber, D. 2009. Glyphosate associations with cereal diseases caused by Fusarium spp. in the Canadian prairies. *European Journal of Agronomy*, 31: 133–143.

Fusarium pathogens cause important diseases, such as root/crown rot and Fusarium head blight (FHB), in cereal crops. These diseases can be caused by similar *Fusarium* spp. Common root rot (CRR) is widespread in the western Canadian Prairies, whereas FHB has potential of becoming an important disease in this region. There are no commercially available cereal cultivars with good resistance to these diseases. It is therefore important to identify agronomic practices that could affect levels of *Fusarium* pathogens in cereals. This review deals primarily with the effects of tillage systems and glyphosate use on the development of FHB and CRR in wheat and barley in eastern Saskatchewan. Although the FHB study in 1999–2002 indicated that environment was the most important factor determining FHB development, previous glyphosate use and tillage practice were among the production factors with the greatest association with FHB. Overall, disease was highest in crops under minimum-till management. Previous glyphosate use was consistently associated with higher FHB levels caused by the most important FHB pathogens, *Fusarium avenaceum* and *Fusarium graminearum. Cochliobolus sativus*, the most common CRR pathogen, was negatively associated with previous glyphosate use, while *F. avenaceum,F. graminearum*, and other fungi were positively associated, suggesting that glyphosate might cause changes in fungal communities. The occurrence and isolation of *F. avenaceum* from cereal residues were greater under reduced-till than conventional-till while *C. sativus* was most common under conventional-till, and *F. graminearum* was lowest under zero-till. Previous glyphosate applications were again correlated positively with *F. avenaceum* and negatively with *C. sativus*. These observations agreed with results from the FHB and CRR studies. These are the first studies that established a relationship between previous glyphosate use and increased *Fusarium* infection of spikes and subcrown internodes of wheat and barley, or *Fusarium* colonization of crop residues. However, because of the close association between noncereal crops, reduced tillage and glyphosate use, it was not possible to completely separate the effects of these factors on *Fusarium* infections. Determining the relative contribution of these popular production trends to the development of diseases caused by *Fusarium* spp. are essential for devising appropriate agronomic recommendations to prevent their further spread in western Canada, and to reduce the impact that these diseases are having in areas where they are already established. The consistent association between previous glyphosate use and

Fusarium infections also warrants further research to elucidate the nature of this association and the underlying mechanisms determining these effects.

https://goo.gl/TZlCxG

Kremer, R.; Means, N. 2009. Glyphosate and glyphosate-resistant crop interactions with rhizosphere microorganisms. *European Journal of Agronomy,* 31 (3): 153-161.

Current crop production relies heavily on transgenic, glyphosate-resistant (GR) cultivars. Widespread cultivation of transgenic crops has received considerable attention. Impacts of glyphosate on rhizosphere microorganisms and activities are reviewed based on published and new data from long-term field projects documenting effects of glyphosate applied to GR soybean and maize. Field studies conducted in Missouri, U.S.A. during 1997–2007 assessed effects of glyphosate applied to GR soybean and maize on root colonization and soil populations of *Fusarium* and selected rhizosphere bacteria. Frequency of root-colonizing *Fusarium* increased significantly after glyphosate application during growing seasons in each year at all sites. Roots of GR soybean and maize treated with glyphosate were heavily colonized by *Fusarium* compared to non-GR or GR cultivars not treated with glyphosate. Microbial groups and functions affected by glyphosate included Mn transformation and plant availability; phytopathogen–antagonistic bacterial interactions; and reduction in nodulation. Root-exuded glyphosate may serve as a nutrient source for fungi and stimulate propagule germination. The specific microbial indicator groups and processes were sensitive to impacts of GR crops and are part of an evolving framework in developing polyphasic microbial analyses for complete assessment of GR technology that is more reliable than single techniques or general microbial assays.

http://www.sciencedirect.com/science/article/pii/S1161030109000641

Zobiole, L.; Oliveira, R.; Visentainer, J.; Kremer, R.; Bellaloui, N.; Yamada, T. 2010. Glyphosate affects seed composition in glyphosate-resistant soybean. *J. Agric. Food Chem.,* 58 (7), 4517-4522.

The cultivation of glyphosate-resistant (GR) soybeans has continuously increased worldwide in recent years mainly due to the importance of glyphosate in current weed management systems. However, not much has been done to understand eventual effects of glyphosate application on GR soybean physiology, especially those related to seed composition with potential effects on human health. Two experiments were conducted to evaluate the effects of glyphosate application on GR soybeans compared with its near-isogenic non-GR parental lines. Results of the first experiment showed that glyphosate application resulted in significant decreases in shoot nutrient concentrations, photosynthetic parameters, and biomass production. Similar trends were observed for the second experiment, although glyphosate application significantly altered seed nutrient concentrations and polyunsaturated fatty acid percentages. Glyphosate resulted in significant decreases in polyunsaturated linoleic acid (18:2n-6) (2.3% decrease) and linolenic acid (18:3n-3) (9.6% decrease) and a significant increase in monounsaturated fatty acids 17:1n-7 (30.3% increase) and 18:1n-7 (25% increase). The combined observations of decreased photosynthetic parameters and low nutrient availability in glyphosate-treated plants may explain potential adverse effects of glyphosate in GR soybeans.

http://www.ncbi.nlm.nih.gov/pubmed/20307082

Zobiole, L.; Bonini, E.; Oliveira, R.; Kremer, R. ; Ferrarese, O. 2010. Glyphosate affects lignin content and amino acid production in glyphosate-resistant soybean. *Acta Physiologiae Plantarum*, 32, 831–837.

Farmers report that some glyphosate-resistant soybean varieties are visually injured by glyphosate.

Glyphosate is the main herbicide that directly affects the synthesis of secondary compounds. In this work, we evaluated the effect of increasing rates of glyphosate on lignin and amino acid content, photosynthetic parameters and dry biomass in the early maturity group cultivar BRS 242 GR soybean. Plants were grown in half-strength complete nutrient solution and subjected to various rates of glyphosate either as a single or in sequential applications. All parameters evaluated were affected by increasing glyphosate rates. The effects were more pronounced as glyphosate rates increased, and were more intense with a single total application than sequential applications at lower rates.

http://link.springer.com/article/10.1007%2Fs11738-010-0467-0

Zobiole, L.; Oliveira, R.; Kremer, R.; Constantin, J.; Yamada, T.; Castro, C.; Oliveira, F.; Oliveira, A.; 2010. Effect of glyphosate on symbiotic N2 fixation and nickel concentration in glyphosate-resistant soybeans. *Applied Soil Ecology*, 44: 176-180.

Decreased biological nitrogen fixation in glyphosate-resistant (GR) soybeans has been attributed directly to toxicity of glyphosate or its metabolites, to N2-fixing microorganisms. As a strong metal chelator, glyphosate could influence symbiotic N2 fixation by lowering the concentration of nickel (Ni) that is essential for the symbiotic microorganisms. Evaluation of different cultivars grown on different soil types at the State University of Maringa´ , PR, Brazil during the summer 2008 revealed, significant decreases in photosynthetic parameters (chlorophyll, photosynthetic rate, transpiration and stomatal conductance) and nickel content with glyphosate use (single or sequential application). This work demonstrated that glyphosate can influence the symbiotic N2 fixation by lowering nickel content available to the symbiotic microorganisms.

Full article available at https://naldc.nal.usda.gov/download/39648/

Zobiole, L.; Kremer, R.; Oliveira, R.; Constantin, J. 2011. Glyphosate affects micro-organisms in rhizospheres of glyphosate-resistant soybeans. *Journal of Applied Microbiology*, 110: 118-127.

Aims: Glyphosate-resistant (GR) soybean production increases each year because of the efficacy of glyphosate for weed management. A new or 'second' generation of GR soybean (GR2) is now commercially available for farmers that is being promoted as higher yielding relative to the previous, 'first generation' (GR1) cultivars. Recent reports show that glyphosate affects the biology and ecology of rhizosphere micro-organisms in GR soybean that affect yield. The objective of this research was to evaluate the microbiological interactions in the rhizospheres of GR2 and GR1 soybean and the performance of the cultivars with different rates of glyphosate applied at different growth stages.
Methods and Results: A greenhouse study was conducted using GR1 and GR2 soybean cultivars grown in a silt loam soil. Glyphosate was applied at V2, V4 and V6 growth stages at three rates. Plants harvested at R1 growth stage had high root colonization by Fusarium spp.; reduced rhizosphere fluorescent pseudomonads, Mn-reducing bacteria, and indoleacetic acid–producing rhizobacteria; and reduced shoot and root biomass.
Conclusions: Glyphosate applied to GR soybean, regardless of cultivar, negatively impacts the complex interactions of microbial groups, biochemical activity and root growth that can have subsequent detrimental effects on plant growth and productivity.
Significance and Impact of the Study: The information presented here will be crucial in developing strategies to overcome the potential detrimental effects of glyphosate in GR cropping systems.

Full article available at http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2672.2010.04864.x/full

3.2.2 Agronomic damages in adjacent and/or subsequent crops

Studies show that, due to the drift, in the form of airborne microdrops and/or due to their transportation in superficial waters or, further, by means of their accumulation in the soil (affecting the rhizosphere and mycorrhizas), the glyphosate, 2,4-D, dicamba and other herbicides may cause relevant agronomic damages both for the culture where they are applied and also in subsequent or adjacent crops.

Eker, S.; Ozturk, L.; Yazici, A.; Erenoglu, B.; Romheld, V.; Cakmak, I. 2006. Foliar-applied glyphosate substantially reduced uptake and transport of iron and manganese in sunflower (*Helianthus annuus L.*) plants. *J. Agric. Food Chem.*, 54 (26). pp 10019-10025.

Evidence clearly shows that cationic micronutrients in spray solutions reduce the herbicidal effectiveness of glyphosate for weed control due to the formation of metal-glyphosate complexes. The formation of these glyphosate-metal complexes in plant tissue may also impair micronutrient nutrition of nontarget plants when exposed to glyphosate drift or glyphosate residues in soil. In the present study, the effects of simulated glyphosate drift on plant growth and uptake, translocation, and accumulation (tissue concentration) of iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) were investigated in sunflower (Helianthus annuus L.) plants grown in nutrient solution under controlled environmental conditions. Glyphosate was sprayed on plant shoots at different rates between 1.25 and 6.0% of the recommended dosage (i.e., 0.39 and 1.89 mM glyphosate isopropylamine salt). Glyphosate applications significantly decreased root and shoot dry matter production and chlorophyll concentrations of young leaves and shoot tips. The basal parts of the youngest leaves and shoot tips were severely chlorotic. These effects became apparent within 48 h after the glyphosate spray. Glyphosate also caused substantial decreases in leaf concentration of Fe and Mn while the concentration of Zn and Cu was less affected. In short-term uptake experiments with radiolabeled Fe (59Fe), Mn (54Mn), and Zn (65Zn), root uptake of 59Fe and 54Mn was significantly reduced in 12 and 24 h after application of 6% of the recommended dosage of glyphosate, respectively. Glyphosate resulted in almost complete inhibition of root-to-shoot translocation of 59Fe within 12 h and 54Mn within 24 h after application. These results suggest that glyphosate residues or drift may result in severe impairments in Fe and Mn nutrition of nontarget plants, possibly due to the formation of poorly soluble glyphosate-metal complexes in plant tissues and/or rhizosphere interactions.

http://www.ncbi.nlm.nih.gov/pubmed/17177536

Cakmak I., Yazici A., Tutus Y., Ozturk L. (2009) Glyphosate reduced seed and leaf concentrations of calcium, manganese, magnesium, and iron in non-glyphosate resistant soybean. *Eur J Agron,* 31(3):114-9.

Greenhouse experiments were conducted to study the effects of glyphosate drift on plant growth and concentrations of mineral nutrients in leaves and seeds of non-glyphosate resistant soybean plants (*Glycine max*, L.). Glyphosate was sprayed on plant shoots at increasing rates between 0.06 and 1.2% of the recommended application rate for weed control. In an experiment with 3-week-old plants, increasing application of glyphosate on shoots significantly reduced chlorophyll concentration of the young leaves and shoots dry weight, particularly the young parts of plants. Concentration of shikimate due to increasing glyphosate rates was nearly 2-fold for older leaves and 16-fold for younger leaves

compared to the control plants without glyphosate spray. Among the mineral nutrients analyzed, the leaf concentrations of potassium (K), phosphorus (P), copper (Cu) and zinc (Zn) were not affected, or even increased significantly in case of P and Cu in young leaves by glyphosate, while the concentrations of calcium (Ca), manganese (Mn) and magnesium (Mg) were reduced, particularly in young leaves. In the case of Fe, leaf concentrations showed a tendency to be reduced by glyphosate. In the second experiment harvested at the grain maturation, glyphosate application did not reduce the seed concentrations of nitrogen (N), K, P, Zn and Cu. Even, at the highest application rate of glyphosate, seed concentrations of N, K, Zn and Cu were increased by glyphosate. By contrast, the seed concentrations of Ca, Mg, Fe and Mn were significantly reduced by glyphosate. These results suggested that glyphosate may interfere with uptake and retranslocation of Ca, Mg, Fe and Mn, most probably by binding and thus immobilizing them. The decreases in seed concentration of Fe, Mn, Ca and Mg by glyphosate are very specific, and may affect seed quality.

http://www.sciencedirect.com/science/article/pii/S1161030109000665

Egan, J.; Barlow, K.; Mortensen, D. 2014. A Meta-Analysis on the Effects of 2,4-D and Dicamba Drift on Soybean and Cotton. *Weed Science*, 62(1):193-206. 2014.

Commercial introduction of cultivars of soybean and cotton genetically modified with resistance to the synthetic auxin herbicides dicamba and 2,4-D will allow these compounds to be used with greater flexibility but may expose susceptible soybean and cotton cultivars to nontarget herbicide drift. From past experience, it is well known that soybean and cotton are both highly sensitive to low-dose exposures of dicamba and 2,4-D. In this study, a meta-analysis approach was used to synthesize data from over seven decades of simulated drift experiments in which investigators treated soybean and cotton with low doses of dicamba and 2,4-D and measured the resulting yields. These data were used to produce global dose–response curves for each crop and herbicide, with crop yield plotted against herbicide dose. The meta-analysis showed that soybean is more susceptible to dicamba in the flowering stage and relatively tolerant to 2,4-D at all growth stages. Conversely, cotton is tolerant to dicamba but extremely sensitive to 2,4-D, especially in the vegetative and preflowering squaring stages. Both crops are highly variable in their responses to synthetic auxin herbicide exposure, with soil moisture and air temperature at the time of exposure identified as key factors. Visual injury symptoms, especially during vegetative stages, are not predictive of final yield loss. Global dose–response curves generated by this meta-analysis can inform guidelines for herbicide applications and provide producers and agricultural professionals with a benchmark of the mean and range of crop yield loss that can be expected from drift or other nontarget exposures to 2,4-D or dicamba.

http://www.bioone.org/doi/abs/10.1614/WS-D-13-00025.1

Carvalho, F.; Souza, B.; França, A.; Ferreira, E.; Franco, M.; Kasuya, M.; Ferreira, F. 2014. Glyphosate drift affects arbuscular mycorrhizal association in coffee. *Planta daninha*, vol.32 nº 4, Viçosa.

Mycorrhizal association promotes better survival and nutrition of colonized seedling on field, and consequently, increasing of productivity. However, the weed management can interfere on this association, due to incorrect use of glyphosate. This work has assessed the effects of glyphosate drift on the growth and nutrition of arabica coffee plants (Catuaí Vermelho - IAC 99) colonized with arbuscular mycorrhizal fungi (AMF). The experiment was conducted in 2 x 5 factorial scheme, and included inoculated and non-inoculated plants, and five glyphosate subdoses (0.0, 57.6, 115.2, 230.4, and 460.8 g ha^{-1} of glyphosate), in randomized blocks with five replication. The inoculation was carried during the greenhouse phase of seedlings production with a mixture of *Rhizophagus clarus* and *Gigaspora margarita*, and after to transplanting, when the plants had seven pairs of leaves, glyphosate subdoses were applied. The product caused intoxication in up to 60% of non-inoculated

and 45% on inoculated plants, when the highest dose of 460.8 g a.e. ha^{-1} was applied. A negative effect was noted on the growth and phosphorus content of coffee plants, this effect increased depending on glyphosate subdose, but regardless of inoculation. Glyphosate drift reduces the growth and nutrition of plants colonized by species of AMF and native fungi, negatively affecting root colonization of plants treated.

Full article available at http://www.scielo.br/pdf/pd/v32n4/13.pdf

3.3 Transgenic dissemination in the agricultural system: when technology is a pest[37]

In some cases, the transgene inserted into the GMP, when introgressed in genetically related species and considered pests (or weedy[38]), may provide an adaptive advantage to it, generating severe management problems. In fact, the weeds supposedly controlled by the herbicide the HT plant is tolerant to survive to the treatment, making the technology unfeasible. In addition, succession of maize, soy and RR cotton crops, seed which have fallen in a harvest may germinate in parallel with the new planting, creating control difficulties as they will also be resistant to glyphosates or to herbicides involved with that and with other technologies (as the case of glufosinate, in Liberty Link crops).

The articles listed below illustrate the mechanisms responsible for agronomic problems resulting from the transfer of resistance transgene, from the intended crops to some ruderal species intended to be controlled.

Hall, L.; Topinka, K.; Huffman, J.; Davis, L.; Good, A. 2000. Pollen flow between herbicide-resistant Brassica napus is the cause of multiple-resistant B-napus volunteers. *Weed Science,* 48: 688-694.

37 It should be noted that part shares a common theme with item 3.2 of part 3 with respect to escape of transgenes in genetically, closely related and wild species. Sometimes the distinction between these wild / natural populations and those ruderal / adventitious is ambiguous, particularly in case of feral populations that are just populations of domesticated plants in renaturation process.

38 The ruderal expression employed throughout this publication is to the effect proposed by Schneider (2007) (Schneider, A. A. *A flora naturalizada no estado do Rio Grande do Sul, Brasil: herbáceas subespontâneas. Biociências*, Porto Alegre, v. 15, nº 2, p. 257-268, jul. 2007) and relates to plant species that grow without cultivation and without human care, encompassing both native and naturalized species. Unlike the term "harmful", ruderal has no value judgment and rejects the false premise that any plant other than the object culture would be harmful, which is not true of the natural systems that have diversity as an essential inherent element to the homeostasis.

A field in which *Brassica napus* volunteers were not controlled by several applications of glyphosate was investigated in 1998. This field had been planted with glufosinateresistant and imidazolinone-resistant *B. napus* in 1997 and was adjacent to a field that had grown glyphosate-resistant *B. napus.* Mature volunteer *B. napus* were collected on a 50- by 100-m grid in the field. Progeny from 34 volunteers were sprayed with glyphosate at 440 g ae ha_1, and the survivors were sprayed with either glufosinate or imazethapyr at 400 or 50 g ai ha_1, respectively. Where seed numbers permitted (14 volunteers), seedlings were also sprayed sequentially with glyphosate, glufosinate, and imazethapyr, at 440 g ae ha_1, 400 g ai ha_1, and 50 g ai ha_1, respectively. In total, 15 volunteers had progeny that were between 66 and 82% resistant to glyphosate, consistent with the predicted 3:1 resistant : susceptible ratio. Volunteer *B. napus* plants with glyphosate-resistant seedlings were most common close to the putative pollen source; however, a plant with glyphosate-resistant progeny was collected 500 m from the adjacent field edge. Seedlings from all nine volunteers collected from the glufosinate-resistant area showed multiple resistance to glyphosate and glufosinate, whereas seedlings from 10 of 20 volunteers collected from the imidazolinone-resistant area showed resistance to imazethapyr and glyphosate. DNAextraction and restriction fragment length polymorphism (RFLP) analysis of seedlings confirmed that mature *B. napus* volunteers were hybrids resulting from pollen transfer rather than inadvertent seed movement between fields. Two seedlings from the 924 screened were resistant to all three herbicides. Progeny from these selfpollinated individuals were resistant to glyphosate and glufosinate at the predicted 3:1 resistant : susceptible ratio and resistant to imazethapyr at the predicted 15:1 resistant : susceptible ratio. Sequential crossing of three herbicide-resistant varieties is the most likely explanation for the observed multiple herbicide resistance. Integrated management techniques, including suitable crop and herbicide rotations, herbicide mixtures, and nonchemical controls should be used to reduce the incidence and negative effect of *B. napus* volunteers with multiple herbicide resistance.

https://goo.gl/ZLg3HD

Arnaud, J.; Viard, F.; Delescluse, M.; Cuguen, J. 2003. Evidence for gene flow via seed dispersal from crop to wild relatives in *Beta vulgaris* (Chenopodiaceae): consequences for the release of genetically modified crop species with weedy lineages. *Proc. R. Soc. Lond. B*, 270, 1565–1571.

Gene flow and introgression from cultivated to wild plant populations have important evolutionary and ecological consequences and require detailed investigations for risk assessments of transgene escape into natural ecosystems. Sugar beets (Beta vulgaris ssp. vulgaris) are of particular concern because: (i) they are cross-compatible with their wild relatives (the sea beet, B. vulgaris ssp. maritima); (ii) crop-to-wild gene flow is likely to occur via weedy lineages resulting from hybridization events and locally infesting fields. Using a chloroplastic marker and a set of nuclear microsatellite loci, the occurrence of crop-to-wild gene flow was investigated in the French sugar beet production area within a 'contact-zone' in between coastal wild populations and sugar beet fields. The results did not reveal large pollen dispersal from weed to wild beets. However, several pieces of evidence clearly show an escape of weedy lineages from fields via seed flow. Since most studies involving the assessment of transgene escape from crops to wild outcrossing relatives generally focused only on pollen dispersal, this last result was unexpected: it points out the key role of a long-lived seed bank and highlights support for transgene escape via man-mediated long-distance dispersal events.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1691408/pdf/12908976.pdf

Gealy, D.; Mitten, D.; Rutger, J. 2003. Gene flow between red rice (*Oryza sativa*) and herbicide-resistant rice (*O. sativa*)-Implications for weed management. *Weed Technology,* vol. 17: 627-645.

Red rice has long been a troublesome, conspecific weed of cultivated rice. Rice varieties carrying certain herbicide-resistant traits acquired through genetic modification (herbicide-resistant varieties) now offer new options for red rice control. In concert with this innovation is the risk of gene flow, which can result in the transfer of that specific herbicide resistance to red rice and thus render this weed control measure ineffective. Gene flow in concept is simple, however, the parameters that determine the establishment of a new trait in a weed population are complex. Cross-pollination to make hybrid seed and the subsequent fate of those hybrid families in the general weed population are some of the biological factors that influence gene flow between red rice and cultivated rice. Natural outcrossing among rice plants is generally low. Most of the pollen dispersal studies published to date indicated that rice 3 rice outcrossing rates were less than 1.0%. Numerous reports summarized in this study suggest that outcrossing rates between rice and red rice can be highly variable but usually are similar to or lower than this level. However, once hybrids form, they may introgress into a red rice population within only a few generations. If hybrid seed families are to persist and establish herbicide-resistant red rice populations, they must successfully compete in the crop–weed complex. The ability to survive a herbicide applied to a herbicide-resistant rice variety would be a strong selective advantage for these hybrid families. Thus, the well-established principles of weed resistance management appear to be relevant for herbicide-resistant crop systems and should be used in combination with practices to minimize coincident flowering to mitigate the potential impact of gene flow from herbicide-resistant rice into red rice. For the rice–red rice crop–weed complex, there are both biological factors and agricultural practices that can work together to preserve these new weed control options.

Full article available at http://naldc.nal.usda.gov/naldc/download.xhtml?id=54993&content=PDF

Messeguer, J.; Marfa, V.; Catala, M.; Guiderdoni, E.; Melé, E. 2004. A field study of pollen-mediated gene flow from Mediterranean GM rice to conventional rice and the red rice weed. *Molecular Breeding*, 13 (1), 103- 112.

The objective of this study was to assess the frequency of pollen-mediated gene flow from a transgenic rice line, harbouring the *gusA* and the *bar* genes encoding respectively, _-glucuronidase and phosphinothricin acetyl transferase as markers, to the red rice weed and conventional rice in the Spanish *japonica* cultivar Senia. A circular field trial design was set up to investigate the influence of the wind on the frequency of pollination of red rice and conventional rice recipient plants with the transgenic pollen. Frequencies of gene flow based on detection of herbicide resistant, GUS positive seedlings among seed progenies of recipient plants averaged over all wind directions were 0.036 _ 0.006% and 0.086 _ 0.007 for red rice and conventional rice, respectively. However, for both red rice and conventional rice, a clear asymmetric distribution was observed with pollination frequency favoured in plants placed under the local prevailing winds. Southern analyses confirmed the hemizygous status and the origin of the transgenes in progenies of surviving, GUS positive plants. Gene flow detected in conventional rice planted at 1, 2, 5 and 10 m distance revealed a clear decrease with increasing distance which was less dramatic under the prevailing wind direction. Consequences of these findings for containment of gene flow from transgenic rice crops to the red rice weed are discussed. The precise determination of the local wind conditions at flowering time and pollination day time appear to be of primary importance for setting up suitable isolation distances.

http://link.springer.com/article/10.1023%2FB%3AMOLB.0000012285.39859.9d

Légère, A. 2005. Risks and consequences of gene flow from herbicide-resistant crops: canola (*Brassica napus* L.) as a case study. *Pest Manag. Sci.,* 61 (3): 292-300.

Data from the literature and recent experiments with herbicide-resistant (HR) canola (Brassica napus L) repeatedly confirm that genes and transgenes will flow and hybrids will form if certain conditions are met. These include sympatry with a compatible relative (weedy, wild or crop), synchrony of flowering, successful fertilization and viable offspring. The chance of these events occurring is real; however, it is generally low and varies with species and circumstances. Plants of the same species (non-transgenic or with a different HR transgene) in neighbouring fields may inherit the new HR gene, potentially generating plants with single and multiple HR. For canola, seed losses at harvest and secondary dormancy ensures the persistence over time of the HR trait(s) in the seed bank, and the potential presence of crop volunteers in subsequent crops. Although canola has many wild/weedy relatives, the risk of gene flow is quite low for most of these species, except with Brassica rapa L. Introgression of genes and transgenes in B rapa populations occurs with apparently little or no fitness costs. Consequences of HR canola gene flow for the agro-ecosystem include contamination of seed lots, potentially more complex and costly control strategy, and limitations in cropping system design. Consequences for non-agricultural habitats may be minor but appear largely undocumented.

http://www.ncbi.nlm.nih.gov/pubmed/15593291

Zelaya, I.; Owen, M.; VanGessel, M. 2007. Transfer of glyphosate-resistance: Evidence of hybridisation in *Conyza* (Asteraceae). *American Journal of Botany*, 94: 660-673.

Transfer of herbicide resistance genes between crops and weeds is relatively well documented; however, far less information exists for weed-to-weed interactions. The hybridization between the weedy diploids Conyza canadensis (2n ¼ 18) and C. ramosissima (2n ¼ 18) was investigated by monitoring transmission of the allele conferring resistance to N-phosphonomethyl glycine (glyphosate). In a multivariate quantitative trait analysis, we described the phylogenic relationship of the plants, whereas we tested seed viability to assess potential postzygotic reproductive barriers (PZRB) thus affecting the potential establishment of hybrid populations in the wild. When inflorescences were allowed to interact freely, approximately 3% of C. ramosissima or C. canadensis ova were fertilized by pollen of the opposing species and produced viable seeds; > 95% of the ova were fertilized under no-pollen competition conditions (emasculation). The interspecific Conyza hybrid (F^H_1) demonstrated an intermediate phenotype between the parents but superior resistance to glyphosate compared to the resistant C. canadensis parent. Inheritance of glyphosate resistance in the selfed F^H_1 (F^H_2) followed the partially dominant nuclear, single-gene model; F^H_1 backcrosses confirmed successful introgression of the resistance allele to either parent. Negligible PZRB were observed in the hybrid progenies, confirming fertility of the C. canadensis 3 C. ramosissima nothotaxa. The implications of introgressive hybridization for herbicide resistance management and taxonomy of Conyza are discussed.

Full article available at http://www.amjbot.org/content/94/4/660.long

Song, X.; Liu, L.; Wang, Z.; Qiang, S. 2009. Potential gene flow from transgenic rice (*Oryza sativa L.*) to different weedy rice (*Oryza sativa f. spontanea*) accessions based on reproductive compatibility. *Pest Management Science, v*ol 65 (8): 862-9.

Background: The possibility of gene flow from transgenic crops to wild relatives may be affected by reproductive capacity between them. The potential gene flow from two transgenic rice lines containing the bar gene to five accessions of weedy rice (WR1-WR5) was determined through examination of reproductive compatibility under controlled pollination.
Results: The pollen grain germination of two transgenic rice lines on the stigma of all weedy rice, rice pollen tube growth down the style and entry into the weedy rice ovary were similar to self-

pollination in weedy rice. However, delayed double fertilisation and embryo abortion in crosses between WR2 and Y0003 were observed. Seed sets between transgenic rice lines and weedy rice varied from 8 to 76%. Although repeated pollination increased seed set significantly, the rank of the seed set between the weedy rice accessions and rice lines was not changed. The germination rates of F(1) hybrids were similar or greater compared with respective females. All F(1) plants expressed glufosinate resistance in the presence of glufosinate selection pressure.
Conclusions: The frequency of gene flow between different weedy rice accessions and transgenic herbicide-resistant rice may differ owing to different reproductive compatibility. This result suggests that, when wild relatives are selected as experimental materials for assessing the gene flow of transgenic rice, it is necessary to address the compatibility between transgenic rice and wild relatives.

http://www.ncbi.nlm.nih.gov/pubmed/19418443

Song, X.; Wang. Z.; Qiang, S. 2011. Agronomic performance of F1, F2 and F3 hybrids between weedy rice and transgenic glufosinate-resistant rice. *Pest Management Science,* 67(8):921-31. doi: 10.1002/ps.2132. Epub 2011 Mar 2.

Background: Studies of hybrid fitness, of which agronomic performance may be an indicator, can help in evaluating the potential for introgression of a transgene from a transgenic crop to wild relatives. The objective of this study was to assess the agronomic performance of reciprocal hybrids between two transgenic glufosinate-resistant rice lines, Y0003 and 99-t, and two weedy rice accessions, WR1 and WR2, in the greenhouse.
Results: F1 hybrids displayed heterosis in height, flag leaf area and number of spikelets per panicle. The agronomic performance of F1 between WR1 and Y0003 was not affected by crossing direction. The tiller and panicle numbers of F1 individuals were higher than their F2 counterparts. However, these traits did not change significantly from the F2 to the F3 generation or in hybrids with weedy rice as maternal or paternal plants. For all hybrids, the in vitro germination rates of fresh pollen were similar and significantly lower than those of their parents, seed sets were similar to or of lower value than those of weedy rice parents and seed shattering characteristics were partially suppressed, but the survival of hybrids over winter in the field was similar to that of weedy rice parents. All F1, F2 and F3 hybrids had similar composite agronomic performance to weedy rice parents.
Conclusion: There was no significant decrease in the composite agronomic performance of any of the hybrids compared with weedy rice. This implies that gene flow from transgenic cultivated rice to weedy rice could occur under natural conditions.

http://www.ncbi.nlm.nih.gov/pubmed/21370396

In some cases, the transgene transfer into ruderal species considered as pests (weedy) may result in adaptive advantage of such plants, even without using the selection agent (the herbicide associated to a certain HT plant). Thus, the transgene simply “scapes in the nature”, with consequences that are difficult to predict in term of damages to the environment in the medium and long term.

Warwick, S.; Legere, A.; Simard, M.; James, T. 2008. Do escaped transgenes persist in nature? The case of a herbicide resistance transgene in a weedy Brassica rapa population. *Molecular Ecol., v*ol. 17 (5): 1387-95.

The existence of transgenic hybrids resulting from transgene escape from genetically modified (GM) crops to wild or weedy relatives is well documented but the fate of the transgene over time in recipient wild species populations is still relatively unknown. This is the first report of the persistence and apparent introgression, i.e. stable incorporation of genes from one differentiated gene pool into another, of an herbicide resistance transgene from *Brassica napus* into the gene pool of its weedy relative, *Brassica rapa*, monitored under natural commercial field conditions. Hybridization between glyphosate-resistant [herbicide resistance (HR)] *B. napus* and *B. rapa* was first observed at two Québec sites, Ste Agathe and St Henri, in 2001. *B. rapa* populations at these two locations were monitored in 2002, 2003 and 2005 for the presence of hybrids and transgene persistence. Hybrid numbers decreased over the 3-year period, from 85 out of ~200 plants surveyed in 2002 to only five out of 200 plants in 2005 (St Henri site). Most hybrids had the HR trait, reduced male fertility, intermediate genome structure, and presence of both species-specific amplified fragment length polymorphism markers. Both F1 and backcross hybrid generations were detected. One introgressed individual, i.e. with the HR trait and diploid ploidy level of *B. rapa,* was observed in 2005. The latter had reduced pollen viability but produced ~480 seeds. Forty-eight of the 50 progeny grown from this plant were diploid with high pollen viability and 22 had the transgene (1:1 segregation). These observations confirm the persistence of the HR trait over time. Persistence occurred over a 6-year period, in the absence of herbicide selection pressure (with the exception of possible exposure to glyphosate in 2002), and in spite of the fitness cost associated with hybridization.

http://www.ncbi.nlm.nih.gov/pubmed/17971090

Dlugosch, K.; Whitton, J.; 2008. Can we stop transgenes from taking a walk on the wild side? *Molecular Ecology*, 17(5):1167-9. doi: 10.1111/j.1365-294X.2008.03663.x.

Whether the potential costs associated with broad-scale use of genetically modified organisms (GMOs) outweigh possible benefits is highly contentious, including within the scientific community. Even among those generally in favour of commercialization of GM crops, there is nonetheless broad recognition that transgene escape into the wild should be minimized. But is it possible to achieve containment of engineered genetic elements in the context of large scale agricultural production? In a previous study, Warwick et al. (2003) documented transgene escape via gene flow from herbicide resistant (HR) canola (Brassica napus) into neighbouring weedy B. rapa populations (Fig. 1) in two agricultural fields in Quebec, Canada. In a follow-up study in this issue of Molecular Ecology, Warwick et al. (2008) show that the transgene has persisted and spread within the weedy population in the absence of selection for herbicide resistance. Certainly a trait like herbicide resistance is expected to spread when selected through the use of the herbicide, despite potentially negative epistatic effects on fitness. However, Warwick et al.'s findings suggest that direct selection favouring the transgene is not required for its persistence. So is there any hope of preventing transgene escape into the wild?

Full article available at http://onlinelibrary.wiley.com/doi/10.1111/j.1365-294X.2008.03663.x/pdf

Wang, w.; Xia, H.; Yang, X.; Xu, T.; Si, H.; Cai, X.; Wang, F.; Su, J.; Snow, A.; Lu, B-R. 2013. A novel 5-enolpyruvoylshikimate-3-phosphate (EPSP) synthase transgene for glyphosate resistance stimulates growth and fecundity in weedy rice (*Oryza sativa*) without herbicide. *New Phytologist,* doi: 10.1111/nph.12428.

Understanding evolutionary interactions among crops and weeds can facilitate effective weed management. For example, gene flow from crops to their wild or weedy relatives can lead to rapid evolution in recipient populations. In rice (Oryza sativa), transgenic herbicide resistance is expected to spread to conspecific weedy rice (Oryza sativa f. spontanea) via hybridization. Here, we studied fitness effects of transgenic over-expression of a native 5-enolpyruvoylshikimate-3-phosphate synthase (epsps) gene developed to confer glyphosate resistance in rice. Controlling for genetic background, we examined physiological traits and field performance of crop-weed hybrid lineages

that segregated for the presence or absence of this novel epsps transgene. Surprisingly, we found that transgenic F2 crop-weed hybrids produced 48-125% more seeds per plant than nontransgenic controls in monoculture- and mixed-planting designs without glyphosate application. Transgenic plants also had greater EPSPS protein levels, tryptophan concentrations, photosynthetic rates, and per cent seed germination compared with nontransgenic controls. Our findings suggest that over-expression of a native rice epsps gene can lead to fitness advantages, even without exposure to glyphosate. We hypothesize that over-expressed epsps may be useful to breeders and, if deployed, could result in fitness benefits in weedy relatives following transgene introgression.

http://www.ncbi.nlm.nih.gov/pubmed/23905647

4 The impossible coexistence

Gene flow through cross fertilization (pollination), dispersion of seeds or HGT in crops that are subsequent to GM crops represent natural factors[39], out of the farmer's and the biotechnology companies' control, that make impossible the coexistence of GM and non-GM plants in the fields.

At the same time, whilesome researchers believe in the possibility to control the biological factors involved in the genetic contamination (isolation distances, reserve areas, use of GURTs etc.), others state that in current situation it is impossible to avoid contamination. Coexistence will always imply a certain level of undesirable gene flow. In this point there is consensus in the scientific community concerning the impossibility to guarantee null contamination in conventional agrifood chains. There are reasons ranging from biological to elements of the socioeconomic nature associated to the expansion of the seeds control and to the accumulation of royalties/ patents charging the use of registered technologies.

Devos, Y. ; Reheul, D. ; Schrijver, A. ; Cors, F. ; Moens, W. 2004. Management of herbicide-tolerant oilseed rape in Europe: a case study on minimizing vertical gene flow. *Environ Biosafety Res.,* 3(3):135-48.

The potential commercialization of genetically modified herbicide-tolerant (GMHT) oilseed rape in Europe raises various concerns about their potential environmental and agronomic impacts, especially those associated with the escape of transgenes. Pollen of oilseed rape can be dispersed in space, resulting in the fertilization of sympatric compatible wild relatives (e.g. Brassica rapa) and oilseed rape cultivars grown nearby (GM and/or non-GM Brassica napus). The spatial and

39 Check with item 3 of Part 3 for more references on gene flow between non-agricultural related species.

temporal dispersal of seeds of oilseed rape may lead to feral oilseed rape populations outside the cropped areas and oilseed rape volunteers in subsequent crops in the rotation. The incorporation of a HT trait(s) may increase the fitness of the recipient plants, making them more abundant and persistent, and may result in weeds that are difficult to control by the herbicide(s) to which they are tolerant. Vertical gene flow from transgenic oilseed rape to non-GM counterparts may also have an impact on farming and supply chain management, depending on labelling thresholds for the adventitious presence of GM material in non-GM products. Given the extent of pollen and seed dispersal in oilseed rape, it is obvious that the safe and sound integration of GMHT oilseed rape in Europe may require significant on-farm and off-farm management efforts. Crucial practical measures that can reduce vertical gene flow include (1) isolating seed production of Brassica napus, (2) the use of certified seed, (3) isolating fields of GM oilseed rape, (4) harvesting at the correct crop development stage with properly adjusted combine settings, (5) ensuring maximum germination of shed seeds after harvest, (6) controlling volunteers in subsequent crops, and (7) keeping on-farm records. The implementation of the recommended practices may, however, be difficult, entailing various challenges.

http://www.ncbi.nlm.nih.gov/pubmed/15901096

Hofmann, F.; Otto, M.; Wosniok, W. 2014. Maize pollen deposition in relation to distance from the nearest pollen source under common cultivation - results of 10 years of monitoring (2001 to 2010). *Environmental Sciences Europe,* 26:24.

Background: Information on pollen dispersal is essential for the risk assessment and management of genetically modified organisms (GMOs) such as Bt maize. We analyzed data on maize pollen deposition at 216 sites in Germany, Switzerland, and Belgium from 2001 to 2010. All data were collected using the same standardized sampling method. The distances between sampling site and the nearest maize field ranged from within the field to 4.45 km.
Results: Maize pollen deposition was negatively correlated with distance from the nearest pollen source. The highest pollen deposition was within the field, but depositions of several thousand pollen grains per square meter were recorded over the kilometer range. A power function model most accurately described the relationship between deposition and distance from the nearest pollen source, rather than the exponential model currently used in EU risk assessment and management, which underestimates exposure for distances greater than 10 m. Regression analysis confirmed the high significance of the power relationship. The large variation in pollen deposition at a given distance reflected the influences of wind direction and other meteorological and site conditions. Plausible variations of single values and the predicted mean pollen count at a given distance were expressed by confidence intervals.
Conclusions: The model described here allows estimations of pollen deposition in relation to distance from the nearest field; therefore, it will be valuable for the risk assessment and management of GMOs. Our results indicate that buffer zones in the kilometer range are required to prevent harmful exposure of non-target organisms to GMOs.

Full article available at http://www.enveurope.com/content/26/1/24

REV – Price, B.; Cotter, J. 2014. The GM Contamination Register: a review of recorded contamination incidents associated with genetically modified organisms (GMOs), 1997–2013. *International Journal of Food Contamination,* 1:5.

Background: Since large-scale commercial planting of genetically modified (GM) crops began in 1996, a concern has been that non-GM crops may become contaminated by GM crops and that wild or weedy relatives of GM crops growing outside of cultivated areas could become contaminated. The GM Contamination Register contains records of GM contamination incidents since 1997 and forms a unique database. By the end of 2013, 396 incidents across 63 countries had been recorded.

Results: Analysis of the Register database reveals rice has the highest number of GM contamination incidents of all crops (accounting for a third of incidents), despite there being no commercial growing of GM rice anywhere in the world. The majority of these incidents derive from two distinct cases of contamination of unauthorised GM rice lines, LLRICE from the USA and BT63 rice from China. Maize accounts for 25% of GM contamination incidents, whilst soya and oilseed rape account for approximately 10% of incidents. Although factors such as acreage grown, plant biology, designation as a food or non food crop and degree of international trading can potentially affect the frequency and extent of contamination, it is not possible to determine which are dominant. The Register records a total of nine cases of contamination from unauthorised GM lines, i.e. those at the research and development stage with no authorisation for commercial cultivation anywhere in the world. An important conclusion of this work is that GM contamination can occur independently of commercialisation. Some of these cases, notably papaya in Thailand, maize in Mexico and grass in USA have continued over a number of years and are ongoing, whilst other contamination cases such as Bt10 maize and pharmaceutical-producing GM crops occur only with a single year. The route(s) of contamination are often unclear.
Conclusions: The detection of GMO contamination is dependent on both routine and targeted monitoring regimes, which appears to be inconsistent from country to country, even within the EU. The lack of an analytical methodology for the detection of GM crops at the field trial stage (i.e. pre-commercialisation) can hamper efforts to detect any contamination arising from such GM lines.

Full article available at http://link.springer.com/article/10.1186%2Fs40550-014-0005-8

4.1 Pollen mediated contamination of non-GM cultures

Depending on the species, the climate conditions and the mosaic of the considered crops, gene flow through pollination may occur between individuals separated by great distances. Each species will present its particularities concerning the potential to contaminate through cross fertilization. However, the history of contaminations over the world shows that no conventional variety having transgenic equivalents will be protected against gene flow through pollination. In addition, there are other factors related to the human behavior which increase the risks of contamination.

Rieger, M.; Lamond, M.; Preston, C.; Powles, S.; Roush, R. 2002. Pollen-mediated movement of herbicide resistance between commercial canola fields. *Science*, 296 (5577): 2386-2388.

There is considerable public and scientific debate for and against genetically modified (GM) crops. One of the first GM crops, *Brassica napus* (oilseed rape or canola) is now widely grown in North America, with proposed commercial release into Australia and Europe. Among concerns of opponents to these crops are claims that pollen movement will cause unacceptable levels of gene flow from GM to non-GM crops or to related weedy species, resulting in genetic pollution of the environment. Therefore, quantifying pollen-mediated gene flow is vital for assessing the environmental impact of GM crops. This study quantifies at a landscape level the gene flow that occurs from herbicide-resistant

canola crops to nearby crops not containing herbicide resistance genes.

Full article available at http://www.sciencemag.org/content/296/5577/2386.long

Watrud, L.; Lee, E.; Fairbrother, A.; Burdick, C.; Reichman, J.; Bollman, M.; Storm, M.; King, G.; Van de Water, P. 2004. Evidence for landscape-level, pollen-mediated gene flow from genetically modified creeping bentgrass with CP4 EPSPS as a marker. *PNAS*, vol. 101 (40). 14533-14538.

Sampling methods and results of a gene flow study are described that will be of interest to plant scientists, evolutionary biologists, ecologists, and stakeholders assessing the environmental safety of transgenic crops. This study documents gene flow on a landscape level from creeping bentgrass (*Agrostis stolonifera* L.), one of the first wind-pollinated, perennial, and highly outcrossing transgenic crops being developed for commercial use. Most of the gene flow occurred within 2 km in the direction of prevailing winds. The maximal gene flow distances observed were 21 km and 14 km in sentinel and resident plants, respectively, that were located in primarily nonagronomic habitats. The selectable marker used in these studies was the *CP4 EPSPS* gene derived from *Agrobacterium* spp. strain CP4 that encodes 5-enol-pyruvylshikimate-3-phosphate synthase and confers resistance to glyphosate herbicide. Evidence for gene flow to 75 of 138 sentinel plants of *A. stolonifera* and to 29 of 69 resident *Agrostis* plants was based on seedling progeny survival after spraying with glyphosate in greenhouse assays and positive TraitChek, PCR, and sequencing results. Additional studies are needed to determine whether introgression will occur and whether it will affect the ecological fitness of progeny or the structure of plant communities in which transgenic progeny may become established.

Full article available at http://www.pnas.org/content/101/40/14533.long

Galeano, P.; Debat, C.; Ruibal, F.; Fraguas, L.; Galván, G. 2010. Cross-fertilization between genetically modified and nongenetically modified maize crops in Uruguay. *Env. Biosafety Res.,* vol 9 (3): 147-154.

The cultivation of transgenic (GM) Bt maize (events Mon810 and Bt11) is permitted in Uruguay. Local regulations ensures that 10% of the crop should be a non-GM cultivar as reservation area for biodiversity, and the distance from other non-GM maize crops should be more than 250 m in order to avoid cross-pollination. However, the degree of cross-pollination between maize crops in Uruguay is unknown. The level of contamination with Bt is a relevant issue for organic farming, *in-situ* conservation of genetic resources, and seed production. In this research the occurrence and frequency of cross-pollination between commercial GM and non-GM maize crops in Uruguay was assessed. The methodology comprised field sampling and detection using DAS-ELISA and PCR. Five field-pair situations of GM and non-GM nearly grown with potential risk of pollen contamination were identified, regarding the distance between crops and similarity between sowing dates. Among these five situations, Bt contamination was found in three cases. The detected transgenic events were coincident with the event in each potential source of contamination. Frequencies of transgenic events in the offspring of non-GM crops were estimated as 1/60, 1/40 and 1/255 for three field situations with distances of respectively 40, 100 and 330 m from the source of contamination. This is a first indication that GM contamination may frequently occur in Uruguay if isolation by distance and/or time is not provided. These findings contribute to evaluate the applicability of regulated co-existence policy.

http://www.ncbi.nlm.nih.gov/pubmed/21975255

4.2 Transgene dissemination through feral populations

Depending on the species, the escape of transgenes in populations of ruderal plants associated to commercial transgenic crops (or adjacent to them) occurs - and presents the probability to remain in time - in variable degrees. But when this occurs, and beyond the environmental thus generated, such populations become reservoirs of transgenes which may increase their dissemination, whether by means of pollen or seeds. This reaches even conventional crops relatively isolated – generating coexistence and relationship problems between neighbors.

Ellstrand, N. 2001. When transgenes wander, should we worry? *Plant Physiol.*, 125:1543–1545.

Without summary.

Full article available at http://www.plantphysiol.org/content/125/4/1543.full.pdf+html

Simard, M.; Legere, A.; Warwick, S. 2006. Transgenic *Brassica napus* fields and *Brassica rapa* weeds in Quebec sympatry and weed-crop in situ hybridization. *Canadian Journal of Botany,* 84: 1842-1851.

Hybridization between the herbicide-resistant transgenic crop Brassica napus L. (canola) and its weedy relative Brassica rapa L. (bird rape) has been documented in Quebec. Our goal was to evaluate the actual hybridization potential based on range overlap and actual in situ hybridization rates. This was done by mapping *B. napus* canola fields, comparing them with the sampling locations of *B. rapa* herbarium specimens from Quebec, gathering information on the presence of *B. rapa* in certified canola seed production fields, and surveying for *B. rapa* populations located in, or close to *B. napus* field margins. Progeny from these populations were screened for herbicide resistance (HR) and for the presence of the HR transgene. Two fields were also selected to evaluate *B. rapa* density effects on hybridization rates. Significant sympatry was observed in several areas of the province; hybridization occurred in all eight populations (1.1% to 17.5% hybrid seed) located in field margins and in one (1.1%) out of three populations located less than 10 m from a *B. napus* field. Hybridization rates decreased exponentially as *B. rapa* density increased, but interplant rates (0% to 68%) were highly variable. Environmental problems could be generated by the release of *B. napus* crops with traits conferring fitness benefits in nonmanaged areas.

http://www.nrcresearchpress.com/doi/abs/10.1139/b06-135#.VMJ34fldWt8

Knispel, A.; McLachlan, S.; Van Acker, R.; Friesen, L. 2008. Gene flow and multiple herbicide resistance in escaped canola populations. *WeedScience*, 56, 72-80.

Gene flow among herbicide-resistant (HR) canola varieties can lead to the development of multiple HR canola plants, creating volunteer canola management challenges for producers. In western Canada, escaped populations of HR canola are ubiquitous outside of cultivated fields, yet the extent of gene flow resulting in herbicide resistance trait stacking in individuals within these populations remains unknown. The objectives of this study were to document the presence of single and multiple herbicide resistance traits and assess the extent of gene flow within escaped canola populations. Seed was collected from 16 escaped canola populations along the verges of fields and roadways in four agricultural regions in southern Manitoba from 2004 to 2006. Glyphosate resistance was found in 14 (88%) of these populations, glufosinate resistance in 13 (81%) populations, and imidazolinone resistance in five (31%) populations. Multiple herbicide resistance was observed at levels consistent with previously published canola outcrossing rates in 10 (62%) of the tested populations. In 2005 and 2006, maternal plants from two escaped populations were tested using trait indicator test strips for glyphosate and glufosinate resistance to confirm outcrossing events. In 2005, two of 13 tested maternal plants with single herbicide resistance traits produced progeny with both glyphosate and glufosinate resistance. In 2006, of 21 tested plants, 10 single HR maternal plants produced multiple HR progeny, and five nonresistant maternal plants produced resistant offspring. This is the first report indicating that intraspecific gene flow results in stacking of herbicide resistance traits in individuals within escaped canola populations, confirming that multiple HR canola volunteers are not confined to agricultural fields. Results of this study suggest that escaped populations of crop plants can contribute to the spread of genetically engineered novel traits, which has important implications for containment, especially for highly controversial pharmaceutical and industrial traits in crop plants.

http://www.bioone.org/doi/abs/10.1614/WS-07-097.1?journalCode=wees

Knispel A.; McLachlan, S. 2010. Landscape-scale distribution and persistence of genetically modified oilseed rape (*Brassica napus*) in Manitoba, Canada. *Environ Sci Pollut Res Int.*, 17(1):13-25. doi: 10.1007/s11356-009-0219-0.

Background, Aim and Scope: Genetically modified herbicide-tolerant (GMHT) oilseed rape (OSR; Brassica napus L.) was approved for commercial cultivation in Canada in 1995 and currently represents over 95% of the OSR grown in western Canada. After a decade of widespread cultivation, GMHT volunteers represent an increasing management problem in cultivated fields and are ubiquitous in adjacent ruderal habitats, where they contribute to the spread of transgenes. However, few studies have considered escaped GMHT OSR populations in North America, and even fewer have been conducted at large spatial scales (i.e. landscape scales). In particular, the contribution of landscape structure and large-scale anthropogenic dispersal processes to the persistence and spread of escaped GMHT OSR remains poorly understood. We conducted a multi-year survey of the landscape-scale distribution of escaped OSR plants adjacent to roads and cultivated fields. Our objective was to examine the long-term dynamics of escaped OSR at large spatial scales and to assess the relative importance of landscape and localised factors to the persistence and spread of these plants outside of cultivation.
Materials and Methods: From 2005 to 2007, we surveyed escaped OSR plants along roadsides and field edges at 12 locations in three agricultural landscapes in southern Manitoba where GMHT OSR is widely grown. Data were analysed to examine temporal changes at large spatial scales and to determine factors affecting the distribution of escaped OSR plants in roadside and field edge habitats within agricultural landscapes. Additionally, we assessed the potential for seed dispersal between escaped populations by comparing the relative spatial distribution of roadside and field edge OSR.
Results: Densities of escaped OSR fluctuated over space and time in both roadside and field edge habitats, though the proportion of GMHT plants was high (93-100%). Escaped OSR was positively affected by agricultural landscape (indicative of cropping intensity) and by the presence of an adjacent field planted to OSR. Within roadside habitats, escaped OSR was also strongly associated with large-scale variables, including road surface (indicative of traffic intensity) and distance to the nearest grain elevator. Conversely, within field edges, OSR density was affected by localised crop management practices such as mowing, soil disturbance and herbicide application. Despite the proximity of roadsides and field edges, there was little evidence of spatial aggregation among escaped OSR populations in these two habitats, especially at very fine spatial scales (i.e. <100 m), suggesting

that natural propagule exchange is infrequent.
Discussion: Escaped OSR populations were persistent at large spatial and temporal scales, and low density in a given landscape or year was not indicative of overall extinction. As a result of ongoing cultivation and transport of OSR crops, escaped GMHT traits will likely remain predominant in agricultural landscapes. While escaped OSR in field edge habitats generally results from local seeding and management activities occurring at the field-scale, distribution patterns within roadside habitats are determined in large part by seed transport occurring at the landscape scale and at even larger regional scales. Our findings suggest that these large-scale anthropogenic dispersal processes are sufficient to enable persistence despite limited natural seed dispersal. This widespread dispersal is likely to undermine field-scale management practices aimed at eliminating escaped and in-field GMHT OSR populations.
Conclusions: Agricultural transport and landscape-scale cropping patterns are important determinants of the distribution of escaped GM crops. At the regional level, these factors ensure ongoing establishment and spread of escaped GMHT OSR despite limited local seed dispersal. Escaped populations thus play an important role in the spread of transgenes and have substantial implications for the coexistence of GM and non-GM production systems.
Recommendations and Perspectives: Given the large-scale factors driving the spread of escaped transgenes, localised co-existence measures may be impracticable where they are not commensurate with regional dispersal mechanisms. To be effective, strategies aimed at reducing contamination from GM crops should be multi-scale in approach and be developed and implemented at both farm and landscape levels of organisation. Multiple stakeholders should thus be consulted, including both GM and non-GM farmers, as well as seed developers, processors, transporters and suppliers. Decisions to adopt GM crops require thoughtful and inclusive consideration of the risks and responsibilities inherent in this new technology.

http://www.ncbi.nlm.nih.gov/pubmed/19588180

Heuberger, S.; Ellers-Kirk, C.; Tabashnik, B.; Carrière, Y. 2010. Pollen- and Seed-Mediated Transgene Flow in Commercial Cotton Seed Production Fields. *PLOS ONE,* 5(11): e14128.

Background: Characterizing the spatial patterns of gene flow from transgenic crops is challenging, making it difficult to design containment strategies for markets that regulate the adventitious presence of transgenes. Insecticidal *Bacillus thuringiensis* (Bt) cotton is planted on millions of hectares annually and is a potential source of transgene flow.
Methodology/Principal Findings: Here we monitored 15 non-Bt cotton (*Gossypium hirsutum*, L.) seed production fields (some transgenic for herbicide resistance, some not) for gene flow of the Bt cotton *cry1Ac* transgene. We investigated seed-mediated gene flow, which yields adventitious Bt cotton plants, and pollen-mediated gene flow, which generates outcrossed seeds. A spatially-explicit statistical analysis was used to quantify the effects of nearby Bt and non-Bt cotton fields at various spatial scales, along with the effects of pollinator abundance and adventitious Bt plants in fields, on pollen-mediated gene flow. Adventitious Bt cotton plants, resulting from seed bags and planting error, comprised over 15% of plants sampled from the edges of three seed production fields. In contrast, pollen-mediated gene flow affected less than 1% of the seed sampled from field edges. Variation in outcrossing was better explained by the area of Bt cotton fields within 750 m of the seed production fields than by the area of Bt cotton within larger or smaller spatial scales. Variation in outcrossing was also positively associated with the abundance of honey bees.
Conclusions/Significance: A comparison of statistical methods showed that our spatially-explicit analysis was more powerful for understanding the effects of surrounding fields than customary models based on distance. Given the low rates of pollen-mediated gene flow observed in this study, we conclude that careful planting and screening of seeds could be more important than field spacing for limiting gene flow.

Full article available at https://goo.gl/311cTH

Park, K.; Lee, B.; Kim, C.; Kim, D.; Park, J.; Ko, E.; Jeong, S.; Choi, K.; Yoon, W.; Kim, H. 2010. Monitoring the occurrence of genetically modified maize at a grain receiving port and along transportation routes in the Republic of Korea. *Food Control*, 21(4): 456-461.

The cultivation area of genetically modified (GM) crops is increasing all over the world. Though no land in the Republic of Korea is currently used for the cultivation of GM crops, GM crop imports for food and foraging purposes are continuously increasing. This may promote the unintentional escape of GM crops. This study was conducted to investigate whether imported GM maize is released into our environment during the transportation of grain in the Republic of Korea. Based on PCR analysis, most of the maize grains in the forage products were GM, and about 50% of the grains were germinated. Monitoring was conducted in two major grain receiving ports, 15 feed manufacturing plants, and 14 livestock barns in five provinces of the Republic of Korea from July to September 2007. We found many spilled maize grains around open storage areas of ports and along truck transportation routes near feed manufacturing plants. Established maize plants were not found at or around Incheon port. However, we found 18 established maize plants at the Gunsan port, 15 of which were GM. We also found eight GM maize plants around four feed manufacturing plants and in two livestock barns. Based on the event-specific PCR analysis, three maize events (NK603, Mon810, and TC1507) were identified. Though several GM maize plants were found around the port and feed manufacturing plants, most of these facilities were located inside the industrial park and were far from cultivated fields, likely rendering the impact of these GM maize on the natural environments negligible. However, most of the livestock barns were close to cultivated areas. Moreover, maize plants were cultivated for food or feed near some livestock barns. This practice may facilitate gene flow from GM maize to non-GM maize plants. Therefore, continuous monitoring is necessary to detect the occurrence of GM maize, and appropriate action should be taken to prevent genetic admixture in our environment.

https://goo.gl/9QCKYj

Schafer, M.; Ross, A.; Londo, J.; Burdick, C.; Lee, E.; *et al*. 2011. The Establishment of Genetically Engineered Canola Populations in the U.S. *PLOS ONE*, 6(10): e25736. doi:10.1371/journal.pone.0025736.

Concerns regarding the commercial release of genetically engineered (GE) crops include naturalization, introgression to sexually compatible relatives and the transfer of beneficial traits to native and weedy species through hybridization. To date there have been few documented reports of escape leading some researchers to question the environmental risks of biotech products. In this study we conducted a systematic roadside survey of canola (*Brassica napus)* populations growing outside of cultivation in North Dakota, USA, the dominant canola growing region in the U.S. We document the presence of two escaped, transgenic genotypes, as well as non-GE canola, and provide evidence of novel combinations of transgenic forms in the wild. Our results demonstrate that feral populations are large and widespread. Moreover, flowering times of escaped populations, as well as the fertile condition of the majority of collections suggest that these populations are established and persistent outside of cultivation.

Full article available at https://goo.gl/LxGeR7

Schoenenberger, N.; D'Andrea, L. 2012. Surveying the occurrence of subspontaneous glyphosate tolerant genetically engineered *Brassica napu*s L. (Brassicaceae) along Swiss railways. *Environmental Sciences Europe*, 24:23.

Background: Railway tracks represent a highly interlinked habitat with numerous possibilities for accidental entry of oilseed rape due to seed spill during transportation. Moreover, glyphosate is regularly employed to control the vegetation, increasing the possibility of establishment for plants resistant to it. We surveyed the presence of genetically engineered glyphosate tolerant oilseed rape (*Brassica napus*) with a focus on the most important Swiss railway stations. Our objective was

to detect accidental establishment of transgenic plants, since Switzerland does not import nor cultivate transgenic oilseed rape.
Results: Seventy-nine railway areas were sampled in Switzerland and the Principality of Liechtenstein, and the feral presence of oilseed rape was detected in 58 of them. A total of 2403 individuals were tested for genetic modification using commercially available immunologic test kits. In four localities, one located in Lugano and three in the area of Basel, a total of 50 plants expressing the CP4 EPSPS protein were detected. In two of the localities, survival of herbicide applications was observed. The populations were probably introduced through contaminated seed spills from freight trains, or during the transfer of goods from cargo ships to trains.
Conclusions: Railways represent an ideal system for herbicide resistant transgenic plants to establish and spread as a result of high selective pressure in favour of herbicide resistance with consequent increased difficulties to keep the infrastructure free of weeds. Crop-to-wild gene flow can occur as several sexually compatible species which are congeneric or in allied genera to oilseed rape were found growing sympatrically. Moreover, the capillary presence of railways in the agricultural landscape provides a putative source of contamination of GE-free agriculture. Our results suggests that carefully adapted monitoring designs may be set in order to detect introduction events that can lead to rapid establishment and growing populations as the accepted contamination thresholds are likely to be biologically insufficient to prevent further environmental contamination.

Full article available at http://www.enveurope.com/content/24/1/23

Waminal, N. ; Ryu, K. ; Choi, S.; Kim, H. 2013. Randomly detected genetically modified (GM) maize (*Zea mays L.*) near a transport route revealed a fragile 45S rDNA phenotype. *PLOS ONE*, 8(9):e74060. doi: 10.1371/journal.pone.0074060. eCollection 2013.

Monitoring of genetically modified (GM) crops has been emphasized to prevent their potential effects on the environment and human health. Monitoring of the inadvertent dispersal of transgenic maize in several fields and transport routes in Korea was carried out by qualitative multiplex PCR, and molecular analyses were conducted to identify the events of the collected GM maize. Cytogenetic investigations through fluorescence in situ hybridization (FISH) of the GM maize were performed to check for possible changes in the 45S rDNA cluster because this cluster was reported to be sensitive to replication and transcription stress. Three GM maize kernels were collected from a transport route near Incheon port, Korea, and each was found to contain NK603, stacked MON863 x NK603, and stacked NK603 x MON810 inserts, respectively. Cytogenetic analysis of the GM maize containing the stacked NK603 x MON810 insert revealed two normal compact 5S rDNA signals, but the 45S rDNA showed a fragile phenotype, demonstrating a "beads-on-a-string" fragmentation pattern, which seems to be a consequence of genetic modification. Implications of the 45S rDNA cluster fragility in GM maize are also discussed.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3767626/

Schulze, J.; Frauenknecht, T.; Brodmann, P.; Bagutti, C. 2014. Unexpected diversity of feral genetically modified oilseed rape (*Brassica napus* L.) despite a cultivation and import ban in Switzerland. *PLOS ONE*, 9(12): e114477.

Despite cultivation and seed import bans of genetically modified (GM) oilseed rape (*Brassica napus* L.), feral GM plants were found growing along railway lines and in port areas at four sites in Switzerland in 2011 and 2012. All GM plants were identified as glyphosate-resistant GM event GT73 (Roundup Ready, Monsanto). The most affected sites were the Rhine port of Basel and the St. Johann freight railway station in Basel. To assess the distribution and intra- and interspecific outcrossing of GM oilseed rape in more detail, we monitored these two sites in 2013. Leaves and seed pods of feral oilseed rape plants, their possible hybridization partners and putative hybrid

plants were sampled in monthly intervals and analysed for the presence of transgenes by real-time PCR. Using flow cytometry, we measured DNA contents of cell nuclei to confirm putative hybrids. In total, 2787 plants were sampled. The presence of GT73 oilseed rape could be confirmed at all previously documented sampling locations and was additionally detected at one new sampling location within the Rhine port. Furthermore, we found the glufosinate-resistant GM events MS8xRF3, MS8 and RF3 (all traded as InVigor, Bayer) at five sampling locations in the Rhine port. To our knowledge, this is the first time that feral MS8xRF3, MS8 or RF3 plants were detected in Europe. Real-time PCR analyses of seeds showed outcrossing of GT73 into two non-GM oilseed rape plants, but no outcrossing of transgenes into related wild species was observed. We found no hybrids between oilseed rape and related species. GM plants most frequently occurred at unloading sites for ships, indicating that ship cargo traffic is the main entry pathway for GM oilseed rape. In the future, it will be of major interest to determine the source of GM oilseed rape seeds.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4252112/

4.3 Contamination of conventional crops subsequent to the planting of transgenic varieties

Operating aspects resulting from the emergence of seeds which remain in the soil – and grow in the subsequent crop are another potential source of contamination. Transgenic seeds which fall in the area during the harvest of a certain crop germinate in the following crop and persist along with the subsequent crop because they tolerate the herbicide which would supposedly guarantee the area was cleaned[40]. This difficulty the management and decreases the profitability of the crops, in addition to affecting the final value of the crop (higher impurity indexes will be detected by the time the seeds are classified).

D'Hertefeldt, T.; Jørgensen, R.; Pettersson, L. 2008. Long term persistence of GM oilseed rape in the seed bank. *Biology Letters,* 4 (3): 314-7.

Coexistence between genetically modified (GM) and non-GM plants is a field of rapid development and considerable controversy. In crops, it is increasingly important to understand and predict the GM volunteer emergence in subsequent non-GM crops. Theoretical models suggest recruitment from the seedbank over extended periods, but empirical evidence matching these predictions has been scarce. Here, we provide evidence of long-term GM seed persistence in conventional agriculture. Ten years after a trial of GM herbicide-tolerant oilseed rape, emergent seedlings were collected and tested for herbicide tolerance. Seedlings that survived the glufosinate herbicide (15 out of 38 volunteers) tested positive for at least one GM insert. The resulting density was equivalent

40 This agronomic problem is very present in Brazil, where corn-soybean rotation is the dominant production system in the main growing areas (Midwest). In the USA, where the practice is limited by climate winter conditions, unfavorable successive plantings, the problem is less relevant. See news story developed by Embrapa at <https://www.embrapa.br/busca-de-noticias/-/noticia/1920015/milho-voluntario-rr-pode-se-tornar-planta-daninha-para-soja>.

to 0.01 plantsm^{-2}, despite complying with volunteer reduction recommendations. These results are important in relation to debating and regulating coexistence of GM and non-GM crops, particularly for planting non-GM crops after GM crops in the same field.

Full article available at http://rsbl.royalsocietypublishing.org/content/4/3/314.full

Munier, D. ; Brittan, K. ; Lanini, W. 2012. Seed bank persistence of genetically modified canola in California. *Environ Sci Pollut Res Int.,* 19(6):2281-4.

Introduction: Canola, which is genetically modified (GM) for tolerance to glyphosate, has the potential to become established as a new glyphosate resistant weed, thus reducing the effectiveness of glyphosate.
Methods: Volunteer from dormant canola seeds produced thousands of plants per hectare in the fourth year (2011) following a 2007 crop harvest. This occurred with no additional canola seed production since the 2007 harvest.
Results: Volunteer plants following harvests of annual crops are typically only a problem for the first year after harvest. In California, glyphosate is the core herbicide on over a million hectares of high value row, tree, and vine crops and new glyphosate resistant weeds reduce the effectiveness of glyphosate.
Conclusions: The combination of dormant seed and herbicide resistance makes GM glyphosate-resistant canola a new and difficult California weed which was first observed in the winter of 2009.

Full article available at https://goo.gl/igTbF4

4.4 Contamination over the agrifood chain

In addition to the biological aspects, the issues related to the coexistence between transgenic and non-transgenic products require consideration to socioeconomic aspects. With this respect, studies indicate cases of contamination identified over the agrifood chain which have generated disputes between the several actors and causing damages difficult to be measured, affections particularly to those who are not willing to plant/trade/consume GM products are presented.

Friesen, L.; Nelson, A.; Van Acker, R. 2003. Evidence of contamination of pedigreed canola (*Brassica napus*) seed lots in western Canada with genetically engineered herbicide resistance traits. *Agronomy Journal,* 95 (5), 1342-1347.

The objective of this study was to survey pedigreed canola L.) seedlots for contaminating herbicide resistance traits because of complaints from farmers regarding glyphosate [*N*-(phosphonomethyl) glycine]-resistant canola volunteers occurring unexpectedly in their fields at densities and in patterns that suggested that pollen-mediated gene flow from neighboring fields in previous was not the source of contamination. Twenty-seven unique, commercial certified canola seedlot samples were collected. Glyphosate-resistant seedlot samples were not collected. Canola samples were planted in the field, and when the canola had two to four true leaves, glypho- sate, glufosinate [2-amino-4-(hydroxymethylphosphinyl) butanoic acid], and thifensulfuron {methyl 3-[[[[(4-methoxy-6-

methyl-1,3,5-triazin-2yl) amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylate} herbicides were applied. Surviving canola plantswere counted.Of the 27 seedlots, 14 had contamination levels above 0.25% and therefore failed 99.75% cultivar purity guideline for certified canola seed. Three seed lots had glyphosate resistance contamination levels in excess of 2.0%. Unexpected contamination (even at 0.25%) can cause problems for producers that practice direct seeding and depend on glyphosate for nonselective, broad-spectrum weed control. To avoid unexpected problems and costs, it is important that farmers are cognizant of the high probability that pedigreed canola seedlots are cross-contaminated with the various herbicide resistance traits.

Full article available at http://kurt-schweizer.ch/home/gentech/RapsKanadaVanAcker.pdf

COM – Macilwain, C. 2005. US launches probe into sales of unapproved transgenic corn. *Nature*, Vol 434, p.423.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/15791213

Binimelis, R. 2008. Coexistence of plants and coexistence of farmers: is an individual choice possible? *Journal of Agricultural and Environmental Ethics*, DOI10.1007/s10806-008-9099-4.

The introduction of genetically modified organisms (GMOs) in Europe has been characterized by controversy. In 2002, the European Union introduced the concept of "coexistence" as a compromise solution that, through the establishment of science-based technical measures, should allow the market to operate freely while reducing policy conflicts on GMOs. However, the concept remains highly contested and the technical measures difficult to apply. This paper presents qualitative research on the conceptualization and implementation of the coexistence framework in two regions of Spain (Catalonia and Aragon), where 42% and 55% of maize was GM in 2006, respectively. In this context, the concept of coexistence and its proposed implementation both fail to resolve previous conflicts and actually work to generate new ones through the individualization of choice and impacts. Considerations of the social conditions in which the technology and the management measures are implemented were not taken into account. This resulted in the promotion of biotechnological agriculture over other alternatives.

Full article available at http://stopogm.net/sites/stopogm.net/files/choicebinimelis.pdf

The seed exchange practices between family farmers and traditional communities over the world – base of significant part of the existing agrobiodiversity – are also threatened by the free circulation of transgenic varieties. In addition, such practices represent a risk factor for the involuntary dissemination of transgenes over local varieties, such as reported in Mexico and in several other sites of the planet.

Dyer, G.; Serratos-Hernández, J.; Perales, H.; Gepts, P.; Piñeyro-Nelson, A.; *et al*. 2009.

Dispersal of Transgenes through Maize Seed Systems in Mexico. *PLOS ONE,* 4(5): e5734. doi:10.1371/journal.pone.0005734.

Objectives: Current models of transgene dispersal focus on gene flow via pollen while neglecting seed, a vital vehicle for gene flow in centers of crop origin and diversity. We analyze the dispersal of maize transgenes via seeds in Mexico, the crop's cradle.
Methods: We use immunoassays (ELISA) to screen for the activity of recombinant proteins in a nationwide sample of farmer seed stocks. We estimate critical parameters of seed population dynamics using household survey data and combine these estimates with analytical results to examine presumed sources and mechanisms of dispersal.
Results: Recombinant proteins Cry1Ab/Ac and CP4/EPSPS were found in 3.1% and 1.8% of samples, respectively. They are most abundant in southeast Mexico but also present in the west-central region. Diffusion of seed and grain imported from the United States might explain the frequency and distribution of transgenes in west-central Mexico but not in the southeast.
Conclusions: Understanding the potential for transgene survival and dispersal should help design methods to regulate the diffusion of germplasm into local seed stocks. Further research is needed on the interactions between formal and informal seed systems and grain markets in centers of crop origin and diversification.

Full article available at https://goo.gl/mhq1md

Iversen, M.; Grønsberg, I.; Van Den Berg, J.; Fischer, K.; Aheto, D.; Bøhn, T. 2014. Detection of transgenes in local maize varieties of small-scale farmers in eastern cape, South Africa. *PLOS ONE*, 9(12):e116147. doi: 10.1371/journal.pone.0116147.

Small-scale subsistence farmers in South Africa have been introduced to genetically modified (GM) crops for more than a decade. Little is known about i) the extent of transgene introgression into locally recycled seed, ii) what short and long-term ecological and socioeconomic impacts such mixing of seeds might have, iii) how the farmers perceive GM crops, and iv) to what degree approval conditions are followed and controlled. This study conducted in the Eastern Cape, South Africa, aims primarily at addressing the first of these issues. We analysed for transgenes in 796 individual maize plants (leaves) and 20 seed batches collected in a village where GM insect resistant maize was previously promoted and grown as part of an governmental agricultural development program over a seven year period (2001-2008). Additionally, we surveyed the varieties of maize grown and the farmers' practices of recycling and sharing of seed in the same community (26 farmers were interviewed). Recycling and sharing of seeds were common in the community and may contribute to spread and persistence of transgenes in maize on a local or regional level. By analysing DNA we found that the commonly used transgene promoter p35s occurred in one of the 796 leaf samples (0.0013%) and in five of the 20 seed samples (25%). Three of the 20 seed samples (15%) included herbicide tolerant maize (NK603) intentionally grown by the farmers from seed bought from local seed retailers or acquired through a currently running agricultural development program. The two remaining positive seed samples (10%) included genes for insect resistance (from MON810). In both cases the farmers were unaware of the transgenes present. In conclusion, we demonstrate that transgenes are mixed into seed storages of small-scale farming communities where recycling and sharing of seeds are common, i.e. spread beyond the control of the formal seed system.

Full article available at http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0116147

Part 3
Risks to the environment associated to the growth and/or use of transgenic plants

The synthesis of Bt toxins and the tolerance to herbicides are the characteristics incorporated through genetic modification to the vast majority of transgenic plants, in study and commercially released in the planet.

Such characteristics, which in the real world also involve combinations in pyramided events, will be separately addressed in this chapter.

The selected articles show that, in all of the cases, the available studies do not allow to support the hypothesis of absence of environmental risks. The analyses are fragile, the samples are insufficient, the methods are questionable, but, basically, the complexity of the involved ecological relationships is not covered by the research design.

Issues related to environmental impacts must be addressed based on appropriate references and covering the range of alternatives which extends from the absence of technologies to the examination of the impacts caused by products and technological packages associated to them. Thus, when some scientists or political actors prefer to compare environmental impacts resulting from the use of transgenic plants with (and only with) those generated in agricultural systems managed with the intensive contribution of pesticides, leaving aside environment-friendly managements, of agroecological basis, they are evidently deciding for not taking into consideration the entire set of real possibilities.

In the spirit of this book, it is assumed that the evaluation of environmental impacts must also consider alternative references, involving agricultural-ecological systems of agroecological or organic basis. In addition to being scientifically justified, such references are better adjusted to the sustainability subject and, therefore, of the compared evaluation of socioenvironmental impacts resulting from the concerned technologies.

Wolfenbarger, L.; Phifer, P. 2000. The ecological risks and benefits of genetically engineered plants. *Science*, Vol 290.

Discussions of the environmental risks and benefits of adopting genetically engineered organisms are highly polarized between pro- and anti-biotechnology groups, but the current state of our knowledge is frequently overlooked in this debate. A review of existing scientific literature reveals that key experiments on both the environmental risks and benefits are lacking. The complexity of ecological systems presents considerable challenges for experiments to assess the risks and benefits and inevitable uncertainties of genetically engineered plants. Collectively, existing studies emphasize that these can vary spatially, temporally, and according to the trait and cultivar modified.

http://www.sciencemag.org/content/290/5499/2088

1 Environmental risks associated to the use of Bt plants

1.1 The molecular specificity and the mode of action of Bt proteins are not fully understood yet

The companies which develop and trade transgenic plants state that the main advantage of the Bt technology would be associated to the specificity of action of the Cry proteins. By attaching to specific sites and receptors of the digestive systems of certain insects, the Cry1 would have lethal effect only over some insects of the Lepidoptera order, while the Cry3 would be specific to Coleoptera. Thus, side effects and damages to non-target organisms would be prevented and Bt technologies would affect only the populations of organisms listed as crop pests.

The scientific revision reveals failure of this premise. A wide set of studies and diverging information certify that the exceptions overcome the supposed specificity rule.

The general assumption promoted by the companies and other supporters of the technology not only does not exist, but there is an accumulation of registers of impacts on organisms supposedly insensitive to certain Cry proteins.

The available knowledge show that the mode of action of such proteins is not fully understood yet. Biological structures present in the organism of the insects seem not to constitute passive and specific receptors, contributing, in contrast, on a relevant way for the effectiveness of such toxins.

Deml, R.; Meise, T.; Dettner, K. 1999. Effects of *Bacillus thuringiensis*δδ-endotoxins on food utilization, growth, and survival of selected phytophagous insects. *Journal of Applied Entomology*, 123, pp. 55-64.

Feeding experiments using the Bacillus thuringiensisδ-endotoxins, CryIA(c) and CryIIIA, were conducted with herbivorous insects from various orders (Lepidoptera, Coleoptera, Homoptera) in the laboratory. The mortality data obtained indicate a species-specific susceptibility of the insects to the toxins whereby the feeding habits of the given animal seem to play a negligible part. An unexpected, severely damaging effect of CryIIIA on caterpillars was established, for the first time. By computing various development and nutritional indices it could be shown that retarded growth of the insects tested may not only be traced back to reduced feeding but also to a decreased utilization of food containing an endotoxin. The insect gut seems to be the site of operation and of storage or complete degradation of the endotoxins because neither in the faeces nor in the haemolymph and fat body, could the toxins and their degradation products, respectively, be detected hitherto by means of gel electrophoreses. An altering effect of the toxins on the gut-microflora pattern is indicated from the first examinations but has to be further confirmed. Finally the applicability of these trials in corresponding examinations of transgenic plants producing B. thuringiensis toxin is dicussed.

https://goo.gl/jc7Nni

Lundgren, J.; Wiedenmann, R. 2002. Coleopteran-specific Cry3Bb toxin from transgenic corn pollen does not affect the fitness of a nontarget species, *Coleomegilla maculata* DeGeer (*Coleoptera*: *Coccinellidae*). *Environmental Entomology*, 31, pp. 1213-1218.

Coleomegilla maculata DeGeer is a polyphagous predator that is important for suppressing pest populations in corn. We evaluated the impact of Cry3Bb-expressing transgenic corn pollen (event MON863) on *C. maculata* fitness parameters in the laboratory. *C. maculata* larvae were fed mixtures of pollen containing 0, 25, 50, 75, or 100% transgenic pollen, aphids, or were not fed; and the duration of each instar and pupal weight were compared among treatments. In a second trial, other *C. maculata* larvae were reared on one of the pollen mixtures or artificial diet; and the duration of larval and pupal stages, pupal weight, adult mobility, adult survivorship, and female fecundity were compared among treatments. There were no differences in any of the fitness parameters among *C. maculata* in the treatments fed different mixtures of pollen. Beetles in the pollen mixture treatments had faster larval development times, greater larval survivorship, and greater pupal weight than the beetles fed only aphids or an artificial diet. We conclude that we did not detect any effects on the fitness of *C. maculata* that ingested pollen from event MON863. However, these results do not necessarily apply to other transgenic crops expressing toxins specific to Coleoptera.

http://www.bioone.org/doi/abs/10.1603/0046-225X-31.6.1213?journalCode=enve

Tsuda, Y.; Nakatani, F.; Hashimoto, K.; Ikawa, S.; Matsuura, C.; Fukada, T.; Sugimoto, K.; Himeno, M. 2003. Cytotoxic activity of *Bacillus thuringiensis* Cry proteins on mammalian cells transfected with cadherin-like Cry receptor gene of *Bombyx mori* (silkworm). *Biochemical Journal*, 369, 697-703.

Bacillus thuringiensis is widely used as a biological pesticide to control insects that either cause damage to crops or transmit disease. That it can also target the model organism *Caenorhabditis elegans* has not only provided exciting new insights into how the toxins produced by the bacterium target their victims but also how target insects counter the attack. Modern approaches such as reverse genetics and microarray technology have revealed novel receptors for the toxins and possible signal transduction pathways induced within the host following intoxication. This article will discuss how these findings fit in with current models and how they might influence future studies.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1223121/

Hussein, H.; Habustová, O.; Sehnal, F. 2005. Beetle–specific *Bacillus thuringiensis* Cry3Aa toxin reduces larval growth and curbs reproduction in *Spodoptera littoralis* (Boisd.). *Pest Management Science*, 61, 1186–1192.

Application of Bacillus thuringiensis tenebrionis (Bt) and expression of the Bt protein Cry3Aa in genetically modified crops are used for targeted control of the Colorado potato beetle Leptinotarsa decemlineata (Say). The Cry3A proteins are selectively toxic for the beetles but the present study describes effects of Cry3Aa on the Egyptian armyworm, Spodoptera littoralis (Boisduval). Cry3Aa expressed in potatoes or added to an agar-base wheat-germ diet reduced the growth of S. littoralis caterpillars and the fertility of adults. The effect of 1.4 mg kg(-1) Cry3Aa in potato leaves was comparable with that of 3.3 mg kg(-1) in the diet. This difference in activity was correlated with better digestibility and higher conversion efficiency of the diet that also supported higher reproduction rate: S. littoralis grown on the potatoes reached a similar size to those on the diet but laid only 702 instead of 1077 eggs per female. Cry3Aa consumption reduced body growth as a consequence of lower food intake without significantly affecting food digestibility and the conversion efficiency of nutrients. The 11% and 5% body weight reductions caused by 1.4 mg kg(-1) Cry3Aa in potatoes and 3.3 mg kg(-1) in the diet, respectively, were associated with 74% and 65% reduction in the number of progeny; S littoralis grown on a diet with 9.1 mg kg(-1) Cry3Aa were 10% smaller and produced no viable progeny. These data suggest that the curtailment of reproduction was not caused by a general shortage of nutrient reserves but by a more direct Cry3Aa effect on the reproduction process.

http://www.ncbi.nlm.nih.gov/pubmed/16152673

REV - Crickmore, N. 2005. Using worms to better understand how *Bacillus thuringiensis* kills insects. *Trends in Microbiology*, Vol.13 No.8.

Bacillus thuringiensis is widely used as a biological pesticide to control insects that either cause damage to crops or transmit disease. That it can also target the model organism *Caenorhabditis elegans* has not only provided exciting new insights into how the toxins produced by the bacterium target their victims but also how target insects counter the attack. Modern approaches such as reverse genetics and microarray technology have revealed novel receptors for the toxins and possible signal transduction pathways induced within the host following intoxication. This article will discuss how these findings fit in with current models and how they might influence future studies.

http://www.cell.com/trends/microbiology/abstract/S0966-842X%2805%2900156-3

Hussein, H.; Habustová, O.; Turanli, F.; Sehnal, F. 2006. Potato expressing beetle–specific *Bacillus thuringienis* Cry3Aa toxin reduces performance of a moth. *Journal of Chemical Ecology*, 32, 1–13.

Expression of the Bacillus thuringiensis beetle-specific toxin Cry3Aa, which renders a genetically modified potato cultivar resistant to the Colorado potato beetle Leptinotarsa decemlineata, exerts a deleterious effect on the polyphagous moth Spodoptera littoralis. The caterpillars of S. littoralis feed less and produce smaller pupae on the genetically modified cultivar (NewLeaf Superior) than on the parental nontransgenic cultivar (Superior). The conversion efficiencies of total dry matter, combustion heat, carbon, and nitrogen from leaves to insect biomass are similar on both cultivars.

In spite of similar food utilization and a relatively small difference in the body mass at pupation, female adults that developed from caterpillars fed on NewLeaf Superior lay a mean of 309 eggs compared to a mean of 713 eggs deposited by females that developed from caterpillars fed on Superior. Because of this difference and a simultaneous reduction in fertility (egg hatchability) from 78 to 48%, a pair of adults that fed as larvae on NewLeaf Superior produces only 148 larvae, whereas a pair of adults that fed as larvae on Superior produces 556 larvae. We suggest that small amounts of Cry3Aa that accumulate in insect tissue and persist until the adult stage are responsible for the decline in reproduction.

http://www.ncbi.nlm.nih.gov/pubmed/16525866

REV – Hilbeck, A.; Schmidt J. 2006. Another view on Bt proteins - How specific are they and what else might they do? *Biopesticides International*, 2: 1-50.

The entomopathogenic bacterium *Bacillus thuringiensis* (Bt) and its toxins are extensively used for pest control purposes in agriculture, forestry and public health programmes since the 1930. In addition to spray formulations, transgenic plants containing Bt genes for the expression of the toxins (Bt plants) are commercially available since the mid 1990s and are grown on an increasing percentage of the global agricultural area. A main reason for the importance of Bt as a pesticide is the assumed environmental safety concluded from the high specificity of its endotoxins (Cry proteins) towards a limited number of target organisms, mostly distinct groups of pest insects. While the mode of action of the Cry toxins in these susceptible target insects is well studied, Bt experts claim that several details are still not understood well enough. Although there is considerable experience with the application and the environmental safety of Bt sprays, a number of research papers were published in the past that did report adverse effects on non-target organisms. These and the widespread use of transgenic Bt plants stimulated us to review the published laboratory feeding studies on effects of Bt toxins and transgenic Bt plants on non-target invertebrates. We describe those reports that documented adverse effects in non-target organisms in more detail and focus on one prominent example, the green lacewing, *Chrysoperla carnea*. Discussing our findings in the context of current molecular studies, we argue firstly that the evidence for adverse effects in non-target organisms is compelling enough that it would merit more research. We further conclude from our in-depth analysis that the published reports studying the effects of Bt toxins from Bt pesticides and transgenic Bt plants on green lacewing larvae provide complementary and not contradictory data. And, finally, we find that the key experiments explaining the mode of action not only in this particular affected non-target species but also in most other affected non-target species are still missing. Considering the steadily increasing global production area of Bt crops, it seems prudent to thoroughly understand how Bt toxins might affect non-target organisms.

https://goo.gl/jI3QON

REV - Bravo, A.; Gill, S.; Soberón, M. 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*, 49, 423–435.

Bacillus thuringiensis Cry and Cyt protein families are a diverse group of proteins with activity against insects of different orders - Lepidoptera, Coleoptera, Diptera and also against other invertebrates such as nematodes. Their primary action is to lyse midgut epithelial cells by inserting into the target membrane and forming pores. Among this group of proteins, members of the 3-Domain Cry family are used worldwide for insect control, and their mode of action has been characterized in some detail. Phylogenetic analyses established that the diversity of the 3-Domain Cry family evolved by the independent evolution of the three domains and by swapping of domain III among toxins. Like other pore-forming toxins (PFT) that affect mammals, Cry toxins interact with specific receptors located on the host cell surface and are activated by host proteases following receptor binding resulting in the formation of a pre-pore oligomeric structure that is insertion competent. In contrast,

Cyt toxins directly interact with membrane lipids and insert into the membrane. Recent evidence suggests that Cyt synergize or overcome resistance to mosquitocidal-Cry proteins by functioning as a Cry-membrane bound receptor. In this review we summarize recent findings on the mode of action of Cry and Cyt toxins, and compare them to the mode of action of other bacterial PFT. Also, we discuss their use in the control of agricultural insect pests and insect vectors of human diseases.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1857359/

Jimenez-Juarrez, N.; Munoz-Garay, C.; G**ó**mez, I.; Saab-Rincon, G.; Damina-Almazo, J.; Gill, S.; Soberón, M.; Bravo, A. 2007. *Bacillus thuringiensis* Cry1Ab mutants affecting oligomer formation are non-toxic to *Manduca sexta* larvae. *The Journal of Biological Chemistry*, 282, pp. 21222-21229.

Pore-forming toxins are biological weapons produced by a variety of living organisms, particularly bacteria but also by insects, reptiles, and invertebrates. These proteins affect the cell membrane of their target, disrupting permeability and leading eventually to cell death. The pore-forming toxins typically transform from soluble, monomeric proteins to oligomers that form transmembrane channels. The Cry toxins produced by Bacillus thuringiensis are widely used as insecticides. These proteins have been recognized as pore-forming toxins, and their primary action is to lyse midgut epithelial cells in their target insect. To exert their toxic effect, a prepore oligomeric intermediate is formed leading finally to membrane-inserted oligomeric pores. To understand the role of Cry oligomeric pre-pore formation in the insecticidal activity we isolated point mutations that affected toxin oligomerization but not their binding with the cadherin-like, Bt-R(1) receptor. We show the helix alpha-3 in domain I contains sequences that could form coiled-coil structures important for oligomerization. Some single point mutants in this helix bound Bt-R(1) receptors with similar affinity as the wild-type toxin, but were affected in oligomerization and were severally impaired in pore formation and toxicity against Manduca sexta larvae. These data indicate the pre-pore oligomer and the toxin pore formation play a major role in the intoxication process of Cry1Ab toxin in insect larvae.

Full article available at http://www.jbc.org/content/282/29/21222.long

REV - Pigott, C.; Ellar, D. 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiology and Molecular Biology Reviews*, 71 (2): 255–281.

Bacillus thuringiensis produces crystalline protein inclusions with insecticidal or nematocidal properties. These crystal (Cry) proteins determine a particular strain's toxicity profile. Transgenic crops expressing one or more recombinant Cry toxins have become agriculturally important. Individual Cry toxins are usually toxic to only a few species within an order, and receptors on midgut epithelial cells have been shown to be critical determinants of Cry specificity. The best characterized of these receptors have been identified for lepidopterans, and two major receptor classes have emerged: the aminopeptidase N (APN) receptors and the cadherin-like receptors. Currently, 38 different APNs have been reported for 12 different lepidopterans. Each APN belongs to one of five groups that have unique structural features and Cry-binding properties. While 17 different APNs have been reported to bind to Cry toxins, only 2 have been shown to mediate toxin susceptibly in vivo. In contrast, several cadherin-like proteins bind to Cry toxins and confer toxin susceptibility in vitro, and disruption of the cadherin gene has been associated with toxin resistance. Nonetheless, only a small subset of the lepidopteran-specific Cry toxins has been shown to interact with cadherin-like proteins. This review analyzes the interactions between Cry toxins and their receptors, focusing on the identification and validation of receptors, the molecular basis for receptor recognition, the role of the receptor in resistant insects, and proposed models to explain the sequence of events at the cell surface by which receptor binding leads to cell death.

http://www.ncbi.nlm.nih.gov/pubmed/17554045

Schmidt, J.; Braun, C.; Whitehouse, L.; Hilbeck, A. 2009. Effects of activated Bt transgene products (Cry1Ab, Cry3Bb) on immature stages of the ladybird *Adalia bipunctata* in Laboratory Ecotoxicity Testing. *Arch Environ Contam Toxicol*, 56:221–228.

Insect-active Bacillus thuringiensis (Bt) proteins are expressed by several transgenic crop plants to control certain pests, but nontarget organisms such as ladybirds also can be exposed to these proteins in the field. We developed an improved ecotoxicity testing protocol and conducted feeding trials in a laboratory setting to test for possible adverse effects of different concentrations of microbially produced trypsin-activated Cry1Ab and Cry3Bb toxins on the coccinellid Adalia bipunctata. Larval/pupal mortality, development time, and overall body mass accumulation were recorded. Even at the lowest concentration (5 microg/ml), A. bipunctata larvae fed with the lepidopteran-active Cry1Ab toxin exhibited significantly higher mortality than the control group. In experiments with the coleopteran-active Cry3Bb, only a concentration of 25 microg/ml resulted in a marginally significantly higher mortality compared to the control. Both experiments revealed a slight decline in mortality at the highest concentration of 50 microg/ml, though this was statistically significant only in the Cry1Ab treatment. No differences were detected for development time and body mass of newly emerged adults. Dilutions of the expression vector pBD10--used as a control to exclude effects of the toxin production method--at concentrations between 10 and 100 microg/ml revealed no significant effects on either of the studied parameters. This suggests that the increased mortality of larvae in the toxin feeding trials was caused directly by the activated Bt toxins and raises questions regarding their commonly postulated specificity and their mode of action in A. bipunctata. Implications of the reported results for ladybird populations and their biological pest control functions in transgenic crop ecosystems are discussed.

Full article available at http://stopogm.net/sites/stopogm.net/files/cry3Bbschmidt.pdf

REV - Soberón, M.; Gill, S.; Bravo, A. 2009. Signaling versus punching hole: How do *Bacillus thuringiensis* toxins kill insect midgut cells? *Cellular and Molecular Life Science*, 66: 1337-1349.

Cry proteins, produced by Bacillus thuringiensis (Bt), are widely used for the control of insect pests in agriculture as spray products or expressed in transgenic crops, such as maize and cotton. Little was known regarding the mechanism of action of these toxins when the first commercial Bt product was introduced fifty years ago. However, research on the mechanism of action over the last two decades has enhanced our knowledge of toxin interaction with membrane receptors and their effects in insect midgut cells. All this information allowed for the rational design of improved toxins with higher toxicity or toxins that overcome insect resistance, which could compromise Bt use and effectiveness in the field. In this review we discuss and evaluate the different models of the mode of action of Cry toxins, including a discussion about the role of various receptors in toxin action.

http://www.ncbi.nlm.nih.gov/pubmed/19132293

The bibliography also points out synergies and interactions between the several Cry proteins, as well as of these with other toxins naturally synthesized by *Bacillus thuringiensis*.

Pérez, C.; Fernandez, L.; Sun, J.; Folch, J.; Gill, S.; Soberón, M.; Bravo, A. 2005. *Bacillus thuringiensis* subsp. israelensis Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. *PNAS*, 102(51):18303–18308.

Bacillus thuringiensis subsp. israelensis produces crystal proteins, Cry (4Aa, 4Ba, 10Aa, and 11Aa)

and Cyt (1Aa and 2Ba) proteins, toxic to mosquito vectors of human diseases. Cyt1Aa overcomes insect resistance to Cry11Aa and Cry4 toxins and synergizes the toxicity of these toxins. However, the molecular mechanism of synergism remains unsolved. Here, we provide evidence that Cyt1Aa functions as a receptor of Cry11Aa. Sequential-binding analysis of Cyt1Aa and Cry11Aa revealed that Cyt1Aa binding to Aedes aegypti brush border membrane vesicles enhanced the binding of biotinylated-Cry11Aa. The Cyt1Aa- and Cry11Aa-binding epitopes were mapped by means of the yeast two-hybrid system, peptide arrays, and heterologous competition assays with synthetic peptides. Two exposed regions in Cyt1Aa, loop beta6-alphaE and part of beta7, bind Cry11Aa. On the other side, Cry11Aa binds Cyt1Aa proteins by means of domain II-loop alpha8 and beta-4, which are also involved in midgut receptor interaction. Characterization of single-point mutations in Cry11Aa and Cyt1Aa revealed key Cry11Aa (S259 and E266) and Cyt1Aa (K198, E204 and K225) residues involved in the interaction of both proteins and in synergism. Additionally, a Cyt1Aa loop beta6-alphaE mutant (K198A) with enhanced synergism to Cry11Aa was isolated. Data provided here strongly indicates that Cyt1Aa synergizes or suppresses resistance to Cry11Aa toxin by functioning as a membrane-bound receptor. Bacillus thuringiensis subsp. israelensis is a highly effective pathogenic bacterium because it produces a toxin and also its functional receptor, promoting toxin binding to the target membrane and causing toxicity.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1317914/

REV – Then, C. 2010. Risk assessment of toxins derived from *Bacillus thuringiensis* - synergism, efficacy, and selectivity. *Environ Sci Pollut Res Int*, 17:791-797.

Background, aim, and scope: This review deals with publications concerning the mode of action of Bt proteins and their potential synergism with extrinsic factors. The aim was to assess the impact of those factors especially regarding selectivity and efficacy of Bt toxins and to discuss possible gaps in current risk assessment of genetically engineered plants expressing Bt toxins.Main features: The review shows that several extrinsic factors are able to influence the selectivity and efficacy of Bt toxins. The findings are seen as being relevant for risk assessment in Bt plants. This conclusion is derived by discussing current state of knowledge about the mode of action of Bt proteins, unexpected effects on non-target organism, and the way how modified Bt toxins are expressed in genetically engineered plants.Results: Several publications have been identified that show that certain factors and synergism can impact efficacy and selectivity of Bt toxins. These extrinsic factors are various and include other Bt toxins or parts from the spore of *Bacillus thuringiensis* as well as certain enzymes, environmental stress, non-pathogenic microorganisms, and infectious diseases.Discussion: Research on the underlying mechanism of observed synergism might help to explain some of the effects found in non-target organisms. In general, possible synergism of Bt toxins with extrinsic factors can be relevant for risk assessment of genetically engineered Bt plants since they expose a modified Bt toxin to the environment under various conditions and over a long period of time.Conclusions: Risk assessment of genetically engineered plants should put into question the general assumption of a high selectivity and a linear dose–response relationship in the toxicity of Bt proteins. Both selectivity and efficacy can be influenced by synergism, which can provoke unexpected and undesired effects in non-target organisms.Perspectives: It is suggested that systematic research be promoted on synergism between Bt toxins and potential extrinsic factors that could impact the spectrum of susceptible organisms. This research should become a prerequisite for risk assessment of Bt plants.

Full article available at https://goo.gl/dTf5Cl

REV – Soberón, M.; Pardo, L.; Muñóz-Garay, C.; Sánchez, J.; Gómez, I.; Porta, H.; Bravo, A. 2010. Pore formation by Cry toxins. *Advances in Experimental Medicine and Biology*, 677:127-42.

Bacillus thuringiensis (Bt) bacteria produce insecticidal Cry and Cyt proteins used in the biological control of different insect pests. In this review, we will focus on the 3d-Cry toxins that represent

the biggest group of Cry proteins and also on Cyt toxins. The 3d-Cry toxins are pore-forming toxins that induce cell death by forming ionic pores into the membrane of the midgut epithelial cells in their target insect. The initial steps in the mode of action include ingestion of the protoxin, activation by midgut proteases to produce the toxin fragment and the interaction with the primary cadherin receptor. The interaction of the monomeric CrylA toxin with the cadherin receptor promotes an extra proteolytic cleavage, where helix alpha-1 of domain I is eliminated and the toxin oligomerization is induced, forming a structure of 250 kDa. The oligomeric structure binds to a secondary receptor, aminopeptidase N or alkaline phosphatase. The secondary receptor drives the toxin into detergent resistant membrane microdomains formingpores that cause osmotic shock, burst of the midgut cells and insect death. Regarding to Cyt toxins, these proteins have a synergistic effect on the toxicity of some Cry toxins. Cyt proteins are also proteolytic activated in the midgut lumen of their target, they bind to some phospholipids present in the mosquito midgut cells. The proposed mechanism of synergism between Cry and Cyt toxins is that Cyt1Aa function as a receptor for Cry toxins. The Cyt1A inserts into midgut epithelium membrane and exposes protein regions that are recognized by Cry11Aa. It was demonstrated that this interaction facilitates the oligomerization of Cry11Aa and also its pore formation activity.

http://www.ncbi.nlm.nih.gov/pubmed/20687486

1.2 Negative impacts of the Bt technology on Non-Target Organisms (NTOs)

Uncertainties related to the mode of action of Cry toxins recommend a careful evaluation of the impacts on the so-called "non-target organisms" (NTOs). Representing a wide range of beings potentially submitted to negative impacts of the technology (not included among the crop pests), the NTOs require evaluation protocols adjusted to their singularities, comprising the different environments where they establish themselves.

Acute toxicity, subchronic toxicity, metabolic changes which influence the fitness of certain species, their population dynamics and the negative effects on the involved set, resulting from population fluctuations and including changes to the nutritional quality of the transformed plants[41], are included among the items to be considered in the environmental risk evaluations.

The set of studies referred to in this chapter include a number of cases[42] of negative impacts on NTOs resulting from the use of Bt plants.

41 In section 2.3 of Part 1, articles that exemplify nutritional and compositional differences between Bt and conventional plants are included.

42 Current knowledge about potential impacts of Cry toxins on ONAs suggest that the studies listed here represent only "the tip of the iceberg" and are illustrative of something that happens to hundreds of species of arthropods in the various countries that adopt the Bt technology.

Some situations incorporate divergences of scientific interpretation about the relationship between causes and consequences. In fact, some controversies related to the real environmental impact of the technology over some insect species are maintained since the previous decade. Attention should be given to the significant examples of the Monarch butterfly[43] (*Danaus plexippus*), the neuroptera larva (*Chrysoperla carnea)* and ladybug *Adalia bipunctata*.

REV – Hilbeck, A. 2001. Implications of transgenic, insecticidal plants for insect and plant biodiversity. *Perspectives in Plant Ecology, Evolution and Systematics*, 4(1), 43-61.

Genetically modified plants are widely grown predominantly in North America and to a lesser extent in Australia, Argentina and China but their regions of production are expected to spread soon beyond these limited areas also reaching Europe where great controversy over the application of gene technology in agriculture persists. Currently, several cultivars of eight major crop plants are commercially available including canola, corn, cotton, potato, soybean, sugar beet, tobacco and tomato, but many more plants with new and combined multiple traits are close to registration. While currently agronomic traits (herbicide resistance, insect resistance) dominate, traits conferring "quality" traits (altered oil compositions, protein and starch contents) will begin to dominate within the next years. However, economically the most promising future lies in the development and marketing of crop plants expressing pharmaceutical or "nutraceuticals" (functional foods), and plants that express a number of different genes. From this it is clear that future agricultural and, ultimately, also natural ecosystems will be challenged by the large-scale introduction of entirely novel genes and gene products in new combinations at high frequencies all of which will have unknown impacts on their associated complex of non-target organisms, i.e. all organisms that are not targeted by the insecticidal protein. In times of severe global decline of biodiversity, pro-active precaution is necessary and careful consideration of the likely expected effects of transgenic plants on biodiversity of plants and insects is mandatory.
In this paper possible implications of non-target effects for insect and plant biodiversity are discussed and a case example of such non-target effects is presented. In a multiple year research project, tritrophic and bitrophic effects of transgenic corn, expressing the gene from *Bacillus thuringiensis* (Bt-corn) that codes for the high expression of an insecticidal toxin (Cry1Ab), on the natural enemy species, *Chrysoperla carnea* (the green lacewing), was investigated. In these laboratory trials, we found prey-mediated effects of transgenic Bt-corn causing significantly higher mortality of *C. carnea* larvae. In further laboratory trials, we confirmed that the route of exposure (fed directly or via a herbivorous prey) and the origin of the Bt (from transgenic plants or incorporated into artificial diet) strongly influenced the degree of mortality. In choice feeding trials where *C. carnea* could choose between *Spodoptera littoralis* fed transgenic Bt-corn and *S. littoralis* fed non-transgenic corn, larger instars showed a significant preference for *S. littoralis* fed non-transgenic corn while this was not the case when the choice was between Bt- and isogenic corn fed aphids. Field implications of these findings could be multifold but will be difficult to assess because they interfere in very intricate ways with complex ecosystem processes that we still know only very little about. The future challenge in pest management will be to explore how transgenic plants can be incorporated as safe and effective components of IPM systems and what gene technology can contribute to the needs of a modern sustainable agriculture that avoids or reduces adverse impacts on biodiversity? For mainly economically motivated resistance management purposes, constitutive high expression of Bt-toxins in transgenic plants is promoted seeking to kill almost 100% of all susceptible (and if possible heterozygote resistant) target pest insects. However, for pest management this is usually

43 In section 4.2.1 of Part 5, relevant articles on this scientific controversy are included.

not necessary. Control at or below an established economic injury level is sufficient for most pests and cropping systems. It is proposed that partially or moderately resistant plants expressing quantitative rather than single gene traits and affecting the target pest sub-lethally may provide a more meaningful contribution of agricultural biotechnology to modern sustainable agriculture. Some examples of such plants produced through conventional breeding are presented. Non-target effects may be less severe allowing for better incorporation of these plants into IPM or biological control programs using multiple control strategies, thereby, also reducing selection pressure for pest resistance development.

http://www.sciencedirect.com/science/article/pii/S1433831904700396

REV – Marvier, M.; McCreedy, C.; Regetz, J.; Kareiva, P. 2007. A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. *Science*, 316(5830): 1475-1477.

Although scores of experiments have examined the ecological consequences of transgenic Bacillus thuringiensis (Bt) crops, debates continue regarding the nontarget impacts of this technology. Quantitative reviews of existing studies are crucial for better gauging risks and improving future risk assessments. To encourage evidence-based risk analyses, we constructed a searchable database for nontarget effects of Bt crops. A meta-analysis of 42 field experiments indicates that nontarget invertebrates are generally more abundant in Bt cotton and Bt maize fields than in nontransgenic fields managed with insecticides. However, in comparison with insecticide-free control fields, certain nontarget taxa are less abundant in Bt fields.

Full article available at http://stopogm.net/sites/stopogm.net/files/metamarvier.pdf

1.2.1 Negative effects on NTOs which directly feed themselves from Bt plant material

One of the greatest changes posed to the impacts assessment of the Bt technologies over organisms established in the agricultural-ecological systems affected by them lies in the elaboration of research methodologies properly comprising the set of potential effects related to the massive dispersion of the toxins.

If on the one side there are signs of acute toxicity, easily detectable, on the other hand, and more detrimental at long term, are the chronic effects of the underdosages.

The articles below indicate – based on bioassays and field experiments – negative impacts observed on NTOs (insects, aquatic arthropods, soil organisms) fed with vegetal material of Bt transgenic origin (including pollen).

COM – Losey, J.; Rayor, L.; Carter, M. 1999. Transgenic pollen harms monarch larvae. *Nature*, 399 (6733): 214.

Without summary.

Full article available at http://www.nature.com/scitable/content/transgenic-pollen-harms-monarch-larvae-97961

Jesse, L.; Obrycki, J. 2000. Field deposition of Bt transgenic corn pollen: lethal effects on the monarch butterfly. *Oecologia*, 125:241–248. doi: 10.1007/s004420000502.

We present the first evidence that transgenic Bacillus thuringiensis (Bt) corn pollen naturally deposited on Asclepias syriaca; common milkweed, in a corn field causes significant mortality of Danaus plexippus L. (Lepidoptera: Danaidae) larvae. Larvae feeding for 48 h on A. syriaca plants naturally dusted with pollen from Bt corn plants suffered significantly higher rates of mortality at 48 h (20±3%) compared to larvae feeding on leaves with no pollen (3±3%), or feeding on leaves with non-Bt pollen (0%). Mortality at 120 h of D. plexippus larvae exposed to 135 pollen grains/cm(2) of transgenic pollen for 48 h ranged from 37 to 70%. We found no sub-lethal effects on D. plexippus adults reared from larvae that survived a 48-h exposure to three concentrations of Bt pollen. Based on our quantification of the wind dispersal of this pollen beyond the edges of agricultural fields, we predict that the effects of transgenic pollen on D. plexippus may be observed at least 10 m from transgenic field borders. However, the highest larval mortality will likely occur on A. syriaca plants in corn fields or within 3 m of the edge of a transgenic corn field. We conclude that the ecological effects of transgenic insecticidal crops need to be evaluated more fully before they are planted over extensive areas.

http://www.ncbi.nlm.nih.gov/pubmed/24595835

Hellmich, R.; Siegfried, B.; Sears, M.; Stanley-Horn, D.; Daniels, M.; Mattila, H.; Spencer, T.; Bidne, K.; Lewis, L. 2001. Monarch larvae sensitivity to *Bacillus thuringiensis*- purified proteins and pollen. *PNAS*, 98 (21) 11925-11930.

Laboratory tests were conducted to establish the relative toxicity of *Bacillus thuringiensis* (*Bt*) toxins and pollen from *Bt* corn to monarch larvae. Toxins tested included Cry1Ab, Cry1Ac, Cry9C, and Cry1F. Three methods were used: (*i*) purified toxins incorporated into artificial diet, (*ii*) pollen collected from *Bt* corn hybrids applied directly to milkweed leaf discs, and (*iii*) *Bt* pollen contaminated with corn tassel material applied directly to milkweed leaf discs. Bioassays of purified *Bt* toxins indicate that Cry9C and Cry1F proteins are relatively nontoxic to monarch first instars, whereas first instars are sensitive to Cry1Ab and Cry1Ac proteins. Older instars were 12 to 23 times less susceptible to Cry1Ab toxin compared with first instars. Pollen bioassays suggest that pollen contaminants, an artifact of pollen processing, can dramatically influence larval survival and weight gains and produce spurious results. The only transgenic corn pollen that consistently affected monarch larvae was from Cry1Ab event 176 hybrids, currently <2% corn planted and for which re-registration has not been applied. Results from the other types of *Bt* corn suggest that pollen from the Cry1Ab (events Bt11 and Mon810) and Cry1F, and experimental Cry9C hybrids, will have no acute effects on monarch butterfly larvae in field settings.

Full article available at http://www.pnas.org/content/98/21/11925.full.pdf+html

Zangerl, A.; McKenna, D.; Wraight, C.; Carroll, M.; Ficarello, P.; Warner, R.; Berenbaum, M. 2001. Effects of exposure to event 176 *Bacillus thuringiensis* corn pollen on monarch and

black swallowtail caterpillars under field conditions. *PNAS*, 98: 11908-11912.

The widespread planting of corn genetically modified to produce *Bacillus thuringiensis* endotoxin has led to speculation that pollen from these fields might adversely affect nearby nontarget lepidopterans. A previous study of *Bt* corn engineered with Monsanto event 810 failed to detect an effect of pollen exposure on the black swallowtail, *Papilio polyxenes*, in either the field or the laboratory. Here, we report results of a field study investigating the impact of exposure to pollen from a *Bt* corn hybrid containing Novartis event 176 on two species of Lepidoptera, black swallowtails and monarch butterflies, *Danaus plexippus*. Nearly half of the 600 monarch larvae died within the first 24 h; this and subsequent mortality was not associated with proximity to *Bt* corn and may have been due in part to predation. Survivorship of black swallowtails was much higher than that of the monarchs and was also independent of proximity to the transgenic corn. However, despite five rainfall events that removed much of the pollen from the leaves of their host plants during the experiment, we observed a significant reduction in growth rates of black swallowtail larvae that was likely caused by pollen exposure. These results suggest that *Bt* corn incorporating event 176 can have adverse sublethal effects on black swallowtails in the field and underscore the importance of event selection in reducing environmental impacts of transgenic plants.

Full article available at https://naldc.nal.usda.gov/download/39648/

Zwahlen, C.; Hilbeck, A.; Howald, R.; Nentwig, W. 2003. Effects of transgenic Bt corn litter on the earthworm *Lumbricus terrestris*. Molecular Ecology, 12 (8):1077 1086.

A 200-day study was carried out to investigate the impact of transgenic Bacillus thuringiensis (Bt) corn on immature and adult Lumbricus terrestris in the field and in the laboratory. Another objective of this study was to develop test methods that could be used for standard testing of the impact of transgenic plants on different earthworm species in the field and in the laboratory. For this purpose two different experiments were involved, a laboratory experiment with adult L. terrestris and a field experiment with immature L. terrestris. No lethal effects of transgenic Bt corn on immature and adult earthworms were observed. Immature L. terrestris in the field had a very similar growth pattern when fed either (Bt+) or (Bt-) corn litter. No significant differences in relative weights of (Bt+) and (Bt-) corn-fed adult L. terrestris were observed during the first 160 days of the laboratory trial, but after 200 days adult L. terrestris had a significant weight loss of 18% of their initial weight when fed (Bt+) corn litter compared to a weight gain of 4% of the initial weight of (Bt-) corn-fed earthworms. Further studies are necessary to see whether or not this difference in relative weight was due to the Bt toxin or other factors discussed in the study. Degradation of Cry1Ab toxin in corn residues was significantly slower in the field than at 10 degrees C in the laboratory. Enzyme-linked immunosorbent assay results indicated that earthworms in both experiments were exposed to the Bt toxin throughout the whole experimental time.

http://www.ncbi.nlm.nih.gov/pubmed/12753225

Dively, G.; Rose, R.; Sears, M.; Hellmich, R.; Stanley-Horn, D.; Calvin, D.; Russo, J.; Anderson, P. 2004. Effects on monarch butterfly larvae (*Lepidoptera: Danaidae*) after continuous exposure to Cry1Ab expressing corn during anthesis. *Environmental Entomology*, 33: 1116-1125.

Effects on monarch butterfly, *Danaus plexippus* L., after continuous exposure of larvae to natural deposits of *Bacillus thuringiensis* (Bt) and non-Bt pollen on milkweed, were measured in five studies. First instars were exposed at 3Ð4 and 6Ð7 d after initial anthesis, either directly on milkweed plants in commercial corn-fields or in the laboratory on leaves collected from milkweeds in corn plots. Pollen exposure levels ranging from 122 to 188 grains/cm2/d were similar to within-field levels that monarch butterfly populations might experience in the general population of cornÞelds.

Results indicate that 23.7% fewer larvae exposed to these levels of Bt pollen during anthesis reached the adult stage.A risk assessment procedure used previously was updated with a simulation model estimating the proportion of second-generation monarch butterflies affected. When considered over the entire range of the Corn Belt, which represents only 50% of the breeding population, the risk to monarch butterßy larvae associated with long-term exposure to Bt corn pollen is 0.6% additional mortality. Exposure also prolonged the developmental time of larvae by 1.8 d and reduced the weights of both pupae and adults by 5.5%. The sex ratio and wing length of adults were unaffected. The ecological significance of these sublethal effects is discussed relative to generation mortality and adult performance.

Full article available at http://www.bioone.org/doi/abs/10.1603/0046-225X-33.4.1116?journalCode=enve&

Yoichi, S.; Mami, T. 2005. Effects of transgenic Bt corn pollen on a non-target lycaenid butterfly, *Pseudozizeeria maha. Applied Entomology and Zoology*, 40 (1): 151–159.

To evaluate the effect of pollen released from transgenic insecticidal corn on non-target lepidopteran insects, corn pollen deposition density on the leaves of sunflower and black nightshade was measured near a cornfield. At 12 d from the start of anthesis, the highest cumulative pollen density on leaves was approximately 160 grains per cm^2 at 1m from the edge of the cornfield, falling to 20 grains at 5 m and less than 10 grains at 10m. The pollen density calculated using a mathematical model in a previous study evidently had overestimated values. To evaluate precisely the effect of corn pollen expressing Bacillus thuringiensis (Bt) endotoxin (Cry1Ab) on the survival of lepidopteran larvae, we improved the bioassay methods using the pale grass blue, Pseudozizeeria maha, the leaf disc of the wood sorrel, Oxalis corniculata, and transgenic Bt corn (Event-176). When the surface of the leaf was pretreated with a small amount of 80% acetone solution, the preselected pollen dose was successfully applied onto the leaf disc. Larval survival of P. maha was significantly affected at pollen density of more than 20 grains per cm^2 on the leaf disc. It is unlikely that pollens from Bt corn expressing Cry1Ab have wide-scaled deleterious effects on non-target P. maha near cornfields, because of low pollen deposition dose on the leaves.

http://ci.nii.ac.jp/naid/10014474578/en

Lang, A.; Vojtech, E. 2006. The effects of pollen consumption of transgenic Bt maize on the common swallowtail, *Papilio machaon* L. (*Lepidoptera, Papilionidae*). *Basic and Applied Ecology*, 7: 296-306.

Effects of exposure to maize pollen of event Bt176 (cultivar "Navares") on the larvae of the European common swallowtail (*Papilio machaon* L.) were studied in the laboratory. First instar larvae were exposed to different pollen densities applied to leaf disks of *Pastinaca sativa* L. for 48 h. Pollen densities applied in this study were in the range recorded from the field. Larvae which were exposed to higher Bt maize pollen densities consumed more pollen and had a lower survival rate. The LD_{50} with regard to larvae surviving to adulthood was 13.72 pollen grains consumed by first-instar larva. Uptake of Bt maize pollen led to a reduced plant consumption, to a lower body weight, and to a longer development time of larvae. Effects on pupal weight and duration of the pupal period were present but less pronounced and smaller than effects on larvae. Larvae having consumed Bt-maize pollen as first instars had a lower body weight as adult females and smaller forewings as adult males. We conclude that possible effects of Bt maize on European butterflies and moths must be evaluated more rigorously before Bt maize should be cultivated over large areas.

http://www.sciencedirect.com/science/article/pii/S1439179105001246

Rosi-Marshall, E.; Tank, J.; Royer, T.; Whiles, M.; Evans-White, M.; Chambers, C.; Griffiths, N.; Pokelsek, J.; Stephen, M. 2007. Toxins in transgenic crop byproducts may affect headwater stream ecosystems. *PNAS*, 104(41): 16204-16208.

Corn (*Zea mays* L.) that has been genetically engineered to produce the Cry1Ab protein (Bt corn) is resistant to lepidopteran pests. Bt corn is widely planted in the midwestern United States, often adjacent to headwater streams. We show that corn byproducts, such as pollen and detritus, enter headwater streams and are subject to storage, consumption, and transport to downstream water bodies. Laboratory feeding trials showed that consumption of Bt corn byproducts reduced growth and increased mortality of nontarget stream insects. Stream insects are important prey for aquatic and riparian predators, and widespread planting of Bt crops has unexpected ecosystem-scale consequences.

Full article available at http://www.pnas.org/content/104/41/16204.full

Bøhn, T.; Primicerio, R.; Hessen, D.; Traavik, T. 2008. Reduced fitness of *Daphnia magna* fed a Bt-transgenic maize variety. *Archives of Environmental Contamination and Toxicology*, DOI 10.1007/s00244-008-9150-5.

Genetically modified (GM) maize expressing the Bt-toxin Cry1Ab (Bt-maize) was tested for effects on survival, growth, and reproduction of the water flea Daphnia magna, a crustacean arthropod commonly used as a model organism in ecotoxicological studies. In three repeated experiments, D. magna were fed 100% ground maize in suspension, using either GM or isogenic unmodified (UM) maize. D. magna fed GM-maize showed a significantly reduced fitness performance: The mortality was higher, a lower proportion of females reached sexual maturation, and the overall egg production was lower compared to D. magna fed UM isogenic maize. We conclude that the tested variety of Bt-maize and its UM counterpart do not have the same quality as food sources for this widely used model organism. The combination of a reduced fitness performance combined with earlier onset of reproduction of D. magna fed Bt-maize indicates a toxic effect rather than a lower nutritional value of the GM-maize.

http://www.ncbi.nlm.nih.gov/pubmed/18347840

Mason, C.; Sheldon, J.; Pesek, J.; Bacon, H.; Gallusser, R.; Radke, G.; Slabaugh, B. 2008. Assessment of *Chrysoperla plorabunda* longevity, fecundity, and egg viability when adults are fed transgenic Bt corn pollen. *Journal of Agricultural and Urban Entomology*, 25(4): 265–278.

The widespread planting of transgenic corn containing Bacillus thuringiensis (Bt) Cry endotoxin in its tissues for insect pest control raises the potential for influence on many non-target species including pollenfeeding species of Chrysopidae. This study was conducted to assess fitness parameters associated with longevity, fecundity, and egg viability of adult Chrysoperla plorabunda (Fitch) (Neuroptera: Chrysopidae) when fed Bt corn pollen. Bt products tested with their respective non-Bt near-isolines were Event 176 (Cry1Ab), MON810 (Cry1Ab), and TC1507 (Cry1F). Females fed pollen from Event 176 lived significantly longer than those fed pollen from its non-Bt near-isoline. Males fed pollen from TC1507 showed a trend for living longer than males fed its non-Bt near-isoline pollen, but there was no difference for females regarding this event. The mean number of eggs produced per female per day was significantly less for those fed MON810 pollen compared with females fed pollen from the non-Bt near-isoline. Total egg production was significantly less for females fed MON810 pollen vs. females fed pollen from its non-Bt near-isoline.

http://www.bioone.org/doi/abs/10.3954/1523-5475-25.4.265

Höss, S.; Arndt, M.; Baumgarte, S.; Tebbe, C.; Nguyen, H.; Jehle, J. 2008. Effects of transgenic corn and Cry1Ab protein on the nematode, *Caenorhabditis elegans*. *Ecotoxicology and Environmental Safety*, 70 (2): 334-340.

The effects of the insecticidal Cry1Ab protein from Bacillus thuringiensis (Bt) on the nematode, Caenorhabditis elegans, were studied with soil from experimental fields cultivated with transgenic Bt corn (MON810) and with trypsinized Cry1Ab protein expressed in Escherichia coli. The content of Cry1Ab protein was above the detection limit of an ELISA test in only half of the soil samples obtained from transgenic plots, ranging from 0.19 to 1.31 ng g_1 dry weight. In a laboratory bioassay, C. elegans was exposed to rhizosphere and bulk soil from fields with isogenic or transgenic corn or to solutions of Cry1Ab protein (0, 24, 41, 63, 118, and 200 mgl_1) over a period of 96 h, with growth and reproduction serving as the test parameters. Nematode reproduction and growth were significantly reduced in rhizosphere and bulk soil of Bt corn compared with soil from isogenic corn and were significantly correlated with concentrations of the Cry1Ab protein in the soil samples. Moreover, the toxicity of pure Cry1Ab protein to the reproduction and growth of C. elegans was concentration-dependent. As significant inhibition occurred at relatively high concentrations of the Cry1Ab protein (41 mgl_1), the effects of the soil samples from Bt corn could not be assigned directly to the toxicity of the Cry1Ab protein. The results demonstrate that bioassays with the nematode, C. elegans, provide a promising tool for monitoring the potential effects of Bt toxins in aqueous médium and soils.

Full article available at http://stopogm.net/sites/stopogm.net/files/caenorhabditishoess.pdf

Schmidt, J.; Braun, C.; Whitehouse, L.; Hilbeck, A. 2009. Effects of activated Bt transgene products (Cry1Ab, Cry3Bb) on immature stages of the ladybird *Adalia bipunctata* in Laboratory Ecotoxicity Testing. *Arch Environ Contam Toxicol*, 56:221–228.

Insect-active Bacillus thuringiensis (Bt) proteins are expressed by several transgenic crop plants to control certain pests, but nontarget organisms such as ladybirds also can be exposed to these proteins in the field. We developed an improved ecotoxicity testing protocol and conducted feeding trials in a laboratory setting to test for possible adverse effects of different concentrations of microbially produced trypsin-activated Cry1Ab and Cry3Bb toxins on the coccinellid Adalia bipunctata. Larval/pupal mortality, development time, and overall body mass accumulation were recorded. Even at the lowest concentration (5 microg/ml), A. bipunctata larvae fed with the lepidopteran-active Cry1Ab toxin exhibited significantly higher mortality than the control group. In experiments with the coleopteran-active Cry3Bb, only a concentration of 25 microg/ml resulted in a marginally significantly higher mortality compared to the control. Both experiments revealed a slight decline in mortality at the highest concentration of 50 microg/ml, though this was statistically significant only in the Cry1Ab treatment. No differences were detected for development time and body mass of newly emerged adults. Dilutions of the expression vector pBD10--used as a control to exclude effects of the toxin production method--at concentrations between 10 and 100 microg/ml revealed no significant effects on either of the studied parameters. This suggests that the increased mortality of larvae in the toxin feeding trials was caused directly by the activated Bt toxins and raises questions regarding their commonly postulated specificity and their mode of action in A. bipunctata. Implications of the reported results for ladybird populations and their biological pest control functions in transgenic crop ecosystems are discussed.

Full article available at http://stopogm.net/sites/stopogm.net/files/cry3Bbschmidt.pdf

REV - Lang, A.; Otto, M. 2010. A synthesis of laboratory and field studies on the effects of transgenic *Bacillus thuringiensis* (Bt) maize on non-target *Lepidoptera*. *Entomologia Experimentalis et Applicata*, 135: 121–134, 2010.

One of the major applications of transgenic crops in agriculture are the so-called Bacillus thuringiensis Berliner (Bt) plants, in particular Bt maizes, which produce insecticidal Cry proteins that target specific orders, such as the Lepidoptera or Coleoptera. We reviewed publications that reported on the direct toxic effects of Bt-maize and/or Cry proteins of current Bt-maize events on larvae of non-target butterflies and moths (Lepidoptera). In total, 20 peer-reviewed publications were identified, of which 16 papers contributed laboratory-based data and seven field-based data. An adverse effect on caterpillars was recorded in 52% of all laboratory-based and in 21% of all field-based observations. The variables most often studied and having the highest occurrence of effects were larval survival, body mass, and developmental time. Parameters of the adult stage were under-represented in the studies. Overall, 11 lepidopteran species were tested. The majority of the studies originated from the USA, with the Monarch butterfly being the most studied, whereas other species and other parts of the world were widely neglected. Laboratory experiments were often run under unrealistic conditions from an ecological point of view. Although the papers we reviewed indicated a potential hazard for Lepidoptera that are exposed to and feed on lepidopteran-specific Bt-maize pollen, a general conclusion on the level of risk for butterflies and moths cannot as yet be drawn. A comprehensive risk characterization would require thorough hazard identification, exposure assessment, and impact assessment. However, our review showed that even the basic level of hazard characterization is as yet incomplete. Reasons for this are the still-limited numbers of publications and concurrent lack of knowledge, the restriction of data to only a few species, the over-representation of North American species, and the identified limitations of both laboratory and field experiments. The findings of this review suggest that more realistic, ecologically meaningful, and detailed experiments and analyses are crucial to improve the present assessment of Bt-maize cultivation effects on Lepidoptera.

Full article available at http://onlinelibrary.wiley.com/doi/10.1111/j.1570-7458.2010.00981.x/pdf

Jensen, P.; Dively, G.; Swan, C.; Lamp, W. 2010. Exposure and nontarget effects of transgenic Bt corn debris in streams. *Environmental Entomology*, 39 (2) 707-714.

Corn (Zea mays L.) transformed with a gene from the bacterium Bacillus thuringiensis (Bt) comprises 49% of all corn in the United States. The input of senesced corn tissue expressing the Bt gene may impact stream-inhabiting invertebrates that process plant debris, especially trichopteran species related to the target group of lepidopteran pests. Our goal was to assess risk associated with transgenic corn debris entering streams. First, we show the input of corn tissue after harvest was extended over months in a stream. Second, using laboratory bioassays based on European corn borer [Ostrinia nubilalis (Hübner)], we found no bioactivity of Cry1Ab protein in senesced corn tissue after 2 wk of exposure to terrestrial or aquatic environments. Third, we show that Bt near-isolines modify growth and survivorship of some species of invertebrates. Of the four nontarget invertebrate species fed Bt near-isolines, growth of two closely related trichopterans was not negatively affected, whereas a tipulid crane fly exhibited reduced growth rates, and an isopod exhibited reduced growth and survivorship on the Cry1Ab near-isoline but not on the stacked Cry1Ab + Cry3Bb1 near-isoline. Because of lack of evidence of bioactivity of Bt after 2 wk and because of lack of nontarget effects on the stacked near-isoline, we suggest that tissue-mediated differences, and not the presence of the Cry1Ab protein, caused the different responses among the species. Overall, our results provide evidence that adverse effects to aquatic nontarget shredders involve complex interactions arising from plant genetics and environment that cannot be ascribed to the presence of Cry1Ab proteins.

Full article available at http://ee.oxfordjournals.org/content/ee/39/2/707.full.pdf

Bøhn, T.; Traavik, T.; Primicerio, R. 2010. Demographic responses of *Daphnia magna* fed transgenic Bt-maize. *Ecotoxicology*, 19(2): 419-430.

The food/feed quality of a variety of genetically modified (GM) maize expressing Cry1Ab Bt-toxin was tested over the life-cycle of *Daphnia magna*, an arthropod commonly used as model organism in ecotoxicological studies. Demographic responses were compared between animals fed GM or unmodified (UM) near isogenic maize, with and without the addition of predator smell. Age-specific data on survival and birth rates were integrated and analysed using life tables and Leslie matrices. Survival, fecundity and population growth rate (PGR) data generally disfavoured transgenic Bt-maize as feed for *D. magna* compared to animals fed the unmodified (UM) near isogenic line of maize. Decomposition of age-specific effects revealed that the most important contributions to a reduced PGR in the GM-fed group came from both fecundity and survival differences early in life. We conclude that juvenile and young adult stages are the most sensitive experimental units and should be prioritized in future research. These stages are often omitted in toxicological/ecotoxicological studies and in feeding trials.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2811247/

Hilbeck, A.; McMillan, J.; Meier, M.; Humbel, A.; Schlaepfer-Miller, J.; Trtikova, M. 2012. A controversy re-visited: Is the coccinellid *Adalia bipunctata* adversely affected by Bt toxins? *Environmental Sciences Europe*, 24(10).

Background: In 2008/2009, Schmidt and colleagues published a study reporting lethal effects of the microbial Bt toxins Cry1Ab and Cry3Bb on the coccinellid biological control organisms *Adalia bipunctata*. Based on this study, in concert with over 30 other publications, Mon810 cultivation was banned in Germany in 2009. This triggered two commentaries and one experimental study all published in the journal '*Transgenic Research*' that question the scientific basis of the German ban or claim to disprove the adverse effects of the Bt toxins on *A. bipunctata* reported by Schmidt and colleagues, respectively. This study was undertaken to investigate the underlying reasons for the different outcomes and rebuts the criticism voiced by the two other commentaries.
Results: It could be demonstrated that the failure to detect an adverse effect by Alvarez-Alfageme and colleagues is based on the use of a significantly different testing protocol. While Schmidt and colleagues exposed and fed larvae of *A. bipunctata* continuously, Alvarez-Alfageme and colleagues applied an exposure/recovery protocol. When this exposure/recovery protocol was applied to a highly sensitive target insect, *Ostrinia nubilalis*, the lethal effect was either significantly reduced or disappeared altogether. When repeating the feeding experiments with the Bt toxin Cry1Ab using a combined protocol of both previous studies, again, a lethal effect on *A. bipunctata* larvae was observed. ELISA tests with Bt-toxin fed larvae and pupae confirmed ingestion of the toxin.
Conclusions: The new data corroborates earlier findings that Cry1Ab toxin increases mortality in *A. bipunctata* larvae. It was also shown that the different applied testing protocols explained the contrasting results.

Full article available at http://www.enveurope.com/content/24/1/10

MOD –Holst, N.; Lang, A.; Lövei, G.; Otto, M. 2013. Increased mortality is predicted of *Inachis io* larvae caused by Bt-maize pollen in European farmland. *Ecological Modelling*, 250: 126133.

A potential environmental risk of the field cultivation of insect-resistant (Bt-toxin expressing) transgenic maize (*Zea mays*) is the consumption of Bt-containing pollen by herbivorous larvae of butterflies (Lepidoptera). Maize is wind-pollinated, and at flowering time large amounts of pollen can be deposited on various plants growing in the landscape, leading to inadvertent ingestion of toxic pollen with plant biomass consumed by these butterfly larvae. To examine the possible effect of this coincidence, we focused our study on the protected butterfly *Inachis io* and two regions of Europe. Using climatic records, maize and butterfly phenology data, we built a simulation model of the butterfly's annual life cycle, overlaid with the phenology of maize pollen deposition on the leaves of the food plant *Urtica dioica*, and linked these with the dose–response curve of *I. io* larvae

to Bt-maize pollen (event MON810). The simulations indicated that in Northern Europe, where *I. io* is univoltine, Bt-maize pollen would not be present on the food plant at the same time as the *I. io* larvae. However, in Central and Southern Europe, where *I. io* is bivoltine, Bt-maize pollen and the second generation *I. io* larvae would coincide, and an increased mortality of the larvae was predicted. This prediction differs from earlier studies which predicted negligible effect of field-grown Bt-maize on *I. io* larvae. Our model is an improvement over previous efforts since it is based on more detailed, empirical data, includes more biological detail, and provides explicit estimation of all model parameters. The model is open-source software and is available for re-use and for modelling the effects on other species or regions.

http://www.sciencedirect.com/science/article/pii/S0304380012005315

Linn, M.; Moore, P. 2014. The effects of Bt corn on rusty crayfish (*Orconectes rusticus*) growth and survival. *Arch Environ Contam Toxicol*, 67(3):436-43. doi: 10.1007/s00244-014-0061-3.

Bt crops are one of the most commonly used genetically modified crops worldwide. Bt crops contain a gene that is derived from the bacteria Bacillus thuringiensis, which produces the Cry1Ab toxin. Bt corn that contains the Cry1Ab toxin is used throughout the Midwest United States to control crop pests such as the European corn borer (Ostrinia nubilalis). Headwater streams in regions known for intensive agriculture receive Bt corn detritus after the fall harvest, which is then consumed by a diverse community of stream invertebrates. The rusty crayfish (Orconectes rusticus) is a common invertebrate detritivore in these headwater streams. Both isogenic and Bt corn were grown under the controlled environmental conditions of a greenhouse and, after senescence, were tested for nutritional equality. Rusty crayfish were exposed to one of several detrital treatments composed of Bt corn, Bt corn plus American sycamore (Platanus occidentalis), isogenic corn alone, isogenic corn plus P. occidentalis, or P. occidentalis alone for 8 weeks. Both strains of corn were grown under the controlled environmental conditions in a greenhouse and were tested for nutritional equality after senescence. Crayfish were housed in live streams with a water temperature of 12.8 °C and a 12:12 h light-to-dark photoperiod. Survival and growth of animals within each experimental treatment were monitored each week. After 8 weeks of exposure, there was no statistically significant difference in growth between crayfish in Bt and isogenic treatments. However, survivorship was 31 % lower in the Bt treatment compared with the isogenic treatment. These results suggest that the Bt corn and isogenic corn were of equivalent nutritional value but that Bt corn does have a toxic effect on rusty crayfish during long-term exposure.

http://www.ncbi.nlm.nih.gov/pubmed/25001246

Difficult to be noted in laboratories, subchronic impacts can be consolidated by means of environmental changes involving the affected organisms and their relationship with the environment.

In this context, among the few studies conducted in the sociobiology and etiology fields, are damage evaluations on populations of domestic bees. The results indicate environmental changes which result in damages and imbalances of population order.

Prasifka, P.; Hellmich, R.; Prasifka, J.; Lewis, L. 2007. Effects of Cry1Ab-expressing corn anthers

on the movement of monarch butterfly larvae. *Environmental Entomology*, 36 (1): 228-33.

Decreased larval feeding and weight of the monarch butterfly, Danaus plexippus L., have been detected after 4 d of exposure in the laboratory to a high density of Bacillus thuringiensis (Bt)-expressing anthers. One hypothesis is that larvae exposed to Bt anthers exhibit increased wandering, resulting in less feeding and lower weight gain. To test this hypothesis, 2-d-old monarch butterfly larvae exposed to milkweed leaf disks with no anthers, anthers that express Bt (Cry1Ab, event MON810), or other non-Bt anthers were observed using a video-tracking system. As had been shown in previous studies, larvae exposed to Bt anthers fed less and gained less weight than larvae exposed to non-Bt or no anthers, yet there was no evidence of feeding on anthers. Total distance moved, maximum displacement from release point, percentage of time spent moving or near anthers, or mean turn angle did not differ across treatments. However, larvae exposed to Bt anthers spent more time off milkweed leaf disks than those exposed to no anthers and were more likely to move off the leaf than larvae exposed to non-Bt anthers. Results suggest that larvae exposed to Bt anthers behave differently and that ingestion may not be the only way Bt can affect nontarget insects like the monarch butterfly.

Full article available at http://ee.oxfordjournals.org/content/ee/36/1/228.full.pdf

Ramirez-Romero, R.; Desneux, N.; Decourtye, A.; Chaffiol, A.; Pham-Delègue, M. 2008. Does Cry1Ab protein affect learning performances of the honey bee *Apis mellifera* L. (*Hymenoptera*, *Apidae*)? *Ecotoxicology and Environmental Safety*, 70 (3): 327 333.

Genetically modified Bt crops are increasingly used worldwide but side effects and especially sublethal effects on beneficial insects remain poorly studied. Honey bees are beneficial insects for natural and cultivated ecosystems through pollination. The goal of the present study was to assess potential effects of two concentrations of Cry1Ab protein (3 and 5000 ppb) on young adult honey bees. Following a complementary bioassay, our experiments evaluated effects of the Cry1Ab on three major life traits of young adult honey bees: (a) survival of honey bees during sub-chronic exposure to Cry1Ab, (b) feeding behaviour, and (c) learning performance at the time that honey bees become foragers. The latter effect was tested using the proboscis extension reflex (PER) procedure. The same effects were also tested using a chemical pesticide, imidacloprid, as positive reference. The tested concentrations of Cry1Ab protein did not cause lethal effects on honey bees. However, honey bee feeding behaviour was affected when exposed to the highest concentration of Cry1Ab protein, with honey bees taking longer to imbibe the contaminated syrup. Moreover, honey bees exposed to 5000 ppb of Cry1Ab had disturbed learning performances. Honey bees continued to respond to a conditioned odour even in the absence of a food reward. Our results show that transgenic crops expressing Cry1Ab protein at 5000 ppb may affect food consumption or learning processes and thereby may impact honey bee foraging efficiency. The implications of these results are discussed in terms of risks of transgenic Bt crops for honey bees.

Full article available at http://stopogm.net/sites/stopogm.net/files/hymenopteraramirez.pdf

Han, P.; Niu, C-Y.; Lei, C-L.; Cui, J-J.; Desneux, N. 2010. Quantification of toxins in a Cry1Ac + CpTI cotton cultivar and its potential effects on the honey bee *Apis mellifera* L. *Ecotoxicology*, 19(8):1452-9.

Transgenic Cry1Ac + CpTI cotton (CCRI41) is increasingly planted throughout China. However, negative effects of this cultivar on the honey bee Apis mellifera L., the most important pollinator for cultivated ecosystem, remained poorly investigated. The objective of our study was to evaluate the potential side effects of transgenic Cry1Ac + CpTI pollen from cotton on young adult honey bees A. mellifera L. Two points emphasized the significance of our study: (1) A higher expression level of insecticidal protein Cry1Ac in pollen tissues was detected (when compared with previous

reports). In particular, Cry1Ac protein was detected at 300 ± 4.52 ng g(-1) [part per billion (ppb)] in pollen collected in July, (2) Effects on chronic mortality and feeding behaviour in honey bees were evaluated using a no-choice dietary feeding protocol with treated pollen, which guarantee the highest exposure level to bees potentially occurring in natural conditions (worst case scenario). Tests were also conducted using imidacloprid-treated pollen at a concentration of 48 ppb as positive control for sublethal effect on feeding behaviour. Our results suggested that Cry1Ac + CpTI pollen carried no lethal risk for honey bees. However, during a 7-day oral exposure to the various treatments (transgenic, imidacloprid-treated and control), honey bee feeding behaviour was disturbed and bees consumed significantly less CCRI41 cotton pollen than in the control group in which bees were exposed to conventional cotton pollen. It may indicate an antifeedant effect of CCRI41 pollen on honey bees and thus bees may be at risk because of large areas are planted with transgenic Bt cotton in China. This is the first report suggesting a potential sublethal effect of CCRI41 cotton pollen on honey bees. The implications of the results are discussed in terms of risk assessment for bees as well as for directions of future work involving risk assessment of CCRI41 cotton.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2995320/

1.2.2 Negative effects on NTOs which indirectly consume Bt plant material (through trophic chains)

In addition to potential impacts related to the direct consumption of Bt vegetal material, studies show that there are damages related to the indirect consumption, involving the accumulation of the toxins over the trophic chains. This is due to the fact that the Bt toxins remain biologically active (they are, then, accessed by non-phytophagous predators). It is also noted that changes to the nutritional quality of the preys and hosts of parasitoids (resulting from these organisms feeding on Bt material) may generate negative impacts on species located in upper scales of the trophic and ecological webs.

Hilbeck, A.; Baumgartner, M.; Fried, P.; Bigler, F. 1998. Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla cornea* (*Neuroptera: Chrysopidae*). *Environmental Entomology*, 276: 480-487.

Laboratory feeding experiments using transgenic *Bacillus thuringiensis* variety *kurstaki* (Berliner) corn plants have been carried out to study the effects of *B. thuringiensis*-fed herbivores (i.e., prey), on the predator *Chrysoperla carnea* Stephens. Host plants were a transgenic *B. thuringiensis*-expressing (CrylAb) corn hybrid and the corresponding untransformed, *B. thuringiensis*-free corn hybrid. Two different prey species were used in the experiments, the European corn borer, *Ostrinia nubilalis* (Hübner) (lepidopterous target pest), and *Spodoptera littoralis* (Boisduval) (lepidopterous nontarget pest for *B. thuringiensis*). The objectives were to quantify the effects of *B. thuringiensis*-fed prey on chrysopid immature development and to determine whether observed effects were caused by sick, suboptimal prey (indirect effects) or associated with *B. thuringiensis*-related causes (direct effects). Mean total immature mortality for chrysopid larvae raised on *B. thuringiensis*-fed prey was 62% compared with 37% when raised on *B. thuringiensis*-free prey. There was no significant difference in

mortality between chrysopid larvae reared on *B. thuringiensis*-fed *O. nubilalis* or *B. thuringiensis*-fed *S. littoralis*. Similarly, no significant difference in mortality was detected when chrysopid larvae were raised on *B. thuringiensis*-free *O. nubilalis* or *B. thuringiensis*-free *S. littoralis*. Development time of chrysopid larvae was prolonged when *B. thuringiensis*-fed *O. nubilalis* was given to the predators but not for *B. thuringiensis*-fed *S. littoralis*. Although some unnoticed adverse effects in *S. littoralis* may have occurred because of the *B. thuringiensis* corn, our results suggest that the reduced fitness of chrysopid larvae was associated with *B. thuringiensis*. The prolonged development time of chrysopid larvae raised on *B. thuringiensis*-fed *O. nubilalis* was probably because of a combined effect of *B. thuringiensis* exposure and nutritional deficiency caused by sick prey.

https://goo.gl/8vnWb8

Hilbeck, A.; Moar, W.; Pusztai-Carey, M.; Filippini, A.; Bigler, F. 1999. Prey-mediated effects of Cry1Ab toxin and protoxin and Cry2A protoxin on the predator *Chrysoperla carnea. Entomologia Experimentalis et Applicata*, 91: 305–316.

Laboratory feeding experiments were carried out to study prey-mediated effects of artificial diet containing Bacillus thuringiensis proteins on immature Chrysoperla carnea. Activated Cry1Ab toxin and the protoxins of Cry1Ab and Cry2A were mixed into standard meridic diet for Spodoptera littoralis (Boisduval) larvae at the following concentrations; for Cry1Ab toxin, 25, 50, 100gg–1 diet were used; for Cry1Ab protoxin, the concentration was doubled (50gg–1 diet, 100gg–1 diet and 200gg–1 diet) to give relative comparable levels of toxin concentration. Cry2A protoxin was incorporated into the meridic diet at one concentration only (100gg–1 diet). For the untreated control, the equivalent amount of double distilled water was added to the meridic diet. Individual C.carnea larvae were raised on S.littoralis larvae fed with one of the respective treated meridic diets described above. The objectives were to quantify and compare the resulting effects on mortality and development time of C.carnea with those observed in two previous studies investigating prey-mediated effects of transgenic Cry1Ab toxin-producing corn plants and the other studying effects of Cry1Ab toxin fed directly to C.carnea larvae. Mean total immature mortality for chrysopid larvae reared on B.thuringiensis-fed prey was always significantly higher than in the control (26%). Total immature mortality of C.carnea reared on Cry1Ab toxin 100gg–1 diet-fed prey was highest (78%) and declined with decreasing toxin concentration. Cry1Ab protoxin-exposed C.carnea larvae did not exhibit a dose response. Prey-mediated total mortality of Cry1Ab protoxin-exposed chrysopid larvae was intermediate (46–62%) to Cry1Ab toxin exposed (55–78%) and Cry2A protoxin (47%) exposed C.carnea. In agreement with the previous studies, total development time of C.carnea was not consistently, significantly affected by the Bt-treatments except at the highest Cry1Ab toxin concentration. However, both highest mortality and delayed development of immature C.carnea raised on Cry1Ab toxin 100gg–1 diet – fed prey may have been confounded with an increased intoxication of S.littoralis larvae that was observed at that concentration. At all other B.thuringiensis protein concentrations S.littoralis was not lethally affected. Comparative analysis of the results of this study with those of the two previous studies revealed that in addition to prey/herbivore by B.thuringiensis interactions, also prey/herbivore by plant interactions exist that contribute to the observed toxicity of B.thuringiensis – fed S.littoralis larvae for C.carnea. These findings demonstrate that tritrophic level studies are necessary to assess the long-term compatibility of insecticidal plants with important natural enemies.

https://goo.gl/qSHpvu

Dutton, A.; Klein, H.; Romeis, J.; Bigler, F. 2002. Uptake of Bt-toxin by herbivores feeding on transgenic maize and consequences for the predator *Chrysoperia carnea*. *Ecological Entomology*, 27: 441-7.

1. *Chrysoperla carnea* is an important predatory insect in maize. To assess the ecological effects of

Bt-maize, expressing the Cry1Ab protein, on larvae of this predator, the following factors were examined: (1) the performance of three prey herbivores (*Rhopalosiphum padi*, *Tetranychus urticae*, and *Spodoptera littoralis*) on transgenic Bt and non-transgenic maize plants; (2) the intake of the Cry1Ab toxin by the three herbivores; and (3) the effects on *C. carnea* when fed each of the prey species.
2. The intrinsic rate of natural increase (r_m) was used as a measure of performance for *R. padi* and *T. urticae*. No difference in this parameter was observed between herbivores reared on Bt or non-transgenic plants. In contrast, a higher mortality rate and a delay in development were observed in *S. littoralis* larvae when fed Bt-maize compared with those fed the control maize plants.
3. The ingestion of Cry1Ab toxin by the different herbivores was measured using an immunological assay (ELISA). Highest amounts of Cry1Ab toxin were detected in *T. urticae*, followed by *S. littoralis*, and only trace amounts detected in *R. padi*.
4. Feeding *C. carnea* with *T. urticae*, which were shown to contain the Cry1Ab toxin, or with *R. padi*, which do not ingest the toxin, did not affect survival, development, or weight of *C. carnea*. In contrast, a significant increase in mortality and a delay in development were observed when predators were fed *S. littoralis* larvae reared on Bt-maize.
5. A combined interaction of poor prey quality and Cry1Ab toxin may account for the negative effects observed on *C. carnea* when fed *S. littoralis*. The relevance of these findings to the ecological risks of Bt-maize on *C. carnea* is discussed.

Full article available at http://onlinelibrary.wiley.com/doi/10.1046/j.1365-2311.2002.00436.x/pdf

Ponsard, S.; Gutierrez, A.; Mills, N. 2002. Effect of Bt-toxin (Cry1Ac) in transgenic cotton on the adult longevity of four *heteropteran* predators. *Environmental Entomology*, 31: 1197-1205.

The management of agroecosystems affects intricately linked assemblages of organisms, and nontarget species are not necessarily unimpacted. We examined the effect of *Bt*-cotton and of lepidopteran prey (*Spodoptera exigua* Hübner) that had ingested it on the adult survivorship of four important heteropteran predators of cotton pests. Longevity significantly decreased for *Orius tristicolor* White and *Geocoris punctipes* Say (by 28 and 27% of the control value, respectively), whereas no effect was found for *Nabis* sp. and *Zelus renardii* Kolenati. This finding contrasts with the results of previous studies in which *Orius* spp. and *G. punctipes* were either fed only plant material or nonlepidopteran prey. *S. exigua* is a lepidopteran with low susceptibility to the *Bt* toxin expressed in cotton and therefore exemplifies the possible effect on predators of lepidopteran pests that would become resistant to *Bt.* The importance of *Bt* toxin type, the difference between plants and prey and between different prey species as routes of ingestion of *Bt* toxins, and the need for studies assessing the population and ecosystem-level effects of *Bt* cotton are discussed.

http://www.bioone.org/doi/abs/10.1603/0046-225X-31.6.1197?journalCode=enve

Baur, M.; Boethel, D. 2003. Effect of Bt-cotton expressing Cry1A(c) on the survival and fecundity of two *hymenopteran* parasitoids (*Braconidae*, *Encyrtidae*) in the laboratory. *Biological Control*, 26, 325–332.

We examined the effect of Bt-cotton (Event 531) plants expressing the *Bacillus thuringiensis* δ-endotoxin CryIA(c) on two hymenopteran endoparasitoids, *Cotesia marginiventris* and *Copidosoma floridanum*. In the laboratory, parasitized and unparasitized *Pseudoplusia includens* larvae were reared on foliage from a conventional soybean cultivar (Pioneer 97B61), a conventional cotton cultivar (DPL 5415), or a Bt-cotton cultivar (NuCotn 33B). *C. marginiventris* developed significantly faster within *P. includens* larvae feeding on Pioneer 97B61 and DPL 5415 compared to those feeding on NuCotn 33B. *C. marginiventris* that developed inside *P. includens* larvae feeding on NuCotn 33B suffered reduced longevity, and females had fewer ova. NuCotn 33B also affected the growth and development of *P. includens* parasitized with *C. floridanum* and life history parameters of adult *C. floridanum*. Parasitized and unparasitized *P. includens* developed more slowly when they were fed NuCotn 33B and the prepupae weighed less. Survival of parasitized and unparasitized *P. includens* was lower when larvae were fed NuCotn 33B and some evidence points to higher susceptibility of parasitized caterpillars to intoxication by NuCotn 33B. Fewer *C. floridanum* adults emerged from hosts fed NuCotn 33B, but pupal weight and adult longevity were unaffected. Analysis comparing the two experiments conducted with *C. floridanum* suggests that older NuCotn 33B plants (90–120 days after planting) may affect parasitoid development and adult survival less than younger NuCotn 33B plants (60–90 days after planting). Feeding on NuCotn 33B by *P. includens* affected the survival and development of the two hymenopteran endoparasitoids studied here, and the degree of the effect was similar to that observed with natural resistance found in soybean plants. It remains to be determined if the effects demonstrated here are less than, equal to, or greater than the impact of conventional insecticide applications used in conventional, non-transgenic, cotton.

http://www.sciencedirect.com/science/article/pii/S1049964402001603

REV – Lövei, G.; Arpaia, S. 2005. The impact of transgenic plants on natural enemies: a critical review of laboratory studies. *Entomologia Experimentalis et Applicata*, 114 (1):114.

We reviewed laboratory tests which studied the impact of genetically modified plants on arthropod natural enemies. A total of 18 species of predators and 14 species of parasitoids have been tested, most in only a few experiments. Certain groups (braconid wasps) or species (the green lacewing, *Chrysoperla carnea*) have attracted much effort, while representatives of others, including whole orders (e.g., Diptera), have never had a species tested. We conclude that laboratory tests are not the 'worst case' scenarios intended by the experimental designs, and are not often ecologically realistic: they typically provided ad libitum feeding, no prey choice, single prey type, no combination of stress factors and usually uniform temperatures. None of these are representative of field conditions, yet most could be easily mimicked in more complex laboratory tests. In most cases (94.6%), the studies were unable to indicate the level of power required to detect any impact. Small sample size and large variability are factors that mask all but very large differences in potential effects. For predators, 126 parameters were quantified, most commonly including survival/mortality (37 cases), development time (22), and body mass/size (20). For parasitoids, 128 parameters were quantified, the majority involving lectins or proteinase inhibitors. Most frequent measurements were: fecundity (23 experiments), adult longevity, extent of parasitism (17 each), body size, mortality, and larval development time. An aggregative scoring (summarising all quantified parameters) indicated that the laboratory tests quantified a remarkable number of cases (30% for predators, 39.8% for parasitoids), where the impacts of the genetically modified plant were significantly negative. These involve various parameters, organisms, test methods, and significance levels, but collectively they indicate that the use of genetically modified crops may result in negative effects on the natural enemies of crop pests.

http://onlinelibrary.wiley.com/doi/10.1111/j.0013-8703.2005.00235.x/abstract

Pilcher, C.; Rice, M.; Obrycki, J. 2005. Impact of transgenic *Bacillus thuringiensis* corn and crop phenology on five nontarget arthropods. *Environmental Entomology*, 34(5): 1302-1316.

Large-scale field studies were conducted to determine if temporal plantings of *Bacillus thuringiensis* (Berliner) (*Bt*) corn (event 176 and *Bt*11) would affect the seasonal abundance of the following generalist predators: *Coleomegilla maculata* DeGeer and *Cycloneda munda* (Say) (Coleoptera: Coccinellidae), *Orius insidiosus* (Say) (Heteroptera: Anthocoridae), *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae), and one specialist parasitoid, *Macrocentrus cingulum* Brischke (Hymenoptera: Braconidae). Adult populations were monitored using Pherocon AM yellow sticky traps at three locations in Iowa (1996–1998). At each location, a split-plot design was used with *Bt* and non-*Bt* corn as main plots and three planting dates as the split plots. Few differences in

abundance were observed between *Bt* and non-*Bt* corn for the generalist predators studied. However, *M. cingulum*, a specialist parasitoid of European corn borer, was significantly affected by the presence of *Bt* corn. Densities of adult *M. cingulum* were 29–60% lower in *Bt* corn compared with non-*Bt* corn. Regression analyses indicated *M. cingulum* adults were preferentially recruited to and subsequently increased over time in the non-*Bt* corn treatments at each location within each year. Significant differences were observed among planting dates for all five species. Abundance effects from *Bt* corn on these natural enemies were not unexpected given the foraging and searching behaviors of different species and their varying levels of dependence on the presence of European corn borer.

Full article available at http://ee.oxfordjournals.org/content/ee/34/5/1302.full.pdf

Liu, X-X.; Sun, C-G.; Zhang, Q-W. 2005. Effects of transgenic *Cry1A+CpTI* cotton and Cry1Ac toxin on the parasitoid, *Campoketis chlorideae* (*Hymenoptera*: *Ichneumonidae*). *Insect Science*, 12: 101-107.

The effect of transgenic double genes, *Cry1A+CpTI* cotton and Cry1Ac toxin on the parasitoid, *Campoketis chlorideae* Uchida of cotton bollworm, *Helicoverpa armigera* (Hübner), was investigated in the laboratory. *Helicoverpa armigera* larvae when in the first, second and third instar could not survive if fed on transgenic cotton leaves. Consequently, *C. chlorideae* larvae could not complete their development if parasitizing on such hosts. After *H. armigera* larvae were reared on transgenic or traditional cotton leaves for 12J48 hours, they were parasitized by *C. chlorideae* females. Parasitized larvae continued to feed on transgenic or traditional cotton for 12–48 h. The present results showed that the body weight of larvae of the parasitoids were significantly reduced when parasitized hosts fed on transgenic cotton leaves compared to those fed on traditional cotton. Duration of egg and larvae stage were significantly prolonged, pupal and adult weight of *C. chloridae* was decreased when the host larvae fed on transgenic cotton leaves longer than 48 h. The development duration of *C. chlorideae* pupae on the hosts fed on transgenic cotton leaves in each treatment was not significantly different from those of controls. The longevity of parasitoid females and males fed with a solution containing Cry1Ac toxin was not significantly different with that of the control.

http://onlinelibrary.wiley.com/doi/10.1111/j.1744-7917.2005.00012.x/abstract

Liu, X.; Zhang, Q.; Zhao, J-Z.; Cai, Q.; Xu, H.; Li, J. 2005. Effects of the Cry1Ac toxin of *Bacillus thuringiensis* on *Microplitis mediator*, a parasitoid of the cotton bollworm, *Helicoverpa armigera*. *Entomologia Experimentalist et Applicata*, 114: 205-213.

Interactions between the cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), its larval parasitoid *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae), and the Cry1Ac toxin of *Bacillus thuringiensis* Berliner were evaluated under laboratory conditions. The growth of *H. armigera* larvae was delayed and its pupal rate and pupal weight decreased when they were fed on a diet containing Cry1Ac toxin. Due to the lowered growth rate of the host larvae, the time available for parasitization of *H. armigera* by *M. mediator* increased when the host larvae were reared on a diet containing Cry1Ac toxin at concentrations of 0.5, 1, 2, and 4 μg g^{-1}. The longevity of female and male parasitoids was not significantly affected when newly emerging wasps fed on honey solutions containing three different concentrations of Cry1Ac toxin (125, 250, and 500 μg ml^{-1}). When female parasitoids were fed on honey solutions containing Cry1Ac, their offsprings' egg and larval development period, pupal weight, length of pupation, adult weight, and adult longevity did not change significantly in most of the treatments compared with controls. When the female parasitoids parasitized host larvae that had been fed on a diet containing 0.5, 1, 2, 4, and 8 μg g^{-1} Cry1Ac toxin, their offsprings' eggs and larvae were significantly delayed. Their pupal weight, adult weight, and adult longevity were also significantly less than controls.

http://onlinelibrary.wiley.com/doi/10.1111/j.1570-7458.2005.00248.x/abstract

Zhang, G-F.; Wan, F-H.; Lövei, G.; Liu, W-X.; Guo, J-Y. 2006. Transmission of Bt toxin to the predator *Propylaea japonica* (*Coleoptera*: *Coccinellidae*) through its aphid prey feeding on transgenic Bt cotton. *Environmental Entomology*, 35(1): 143-150.

Laboratory feeding experiments using transgenic *Bacillus thuringiensis* (*Bt*) cotton plants were carried out to evaluate the transmission of *Bt* toxin among trophic levels and the effects of *Bt*-fed herbivorous prey on the coccinellid predator *Propylaea japonica* (Thunberg). The experimental host plants were transgenic *Bt*-expressing cotton cultivars, NuCOTN 33B and GK-12 and one corresponding untransformed isogenic (non-*Bt*) cultivar. The herbivorous prey, cotton aphid *Aphis gossypii* Glover, was not sensitive to *Bt* toxin. Trace amounts of *Bt* toxins (6.0 ng/g fresh mass [FM] in GK-12, 4.0 ng/g FM in NuCOTN 33B) were detected in *A. gossypii* feeding on *Bt* cotton cultivars. *Bt* toxin was detected in ladybirds preying on *Bt*-fed aphids, and its quantity increased as the predatory period extended (5–20 d). Small amounts of *Bt* toxin was also found in newly hatched, unfed coccinellid larvae when their parents fed on NuCOTN 33B-reared aphids (15.0 ng/g FM), but not when the parents were fed on GK-12–reared prey. In experiments assessing life history consequences, mortality was low (mean = 7.9%), confirming that the rearing methods were appropriate. There were no distinct differences in preimaginal mortality between predators reared on *Bt*-fed or *Bt*-free aphids. The preimaginal stages of the ladybird beetles developed faster when reared on prey fed on either *Bt*-cotton cultivar than those fed control prey. There was a trend of more adult malformations when the predator was fed with prey from one (GK-12) but not the other of the *Bt* cotton cultivars than on control prey. There were no significant differences in the preovipositing period or in fecundity. Ladybird beetles preying on *Bt*-reared aphids matured faster and mated more frequently than those fed on *Bt*-free aphids. These results indicate that *Bt* toxin expressed in transgenic cotton cultivars can be transmitted to a higher trophic level through a nontarget pest insect and may alter the biology and behavior of a predatory ladybird. Further work should evaluate the possible long-term, sublethal impacts on the agroenvironment under field conditions.

http://www.bioone.org/doi/abs/10.1603/0046-225X-35.1.143?journalCode=enve

REV – Lövei, G.; Andow, D.; Arpaia, S. 2009. Transgenic insecticidal crops and natural enemies: a detailed review of laboratory studies. *Environmental Entomology*, 38(2): 293-306.

This review uses a data-driven, quantitative method to summarize the published, peer-reviewed literature about the impact of genetically modiÞed (GM) plants on arthropod natural enemies in laboratory experiments. The method is similar to meta-analysis, and, in contrast to a simple author-vote counting method used by several earlier reviews, gives an objective, data-driven summary of existing knowledge about these effects. SigniÞcantly more non-neutral responses were observed than expected at random in 75% of the comparisons of natural enemy groups and response classes. These observations indicate that Cry toxins and proteinase inhibitors often have non-neutral effects on natural enemies. This synthesis identiÞes a continued bias toward studies on a few predator species, especially the green lacewing, *Chrysoperla carnea* Stephens, which may be more sensitive to GM insecticidal plants (16.8% of the quantiÞed parameter responses were signiÞcantly negative) than predators in general (10.9% signiÞcantly negative effects without *C. carnea*). Parasitoids were more susceptible than predators to the effects of both Cry toxins and proteinase inhibitors, with fewer positive effects (18.0%, signiÞcant and nonsigniÞcant positive effects combined) than negative ones (66.1%, signiÞcant and nonsigniÞcant negative effects combined).GMplants can have a positive effect on natural enemies (4.8% of responses were signiÞcantly positive), although signiÞcant negative (21.2%) effects were more common. Although there are data on 48 natural enemy species, the database is still far from adequate to predict the effect of a *Bt* toxin or proteinase inhibitor on natural enemies.

Full article available at http://stopogm.net/sites/stopogm.net/files/ReviewLoeveietal.pdf

Zhou, J.; Xiao, K.; Wei, B.; Wang, Z.; Tian, Y.; Tian, Y.; Song, Q. 2014. Bioaccumulation of Cry1Ab protein from an herbivore reduces anti-oxidant enzyme activities in two spider species. *PLOS ONE*, 9(1): e84724. doi:10.1371/journal.pone.0084724.

Cry proteins are expressed in rice lines for lepidopteran pest control. These proteins can be transferred from transgenic rice plants to non-target arthropods, including planthoppers and then to a predatory spider. Movement of Cry proteins through food webs may reduce fitness of non-target arthropods, although recent publications indicated no serious changes in non-target populations. Nonetheless, Cry protein intoxication influences gene expression in Cry-sensitive insects. We posed the hypothesis that Cry protein intoxication influences enzyme activities in spiders acting in tri-trophic food webs. Here we report on the outcomes of experiments designed to test our hypothesis with two spider species. We demonstrated that the movement of CryAb protein from *Drosophila* culture medium into fruit flies maintained on the CryAb containing medium and from the flies to the spiders *Ummeliata insecticeps* and *Pardosa pseudoannulata*. We also show that the activities of three key metabolic enzymes, acetylcholine esterase (AchE), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) were significantly influenced in the spiders after feeding on Cry1Ab-containing fruit flies. We infer from these data that Cry proteins originating in transgenic crops impacts non-target arthropods at the physiological and biochemical levels, which may be one mechanism of Cry protein-related reductions in fitness of non-target beneficial predators.

Full article available at https://goo.gl/4tAj0O

1.3 Dispersion and persistence of Bt proteins in the environment and imbalances caused in the soil microbiota communities

As exemplified in the articles referred above, the Cry proteins disperse themselves and remain for significant periods in different environments: food chains, soil, water bodies interconnected to the crops, natural and semi-natural ecosystems of the crop surroundings etc.

Exsudations of Cry proteins, by the Bt plants' roots, pollen dissemination and biotransposition of the toxins over the food webs (including the decomposers sphere) are included among the main dispersion means of such insecticide proteins in the environment.

1.3.1 Spread and persistence of BT toxins in trophic chains

Dutton, A.; Klein, H.; Romeis, J.; Bigler, F. 2002. Uptake of Bt-toxin by herbivores feeding on transgenic maize and consequences for the predator *Chrysoperia carnea*. *Ecological Entomology*, 27: 441-7.

1. *Chrysoperla carnea* is an important predatory insect in maize. To assess the ecological effects of Bt-maize, expressing the Cry1Ab protein, on larvae of this predator, the following factors were examined: (1) the performance of three prey herbivores (*Rhopalosiphum padi*, *Tetranychus urticae*, and *Spodoptera littoralis*) on transgenic Bt and non-transgenic maize plants; (2) the intake of the Cry1Ab toxin by the three herbivores; and (3) the effects on *C. carnea* when fed each of the prey species.
2. The intrinsic rate of natural increase (r_m) was used as a measure of performance for *R. padi* and *T. urticae*. No difference in this parameter was observed between herbivores reared on Bt or non-transgenic plants. In contrast, a higher mortality rate and a delay in development were observed in *S. littoralis* larvae when fed Bt-maize compared with those fed the control maize plants.
3. The ingestion of Cry1Ab toxin by the different herbivores was measured using an immunological assay (ELISA). Highest amounts of Cry1Ab toxin were detected in *T. urticae*, followed by *S. littoralis*, and only trace amounts detected in *R. padi*.
4. Feeding *C. carnea* with *T. urticae*, which were shown to contain the Cry1Ab toxin, or with *R. padi*, which do not ingest the toxin, did not affect survival, development, or weight of *C. carnea*. In contrast, a significant increase in mortality and a delay in development were observed when predators were fed *S. littoralis* larvae reared on Bt-maize.
5. A combined interaction of poor prey quality and Cry1Ab toxin may account for the negative effects observed on *C. carnea* when fed *S. littoralis*. The relevance of these findings to the ecological risks of Bt-maize on *C. carnea* is discussed.

http://onlinelibrary.wiley.com/doi/10.1046/j.1365-2311.2002.00436.x/abstract

Harwood, J.; Wallin, W.; Obrycki, J. 2005. Uptake of Bt endotoxins by nontarget herbivores and higher order arthropod predators: molecular evidence from a transgenic corn agroecosystem. *Molecular Ecology*, 14, 2815–2823.

The planting of transgenic crops expressing Bacillus thuringiensis endotoxins is widespread throughout the world; the prolific increase in their application exposes nontarget organisms to toxins designed to control pests. To date, studies have focused upon the effects of Bt endotoxins on specific herbivores and detritivores, without consideration of their persistence within arthropod food webs. Here, we report the first quantitative field evaluation of levels of Bt endotoxin within nontarget herbivores and the uptake by higher order arthropods. Antibody-based assays indicated significant quantities of detectable Cry1Ab endotoxin within nontarget herbivores which feed on transgenic corn (including the corn flea beetle, Chaetocnema pulicaria, Japanese beetle, Popillia japonica and southern corn rootworm, Diabrotica undecimpunctata howardi). Furthermore, arthropod predators (Coccinellidae, Araneae, and Nabidae) collected from these agroecosystems also contained significant quantities of Cry1Ab endotoxin indicating its movement into higher trophic levels. This uptake by predators is likely to have occurred by direct feeding on plant material (in predators which are facultatively phytophagous) or the consumption of arthropod prey which contained these proteins. These data indicate that long-term exposure to insecticidal toxins occurs in the field. These levels of exposure should therefore be considered during future risk assessments of transgenic crops to nontarget herbivores and arthropod predators.

http://www.ncbi.nlm.nih.gov/pubmed/16029480

Obrist, L.; Dutton, A.; Albajes, R.; Bigler, F. 2006. Exposure of arthropod predators to Cry1Ab toxin in Bt maize fields. *Ecological Entomology*, 31, 143–154.

1. To assess the risks of an insect-resistant transgenic plant for non-target arthropods, it is important to investigate the exposure of non-target species to the transgene product. Exposure of predators in the field depends on the toxin levels in food sources, their feeding ecology and that of their prey.
2. To verify the transmission of Cry1Ab toxin through the food chain, and thus exposure of predators in the field, samples from different plant tissues, herbivores, and predators in Bt maize fields in Spain (Event 176) were collected at different periods over the season and the toxin content was measured using ELISA. Complementary laboratory studies were performed with the omnivorous predator *Orius majusculus* to assess the toxin uptake and persistence after feeding on variable Bt-containing food sources.
3. Field results revealed that toxin content in some herbivores was negligible (aphids, thrips, leafhoppers) compared with those in spider mites. The latter herbivore only occurred after pollen shed and contained three times greater toxin levels than Bt maize leaves.
4. Data confirmed that the Bt toxin can be transferred to predators, that is to say to *Orius* spp., *Chrysoperla* spp., and *Stethorus* sp. This only applied when Bt maize pollen or spider mites were available. The passage of Bt toxin to *O. majusculus* via these two food sources was also confirmed in the laboratory. Contrastingly, some predators in the field (hemerobiids, *Nabis* sp., *Hippodamia* sp., *Demetrias* sp.) contained no or negligible toxin levels even when pollen or spider mites were present.
5. Besides essential information for exposure assessment of numerous arthropod predators, this study provides an insight into the feeding ecology of different arthropods in the maize system.

http://onlinelibrary.wiley.com/doi/10.1111/j.0307-6946.2006.00762.x/abstract

Obrist, L.; Dutton, A.; Romeis, J.; Bigler, F. 2006. Biological activity of Cry1Ab toxin expressed by Bt maize following ingestion by herbivorous arthropods and exposure of the predator *Chrysoperla carnea*. *BioControl*, 51: 31-48.

A major concern regarding the deployment of insect resistant transgenic plants is their potential impact on non-target organisms, in particular on beneficial arthropods such as predators. To assess the risks that transgenic plants pose to predators, various experimental testing systems can be used. When using tritrophic studies, it is important to verify the actual exposure of the predator, i.e., the presence of biologically active toxin in the herbivorous arthropod (prey). We therefore investigated the uptake of Cry1Ab toxin by larvae of the green lacewing (Chrysoperla carnea (Stephens); Neuroptera: Chrysopidae) after consuming two Bt maize-fed herbivores (Tetranychus urticae Koch; Acarina: Tetranychidae and Spodoptera littoralis (Boisduval); Lepidoptera: Noctuidae) by means of an immunological test (ELISA) and the activity of the Cry1Ab toxin following ingestion by the herbivores. Moreover, we compared the activity of Cry1Ab toxin produced by Bt maize to that of purified toxin obtained from transformed Escherichia coli, which is recommended to be used in toxicity studies. The activity of the toxin was assessed by performing feeding bioassays with larvae of the European corn borer (Ostrinia nubilalis (Hübner); Lepidoptera: Crambidae), the target pest of Cry1Ab expressing maize. ELISA confirmed the ingestion of Bt toxin by C. carnea larvae when fed with either of the two prey species and feeding bioassays using the target pest showed that the biological activity of the Cry1Ab toxin is maintained after ingestion by both herbivore species. These findings are discussed in the context of previous risk assessment studies with C. carnea. The purified Cry1Ab protein was more toxic to O. nubilalis compared to the plant-derived Cry1Ab toxin when applied at equal concentrations according to ELISA measurements. Possible reasons for these findings are discussed.

http://link.springer.com/article/10.1007%2Fs10526-005-2936-8

Harwood, J.; Samson, R.; Obrycki, J. 2007. Temporal detection of Cry1Ab-endotoxins in *coccinellid* predators from fields of *Bacillus thuringiensis* corn. *Bull Entomol Res*, 97(6):643-8.

The area planted to genetically engineered crops has increased dramatically in the last ten years. This has generated many studies examining non-target effects of bioengineered plants expressing Bacillus thuringiensis endotoxins. To date, most have focused on population-level effects in the field or laboratory evaluation of specific plant-herbivore or plant-herbivore-predator trophic pathways. Using a post-mortem enzyme-linked immunosorbent assay, we examined the uptake of Cry1Ab-endotoxins by predatory coccinellids and the importance of anthesis to this trophic pathway. Adult Coleomegilla maculata, Harmonia axyridis, Cycloneda munda and Coccinella septempunctata contained low, but detectable, quantities of Bt-endotoxin when screened by ELISA. This was most evident in C. maculata, with 12.8% of 775 individuals testing positive for Cry1Ab-endotoxins. Interestingly, the presence of endotoxins in gut samples was not confined to periods around anthesis, but coccinellid adults tested positive two weeks before and up to ten weeks after pollen was shed, suggesting tri-trophic linkages in their food chain facilitates the transfer of endotoxins into higher-order predators. This contrasts with adult Coleomegilla maculata entering overwintering sites where Bt-endotoxins were not detected in gut samples, indicating low levels of persistence of Cry1Ab-endotoxins within coccinellid predators. This study enhances our understanding of complex interactions between transgenic crops and non-target food webs, but further research is required to quantify the significance of specific trophic linkages in the field.

http://www.ncbi.nlm.nih.gov/pubmed/17997879

Douville, M.; Gagné, F.; André, C; Blaise, C. 2009. Occurrence of the transgenic corn cry1Ab gene in freshwater mussels (*Elliptio complanata*) near corn fields: evidence of exposure by bacterial ingestion. *Ecotoxicology and Environmental Safety*, 72: 17-25.

The purpose of this study was to examine the contamination of cry1 and cry1Ab genes from Bacillus thuringiensis and transgenic corn in feral freshwater mussels collected from sites located in proximity of corn fields. In addition, mussels were transplanted for 2 months to a site in the Huron River, upstream to the Richelieu River, which is subject to intensive corn farming. Mussels were significantly contaminated by both genes in their gills, digestive glands, and gonads, as determined by qPCR methodology. Gene sequence analysis confirmed the presence of transgenic corn cry1Ab gene in mussel tissues. In an attempt to explain the presence of the transgene in mussel tissues, heterotrophic bacteria were grown from surface water and sediment samples on agar plates in the Richelieu River in May and August. The transgene was found at two out of six surface water samples and in one sediment sample. The study revealed that exposure to transgenic corn cry1Ab gene in mussels seems to proceed by ingestion of microorganisms during feeding.

Full article available at https://goo.gl/PYgfYF

Zurbrügg, C.; Nentwig, W. 2009. Ingestion and excretion of two transgenic Bt corn varieties by slugs. *Transgenic Res*, 18(2):215-25. doi: 10.1007/s11248-008-9208-1.

The release of transgenic Bacillus thuringiensis (Bt) corn expressing various Cry endotoxins has raised concern that these endotoxins are disseminated in the food web and may adversely affect non-target beneficial organisms, such as predators and organisms of the decomposer food web. We therefore investigated in a laboratory study, whether the Cry1Ab and Cry3Bb1 protein from Bt corn could potentially be transferred to such organisms by measuring the Cry protein content in the two common agricultural slug pests Arion lusitanicus and Deroceras reticulatum and their feces. We measured Cry1Ab and Cry3Bb1 protein concentration in leaves, intestines, and feces of corn leaf-fed slugs using ELISA and determined how much of the ingested protein is excreted by the

slugs. Cry3Bb1 concentration in leaves of DKC5143Bt corn was significantly higher than Cry1Ab concentration in leaves of N4640Bt corn. While slugs were feeding on corn leaves, the Cry3Bb1 and Cry1Ab proteins were found in intestines and feces of both slug species. Bt protein concentrations in intestines of Cry3Bb1 corn-fed slugs were in both slug species higher than in Cry1Ab corn fed slugs, whereas no differences between Cry3Bb1 and Cry1Ab protein in feces were found. After slugs had ceased feeding on Bt corn, Cry1Ab was detectable in fresh slug feces for a significantly longer time and often in higher amounts than the Cry3Bb1. Our results indicate that both Cry proteins are likely to be transferred to higher trophic levels and to the decomposer food web. Since different Bt proteins seem to vary in their degradation, they have different transfer probabilities. This should be considered in risk assessments for non-target arthropods.

http://www.ncbi.nlm.nih.gov/pubmed/18763046

Yu, H.; Romeis, J.; Li, Y.; Li, X.; Wu, K. 2014. Acquisition of Cry1Ac protein by non-target arthropods in Bt soybean fields. *PLOS ONE*, 9(8): e103973. Doi:10.1371/journal.pone.0103973.

Soybean tissue and arthropods were collected in *Bt* soybean fields in China at different times during the growing season to investigate the exposure of arthropods to the plant-produced Cry1Ac toxin and the transmission of the toxin within the food web. Samples from 52 arthropod species/taxa belonging to 42 families in 10 orders were analysed for their Cry1Ac content using enzyme-linked immunosorbent assay (ELISA). Among the 22 species/taxa for which three samples were analysed, toxin concentration was highest in the grasshopper *Atractomorpha sinensis* and represented about 50% of the concentration in soybean leaves. Other species/taxa did not contain detectable toxin or contained a concentration that was between 1 and 10% of that detected in leaves. These Cry1Ac-positive arthropods included a number of mesophyll-feeding Hemiptera, a cicadellid, a curculionid beetle and, among the predators, a thomisid spider and an unidentified predatory bug belonging to the Anthocoridae. Within an arthropod species/taxon, the Cry1Ac content sometimes varied between life stages (nymphs/larvae *vs.* adults) and sampling dates (before, during, and after flowering). Our study is the first to provide information on Cry1Ac-expression levels in soybean plants and Cry1Ac concentrations in non-target arthropods in Chinese soybean fields. The data will be useful for assessing the risk of non-target arthropod exposure to Cry1Ac in soybean.

Full article available at https://goo.gl/1sO4Iw

1.3.2 Dissemination and persistence of Bt toxins in the environment (soil and water)

Studies show that the Bt toxins remain active in the soil for variable periods, expanded in clayey soil and in the presence of agrochemicals related to transgenic plants carrying tolerance to glyphosate and to other herbicides.

Koskella, J.; Stotzky, G. 1997. Microbial utilization of free and clay-bound insecticidal toxins from *Bacillus thuringiensis* and their retention of insecticidal activity after incubation with microbes. *Applied and Environmental Microbiology*, 1997, p. 3561–3568.

The insecticidal toxins produced by Bacillus thuringiensis subspp. kurstaki and tenebrionis were

resistant when bound on clays, but not when free, to utilization by pure and mixed cultures of microbes as sources of carbon and carbon plus nitrogen, and their availability as a nitrogen source was reduced. The bound toxins retained insecticidal activity both before and after exposure to microbes or pronase. The insecticidal activity of the toxins persisted for 40 days (the longest time evaluated) in nonsterile soil continuously maintained at the -33-kPa water tension and room temperature, alternately air dried and rewetted to the -33-kPa water tension, or alternately frozen and thawed, although alternate drying and wetting reduced the activity.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1389248/pdf/hw3561.pdf

Tapp, H.; Stozsky, G. 1998. Persistence of the insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* in soil. *Soil Biology Biochemistry*, 30 (4): 471-476.

The accumulation and persistence of the insecticidal toxins from *Bacillus thuringiensis* may result in environmental hazards, such as toxicity to nontarget species and the selection of toxin-resistant target species. Toxins from *B. thuringiensis* subsp. *kurstaki* were added to three soils [Kitchawan soil (which contains kaolinite but not montmorillonite) unamended or amended with montmorillonite or kaolinite (as an internal control); Mopala soil, which contains montmorillonite and kaolinite; and San Alejo soil, which does not contain montmorillonite but contains kaolinite], and the persistence of the toxins was determined by insect bioassays using the larvae of the tobacco hornworm (*Manduca sexta*). Toxicity varied with the type of soil: the Kitchawan soil, either unamended or amended with kaolinite, remained toxic to the larvae for more than 6 months, maintaining a lethal concentration at which 50% of the larvae were killed (LC_{50}) of 61 to 111 ng 100 μl^{-1} of soil suspension throughout 195 d of incubation. The Kitchawan soil amended with montmorillonite and the Mopala and San Alejo soils showed reduced insecticidal activity after only 35 d (LC_{50} from 104 to 192 ng 100 μl^{-1}). The pH of soils in which insecticidal activity was reduced was higher (5.8 to 7.3) than that of soils in which insecticidal activity was retained (4.9 to 5.1). As microbial activity is greater at higher pH values, more of the toxins may have been degraded by microbes in soils with the higher pH values. This hypothesis was confirmed by the greater loss in insecticidal activity during 234 d when the pH of the Kitchawan soil, unamended or amended to 6% (vol vol^{-1}) with kaolinite, was increased from 4.9 to *ca.* 7.0 by the addition of $CaCO_3$.

http://www.sciencedirect.com/science/article/pii/S003807179700148X

COM – Saxena, D.; Flores, S.; Stozskyk, G. 1999. Transgenic plants: Insecticidal toxin in root exudates from Bt corn. *Nature*, 402: 480.

Bt corn is corn (*Zea mays*) that has been genetically modified to express insecticidal toxins derived from the bacterium *Bacillus thuringiensis* to kill lepidopteran pests feeding on these plants. Here we show that *Bt* toxin is released into the rhizosphere soil in root exudates from *Bt* corn.

http://www.nature.com/nature/journal/v402/n6761/abs/402480a0.html#close

Saxena, D.; Stotzky, G. 2000. Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic Bt corn in vitro and in situ. *FEMS Microbiology Ecology*, 33(1):35-39.

The insecticidal toxin encoded by the cry1Ab gene from Bacillus thuringiensis was released in root exudates from transgenic Bt corn during 40 days of growth in soil amended to 0, 3, 6, 9, or 12% (v/v) with montmorillonite or kaolinite in a plant growth room and from plants grown to maturity in the field. The presence of the toxin in rhizosphere soil was determined by immunological and larvicidal assays. No toxin was detected in any soils from isogenic non-Bt corn or without plants. Persistence of the toxin was apparently the result of its binding on surface-active particles in the

soils, which reduced the biodegradation of the toxin. The release of the toxin could enhance the control of insect pests or constitute a hazard to nontarget organisms, including the microbiota of soil, and increase the selection of toxin-resistant target insects.

Full article available at http://femsec.oxfordjournals.org/content/33/1/35.long

Saxena, D.; Flores, S.; Stotzky, G. 2002. Bt toxin is released in root exudates from 12 transgenic corn hybrids representing three transformation events. *Soil Biology and Biochemistry*, Volume 34, Issue 1, Pages 133–137.

The anti-lepidopteran toxin (Cry1Ab protein) encoded by truncated genes from *Bacillus thuringiensis* was released in the root exudates from all hybrids of Bt corn studied and which represented three transformation events (Bt11, MON810, and 176). In vitro and in situ studies indicated that the toxin released in root exudates accumulates in soil, as it adsorbs and binds rapidly on surface-active particles (e.g. clays and humic substances), and retains insecticidal activity for at least 180 d, the longest time studied. The results indicated that the release of the Cry1Ab protein by roots is a common phenomenon with transgenic Bt corn and is not restricted to only the one Bt corn hybrid (NK4640Bt) and tranformation event (Bt11) studied initially.

Full article available at http://stopogm.net/sites/stopogm.net/files/SAXENAsoil.pdf

Zwahlen, C.; Hilbeck, A.; Gugerli, P.; Nentwig, W. 2003. Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. *Molecular Ecology*, 12 (3): 765-775.

Large quantities of Bacillus thuringiensis (Bt) corn plant residue are left in the field after harvest, which may have implications for the soil ecosystem. Potential impacts on soil organisms will also depend on the persistence of the Bt toxin in plant residues. Therefore, it is important to know how long the toxin persists in plant residues. In two field studies in the temperate corn-growing region of Switzerland we investigated degradation of the Cry1Ab toxin in transgenic Bt corn leaves during autumn, winter and spring using an enzyme-linked immunosorbent assay (ELISA). In the first field trial, representing a tillage system, no degradation of the Cry1Ab toxin was observed during the first month. During the second month, Cry1Ab toxin concentrations decreased to approximately 20% of their initial values. During winter, there was no further degradation. When temperatures again increased in spring, the toxin continued to degrade slowly, but could still be detected in June. In the second field trial, representing a no-tillage system, Cry1Ab toxin concentrations decreased without initial delay as for soil-incorporated Bt plants, to 38% of the initial concentration during the first 40 days. They then continued to decrease until the end of the trial after 200 days in June, when 0.3% of the initial amount of Cry1Ab toxin was detected. Our results suggest that extended pre- and post-commercial monitoring are necessary to assess the long-term impact of Bt toxin in transgenic plant residues on soil organisms.

http://www.ncbi.nlm.nih.gov/pubmed/12675831

Lee, L.; Saxena, D.; Stotzky, G. 2003. Activity of free and clay-bound insecticidal proteins from *Bacillus thuringiensis* subsp. *israelensis* against the Mosquito *Culex pipiens. Applied and Environmental Microbiology*, vol. 69 no. 7 4111-4115.

Bacillus thuringiensis subsp. *israelensis* produces parasporal insecticidal crystal proteins (ICPs) that have larvicidal activity against some members of the order Diptera, such as blackflies

and mosquitoes. Hydrolysis of the ICPs in the larval gut results in four major proteins with a molecular mass of 27, 65, 128, and 135 kDa. Toxicity is caused by synergistic interaction between the 25-kDa protein (proteolytic product of the 27-kDa protein) and one or more of the higher-molecular-mass proteins. Equilibrium adsorption of the proteins on the clay minerals montmorillonite and kaolinite, which are homoionic to various cations, was rapid (<30 min for maximal adsorption), increased with protein concentration and then reached a plateau (68 to 96% of the proteins was adsorbed), was significantly lower on kaolinite than on montmorillonite, and was not significantly affected by the valence of the cation to which the clays were homoionic. Binding of the toxins decreased as the pH was increased from 6 to 11, and there was 35 to 66% more binding in phosphate buffer at pH 6 than in distilled water at pH 6 or 7.2. Only 2 to 12% of the adsorbed proteins was desorbed by two washes with water; additional washings desorbed no more toxins, indicating that they were tightly bound. Formation of clay-toxin complexes did not alter the structure of the proteins, as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the equilibrium supernatants and desorption washes and by dot blot enzyme-linked immunosorbent assay of the complexes, which was confirmed by enhanced chemiluminescence Western blot analysis. Free and clay-bound toxins resulted in 85 to 100% mortality of the mosquito *Culex pipiens*. Persistence of the bound toxins in nonsterile water after 45 days was significantly greater (mortality of 63% ± 12.7%) than that of the free toxins (mortality of 25% ± 12.5%).

Full article available at http://aem.asm.org/content/69/7/4111.full

Saxena, D.; Stewart, C.; Altosaar, I.; Shu, Q.; Stotzky, G. 2004. Larvicidal cry proteins from *Bacillus thuringiensis* are released in root exudates of transgenic *B. thuringiensis* corn, potato, and rice but not of *B. thuringiensis* canola, cotton, and tobacco. *Plant Physiology & Biochemistry*, 42 (5): 383-387.

Larvicidal proteins encoded by cry genes from Bacillus thuringiensis were released in root exudates from transgenic B. thuringiensis corn, rice, and potato but not from B. thuringiensis canola, cotton, and tobacco. Nonsterile soil and sterile hydroponic solution in which B. thuringiensis corn, rice, or potato had been grown were immunologically positive for the presence of the Cry proteins; from B. thuringiensis corn and rice, the soil and solution were toxic to the larva of the tobacco hornworm (Manduca sexta), and from potato, to the larva of the Colorado potato beetle (Leptinotarsa decemlineata), representative lepidoptera and coleoptera, respectively. No toxin was detected immunologically or by larvicidal assay in soil or hydroponic solution in which B. thuringiensis canola, cotton, or tobacco, as well as all near-isogenic non-B. thuringiensis plant counterparts or no plants, had been grown. All plant species had the cauliflower mosaic virus (CaMV) 35S promoter, except rice, which had the ubiquitin promoter from maize. The reasons for the differences between species in the exudation from roots of the toxins are not known. The released toxins persisted in soil as the result of their binding on surface-active particles (e.g. clay minerals, humic substances), which reduced their biodegradation. The release of the toxins in root exudates could enhance the control of target insect pests, constitute a hazard to nontarget organisms, and/or increase the selection of toxin-resistant target insects.

http://www.ncbi.nlm.nih.gov/pubmed/15191740

Stotzky, G. 2004. Persistence and biological activity in soil of the insecticidal proteins from *Bacillus thuringiensis*, especially from transgenic plants. *Plant and Soil*, 266: 77- 89.

Insecticidal proteins produced by various subspecies (*kurstaki, tenebrionis*, and *israelensis*) of *Bacillus thuringiensis* (*Bt*) bound rapidly and tightly on clays, both pure mined clay minerals and soil clays, on humic acids extracted from soil, and on complexes of clay and humic acids. Binding

reduced susceptibility of the proteins to microbial degradation. However, bound proteins retained biological activity. Purified Cry1Ab protein and protein released from biomass of transgenic *Bt* corn and in root exudates of growing *Bt* corn (13 hybrids representing three transformation events) exhibited binding and persistence in soil. Insecticidal protein was also released in root exudates of *Bt* potato (Cry3A protein) and rice (Cry1Ab protein) but not in root exudates of *Bt* canola, cotton, and tobacco (Cry1Ac protein). Vertical movement of CrylAb protein, either purified or in root exudates or biomass of *Bt* corn, decreased as the concentration of the clay minerals, kaolinite or montmorillonite, in soil increased.
Biomass of transgenic *Bt* corn decomposed less in soil than biomass of near-isogenic non-*Bt* corn, possibly because biomass of *Bt* corn had a significantly higher content of lignin than biomass of non-*Bt* corn. Biomass of *Bt* canola, cotton, potato, rice, and tobacco also decomposed less than biomass of the respective near-isogenic non-*Bt* plants. However, the lignin content of these *Bt* plants, whichwas significantly less than that of *Bt* corn, was not significantly different from that of their near-isogenic non-*Bt* counterparts, although it was consistently higher. The Cry1Ab protein had no consistent effects on organisms (earthworms, nematodes, protozoa, bacteria, fungi) in soil or *in vitro*. The Cry1Ab protein was not taken up from soil by non-*Bt* corn, carrot, radish, or turnip grown in soil in which *Bt* corn had been grown or into which biomass of *Bt* corn had been incorporated.

Full article available at https://goo.gl/buiyx6

Baumgarte, S.; Tebbe, C. 2005. Field studies on the environmental fate of the Cry1Ab Bt-toxin produced by transgenic maize (MON810) and its effect on bacterial communities in the maize rhizosphere. *Molecular Ecology*, 14 (8): 2539 2551.

Field studies were done to assess how much of the transgenic, insecticidal protein, Cry1Ab, encoded by a truncated cry1Ab gene from Bacillus thuringiensis (Bt), was released from Bt-maize MON810 into soil and whether bacterial communities inhabiting the rhizosphere of MON810 maize were different from those of the rhizosphere of nontransgenic maize cultivars. Bacterial community structure was investigated by SSCP (single-strand conformation polymorphism) of PCR-amplified 16S rRNA genes from community DNA. Using an improved extraction and detection protocol based on a commercially available ELISA, it was possible to detect Cry1Ab protein extracted from soils to a threshold concentration of 0.07 ng/g soil. From 100 ng of purified Cry1Ab protein added per gram of soil, only an average of 37% was extractable. At both field sites investigated, the amount of Cry1Ab protein in bulk soil of MON810 field plots was always lower than in the rhizosphere, the latter ranging from 0.1 to 10 ng/g soil. Immunoreactive Cry1Ab protein was also detected at 0.21 ng/g bulk soil 7 months after harvesting, i.e. in April of the following year. At this time, however, higher values were found in residues of leaves (21 ng/g) and of roots (183 ng/g), the latter corresponding to 12% of the Cry1Ab protein present in intact roots. A sampling 2 months later indicated further degradation of the protein. Despite the detection of Cry1Ab protein in the rhizosphere of MON810 maize, the bacterial community structure was less affected by the Cry1Ab protein than by other environmental factors, i.e. the age of the plants or field heterogeneities. The persistence of Cry1Ab protein emphasizes the importance of considering post-harvest effects on nontarget organisms.

http://www.ncbi.nlm.nih.gov/pubmed/15969733

Rosi-Marshall, E.; Tank, J.; Royer, T.; Whiles, M.; Evans-White, M.; Chambers, C.; Griffiths, N.; Pokelsek, J.; Stephen, M. 2007. Toxins in transgenic crop byproducts may affect headwater stream ecosystems. *PNAS*, 104(41): 16204-16208.

Corn (*Zea mays* L.) that has been genetically engineered to produce the Cry1Ab protein (Bt corn) is resistant to lepidopteran pests. Bt corn is widely planted in the midwestern United States,

often adjacent to headwater streams. We show that corn byproducts, such as pollen and detritus, enter headwater streams and are subject to storage, consumption, and transport to downstream water bodies. Laboratory feeding trials showed that consumption of Bt corn byproducts reduced growth and increased mortality of nontarget stream insects. Stream insects are important prey for aquatic and riparian predators, and widespread planting of Bt crops has unexpected ecosystem-scale consequences.

Full article available at http://www.pnas.org/content/104/41/16204.full

Douville, M.; Gagné, F.; Blaise, C.; André, C. 2007. Occurrence and persistence of *Bacillus thuringiensis* (Bt) and transgenic Bt corn cry1Ab gene from an aquatic environment. *Ecotoxicology and Environmental Safety*, 66 (2): 195203.

Genetically modified corn crops and suspensions of Bacillus thuringiensis (Bt) are currently used to control pest infestations of insects of the Lepidoptera family. For this purpose, the cry1Ab gene coding for protein delta-endotoxin derived from B. thuringiensis kurstaki (Btk), which is highly toxic to these insects, was inserted and expressed in corn. The aims of this study were to examine the occurrence and persistence of the cry1Ab gene from Btk and Bt corn in aquatic environments near fields where Bt corn was cultivated. First, an optimal DNA preparation and extraction methodology was developed to allow for quantitative gene analysis by real-time polymerase chain reaction (qPCR) in various environmental matrices. Second, surface water and sediment were spiked in vitro with genomic DNA from Bt or Bt corn to evaluate the persistence of cry1Ab genes. Third, soil, sediment, and water samples were collected before seeding, 2 weeks after pollen release, and after corn harvesting and mechanical root remixing in soils to assess cry1Ab gene content. DNA was extracted with sufficient purity (i.e., low absorbance at 230 nm and absence of PCR-inhibiting substances) from soil, sediment, and surface water. The cry1Ab gene persisted for more than 21 and 40 days in surface water and sediment, respectively. The removal of bacteria by filtration of surface water samples did not significantly increase the half-life of the transgene, but the levels were fivefold more abundant than those in unfiltered water at the end of the exposure period. In sediments, the cry1Ab gene from Bt corn was still detected after 40 days in clay- and sand-rich sediments. Field surveys revealed that the cry1Ab gene from transgenic corn and from naturally occurring Bt was more abundant in the sediment than in the surface water. The cry1Ab transgene was detected as far away as the Richelieu and St. Lawrence rivers (82 km downstream from the corn cultivation plot), suggesting that there were multiple sources of this gene and/or that it undergoes transport by the water column. Sediment-associated cry1Ab gene from Bt corn tended to decrease with distance from the Bt cornfield. Sediment concentrations of the cry1Ab gene were significantly correlated with those of the cry1Ab gene in surface water (R=0.83;P=0.04). The data indicate that DNA from Bt corn and Bt were persistent in aquatic environments and were detected in rivers draining farming areas.

http://www.ncbi.nlm.nih.gov/pubmed/16499967

Serratos-Hernández, J-A.; Gómez-Olivares, J-L.; Salinas-Arreortua, N.; Buendía-Rodríguez, E.; Islas-Gutiérrez, F.; de-Ita, A. 2007. Transgenic proteins in maize in the Soil Conservation area of Federal District, Mexico. *Frontiers in Ecology and the Environment*, 5: 247-252.

In 2003, the environmental authorities of the Federal District of Mexico declared that genetically modified organisms were incompatible with ecological agriculture practices established in rural areas south of Mexico City. To ensure compliance with official standards and organic agriculture policies, steps were taken to implement an early warning system for the detection of genetically modified maize in farmers' fields. In our sampling efforts, which were conducted in 2003, transgenic proteins expressed in maize were found in two (0.96%) of 208 samples from farmers' fields, located in two

(8%) of 25 sampled communities. Mexico imports a substantial amount of maize from the US, and due to formal and informal seed networks among rural farmers, there are many potential routes of entrance for transgenic maize into food and feed webs. To sustain agroecological practices, preserve organic agriculture, and conserve maize landraces in the Soil Conservation area of the Mexican Federal District, environmental authorities will need to maintain and update ecological policies such as the "green seal" for organic agriculture, apply alternative technologies such as biofertilizers to enhance plant nutrition, and develop sustainable maize agriculture with the implementation of profitable intercropping systems.

https://goo.gl/cJxK3u

Icoz, I.; Saxena, D.; Andow, D.; Zwahlen, C.; Stotzky, G. 2008. Microbial populations and enzyme activities in soil in situ under transgenic corn expressing cry proteins from *Bacillus thuringiensis*. *J Environ Qual*, 37(2):647-62.

Transgenic Bt crops produce insecticidal Cry proteins that are released to soil in plant residues, root exudates, and pollen and that may affect soil microorganisms. As a continuation of studies in the laboratory and a plant-growth room, a field study was conducted at the Rosemount Experiment Station of the University of Minnesota. Three Bt corn varieties that express the Cry1Ab protein, which is toxic to the European corn borer (Ostrinia nubilalis Hübner), and one Bt corn variety that expresses the Cry3Bb1 protein, which is toxic to the corn rootworm complex (Diabrotica spp.), and their near-isogenic non-Bt varieties were evaluated for their effects on microbial diversity by classical dilution plating and molecular (polymerase chain reaction-denaturing gradient gel electrophoresis) techniques and for the activities of some enzymes (arylsulfatases, acid and alkaline phosphatases, dehydrogenases, and proteases) involved in the degradation of plant biomass. After 4 consecutive years of corn cultivation (2003-2006), there were, in general, no consistent statistically significant differences in the numbers of different groups of microorganisms, the activities of the enzymes, and the pH between soils planted with Bt and non-Bt corn. Numbers and types of microorganisms and enzyme activities differed with season and with the varieties of corn, but these differences were not related to the presence of the Cry proteins in soil. The Cry1Ab protein of Bt corn (events Bt11 and MON810) was detected in most soils during the 4 yr, whereas the Cry3Bb1 protein was not detected in soils of Bt corn (event MON863) expressing the cry3Bb1 gene.

http://www.ncbi.nlm.nih.gov/pubmed/18396552

Tank, J.; Rosi-Marshall, E.; Royer, T.; Whiles, M.; Griffiths, N.; Frauendorf, T.; Treering, D. 2010. Occurrence of maize detritus and a transgenic insecticidal protein (Cry1Ab) within the stream network of an agricultural landscape. *PNAS*.

Widespread planting of maize throughout the agricultural Midwest may result in detritus entering adjacent stream ecosystems, and 63% of the 2009 US maize crop was genetically modified to express insecticidal Cry proteins derived from *Bacillus thuringiensis*. Six months after harvest, we conducted a synoptic survey of 217 stream sites in Indiana to determine the extent of maize detritus and presence of Cry1Ab protein in the stream network. We found that 86% of stream sites contained maize leaves, cobs, husks, and/or stalks in the active stream channel. We also detected Cry1Ab protein in stream-channel maize at 13% of sites and in the water column at 23% of sites. We found that 82% of stream sites were adjacent to maize fields, and Geographical Information Systems analyses indicated that 100% of sites containing Cry1Ab-positive detritus in the active stream channel had maize planted within 500 m during the previous crop year. Maize detritus likely enters streams throughout the Corn Belt; using US Department of Agriculture land cover data, we estimate that 91% of the 256,446 km of streams/rivers in Iowa, Illinois, and Indiana are located within 500 m of a maize field. Maize detritus is common in low-gradient stream channels in northwestern Indiana, and Cry1Ab proteins persist in maize leaves and can be measured in the water column even 6 mo after harvest. Hence, maize detritus, and associated Cry1Ab proteins, are widely

distributed and persistent in the headwater streams of a Corn Belt landscape.

Full article available at http://www.pnas.org/content/107/41/17645.full

Fu, Q.; Chen, S.; Hu, H.; Li, Z.; Han, X.; Wang, P. 2011. Distribution of Bt protein in transgenic cotton soils. [Article in Chinese]. *Ying Yong Sheng Tai Xue Bao*, 22(6):1493-8.

A pot experiment with red soil, yellow brown soil, and yellow cinnamon soil was conducted to detect the Bt protein content in rhizosphere and non-rhizosphere soils at different growth stages of transgenic Bt cotton and common cotton by using enzyme linked immunosorbent assay (ELISA). With the planting of transgenic Bt cotton, the Bt protein content in rhizosphere soil was significantly higher than that in non-rhizosphere soil; while in common cotton soils, there was no significant difference in the Bt protein content between rhizosphere soil and non-rhizosphere soil. At bud stage of transgenic Bt cotton, the Bt protein content in rhizosphere soil was in the order of yellow cinnamon soil > yellow brown soil > red soil, being 144% 121%, and 238% of that in common cotton rhizosphere soil; at florescence stage of transgenic Bt cotton, the Bt protein content in rhizosphere soil was in the order of yellow brown soil > yellow cinnamon soil > red soil, being 156% , 116% , and 197% of that in common cotton rhizosphere soil, respectively. Regardless of planting Bt cotton or common cotton, the Bt protein content in rhizosphere and non-rhizosphere soils had an initial increase with the growth of cotton, peaked at florescence stage, and then decreased. Throughout the whole cotton growth period, the Bt protein content in transgenic Bt cotton rhizosphere soil was higher than that in Bt cotton non-rhizosphere soil, and also, higher than that in common cotton rhizosphere soil, indicating that transgenic Bt cotton could release its Bt protein to rhizosphere soil.

http://www.ncbi.nlm.nih.gov/pubmed/21941750

1.3.3 Imbalances noted in soil communities affected by the Bt growth pressure

Among the risk associated to the presence of Cry toxins in the soil – for significant periods of time and in biologically active forms - it is their unbalancing action over population compositions of the communities established there in.

It is an investigation area still being developed[44]. Here the study of environmental impacts resulting from the growth of Bt plants provides frequently divergent scientific articles. Good part of these studies point out to apparently poor significant disturbs, even with record of changes and damages to relevant functional groups, so that the long term consequences (to the soil health) are still far from being known.

44 In recent years, the social microbiology tends to strengthen as a new approach to the close relations existing in this soil biota.

As referred to in previous chapters, also in this case the environmental and management conditions (type of soil, climate and management practices, such as the presence or not of cover crops or mulch) influence the research results, making generic conclusions unfeasible.

Consequently, the information available point out to the need of careful studies based on appropriate protocols, on a case-by-case basis. It is thus justified the establishment of long term studies, supported in real scenarios, representative of the potentially affected agricultural-ecological systems and biomes, without which the risk evaluations will remain insufficient and inappropriate.

Sun, C.; Chen, L.; Wu, Z.; Zhang, Y.; Zhang, L. 2003. Effect of transgenic Bt rice planting on soil enzyme activities. [Article in Chinese]. *Ying Yong Sgeng Tai Xue Bao*, 14: 2261-2264.

A pot experiment was conducted with silty loam Agrodolf as test soil and with transgenic Bt rice and non-Bt rice as test crops to study the effect of transgenic Bt rice planting on soil urease, phophatase, arylsulfatase, invertase, and dehydrogenase activities. The results showed that Bt toxin could be introduced into soil through root exudates of transgenic Bt rice, and its survival amount in soil varied with time. Compared with non-Bt rice treatment, transgenic Bt rice treatment had a significant decrease (2.47%) of soil urease activity and a significant increase (8.91%) of soil acid phosphatase activity, but no significant change in soil arylsulfatase, invertase, and dehydrogenase activities at the 15th day of emergence. At the 30th day of emergence, the transgenic Bt rice treatment still had a significant decrease of soil urease activity (16.36%) and a significant increase of acid phosphatase activity (35.69%), and no change in invertase activity. It also had significant increase in soil arylsulfatase (19.70%) and dehydrogenase activities (16.83%).

http://www.ncbi.nlm.nih.gov/pubmed/15031930

REV – Bruinsma, M.; Kowalchuk, G.; Veen, J. 2003. Effects of genetically modified plants on microbial communities and processes in soil. *Biology and Fertility of Soils*, 37: 329-337.

The development and use of genetically modified plants (GMPs) has been a topic of considerable public debate in recent years. GMPs hold great promise for improving agricultural output, but the potential for unwanted effects of GMP use is still not fully understood. The majority of studies addressing potential risks of GMP cultivation have addressed only aboveground effects. However, recent methodological advances in soil microbial ecology have allowed research focus to move underground to try to gain knowledge of GMP-driven effects on the microbial communities and processes in soil that are essential to key terrestrial ecosystem functions. This review gives an overview of the research performed to date on this timely topic, highlighting a number of case studies. Although such research has advanced our understanding of this topic, a number of knowledge gaps still prevent full interpretation of results, as highlighted by the failure of most studies to assign a definitively negative, positive or neutral effect to GMP introduction. Based upon our accumulating, yet incomplete, understanding of soil microbes and processes, we propose a synthesis for the case-by-case study of GMP effects, incorporating assessment of the potential plant/

ecosystem interactions, accessible and relevant indicators, and tests for unforeseen effects.

http://link.springer.com/article/10.1007%2Fs00374-003-0613-6

Wu, W-X.; Ye, Q-F.; Min, H. 2004. Effect of straws from Bt-transgenic rice on selected biological activities in water-flooded soil. *European Journal of Soil Biology*, 40 (1): 15-22.

The biochemical properties of soil have often been described as early and sensitive indicators of ecological changes in both natural soil and agroecosystem. In the current study, the impacts of the amendment of Bt-transgenic rice (KMD) straw on biological activities in water-flooded soil were investigated under laboratory conditions and compared with non-transgenic rice (Xiushui 11) straw. The results showed that there were some differences in protease, neutral phosphatase and cellulase activities between soil amended with Bt-transgenic rice straw and non-transgenic rice straw at the early stage of incubation, and none of these differences were persistent. However, differences in dehydrogenase activity, methanogenesis, hydrogen production and anaerobic respiration between soil supplemented with Bt-transgenic rice straw and non-transgenic rice straw were persistent over the course of incubation. Dehydrogenase activity, methanogenesis and anaerobic respiration were considerably lower from sample days 7 to 56, but higher after day 56 in soil amended with Bt-transgenic rice straw. In comparison, the H_2-production in soil containing Bt-transgenic rice straw was significantly lower after day 56. The results demonstrated that the amendment of the Bt-transgenic rice straw altered some important biological properties in water-flooded soil, indicating a shift in microbial populations or a change in the metabolic abilities of the microbial community as a result of substrate availability in soil.

http://www.sciencedirect.com/science/article/pii/S1164556304000044

REV – Dunfield, K.; Germida, J. 2004. Impact of genetically modified crops on soil and plant-associated microbial communities. *Journal of Environmental Quality*, 33 (3), 806-815.

Transgenic or genetically modified plants possess novel genes that impart beneficial characteristics such as herbicide resistance. One of the least understood areas in the environmental risk assessment of genetically modified crops is their impact on soil- and plant-associated microbial communities. The potential for interaction between transgenic plants and plant residues and the soil microbial community is not well understood. The recognition that these interactions could change microbial biodiversity and affect ecosystem functioning has initiated a limited number of studies in the area. At this time, studies have shown the possibility that transgenes can be transferred to native soil microorganisms through horizontal gene transfer, although there is not evidence of this occurring in the soil. Furthermore, novel proteins have been shown to be released from transgenic plants into the soil ecosystem, and their presence can influence the biodiversity of the microbial community by selectively stimulating the growth of organisms that can use them. Microbial diversity can be altered when associated with transgenic plants; however, these effects are both variable and transient. Soil- and plant-associated microbial communities are influenced not only by plant species and transgene insertion but also by environmental factors such as field site and sampling date. Minor alterations in the diversity of the microbial community could affect soil health and ecosystem functioning, and therefore, the impact that plant variety may have on the dynamics of the rhizosphere microbial populations and in turn plant growth and health and ecosystem sustainability, requires further study.

https://dl.sciencesocieties.org/publications/jeq/abstracts/33/3/0806

Castaldini, M.; Turrini, A.; Sbrana, C.; Benedetti, A.; Marchionni, M.; Mocali, S.; Fabiani, A.; Landi, S.; Santomassimo, F.; Pietrangeli, B.; Nuti, M.; Miclaus, N.; Giovannetti, M. 2005. Impact of Bt corn on rhizospheric and soil eubacterial communities and on beneficial

mycorrhizal symbiosis in experimental microcosms. *Applied and Environmental Microbiology*, 71(11): 6719-29.

A polyphasic approach has been developed to gain knowledge of suitable key indicators for the evaluation of environmental impact of genetically modified Bt 11 and Bt 176 corn lines on soil ecosystems. We assessed the effects of Bt corn (which constitutively expresses the insecticidal toxin from *Bacillus thuringiensis*, encoded by the truncated *Cry1Ab* gene) and non-Bt corn plants and their residues on rhizospheric and bulk soil eubacterial communities by means of denaturing gradient gel electrophoresis analyses of 16S rRNA genes, on the nontarget mycorrhizal symbiont *Glomus mosseae*, and on soil respiration. Microcosm experiments showed differences in rhizospheric eubacterial communities associated with the three corn lines and a significantly lower level of mycorrhizal colonization in Bt 176 corn roots. In greenhouse experiments, differences between Bt and non-Bt corn plants were detected in rhizospheric eubacterial communities (both total and active), in culturable rhizospheric heterotrophic bacteria, and in mycorrhizal colonization. Plant residues of transgenic plants, plowed under at harvest and kept mixed with soil for up to 4 months, affected soil respiration, bacterial communities, and mycorrhizal establishment by indigenous endophytes. The multimodal approach utilized in our work may be applied in long-term field studies aimed at monitoring the real hazard of genetically modified crops and their residues on nontarget soil microbial communities.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1287690/

Mulder, C.; Wouterse, M.; Raubuch, M.; Roelofs, W.; Rutgers, M. 2006. Can transgenic maize affect soil microbial communities? *PLOS Computational Biology*, 2(9): e128. DOI: 10.1371/journal.pcbi.0020128.

The aim of the experiment was to determine if temporal variations of belowground activity reflect the influence of the Cry1Ab protein from transgenic maize on soil bacteria and, hence, on a regulatory change of the microbial community (ability to metabolize sources belonging to different chemical guilds) and/or a change in numerical abundance of their cells. Litter placement is known for its strong influence on the soil decomposer communities. The effects of the addition of crop residues on respiration and catabolic activities of the bacterial community were examined in microcosm experiments. Four cultivars of *Zea mays* L. of two different isolines (each one including the conventional crop and its *Bacillus thuringiensis* cultivar) and one control of bulk soil were included in the experimental design. The growth models suggest a dichotomy between soils amended with either conventional or transgenic maize residues. The Cry1Ab protein appeared to influence the composition of the microbial community. The highly enhanced soil respiration observed during the first 72 h after the addition of *Bt*-maize residues can be interpreted as being related to the presence of the transgenic crop residues. This result was confirmed by agar plate counting, as the averages of the colony-forming units of soils in conventional treatments were about one-third of those treated with transgenic straw. Furthermore, the addition of *Bt*-maize appeared to induce increased microbial consumption of carbohydrates in BIOLOG EcoPlates. Three weeks after the addition of maize residues to the soils, no differences between the consumption rate of specific chemical guilds by bacteria in soils amended with transgenic maize and bacteria in soils amended with conventional maize were detectable. Reaped crop residues, comparable to post-harvest maize straw (a common practice in current agriculture), rapidly influence the soil bacterial cells at a functional level. Overall, these data support the existence of short *Bt*-induced ecological shifts in the microbial communities of croplands' soils.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1584322/

Sun, C.; Chen, L.; Wu, Z.; Zhou, L.; Shimizu, H. 2007. Soil persistence of *Bacillus thuringiensis* (Bt) toxin from transgenic Bt cotton tissues and its effect on soil enzyme activities. *Biology and Fertility of Soils*, 43 (5): 617-620.

A silty loam soil was incubated with the leaves and stems of two transgenic Bacillus thuringiensis (Bt) cotton varieties and nontransgenic Bt cotton to study the soil persistence of the Bt toxin from the decomposing transgenic Bt cotton tissues and its effect on soil enzyme activities. The results showed that after Bt cotton tissue amendment, Bt toxin was introduced into soil upon decomposition; about 50% of the introduced Bt toxin persisted in soil for at least 56 days. No Bt toxin was detected in the nontransgenic Bt cotton-amended soil; the amount of Bt toxin was the highest in the soil treated with the residue with the higher Bt toxin content. Activities of soil urease, acid phosphomonoesterase, invertase, and cellulase were stimulated by the addition of Bt cotton tissues, whereas activity of soil arylsulfatase was inhibited. Probably cotton tissue stimulated microbial activity in soil, and as a consequence, enzyme activities of soil were generally increased. This effect can mask any negative effect of the Bt toxin on microbial activity and thus on enzyme activities.

http://link.springer.com/article/10.1007%2Fs00374-006-0158-6

Sarkar, B.; Patra, A.; Purakayastha, T. 2008. Transgenic Bt-Cotton affects enzyme activity and nutrient availability in a sub-tropical inceptisol. *J Agronomy & Crop Science*, ISSN 0931-2250.

We investigated the dynamics of N and P availability in the rhizosphere of Bt and non-Bt cotton crops during their growth. In a net-house pot culture experiment at the Indian Agricultural Research Institute, New Delhi, Bt-cotton (cv. MRC-6301Bt) and its non-transgenic near-isoline (MRC-6301) were grown on a sandy loam soil until maturity. A control (no-crop) treatment was also included. Rhizosphere soil and root samples were collected at 60, 90, and 120 days after sowing (DAS). Soil samples were analysed for dehydrogenase activity, soil respiration, mineral-N and Olsen-P. Results have revealed a significant reduction in dehydrogenase activity ()17 %) and soil respiration ()3.5 %) in the rhizosphere of Bt-cotton over non-Bt isoline. Total mineral-N (NH4+-N + NO_3 -N) in soil was reduced by 14 %, whereas Olsen-P was increased by 8 % because of Bt-cotton. Root biomass yields were not diferente (P > 0.05), but root volume was significantly higher in Bt than non-Bt isoline. Time of sampling strongly (P < 0.05) affected the above parameters, showing their highest values at 60 or 90 DAS. A significant interactive effect of sampling time and treatments was also indicated. Our results suggest that Bt-cotton may constrain the availability of N, but enhances P-availability in these soils.

http://onlinelibrary.wiley.com/doi/10.1111/j.1439-037X.2008.00312.x/abstract

Cheeke, T.; Pace, B.; Rosenstiel, T.; Cruzan, M. 2011. The influence of fertilizer level and spore density on arbuscular mycorrhizal colonization of transgenic Bt 11 maize (Zea mays) in experimental microcosms. *FEMS Microbiol Ecol*, 75(2): 304-312.

Crop plants genetically modified for the expression of Bacillus thuringiensis (Bt) insecticidal toxins have broad appeal for reducing insect damage in agricultural systems, yet questions remain about the impact of Bt plants on symbiotic soil organisms. Here, arbuscular mycorrhizal fungal (AMF) colonization of transgenic maize isoline Bt 11 (expressing Cry1Ab) and its non-Bt parental line (Providence) was evaluated under different fertilizer level and spore density scenarios. In a three-way factorial design, Bt 11 and non-Bt maize were inoculated with 0, 40, or 80 spores of Glomus mosseae and treated weekly with 'No' (0 g L(-1)), 'Low' (0.23 g L(-1)), or 'High' (1.87 g L(-1)) levels of a complete fertilizer and grown for 60 days in a greenhouse. While no difference in AMF colonization was detected between the Bt 11 and Providence maize cultivars in the lower spore/higher fertilizer treatments, microcosm experiments demonstrated a significant reduction in AMF

colonization in Bt 11 maize roots in the 80 spore treatments when fertilizer was limited. These results confirm previous work indicating an altered relationship between this Bt 11 maize isoline and AMF and demonstrate that the magnitude of this response is strongly dependent on both nutrient supply and AMF spore inoculation level.

http://www.ncbi.nlm.nih.gov/pubmed/21198682

Chen, Z.; Chen, L.; Zhang, Y.; Wu, Z. 2011. Microbial properties, enzyme activities and the persistence of exogenous proteins in soil under consecutive cultivation of transgenic cottons (*Gossypium hirsutum L.). Plant Soil and Environment*, UZEI 57 (2): 67-74.

One *Bacillus thuringiensis* (*Bt*) and two stacked *Bt* and cowpea trypsin inhibitor (*Bt* + *CpTI*) cottons and their non-transgenic isolines were consecutively cultivated to investigate the soil persistence of *Cry1Ac* and *CpTI* proteins and their effects on microbial properties and enzyme activities involving C, N, P, and S cycling in soil. Results showed that there were the persistence of *Cry1Ac* and *CpTI* proteins in soil under 4-year consecutive cultivation of transgenic cottons. *Cry1Ac* proteins varied from 6.75 ng/g to 12.01 ng/g and *CpTI* proteins varied from 30.65 to 43.60 ng/g. However, neither of these two proteins was detected in soil under non-transgenic cottons. Soil microbial biomass carbon, microbial activities, and soil enzyme activities (except urease and phosphodiesterase) significantly decreased in soil under transgenic cottons. Correlation analysis showed that most of microbial properties and enzyme activities in soil had a negative relationship with *Cry1Ac* content, while most of them had a positive relationship with *CpTI* content. Our data indicate that consecutive cultivation by genetically modified cottons with *Bt* and *CpTI* genes can result in persistence of *Cry1Ac* and *CpTI* proteins and negatively affect soil microbial and biochemical properties.

Full article available at http://www.agriculturejournals.cz/publicFiles/35214.pdf

Zhou, X.; Liu, N.; Zhao, M.; Li, H.; Zhou, L.; Tang, Z.; Cao, F.; Li, W. 2011. Advances in effects of insecticidal crystal proteins released from transgenic Bt crops on soil ecology. [Article in Chinese]. *Yi Chuan*, 33(5):443-8.

With the large scale cultivation of transgenic crops expressing Bacillus thuringiensis (Bt) insecticidal crystal proteins in the world, the problem of environmental safety caused by these Bt crops has received extensive attention. These insecticidal crystal proteins can be released into the soil continuously in the growing period of Bt plants. If their accumulation of the insecticidal crystal proteins exceeds consumption by insect larvae and degradation by the environmental factors, these insecticidal crystal proteins could constitute a hazard to non-target insects and soil microbiota. There are three main ways to release insecticidal crystal proteins into soil for Bt plants: root exudates, pollen falling, and crop reside returning. The Bt insecticidal crystal proteins released into soil can be adsorbed rapidly by active soil particles and the absorption equilibrium attained within 1-3 h. The adsorption protects Bt insecticidal crystal proteins against soil microbial degradation or enzyme degradation, which leads to remarkable prolong of the persistence of insecticidal activity. The change of soil microorganism species is an important index for evaluating the effect of Bt plants on soil ecology. The research showed that these insecticidal crystal proteins released by the Bt plant root exudates or Bt organism had no toxicity to the soil earthworms, nematodes, protozoa, bacteria and fungi; however, it could reduce the mycelium length of the arbuscular mycorrhizal fungi (AMF) and restrain AMF to form invasion unit. The influencing degree of Bt protein on soil enzyme activity varied with the releasing modes or growth period of Bt crops. Bt Cry1Ab protein can be taken up from soil by parts of following crops; however, different results were obtained with different commercial kits. To better understand the soil ecological evaluation about the insecticidal crystal proteins released from transgenic Bt crops, this review provides a comprehensive overview about the release, adsorption and residue of Bt insecticidal crystal proteins in soil, as well as their effects on soil protozoa, soil microorganism, soil enzyme activity and following crops.

http://www.ncbi.nlm.nih.gov/pubmed/21586391

Tarafdar, J.; Rathore, I.; Shiva, V. 2012. Effect of Bt- transgenic cotton on soil biological health. *Applied Biological Research*, 14 (1): 15-23.

Bt cotton are plants that have been genetically modified to express the insecticidal proteins *Cry 1 Ac* from subspecies of the bacterium, *Bacillus thuringiensis* israelensis (Bt), to control bollworm pest that feed on cotton. There is a persistent environmental concern that transgenic Bt-crops carry genes that have indirect undesirable effect to natural and agro–ecosystem function. We investigated the effect of Bt-cotton (with *Cry 1 Ac* gene) on several microbial and biochemical indicators in fields under subhumid tropical condition. Twenty five fields were selected in the Vidarbha region, India, where Bt-cotton has been growing at least three consecutive years and side by side field of non-transgenic cotton is growing under clay to clay loam soil. Soil from a control (no-crop) treatment was also included from each area to compare the extent of adverse effect of Bt, if any. Samples were analyzed for actinobacteria, fungi and nitrifiers population, biomass carbon (MBC), biomass nitrogen (MBN), biomass phosphorus (MBP) and soil enzyme activities. The result revealed a significant decline in actinobacteria (17%), bacterial (14%) count as well as acid phosphatases (27%), phytase (18%), nitrogenase (23%) and dehydrogenase (12%) activities in Bt cotton compared with non-Bt cotton fields. Fungal and nitrifiers counts and esterase and alkaline phosphatase activities were not affected by the introduction of Bt-cotton in fields. However, significant decline between 8 and 9% in MBC and MBN was also noticed.

Full article available at http://afsafrica.org/wp-content/uploads/2014/08/ABR-14-_1_-02-00-Bt-cotton-Dr-I-Rathore.pdf

Cheeke, T.; Rosenstiel, T.; Cruzan, M. 2012. Evidence of reduced arbuscular mycorrhizal fungal colonization in multiple lines of Bt maize. *American Journal of Botany*, 99(4): 700707.

Premise of the Study: Insect-resistant Bacillus thuringiensis (Bt) maize is widely cultivated, yet few studies have examined the interaction of symbiotic arbuscular mycorrhizal fungi (AMF) with different lines of Bt maize. As obligate symbionts, AMF may be sensitive to genetic changes within a plant host. Previous evaluations of the impact of Bt crops on AMF have been inconsistent, and because most studies were conducted under disparate experimental conditions, the results are difficult to compare.
Methods: We evaluate AMF colonization in nine Bt maize lines, differing in number and type of engineered trait, and five corresponding near-isogenic parental (P) base hybrids in greenhouse microcosms. Plants were grown in 50% local agricultural soil with low levels of fertilization, and AMF colonization was evaluated at 60 and 100 d. Nontarget effects of Bt cultivation on AMF colonization were tested in a subsequently planted crop, Glycine max, which was seeded into soil that had been preconditioned for 60 d with Bt or P maize.
Key Results: We found that Bt maize had lower levels of AMF colonization in their roots than did the non-Bt parental lines. However, reductions in AMF colonization were not related to the expression of a particular Bt protein. There was no difference in AMF colonization in G. max grown in the Bt- or P-preconditioned soil.
Conclusions: These findings are the first demonstration of a reduction in AMF colonization in multiple Bt maize lines grown under the same experimental conditions and contribute to the growing body of knowledge examining the unanticipated effects of Bt crop cultivation on nontarget soil organisms

http://www.ncbi.nlm.nih.gov/pubmed/22473978

2 Environmental risks associated to the use of herbicide-tolerant plants (HT technology)

The so-called herbicide-tolerant plants were genetically modified with views to facilitate the control of weeds in crop areas. In practical terms, this was obtained by inserting transgenes which allowed the GM-HT plants to absorb and metabolize certain herbicides, without causing them lethal. Thus, pesticide baths applied at any moment and as many times as desired over the entire crop area would eliminate the rest of the vegetable coverage, decreasing the competition of the main culture, for light, water and nutrients andradically simplifying weed management and control.

The proponents of the technology stated that, with the HT monocultures weed would be controlled with scarce side effects. Actually, this seems to have occurred in the first years. However, on the medium run, deep modifications started to be verified in the ecosystems. Studies accumulated evidences of impacts beyond issues related to the management of the so-called "weeds". Seed banks in soil decreased and were homogenized, the use of the associated herbicides was extended and substantial changes started to be noted in the communities of organisms established in the agroecosystem soil underground ands waters .

The environmental impacts not only determined the emergence of tolerant and resistant plants (demanding an increase in use and change in active ingredients, of formulations that are more dangerous to the health and to the environment) but brought several implications, negatively affecting communities of terrestrial, aquatic and underground environments of the areas with HT plants.

For these reasons, the risk assessment of HT plants must take into consideration the herbicides associated to them.

The articles below develop such perspective.

2.1 Specific negative impacts of the main herbicides associated to the HT GM crops

In this item, three of the main groups of herbicides for which TH plants were developed (glyphosate, glufosinate ammonium and 2,4-D) are evaluated. Increased highlight is provided to the family of the glyphosate-based herbicides, being for their prevalence in the field and also for the amount of information available. It is important to mention that, despite of the extensive documentation proving their detrimental impacts to human, animal and environmental health, it is still a pesticide classified as being of low toxicity. Recently (after the completion of this book) the IARC (International Agency for Research on Cancer) classified it as possibly cancerous, generating re-evaluation processes which may determine its re-classification or even prohibition for use in Brazil.

2.1.1 Glyphosate and glyphosate-based commercial formulas

In contrast to the advertisement historically developed by the pesticide industry[45] and other supporters of the technology, glyphosate-based herbicides are not biodegradable or of low toxicity to the human health[46] and to the environment.

Endocrine disruption, genotoxicity, mutagenicity and even behavioral changes in populations of organisms in forced contact with the herbicide are related in dozens of studies found in the scientific literature. By means of their systemic action, the large amounts of glyphosate poured in dozens of millions of hectares over the world have caused significant degradation in natural and semi-

45 The company initially responsible for the herbicide Roundup brand, glyphosate-based, was effectively sentenced twice for justice by misleading advertising, once in France and in once in the USA.

46 In items 2.1.1 and 2.2.2 of Part 4 there are additional articles dealing with the health risks associated with the use of herbicides to glyphosate.

natural environments – and of the biocenosis comprising them. The aquatic and semi-aquatic environments are being impacted on a more acute and dramatic way by such herbicides.

Some of the studies listed below report ecotoxicity in model animals (such as urchins, certain amphibians or birds) and mammalians, also covering the field of the risks to the human health.

Damages observed on soil communities suggest evidences of agronomic problems, with socioeconomic impacts that tend to be alarming. In item 3.2 of Part 2 there are further information about the subject worked in the articles presented below.

Santillo, D.; Brown, P.; Leslie, D. 1989. Response of songbirds to glyphosate-induced habitat changes on clearcuts. *Journal of Wildlife Management*. 53: 64–71.

We examined *breeding* bird populations and habitats on glyphosate (nitrogen-phosphonomethyl glycine) (Roundup, Monsanto, St. Louis, Mo.)-treated and untreated clearcuts in north-central Maine. Treatment of clearcuts with glyphosate herbicide reduced the complexity of vegetation through *3 years* post-treatment compared to untreated clearcuts. Total *numbers* of birds, common yellowthroats (Geothlypis trichas), Lincoln's sparrows (Melospiza lincolnii), and alder flycatchers (Empidonax alnorum) were less abundant ($P < 0.05$) on treated clearcuts than on untreated clearcuts. Songbird densities were correlated with habitat complexity, especially hardwood regeneration, foliage height diversity (FHD), and vegetation height. Leaving untreated patches of vegetation and staggering herbicide treatments on large clearcuts will maintain bird populations similar to those of untreated clearcuts.

https://eurekamag.com/research/007/751/007751516.php

Springett, J.; Gray, R. 1992. Effect of repeated low doses of biocides on the earthworm Aporrectodea caliginosa in laboratory culture. *Soil Biology and Biochemistry,* 24: 1739–1744.

The growth rates of *Aporrectodea caliginosa* (Savigny) were measured over a 100-day period in soil in culture chambers which were treated with common biocides singly and in combination. The biocides used were: the fungicide Captan, the herbicide, Glyphosate and the insecticide, Azinphos-methyl. The biocides were applied at intervals of 14 days and each treatment was replicated six times. The results are variable, all biocides depressed growth when applied alone but some combinations reduced the effect of other biocides. Azinphos-methyl and Glyphosate applied alone, reduced growth the most over the 100 days and at all rates of application. Azinphos-methyl applied at the highest rate killed worms. Captan applied alone had the least effect on growth and mortality. In combination, Glyphosate and Captan had a lesser effect than Glyphosate alone. Azinphos-methyl and Captan had less effect than Azinphos-methyl alone. After 100 days the combination of all three biocides reduced growth to the same degree as Glyphosate alone.

http://www.sciencedirect.com/science/article/pii/0038071792901806

Tate, T.; Spurlock, J.; Christian, F. 1997. Effect of glyphosate on the development of *Pseudosuccinea columella* snails. *Archives of Environmental Contamination and Toxicology,* 33: 286-9.

Glyphosate (Roundup) is one of the most commonly used broad-spectrumherbicides with little to no hazard to animals, man, or the environment. Due to its widespread use, there is continuous contamination of the environment in both soil and water with this herbicide. There is a paucity of long-term exposure studies with sublethal concentrationsof glyphosate on aquatic snails. This study was developed to determine the effects of sublethal concentrations of glyphosate on development and survival of Pseudosuccinea columella (intermediate snail host of Fasciola hepatica). This was assessed by continuously exposing three successive generations of snails to varying concentrations (0.1-10mg/L) of glyphosate. Glyphosate had little effect on the first- and second-generation snails. However, third-generation snail embryos exposed to 1.0 mg/L glyphosate developed much faster than other embryos exposed to 0.1 mg/L, 10 mg/L, and 0 mg/L (control). Hatching was inhibited at 10 mg/L and inhibited slightly at 0.1 mg/L. The egg-laying capacity was increased in snails exposed to 0.1 and 10 mg/L. Abnormalities and polyembryony were observed in snails exposed to 0.1 and 10 mg/L. These results indicate that glyphosate does affect snail reproduction and development. This, in turn, could possibly have an effect on the population dynamics of F. hepatica, which could result in increased infections in animals, including man.

http://link.springer.com/article/10.1007%2Fs002449900255

Newmaster, S.; Bell, F.; Vitt, D. 1999. The effects of glyphosate and triclopyr on common bryophytes and lichens in northwestern Ontario. *Canadian Journal of Forest Research*, 29, 1101-1111.

The effects of two silvicultural herbicides (Vision®, Release®) on bryophytes and lichens were studied in a harvested boreal mixedwood ecosystem. A completely randomized design with 115 plots of 1 m^2 allowed direct comparison between herbicides and their effects on community dynamics. Regression models were used to analyze the relationship between herbicide application rates (0.71-6.72 kg active ingredient/ha) and changes in bryophyte and lichen abundance and species richness for 2 years following herbicide application. Results showed that bryophyte and lichen abundance and species richness decreased after herbicide treatments. In general, herbicide applications reduced the diversity of forest mesophytes and weedy colonizers to an ecosystem with only a few species of colonizers. A combination of clustering techniques and ANOVA were used to divide bryophytes and lichens into three ecologically defined response groups: herbicide-tolerant colonizers, semi tolerant long-term stayers from dry open forest, and sensitive forest mesophytes.

http://www.nrcresearchpress.com/doi/abs/10.1139/x99-0 83#.VNTl9Sd4tXg

Relyea, R. 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecological Applications*, 15, 618-627.

Pesticides constitute a major anthropogenic addition to natural communities. In aquatic communities, a great majority of pesticide impacts are determined from singlespecies experiments conducted under laboratory conditions. Although this is an essential protocol to rapidly identify the direct impacts of pesticides on organisms, it prevents na assessment of direct and indirect pesticide effects on organisms embedded in their natural ecological contexts. In this study, I examined the impact of four globally common pesticides (two insecticides, carbaryl [Sevin] and malathion; two herbicides, glyphosate [Roundup] and 2,4-D) on the biodiversity of aquatic communities containing algae and 25 species of animals. Species richness was reduced by 15% with Sevin, 30% with malathion, and 22% with Roundup, whereas 2,4-D had no effect. Both insecticides reduced zooplankton diversity by eliminating cladocerans but not copepods (the latter increased in abundance). The insecticides also reduced the diversity and biomass of predatory insects and had an apparent indirect positive

effect on several species of tadpoles, but had no effect on snails. The two herbicides had no effects on zooplankton, insect predators, or snails. Moreover, the herbicide 2,4-D had no effect on tadpoles. However, Roundup completely eliminated two species of tadpoles and nearly exterminated a third species, resulting in a 70% decline in the species richness of tadpoles. This study represents one of the most extensive experimental investigations of pesticide effects on aquatic communities and offers a comprehensive perspective on the impacts of pesticides when nontarget organisms are examined under ecologically relevant conditions.

Full article available at https://www.whyy.org/91FM/ybyg/relyea2005.pdf

Relyea, R. 2005. The lethal impact of Roundup on aquatic and terrestrial amphibians. *Ecological Applications*, 15 (4): 1118-1124.

The global decline in amphibian diversity has become an international environmental problem with a multitude of possible causes. There is evidence that pesticides may play a role, yet few pesticides have been tested on amphibians. For example, Roundup is a globally common herbicide that is conventionally thought to be nonlethal to amphibians. However, Roundup has been tested on few amphibian species, with existing tests conducted mostly under laboratory conditions and on larval amphibians. Recent laboratory studies have indicated that Roundup may be highly lethal to North American tadpoles, but we need to determine whether this effect occurs under more natural conditions and in post-metamorphic amphibians. I assembled communities of three species of North American tadpoles in outdoor pond mesocosms that contained different types of soil (which can absorb the pesticide) and applied Roundup as a direct overspray. After three weeks, Roundup killed 96–100% of larval amphibians (regardless of soil presence). I then exposed three species of juvenile (post-metamorphic) anurans to a direct overspray of Roundup in laboratory containers. After one day, Roundup killed 68–86% of juvenile amphibians. These results suggest that Roundup, a compound designed to kill plants, can cause extremely high rates of mortality to amphibians that could lead to population declines.

Full article available at http://pbadupws.nrc.gov/docs/ML1434/ML14345A564.pdf

Oliveira, A.; Telles, L.; Hess, R.; Mahecha, G.; Oliveira, C. 2007. Effects of the herbicide Roundup on the epididymal region of drakes *Anas platyrhynchos*. *Reproductive Toxicology*, 23, 182–191.

Exposure to the Roundup has been shown to affect StAR protein and aromatase expression and activity, pointing out that this herbicide may cause adverse effects in animal reproduction by affecting androgen and estrogen synthesis. We tested this hypothesis by investigating the in vivo effects of the Roundup on the testis and epididymal region of drake Anas platyrhynchos. The exposure to the herbicide resulted in alterations in the structure of the testis and epididymal region as well as in the serum levels of testosterone and estradiol, with changes in the expression of androgen receptors restricted to the testis. The harmful effects were more conspicuous in the proximal efferent ductules and epididymal ducts, suggesting higher sensitivity of these segments among the male genital organs. The effects were mostly dose dependent, indicating that this herbicide may cause disorder in the morphophysiology of the male genital system of animals.

http://www.ncbi.nlm.nih.gov/pubmed/17166697

Soso, A.; Barcellos, L.; Ranzani-Paiva, M.; Kreutz, L.; Quevedo, R.; Anziliero, D.; Lima, M.; Silva, L.; Ritter, F.; Bedin, A.; Finco, J. 2007. Chronic exposure to sub-lethal concentration

of a glyphosate based herbicide alters hormone profiles and affects reproduction of female Jundiá (*Rhamdia quelen*). *Environmental Toxicology and Pharmacology*, 23, 308-313.

This work was carried out to verify the effect of a glyphosate-based herbicide on Jundiá hormones (cortisol, 17β-estradiol and testosterone), oocyte and swim-up fry production. Earthen ponds containing Jundiá females were contaminated with glyphosate (3.6mg/L); blood samples were collected from eight females from each treatment immediately before, or at 1, 10, 20, 30 and 40 days following contamination. A typical post-stress rise in cortisol levels was observed at the 20th and 40th days following exposure to glyphosate. At the 40th day, 17β-estradiol was decreased in the exposed females. A similar number of oocytes were stripped out from females from both groups; however, a lower number of viable swim-up fry were obtained from the herbicide exposed females, which also had a higher liver-somatic index (LSI). The results indicate that the presence of glyphosate in water was deleterious to Rhamdia quelen reproduction, altering steroid profiles and egg viability.

http://www.ncbi.nlm.nih.gov/pubmed/21783773

Bringolf, R.; Cope, W.; Mosher, S.; Barnhart, M.; Shea, D. 2007. Acute and chronic toxicity of glyphosate compounds to *glochidia* and juveniles of *Lampsilis siliquoidea* (*Unionidae*). *Environ Toxicol Chem*, 26(10):2094-100.

Native freshwater mussels (family Unionidae) are among the most imperiled faunal groups in the world. Factors contributing to the decline of mussel populations likely include pesticides and other aquatic contaminants; however, there is a paucity of data regarding the toxicity of even the most globally distributed pesticides, including glyphosate, to mussels. Therefore, the toxicity of several forms of glyphosate, its formulations, and a surfactant (MON 0818) used in several glyphosate formulations was determined for early life stages of Lampsilis siliquoidea, a native freshwater mussel. Acute and chronic toxicity tests were performed with a newly established American Society of Testing and Materials (ASTM) standard guide for conducting toxicity tests with freshwater mussels. Roundup, its active ingredient, the technical-grade isopropylamine (IPA) salt of glyphosate, IPA alone, and MON 0818 (the surfactant in Roundup formulations) were each acutely toxic to L. siliquoidea glochidia. MON 0818 was most toxic of the compounds tested and the 48-h median effective concentration (0.5 mg/L) for L. siliquoidea glochidia is the lowest reported for any aquatic organism tested to date. Juvenile L. siliquoidea were also acutely sensitive to MON 0818, Roundup, glyphosate IPA salt, and IPA alone. Technical-grade glyphosate and Aqua Star were not acutely toxic to glochidia or juveniles. Ranking of relative chronic toxicity of the glyphosate-related compounds to juvenile mussels was similar to the ranking of relative acute toxicity to juveniles. Growth data from chronic tests was largely inconclusive. In summary, these results indicate that L. siliquoidea, a representative of the nearly 300 freshwater mussel taxa in North America, is among the most sensitive aquatic organisms tested to date with glyphosate-based chemicals and the surfactant MON 0818.

http://www.ncbi.nlm.nih.gov/pubmed/17867870

Glusczak, L.; Miron, D.; Moraes, B.; Simões, R.; Schetinger, M.; Morsch, V.; Loro, V. 2007. Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, Volume 146, Issue 4, Pages 519–524.

Silver catfish (*Rhamdia quelen*; Teleostei) were exposed to commercial formulation Roundup®, a glyphosate herbicide: 0 (control), 0.2 or 0.4 mg/L for 96 h. Fish exposed to glyphosate showed an increase in hepatic glycogen, but a reduction in muscle glycogen at both concentrations tested. Glucose decreased in liver and increased in muscle of fish at both herbicide concentrations.

Glyphosate exposure increased lactate levels in liver and white muscle at both concentrations. Protein levels increased in liver and decreased in white muscle while levels of ammonia in both tissues increased in fish at both glyphosate concentrations. Specific AChE activity was reduced in brain after treatments, no changes were observed in muscle tissue. Catalase activity in liver did not change during of exposure. Fish exposed to glyphosate demonstrated increased TBARS production in muscle tissue at both concentrations tested. For both glyphosate concentrations tested brain showed a reduction of TBARS after 96 h of exposure. The present results showed that in 96 h, glyphosate changed AChE activity, metabolic parameters and TBARS production. The parameters measured can be used as herbicide toxicity indicators considering environmentally relevant concentration.

http://www.sciencedirect.com/science/article/pii/S1532045607001470

Langiano, V.; Martinez, C. 2008. Toxicity and effects of a glyphosate-based herbicide on the neotropical fish Prochilodus lineatus. *Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology*, 147, 222-231.

The toxicity of Roundup, a glyphosate-based herbicide widely used in agriculture, was determined for the Neotropical fish *Prochilodus lineatus*. The 96 h-LC_{50} of Roundup was 13.69 mg L^{-1}, indicating that this fish is more sensitive to Roundup than rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). These differences should be considered when establishing criteria for water quality and animal well-being in the Neotropical region. Short-term (6, 24 and 96 h) toxicity tests were then performed to evaluate the effects of sub-lethal concentrations of the herbicide (7.5 and 10 mg L^{-1}) to *P. lineatus*. Roundup did not interfere with the maintenance of the ionic balance and there was no significant alteration in plasma cortisol levels in Roundup-exposed fish. However an increase in plasma glucose was noted in fish exposed to 10 mg L^{-1} of the herbicide, indicating a typical stress response. Catalase liver activity also showed an increase in fish exposed to 10 mg L^{-1} of the herbicide, suggesting the activation of antioxidant defenses after Roundup exposure. In addition, Roundup induced several liver histological alterations that might impair normal organ functioning. Therefore, short-term exposure to Roundup at subletal concentrations induced biochemical, physiological and histological alterations in *P. lineatus*.

http://www.sciencedirect.com/science/article/pii/S1532045607002086

Cavalcante, D.; Martinez, C.; Sofia, S. 2008. Genotoxic effects of Roundup® on the fish *Prochilodus lineatus. Mutation Research*, 655 (2008) 41–46.

Glyphosate-based herbicides, such as Roundup, represent the most extensively used herbicides worldwide, including Brazil. Despite its extensive use, the genotoxic effects of this herbicide are not completely understood and studies with Roundup show conflicting results with regard to the effects of this product on the genetic material. Thus, the aim of this study was to evaluate the genotoxic effects of acute exposures (6, 24 and 96 h) to 10 mg L(-1) of Roundup on the neotropical fish Prochilodus lineatus. Accordingly, fish erythrocytes were used in the comet assay, micronucleus test and for the analysis of the occurrence of nuclear abnormalities and the comet assay was adjusted for branchial cells. The results showed that Roundup produces genotoxic damage in erythrocytes and gill cells of P. lineatus. The comet scores obtained for P. lineatus erythrocytes after 6 and 96 h of exposure to Roundup were significantly higher than respective negative controls. For branchial cells comet scores were significantly higher than negative controls after 6 and 24 h exposures. The frequencies of micronucleus and other erythrocyte nuclear abnormalities (ENAs) were not significantly different between Roundup exposed fish and their respective negative controls, for all exposure periods. In conclusion, the results of this work showed that Roundup produced genotoxic effects on the fish species P. lineatus. The comet assay with gill cells showed to be an important

complementary tool for detecting genotoxicity, given that it revealed DNA damage in periods of exposure that erythrocytes did not. ENAs frequency was not a good indicator of genotoxicity, but further studies are needed to better understand the origin of these abnormalities.

http://www.ncbi.nlm.nih.gov/pubmed/18638566

Relyea, R.; Jones, D. 2009. The toxicity of Roundup Original Max to 13 species of larval amphibians. *Environ Toxicol Chem*, 28(9):2004-8.

With the increased use of glyphosate-based herbicides (marketed under several names, including Roundup and Vision), there has been a concomitant increased concern about the unintended impacts that particular formulations containing the popular surfactant polyethoxylated tallowamine (POEA) might have on amphibians. Published studies have examined a relatively small number of anuran species (primarily from Australia and eastern North America) and, surprisingly, no species of salamanders. Using a popular formulation of glyphosate (Roundup Original Max), the goal of the present study was to conduct tests of lethal concentrations estimated to kill 50% of a population after 96 h (LC50(96-h)) on a wider diversity of species from both eastern and western North America. Tests were conducted on nine species of stage 25, larval anurans from three families (Ranidae: Rana pipiens, R. clamitans, R. sylvatica, R. catesbeiana, R. cascadae; Bufonidae: Bufo americanus, B. boreas; and Hylidae: Hyla versicolor, Pseudacris crucifer) and four species of larval salamanders from two families (Ambystomatidae: Ambystoma gracile, A. maculatum, A. laterale; and Salamandridae: Notophthalmus viridescens). For the nine species of larval anurans, LC50(96-h) values ranged from 0.8- to 2.0-mg acid equivalents per liter with relatively little pattern in differential sensitivity among the species or families. The four species of larval salamanders were less sensitive than the anurans, with LC50(96-h) values ranging from 2.7- to 3.2-mg acid equivalents per liter and no substantial differences among the species of salamanders. This work substantially increases the available data on amphibian sensitivity to glyphosate formulations that include either POEA surfactants or the equally moderately to highly toxic surfactants of Roundup Original Max and should be useful for improving future risk assessments.

http://www.ncbi.nlm.nih.gov/pubmed/19405783

Senapati, T.; Mukerjee, A.; Ghosh, A. 2009. Observations on the effect of glyphosate based herbicide on ultra structure (SEM) and enzymatic activity in different regions of alimentary canal and gill of *Channa punctatus* (Bloch). *Journal of Crop and Weed*, 5(1): 236–245.

Glyphosate is the isopropyl amine salt of N-(Phosphonomethyl)-glycine, a broad-spectrum nonselective herbicide, which has been extensively used to control annual and perennial weeds in agricultural, forest and aquatic systems. The ultrastructural changes in different regions of alimentary canal and gill were observed by Scanning Electron Microscopic study on a non-target aquatic teleostea fish, Channa punctatus. Fishes were exposed to herbicide at a dose of 4 mgl^{-1} generally used by farmers to control weeds in water bodies for a period of 45 days in laboratory condition with a control. Severe damage, shrinkage and degeneration of pentagonal cellular contour of stratified epithelial cells (SEC) were observed in gill. Shrinkage of SEC resulting in degeneration of microridges was observed in buccopharynx. Slight necrosed and distorted SEC was observed in oesophagus. Severe mucus secretion was observed in stomach. Erosion on the apical surface of mucosal folds and columnar epithelial cells (CEC) and necrosis of CEC was also noticed in stomach. Obliteration of CEC along its entire length from basement membrane was observed in the intestinal portion. After 45 days treatment by glyphosate protease activity was slightly reduced in stomach and intestine in comparison to control fish. Amylase activity reduced in oesophagus and intestine in treated condition. Lipase activity was also reduced slightly in stomach and intestine of glyphosate treated fish.

http://cropandweed.com/vol5issue1/46.1.html

Albinati, A.; Moreira, E.; Albinati, R.; Carvalho, J.; Lira, A.; Santos, G.; Vida, L. 2009. Histological biomarkers - chronic toxicity for roundup in piauçu (*Leporinus macrocephalus*). *Arq Bras Med Vet Zootec*, 61(3): 621–627.

The use of gills, liver, and kidneys as histological biomarkers was evaluated in a chronic toxicity analysis with herbicide Roundup® in piauçu (L*eporinus. macrocephalus*). The animals were exposed to 1/10 of LC50 (1.58mg/L), during a period of 14 and 28 days. Five animals were used for treatment (days 0, 14, and 28). Hepatic hemorrhage and necrosis and renal congestion were the alterations that presented differences between exposed and non-exposed animals. Among the organs used as histological biomarkers, the liver presented the best results, followed by the kidneys.

Full article available at https://repositorio.ufba.br/ri/bitstream/ri/6321/1/Albinati,%20A.C.L..pdf

Modesto, K.; Martinez, C. 2010. Roundup® causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*. *Chemosphere*, Volume 78, Issue 3, Pages 294–299.

This work aimed to evaluate Roundup® effects on biochemical biomarkers of the neotropical fish *Prochilodus lineatus*. Fish were acutely exposed (6, 24 and 96 h) to 10 mg L^{-1} of Roundup® (RD) or only water (control) and samples of liver, for antioxidants analysis, and brain and muscle, for acetylcholinesterase (AChE) determination, were collected. Fish exposed to RD for 24 h showed reduction on superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, and increased glutathione (GSH) content. After 24 and 96 h, fish of RD group showed increased glutathione-S-transferase (GST) activity and lipid peroxidation. AChE activity was inhibited in brain after 96 h and in muscle after 24 and 96 h of exposure. Thus, acute exposure to RD stimulated the biotransformation pathway, with increased GST, but interfered on the antioxidant defenses, with reduction of SOD and GPx activity, leading to the occurrence of lipid peroxidation. Inhibition of AChE showed that RD acts as a contaminant with anti-AChE action.

http://www.sciencedirect.com/science/article/pii/S0045653509012739

Jayawardena, U.; Rajakaruna, R.; Navaratne, A.; Amerrasinghe, P. 2010. Toxicity of agrochemicals to common hourglass tree frog (*Polypedates cruciger*) in acute and chronic exposure. *International Journal of Agriculture and Biology*, 12, 641-648.

Direct effect of four common agricultural pesticides viz., chlorpyrifos, dimethoate, glyphosate and propanil, on the survival, growth and development of malformations in common hourglass tree frog, *Polypedates cruciger* (Anura: Ranidae) was studied under laboratory conditions in acute and chronic exposure. Acute exposure to high concentrations was carried out to determine the LC50. The 48 h LC50 of the pesticides were within the Pesticide Area Network specified limits, except for propanil. The percentage survival of the tadpoles under chronic exposure to ecologically relevant doses was lower (glyphosate 75%, dimethoate 77.5%, chlorpyrifos 80% & propanil 85%) than the control group (95.5%) and was significantly affected by the concentrations. Exposed tadpoles took more time to metamorphose and were significantly smaller in size than the control tadpoles. They also developed malformations at high frequencies (glyphosate = 69%, dimethoate = 64%, chlorpyrifos = 60%, propanil = 45%). Malformations were mainly kyphosis (hunched back), scoliosis (curvature), skin ulcers and edema. However, severe limb malformations were not observed in the study. Chlorpyrifos had a profound effect even at very low concentrations (0.05 ppm). This study provides the first empirical evidence of a comparative study on the effect of pesticides on an endemic amphibian species in Sri Lanka and underscores the importance of investigation the level of agricultural pesticides in freshwater ecosystems and their effect on non-target organisms.

Full article available at http://www.fspublishers.org/published_papers/28756_..pdf

Kelly, D.; Poulin, R.; Tompkins, D.; Townsend, C. 2010. Synergistic effects of glyphosate formulation and parasite infection on fish malformations and survival. *Journal of Applied Ecology*, 47, 498-504.

1. Anthropogenic pollution and disease can cause both lethal and sub-lethal effects in aquatic species but our understanding of how these stressors interact is often not known. Contaminants can reduce host resistance to disease, but whether hosts are impacted at environmentally relevant concentrations is poorly understood.
2. We investigated the independent and combined effects of exposure to the common herbicide glyphosate and the trematode parasite Telogaster opisthorchis on survival and the development of spinal malformations in juvenile Galaxias anomalus, a New Zealand freshwater fish. We then investigated how exposure to a glyphosate concentration gradient (0Æ36, 3Æ6, 36 mg active ingrediente (a.i.) L)1) affected the production and release of the infective cercarial stage of the parasite by its snail intermediate host Potamopyrgus antipodarum.
3. Survival of juvenile fish was unaffected by exposure to glyphosate alone (at an environmentally relevant concentration; 0Æ36 mg a.i. L)1) or by T. opisthorchis infection alone. However, simultaneous exposure to infection and glyphosate significantly reduced fish survival.
4. Juvenile fish developed spinal malformations when exposed either to infections alone or to infections and glyphosate, with a trend towards greater severity of spinal malformation after exposure to both stressors.
5. All snails exposed to the highest glyphosate concentration (36 mg a.i. L)1) died within 24 h. Snails exposed to a moderate concentration (3Æ6 mg a.i. L)1) produced significantly more T. opisthorchis cercariae than snails in the control group or the low concentration group (0Æ36 mg a.i. L)1; the same concentration as in the fish experiment).
6. Synthesis and applications. This is the first study to show that parasites and glyphosate can act synergistically on aquatic vertebrates at environmentally relevant concentrations, and that glyphosate might increase the risk of disease in fish. Our results have important implications when identifying risks to aquatic communities and suggest that threshold levels of glyphosate currently set by regulatory authorities do not adequately protect freshwater systems.

Full article available at http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2664.2010.01791.x/pdf

Relyea, R. 2012. New effects of Roundup on amphibians: Predators reduce herbicide mortality; herbicides induce antipredator morphology. *Ecological Applications*, 22(2), 2012, pp. 634–647.

The use of pesticides is important for growing crops and protecting human health by reducing the prevalence of targeted pest species. However, less attention is given to the potential unintended effects on nontarget species, including taxonomic groups that are of current conservation concern. One issue raised in recent years is the potential for pesticides to become more lethal in the presence of predatory cues, a phenomenon observed thus far only in the laboratory. A second issue is whether pesticides can induce unintended trait changes in nontarget species, particularly trait changes that might mimic adaptive responses to natural environmental stressors. Using outdoor mesocosms, I created simple wetland communities containing leaf litter, algae, zooplankton, and three species of tadpoles (wood frogs [*Rana sylvatica* or *Lithobates sylvaticus*], leopard frogs [*R. pipiens* or *L. pipiens*], and American toads [*Bufo americanus* or *Anaxyrus americanus*]). I exposed the communities to a factorial combination of environmentally relevant herbicide concentrations (0, 1, 2, or 3 mg acid equivalents [a.e.]/L of Roundup Original MAX) crossed with three predator-cue treatments (no predators, adult newts [*Notophthalmus viridescens*], or larval dragonflies [*Anax junius*]). Without predator cues, mortality rates from Roundup were consistent with past studies. Combined with cues from the most risky predator (i.e., dragonflies), Roundup became less lethal (in direct contrast to past laboratory studies). This reduction in mortality was likely caused by the herbicide stratifying in the water column and predator cues scaring the tadpoles down to the benthos where herbicide concentrations were lower. Even more striking was the discovery that Roundup induced morphological changes in the tadpoles. In wood frog and leopard frog tadpoles, Roundup induced

relatively deeper tails in the same direction and of the same magnitude as the adaptive changes induced by dragonfly cues. To my knowledge, this is the first study to show that a pesticide can induce morphological changes in a vertebrate. Moreover, the data suggest that the herbicide might be activating the tadpoles' developmental pathways used for antipredator responses. Collectively, these discoveries suggest that the world's most widely applied herbicide may have much further-reaching effects on nontarget species than previous considered.

http://www.esajournals.org/doi/abs/10.1890/11-0189.1

Mottier, A.; Kientz-Bouchart, V.; Serpentini, A.; Lebel, J; Jha, A.; Costil, K. 2013. Effects of glyphosate-based herbicides on embryo-larval development and metamorphosis in the Pacific oyster, *Crassostrea gigas*. *Aquat Toxicol*,15;128-129:67-78. doi: 10.1016.

Pesticides may be involved in oyster summer mortality events, not necessarily as a single causative agent but as an additional stressor. In this context, the present study aimed to assess the toxicity of glyphosate, its by-product, aminomethylphosphonic acid (AMPA) and two commercial formulations, Roundup Express(®) (R(EX)) and Roundup Allées et Terrasses(®) (R(AT)), containing glyphosate as the active ingredient, on the early life stages of the Pacific oyster, Crassostrea gigas. The embryotoxicity of these chemicals were quantified by considering both the rates of abnormalities and the arrested development or types of abnormalities in D-shaped larvae after 48 h exposure. The success of metamorphosis was examined in pediveliger larvae exposed for 24 h. Experiments involving both endpoints included range finding experiments for herbicide concentrations ranging from 0.1 to 100,000 µg L(-1). This range was then narrowed down in order to determine precise EC(50) values. Actual concentrations of the herbicide were determined at the beginning and after 48 h (embryotoxicity) and 24 h (metamorphosis) to evaluate the potential temporal variation in the concentrations. During embryo-larval development, no mortalities were recorded at any of the concentrations of glyphosate and AMPA, whereas no embryos or D-shaped larvae could be observed after exposure to 10,000 µg L(-1) of R(EX) or R(AT). Compared with the controls, no effects on embryo-larval development were recorded between 0.1 and 1000 µg L(-1), regardless of the chemical tested. Above a threshold, which varied according to the chemical used, the gradient of herbicide concentrations correlated with a gradient of severity of abnormality ranging from normal larvae to arrested development (an "old embryo" stage). The EC(50) values were 28,315 and 40,617 µg L(-1) for glyphosate and its metabolite, respectively, but much lowered values of 1133 and 1675 µg L(-1) for R(EX) and R(AT), respectively. Metamorphosis tests also revealed a significant difference between molecules, as the EC(50) values exceeded 100,000 µg L(-1) for glyphosate and AMPA but were as low as 6366 and 6060 µg L(-1) for the commercial formulations, which appeared relatively more toxic. Overall, the embryo-larval development of C. gigas was more sensitive to glyphosate-based herbicides compared to various endpoints studied in regulatory model organisms, and embryos and D-shaped larvae were more sensitive compared to pediveliger larvae.

http://www.ncbi.nlm.nih.gov/pubmed/23277103

Cuhra, M.; Traavik, T.; Bøhn, T. 2013. Clone- and age-dependent toxicity of a glyphosate commercial formulation and its active ingredient in *Daphnia magna*. *Ecotoxicology*, 22 (2), 251-262. (DOI: 10.1007/s10646-012-1021-1).

Low levels of glyphosate based herbicide induced significant negative effects on the aquatic invertebrate Daphnia magna. Glyphosate herbicides such as brands of Roundup, are known to be toxic to daphnids. However, published findings on acute toxicity show significant discrepancies and variation across several orders of magnitude. To test the acute effects of both glyphosate and a commercial formulation of Roundup (hereafter Roundup), we conducted a series of exposure experiments with different clones and age-classes of D. magna. The results demonstrated EC(50) (48) values in the low ppm-range for Roundup as well as for the active ingredient (a.i.) isopropylamine salt of glyphosate

(glyphosate IPA) alone. Roundup showed slightly lower acute toxicity than glyphosate IPA alone, i.e. EC(50) values of 3.7-10.6 mg a.i./l, as compared to 1.4-7.2 mg a.i./l for glyphosate IPA. However, in chronic toxicity tests spanning the whole life-cycle, Roundup was more toxic. D. magna was exposed to sublethal nominal concentrations of 0.05, 0.15, 0.45, 1.35 and 4.05 mg a.i./l for 55 days. Significant reduction of juvenile size was observed even in the lowest test concentrations of 0.05 mg a.i./l, for both glyphosate and Roundup. At 0.45 mg a.i./l, growth, fecundity and abortion rate was affected, but only in animals exposed to Roundup. At 1.35 and 4.05 mg a.i./l of both glyphosate and Roundup, significant negative effects were seen on most tested parameters, including mortality. D. magna was adversely affected by a near 100 % abortion rate of eggs and embryonic stages at 1.35 mg a.i./l of Roundup. The results indicate that aquatic invertebrate ecology can be adversely affected by relevant ambient concentrations of this major herbicide. We conclude that glyphosate and Roundup toxicity to aquatic invertebrates have been underestimated and that current European Commission and US EPA toxicity classification of these chemicals need to be revised.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3572389/

Farabaugh, N.; Nowakowski, A. 2014. Behavioral responses of the strawberry poison frog (*Oophaga pumilio*) to herbicide olfactory cues: possible implications for habitat selection and movement in altered landscapes. *Canadian Journal of Zoology,* 92, 979-984.

Recent research has focused on the importance of behavior in mediating the effects of landscape change on amphibian populations and communities. Factors such as chemical contaminants may affect habitat selection and movement of amphibians in human-altered habitats and contribute to landscape-level patterns of distribution and abundance. The objective of this study was to determine if the Strawberry Poison Frog (*Oophaga pumilio* (Schmidt, 1857)) can use olfactory cues to detect and avoid the glyphosate-based herbicide Roundup™. Fifty frogs were captured in the field in Costa Rica and tested in experimental arenas where they were given a choice between a control and an herbicide treatment. Analysis of time spent in treatment areas revealed a significant interaction between sex and treatment. Analyses of choice at the start and end of the trials indicated that sex and cardinal direction were important factors influencing orientation behavior. These results suggest that males and females differed in their behavioral responses, and that male *O. pumilio* may use olfactory cues to detect and avoid areas treated with glyphosate-based herbicide. However, the sampled population was male-biased, which resulted in a lower sample size and lower power to detect an effect for females. Further work is needed to better understand amphibian behavioral responses to herbicides, as well as the role of sex and individual variation in modifying these responses.

http://www.nrcresearchpress.com/doi/abs/10.1139/cjz-2014-0111

Lanctôt, C.; Navarro-Martín, L.; Robertson, C.; Park, B.; Jackman, P.; Pauli, B.; Trudeau, V. 2014. Effects of glyphosate-based herbicides on survival, development, growth and sex ratios of wood frog (*Lithobates sylvaticus*) tadpoles. II: Agriculturally relevant exposures to Roundup WeatherMax® and Vision® under laboratory conditions. *Aquatic Toxicology,* 154, 291-303.

Glyphosate-based herbicides are currently the most commonly used herbicides in the world. They have been shown to affect survival, growth, development and sexual differentiation of tadpoles under chronic laboratory exposures but this has not been investigated under more environmentally realistic conditions. The purpose of this study is (1) to determine if an agriculturally relevant exposure to Roundup WeatherMax®, a relatively new and understudied formulation, influences the development of wood frog tadpoles (*Lithobates sylvaticus*) through effects on the mRNA levels of genes involved in the control of metamorphosis; (2) to compare results to the well-studied Vision® formulation (containing the isopropylamine salt of glyphosate [IPA] and polyethoxylated tallowamine [POEA] surfactant) and to determine which ingredient(s) in the formulations are responsible for potential effects on development; and (3) to compare results to recent field studies

that used a similar experimental design. In the present laboratory study, wood frog tadpoles were exposed to an agriculturally relevant application (i.e., two pulses) of Roundup WeatherMax® and Vision® herbicides as well as the active ingredient (IPA) and the POEA surfactant of Vision®. Survival, development, growth, sex ratios and mRNA levels of genes involved in tadpole metamorphosis were measured. Results show that Roundup WeatherMax® (2.89 mg acid equivalent (a.e.)/L) caused 100% mortality after the first pulse. Tadpoles treated with a lower concentration of Roundup WeatherMax® (0.21 mg a.e./L) as well as Vision® (2.89 mg a.e./L), IPA and POEA had an increased condition factor (based on length and weight measures in the tadpoles) relative to controls at Gosner stage (Gs) 36/38. At Gs42, tadpoles treated with IPA and POEA had a decreased condition factor. Also at Gs42, the effect on condition factor was dependent on the sex of tadpoles and significant treatment effects were only detected in males. In most cases, treatment reduced the normal mRNA increase of key genes controlling development in tadpoles between Gs37 and Gs42, such as genes encoding thyroid hormone receptor beta in brain, glucocorticoid receptor in tail and deiodinase enzyme in brain and tail. We conclude that glyphosate-based herbicides have the potential to alter mRNA profiles during metamorphosis. However, studies in natural systems have yet to replicate these negative effects, which highlight the need for more ecologically relevant studies for risk assessment.

http://www.sciencedirect.com/science/article/pii/S0166445X1400191X

Navarro-Martín, L.; Lanctôt, C.; Jackman, P.; Park, B.; Doe, K.; Pauli, B.; Trudeau, V. 2014. Effects of glyphosate-based herbicides on survival, development, growth and sex ratios of wood frogs (*Lithobates sylvaticus*) tadpoles. I: Chronic laboratory exposures to VisionMax®. *Aquatic Toxicology,* 154, 278-290.

The purpose of this study was to determine if chronic exposure to the glyphosate-based herbicide VisionMax® affects the survival, development, growth, sex ratios and expression of specific genes involved in metamorphosis of wood frog tadpoles (*Lithobates sylvaticus*). We hypothesized that exposure to this herbicide will affect developmental rates by disrupting hormone pathways, sex ratios and/or gonadal morphology. Tadpoles were chronically exposed in the laboratory from Gosner developmental stage 25 to 42 to four different concentrations of VisionMax® (ranging from 0.021 to 2.9 mg acid equivalents/L). Chronic exposures to VisionMax® had direct effects on the metamorphosis of *L. sylvaticus* tadpoles by decreasing development rates, however, there was a decrease in survival only in the group exposed to the highest dose of VisionMax® (2.9 mg a.e./L; from approximately 96% in the control group to 77% in the treatment group). There was a decrease in the number of tadpoles reaching metamorphic climax, from 78% in the control group to 42% in the VisionMax® (2.9 mg a.e./L) group, and a 7-day delay to reach metamorphic climax in the same treatment group. No effects of exposure on sex ratios or gonadal morphology were detected in tadpoles exposed to any of the concentrations of VisionMax® tested. Gene expression analyses in brain and tail tissues demonstrated that exposure to VisionMax® alters the expression of key genes involved in development. Results showed significant interaction (two-way ANOVA, $P < 0.05$) between developmental Gosner stage and treatment in brain *corticotropin-releasing factor*, deiodinase type II (*dio2*) and *glucocorticotiroid receptor* (*grII*) and tail *dio2* and *grII*. This demonstrates that mRNA levels may be differently affected by treatment depending on the developmental stage at which they are assessed. At the same time there was a clear dose–response effect for VisionMax® to increase thyroid hormone receptor β in tadpole brain ($F_{(2,69)} = 3.475$, $P = 0.037$) and tail ($F_{(2,69)} = 27.569$, $P < 0.001$), regardless of developmental stage. Interestingly, delays in development (or survival) were only observed in the group exposed to 2.9 mg a.e./L of VisionMax®, suggesting that tadpoles need to be exposed to a "threshold" concentration of glyphosate-based herbicide to exhibit phenotypic observable effects. We suggest that the upregulation of genes that trigger metamorphosis following VisionMax® herbicide exposure might result from a compensatory response for the delays in development observed. Further studies are needed to determine if disruption of expression of these key genes leads to long-term effects when metamorphs reach adult stages.

http://www.sciencedirect.com/science/article/pii/S0166445X14001763

Moreno, N.; Sofia, S.; Martinez C. 2014. Genotoxic effects of the herbicide Roundup Transorb and its active ingredient glyphosate on the fish *Prochilodus lineatus*. *Environ Toxicol Pharmacol*, 37(1):448-54. doi: 10.1016/j.etap.2013.12.012.

Roundup Transorb (RT) is a glyphosate-based herbicide and despite its wide use around the world there are few studies comparing the effects of the active ingredient with the formulated product. In this context the purpose of this study was to compare the genotoxicity of the active ingredient glyphosate with the formulated product RT in order to clarify whether the active ingredient and the surfactant of the RT formula may exert toxic effects on the DNA molecule in juveniles of fish Prochilodus lineatus. Erythrocytes and gill cells of fish exposed to glyphosate and to RT showed DNA damage scores significantly higher than control animals. These results revealed that both glyphosate itself and RT were genotoxic to gill cells and erythrocytes of P. lineatus, suggesting that their use should be carefully monitored considering their potential impact on tropical aquatic biota.

http://www.ncbi.nlm.nih.gov/pubmed/24448465

Zaller, J.; Heigl, F.; Ruess, L.; Grabmaier, A. 2014. Glyphosate herbicide affects belowground interactions between earthworms and symbiotic mycorrhizal fungi in a model ecosystem. *Scientific Reports*, 4, Article number: 5634. doi:10.1038/srep05634.

Herbicides containing glyphosate are widely used in agriculture and private gardens, however, surprisingly little is known on potential side effects on non-target soil organisms. In a greenhouse experiment with white clover we investigated, to what extent a globally-used glyphosate herbicide affects interactions between essential soil organisms such as earthworms and arbuscular mycorrhizal fungi (AMF). We found that herbicides significantly decreased root mycorrhization, soil AMF spore biomass, vesicles and propagules. Herbicide application and earthworms increased soil hyphal biomass and tended to reduce soil water infiltration after a simulated heavy rainfall. Herbicide application in interaction with AMF led to slightly heavier but less active earthworms. Leaching of glyphosate after a simulated rainfall was substantial and altered by earthworms and AMF. These sizeable changes provide impetus for more general attention to side-effects of glyphosate-based herbicides on key soil organisms and their associated ecosystem services.

Full article available at http://www.nature.com/srep/2014/140709/srep05634/full/srep05634.html

As discussed in documents organized in Part 3 (related to the human and animal health impact of the transgenic plants), the ecotoxicological profile of the glyphosate-based herbicides is not properly represented by the ecotoxicological profile of its active ingredient glyphosate. Studies show that the commercial glyphosate-based herbicides products include other components and its degradation metabolites, being then severely more harmful than the active ingredient solely, with much more expressive toxicological and biological risks. The products actually sprayed on crops, weeds and its sorroundings are mixtures on which the active ingredient depends on adjuvants which, in addition to have their

own toxicity, may potentialize (via synergic effects) the impacts related to the active ingredient itself.

In other words, as glyphosate does not operate on an isolated way, assessments disconsidering such fact are poorly valid. Even so, glyphosate particularly reveals itself as being extremely harmful, being alarming the fact that regulatory bodies dissociate their impacts from the risk assessment of HT GM plants associated to it.

This chapter joins articles showing that the environmental impacts resulting from the use of the commercial product Roundup are more severe than those associated to its active ingredient, the glyphosate.

It is worth mentioning that lab toxicological tests that disconsider the commercial products as such and focus on the active ingredient mystify the results, minimizing then the effects the real world is expose to.

Tsui, M.; Chu, L. 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. *Chemosphere*, 52(7):1189-97.

Glyphosate-based herbicides (e.g. Roundup) are extensively used in the aquatic environment, but there is a paucity of data on the toxicity of the formulated products and the influences by environmental factors. In this study, the acute toxicity of technical-grade glyphosate acid, isopropylamine (IPA) salt of glyphosate, Roundup and its surfactant polyoxyethylene amine (POEA) to Microtox bacterium (Vibrio fischeri), microalgae (Selenastrum capricornutum and Skeletonema costatum), protozoa (Tetrahymena pyriformis and Euplotes vannus) and crustaceans (Ceriodaphnia dubia and Acartia tonsa) was examined and the relative toxicity contributions of POEA to Roundup were calculated. The effects of four environmental factors (temperature, pH, suspended sediment and algal food concentrations) on the acute toxicity of Roundup to C. dubia were also examined. Generally, the toxicity order of the chemicals was: POEA>Roundup>glyphosate acid>IPA salt of glyphosate, while the toxicity of glyphosate acid was mainly due to its high acidity. Microtox bacterium and protozoa had similar sensitivities towards Roundup toxicity (i.e. IC50 from 23.5 to 29.5 mg AE/l). In contrast, microalgae and crustaceans were 4-5 folds more sensitive to Roundup toxicity than bacteria and protozoa. Except photosynthetic microalgae, POEA accounted for more than 86% of Roundup toxicity and the toxicity contribution of POEA was shown to be species-dependent. Increase in pH (6-9) and increase of suspended sediment concentration (0-200 mg/l) significantly increased the toxicity of Roundup to C. dubia, but there were no significant effects due to temperature change and food addition.

http://www.ncbi.nlm.nih.gov/pubmed/12821000

Howe, C.; Berrill, M.; Pauli, B.; Helbing, C.; Werry, K.; Veldhoen, N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. *Environ Toxicol Chem*, 23(8):1928-38.

Glyphosate-based herbicides are among the most widely used pesticides in the world. We compared the acute toxicity of the glyphosate end-use formulation Roundup Original to four North American amphibian species (Rana clamitans, R. pipiens, R. sylvatica, and Bufo americanus) and the toxicity of glyphosate technical, the polyethoxylated tallowamine surfactant (POEA) commonly used in glyphosate-based herbicides, and five newer glyphosate formulations to R. clamitans. For R. clamitans, acute toxicity values in order of decreasing toxicity were POEA > Roundup Original > Roundup Transorb > Glyfos AU; no significant acute toxicity was observed with glyphosate technical material or the glyphosate formulations Roundup Biactive, Touchdown, or Glyfos BIO. Comparisons between the four amphibian species showed that the toxicity of Roundup Original varied with species and developmental stage. Rana pipiens tadpoles chronically exposed to environmentally relevant concentrations of POEA or glyphosate formulations containing POEA showed decreased snout-vent length at metamorphosis and increased time to metamorphosis, tail damage, and gonadal abnormalities. These effects may be caused, in some part, by disruption of hormone signaling, because thyroid hormone receptor beta mRNA transcript levels were elevated by exposure to formulations containing glyphosate and POEA. Taken together, the data suggest that surfactant composition must be considered in the evaluation of toxicity of glyphosate-based herbicides.

http://www.ncbi.nlm.nih.gov/pubmed/15352482

Contardo-Jara, V.; Klingelmann, E.; Wiegand, C. 2009. Bioaccumulation of glyphosate and its formulation Roundup Ultra in *Lumbriculus variegatus* and its effects on biotransformation and antioxidant enzymes. *Environmental Pollution*, 157 (1) 57–63.

The bioaccumulation potential of glyphosate and the formulation Roundup Ultra, as well as possible effects on biotransformation and antioxidant enzymes in Lumbriculus variegatus were compared by four days exposure to concentrations between 0.05 and 5 mg L(-1) pure glyphosate and its formulation. Bioaccumulation was determined using (14)C labeled glyphosate. The bioaccumulation factor (BCF) varied between 1.4 and 5.9 for the different concentrations, and was higher than estimated from logP(ow). Glyphosate and its surfactant POEA caused elevation of biotransformation enzyme soluble glutathione S-transferase at non-toxic concentrations. Membrane bound glutathione S-transferase activity was significantly elevated in Roundup Ultra exposed worms, compared to treatment with equal glyphosate concentrations, but did not significantly differ from the control. Antioxidant enzyme superoxide dismutase was significantly increased by glyphosate but in particular by Roundup Ultra exposure indicating oxidative stress. The results show that the formulation Roundup Ultra is of more ecotoxicological relevance than the glyphosate itself.

http://www.ncbi.nlm.nih.gov/pubmed/18790555

Lipok, J.; Studnik, H.; Gruyaert, S. 2010. The toxicity of Roundup® 360 SL formulation and its main constituents: glyphosate and isopropylamine towards non-target water photoautotrophs. *Ecotoxicol Environ Saf*, 73(7):1681-8. doi: 10.1016/j.ecoenv.2010.08.017.

The toxicity of commercial formulation of Roundup® 360 SL, widely used, nonselective herbicide and its main constituents, glyphosate (PMG), equimolar (1:1) isopropylamine salt of glyphosate (GIPA) and isopropylamine (IPA) was examined towards eight aquatic microphotoautotrophs; seven cyanobacterial strains representing either saline or freshwater communities, and common eukaryotic algae Chlorella vulgaris Beijerinck. Autotrophs were cultured 21 days in their appropriate standard media supplemented with various amounts of Roundup®, glyphosate, GIPA and IPA.

The determination of the growth of examined photoautotrophs was performed by time-course measurements of total chlorophyll content in experimental cultures. The growth rates related to corresponding concentrations of chemicals, the EC(50) values and generation doubling time were determined in order to present the toxicity Roundup® 360 SL formulation and its main constituents. Market available formulation of Roundup® was found to possess toxicity significantly higher than this, attributed to its main constituents; however both these compounds, isopropylamine and glyphosate, also inhibited the growth of examined strains in a dose-dependent manner. Notably, the interpretation of toxicity of the examined substances was found to be significantly dependent on the method of EC(50) calculation. The choice of molar or weight concentration of substances tested separately and in specific formulation was found to be essential in this matter. Due to these findings the EC(50) values were calculated based either on molar or on weight concentrations. Considering Roundup® 360 SL formulation, these values ranged from 10(-3) up to 10(-1) mM and they were one order of magnitude lower than those found for isopropylamine. Quite surprisingly the minimum EC(50) values found for glyphosate did not reach micromolar concentrations, whereas most of the EC(50) values revealed to IPA did not exceed this range. Notably, in all the cases except for Synechocystis aquatilis Sauvageau, isopropylamine alone was indicated as more toxic than glyphosate.

http://www.ncbi.nlm.nih.gov/pubmed/20813408

Moore, L.; Fuentes, L.; Rodgers, J.; Bowerman, W.; Yarrow, G.; Chao, W.; Bridges, W. 2012. Relative toxicity of the components of the original formulation of Roundup to five North American anurans. *Ecotoxicol Environ Saf*, 78:128-33. doi: 10.1016/j.ecoenv.2011.11.025.

The responses of five North American frog species that were exposed in an aqueous system to the original formulation of Roundup were compared. Carefully designed and un-confounded laboratory toxicity tests are crucial for accurate assessment of potential risks from the original formulation of Roundup to North American amphibians in aquatic environments. The formulated mixture of this herbicide as well as its components, isopropylamine (IPA) salt of glyphosate and the surfactant MON 0818 (containing polyethoxylated tallowamine (POEA)) were separately tested in 96 h acute toxicity tests with Gosner stage 25 larval anurans. Rana pipiens, R. clamitans, R. catesbeiana, Bufo fowleri, and Hyla chrysoscelis were reared from egg masses and exposed to a series of 11 concentrations of the original formulation of Roundup herbicide, nine concentrations of MON 0818 and three concentrations of IPA salt of glyphosate in static (non-renewal) aqueous laboratory tests. LC50 values are expressed as glyphosate acid equivalents (ae) or as mg/L for MON 0818 concentrations for comparison between the formulation and components. R. pipiens was the most sensitive of five species with 96 h-LC50 values for formulation tests, for the five species, ranging from 1.80 to 4.22 mg ae/L, and MON 0818 exposures with 96 h-LC50 values ranging from 0.68 to 1.32 mg/L. No significant mortality was observed during exposures of 96 h for any of the five species exposed to glyphosate IPA salt at concentrations up to 100 times the predicted environmental concentration (PEC). These results agree with previous studies which have noted that the surfactant MON 0818 containing POEA contributes the majority of the toxicity to the herbicide formulations for fish, aquatic invertebrates, and amphibians. These study results suggest that anurans are among the most sensitive species, and emphasize the importance of testing the herbicide formulation in addition to its separate components to accurately characterize the toxicity and potential risk of the formulation.

http://www.ncbi.nlm.nih.gov/pubmed/22137360

A number of studies, like the following ones, show that the glyphosate-based herbicides tend to be highly persistent in the soil, water and air, penetrating ecological chains and exacerbating environmental contamination problems.

Kjaer, J.; Olsen, P.; Ullum, M.; Grant, R. 2005. Leaching of glyphosate and amino-methylphosphonic acid from Danish agricultural field sites. *J Environ Qual.*, 34(2):608-20.

Pesticide leaching is an important process with respect to contamination risk to the aquatic environment. The risk of leaching was thus evaluated for glyphosate (N-phosphonomethyl-glycine) and its degradation product AMPA (amino-methylphosphonic acid) under field conditions at one sandy and two loamy sites. Over a 2-yr period, tile-drainage water, ground water, and soil water were sampled and analyzed for pesticides. At a sandy site, the strong soil sorption capacity and lack of macropores seemed to prevent leaching of both glyphosate and AMPA. At one loamy site, which received low precipitation with little intensity, the residence time within the root zone seemed sufficient to prevent leaching of glyphosate, probably due to degradation and sorption. Minor leaching of AMPA was observed at this site, although the concentration was generally low, being on the order of 0.05 microg L(-1) or less. At another loamy site, however, glyphosate and AMPA leached from the root zone into the tile drains (1 m below ground surface [BGS]) in average concentrations exceeding 0.1 microg L(-1), which is the EU threshold value for drinking water. The leaching of glyphosate was mainly governed by pronounced macropore flow occurring within the first months after application. AMPA was frequently detected more than 1.5 yr after application, thus indicating a minor release and limited degradation capacity within the soil. Leaching has so far been confined to the depth of the tile drains, and the pesticides have rarely been detected in monitoring screens located at lower depths. This study suggests that as both glyphosate and AMPA can leach through structured soils, they thereby pose a potential risk to the aquatic environment.

http://www.ncbi.nlm.nih.gov/pubmed/15758114

Peruzzo, P.; Porta, A.; Ronco, A. 2008. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. *Environmental Pollution*, 156, 61-66.

Levels of glyphosate were determined in water, soil and sediment samples from a transgenic soybean cultivation area located near to tributaries streams of the Pergamino-Arrecifes system in the north of the Province of Buenos Aires, Argentina. Field work took into account both the pesticide application and the rains occurring after applications. The pesticide was analysed by HPLC-UV detection, previous derivatization with 9-fluorenylmethylchloroformate (FMOC-Cl). In addition, SoilFug multimedia model was used to analyse the environmental distribution of the pesticides. In the field, levels of glyphosate in waters ranged from 0.10 to 0.70 mg/L, while in sediments and soils values were between 0.5 and 5.0 mg/Kg. Temporal variation of glyphosate levels depended directly on the time of application and the rain events. The results obtained from the application of the model are in accordance with the values found in the field.

http://www.ncbi.nlm.nih.gov/pubmed/18308436

Borggaard, O.; Gimsing, A. 2008. Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. *Pest Manag Sci*, 64(4):441-56.

The very wide use of glyphosate to control weeds in agricultural, silvicultural and urban areas throughout the world requires that special attention be paid to its possible transport from terrestrial to aquatic environments. The aim of this review is to present and discuss the state of knowledge on sorption, degradation and leachability of glyphosate in soils. Difficulties of drawing clear and unambiguous conclusions because of strong soil dependency and limited conclusive investigations are pointed out. Nevertheless, the risk of ground and surface water pollution by glyphosate seems limited because of sorption onto variable-charge soil minerals, e.g. aluminium and iron oxides, and because of microbial

degradation. Although sorption and degradation are affected by many factors that might be expected to affect glyphosate mobility in soils, glyphosate leaching seems mainly determined by soil structure and rainfall. Limited leaching has been observed in non-structured sandy soils, while subsurface leaching to drainage systems was observed in a structured soil with preferential flow in macropores, but only when high rainfall followed glyphosate application. Glyphosate in drainage water runs into surface waters but not necessarily to groundwater because it may be sorbed and degraded in deeper soil layers before reaching the groundwater. Although the transport of glyphosate from land to water environments seems very limited, knowledge about subsurface leaching and surface runoff of glyphosate as well as the importance of this transport as related to ground and surface water quality is scarce.

http://www.ncbi.nlm.nih.gov/pubmed/18161065

Chang, F.; Simci, M.; Capel, P. 2011. Occurrence and fate of the herbicide glyphosate and its degradate aminomethylphosphonic acid in the atmosphere. *Environ Toxicol Chem*, 30(3):548-55. doi: 10.1002/etc.431.

This is the first report on the ambient levels of glyphosate, the most widely used herbicide in the United States, and its major degradation product, aminomethylphosphonic acid (AMPA), in air and rain. Concurrent, weekly integrated air particle and rain samples were collected during two growing seasons in agricultural areas in Mississippi and Iowa. Rain was also collected in Indiana in a preliminary phase of the study. The frequency of glyphosate detection ranged from 60 to 100% in both air and rain. The concentrations of glyphosate ranged from <0.01 to 9.1 ng/m(3) and from <0.1 to 2.5 µg/L in air and rain samples, respectively. The frequency of detection and median and maximum concentrations of glyphosate in air were similar or greater to those of the other high-use herbicides observed in the Mississippi River basin, whereas its concentration in rain was greater than the other herbicides. It is not known what percentage of the applied glyphosate is introduced into the air, but it was estimated that up to 0.7% of application is removed from the air in rainfall. Glyphosate is efficiently removed from the air; it is estimated that an average of 97% of the glyphosate in the air is removed by a weekly rainfall ≥ 30 mm.

http://www.ncbi.nlm.nih.gov/pubmed/21128261

Coupe, R.; Kalkhoff, S.; Capel, P.; Gregoire, C. 2011. Fate and transport of glyphosate and aminomethylphosphonic acid in surface waters of agricultural basins. *Pest Manag Sci.*, 68(1): 16–30.

Background: Glyphosate [N-(phosphonomethyl)glycine] is a herbicide used widely throughout the world in the production of many crops and is heavily used on soybeans, corn and cotton. Glyphosate is used in almost all agricultural areas of the United States, and the agricultural use of glyphosate has increased from less than 10 000 Mg in 1992 to more than 80 000 Mg in 2007. The greatest intensity of glyphosate use is in the midwestern United States, where applications are predominantly to genetically modified corn and soybeans. In spite of the increase in usage across the United States, the characterization of the transport of glyphosate and its degradate aminomethylphosphonic acid (AMPA) on a watershed scale is lacking.
Results: Glyphosate and AMPA were frequently detected in the surface waters of four agricultural basins. The frequency and magnitude of detections varied across basins, and the load, as a percentage of use, ranged from 0.009 to 0.86% and could be related to three general characteristics: source strength, rainfall runoff and flow route.
Conclusions: Glyphosate use in a watershed results in some occurrence in surface water; however, the watersheds most at risk for the offsite transport of glyphosate are those with high application rates, rainfall that results in overland runoff and a flow route that does not include transport through the soil.

http://www.ncbi.nlm.nih.gov/pubmed/21681915

Aparicio, V.; De Gerónimo, E.; Marino, D.; Primost, J.; Carriquiriborde, P.; Costa, J. 2013. Environmental fate of glyphosate and aminomethylphosphonic acid in surface waters and soil of agricultural basins. *Chemosphere,* 93(9):1866-73. doi: 10.1016/j.chemosphere.2013.06.041.

Argentinian agricultural production is fundamentally based on a technological package that combines no-till and glyphosate in the cultivation of transgenic crops. Transgenic crops (soybean, maize and cotton) occupy 23 million hectares. This means that glyphosate is the most employed herbicide in the country, where 180-200 million liters are applied every year. The aim of this work is to study the environmental fate of glyphosate and its major degradation product, aminomethylphosphonic acid (AMPA), in surface water and soil of agricultural basins. Sixteen agricultural sites and forty-four streams in the agricultural basins were sampled three times during 2012. The samples were analyzed by UPLC-MS/MS ESI(+/-). In cultivated soils, glyphosate was detected in concentrations between 35 and 1502 µg kg(-1), while AMPA concentration ranged from 299 to 2256 µg kg(-1). In the surface water studied, the presence of glyphosate and AMPA was detected in about 15% and 12% of the samples analyzed, respectively. In suspended particulate matter, glyphosate was found in 67% while AMPA was present in 20% of the samples. In streams sediment glyphosate and AMPA were also detected in 66% and 88.5% of the samples respectively. This study is, to our knowledge, the first dealing with glyphosate fate in agricultural soils in Argentina. In the present study, it was demonstrated that glyphosate and AMPA are present in soils under agricultural activity. It was also found that in stream samples the presence of glyphosate and AMPA is relatively more frequent in suspended particulate matter and sediment than in water.

http://www.ncbi.nlm.nih.gov/pubmed/23849835

Battaglin, W.; Meyer, M.; Kuivila, K.; Dietze, J. 2014. Glyphosate and its degradation product AMPA occur frequently and widely in U.S. soils, surface water, groundwater, and precipitation. *JAWRA Journal of the American Water Resources Association,* 50:10.1111/jawr.2014.50.issue-2, 275-290.

Glyphosate use in the United States increased from less than 5,000 to more than 80,000 metric tons/yr between 1987 and 2007. Glyphosate is popular due to its ease of use on soybean, cotton, and corn crops that are genetically modified to tolerate it, utility in no-till farming practices, utility in urban areas, and the perception that it has low toxicity and little mobility in the environment. This compilation is the largest and most comprehensive assessment of the environmental occurrence of glyphosate and aminomethylphosphonic acid (AMPA) in the United States conducted to date, summarizing the results of 3,732 water and sediment and 1,018 quality assurance samples collected between 2001 and 2010 from 38 states. Results indicate that glyphosate and AMPA are usually detected together, mobile, and occur widely in the environment. Glyphosate was detected without AMPA in only 2.3% of samples, whereas AMPA was detected without glyphosate in 17.9% of samples. Glyphosate and AMPA were detected frequently in soils and sediment, ditches and drains, precipitation, rivers, and streams; and less frequently in lakes, ponds, and wetlands; soil water; and groundwater. Concentrations of glyphosate were below the levels of concern for humans or wildlife; however, pesticides are often detected in mixtures. Ecosystem effects of chronic low-level exposures to pesticide mixtures are uncertain. The environmental health risk of low-level detections of glyphosate, AMPA, and associated adjuvants and mixtures remain to be determined.

http://onlinelibrary.wiley.com/doi/10.1111/jawr.12159/abstract

Majewski, M.; Coupe, R.; Foreman, W.; Capel, P. 2014. Pesticides in Mississippi air and rain: a comparison between 1995 and 2007. *Environmental Toxicology and Chemistry*, 33(6):1283-1293.

A variety of current-use pesticides were determined in weekly composite air and rain samples collected during the 1995 and 2007 growing seasons in the Mississippi Delta (MS, USA) agricultural region. Similar sampling and analytical methods allowed for direct comparison of results. Decreased overall pesticide use in 2007 relative to 1995 generally resulted in decreased detection frequencies in air and rain; observed concentration ranges were similar between years, however, even though the 1995 sampling site was 500 m from active fields whereas the 2007 sampling site was within 3 m of a field. Mean concentrations of detections were sometimes greater in 2007 than in 1995, but the median values were often lower. Seven compounds in 1995 and 5 in 2007 were detected in ≥50% of both air and rain samples. Atrazine, metolachlor, and propanil were detected in ≥50% of the air and rain samples in both years. Glyphosate and its degradation product, aminomethyl-phosphonic acid (AMPA), were detected in ≥75% of air and rain samples in 2007 but were not measured in 1995. The 1995 seasonal wet depositional flux was dominated by methyl parathion (88%) and was >4.5 times the 2007 flux. Total herbicide flux in 2007 was slightly greater than in 1995 and was dominated by glyphosate. Malathion, methyl parathion, and degradation products made up most of the 2007 nonherbicide flux.

http://www.ncbi.nlm.nih.gov/pubmed/24549493

Mercurio, P.; Flores, F.; Mueller, J.; Carter, S.; Negri, A. 2014. Glyphosate persistence in seawater. *Marine Pollution Bulletin*, 85(2): 385-390.

Glyphosate is one of the most widely applied herbicides globally but its persistence in seawater has not been reported. Here we quantify the biodegradation of glyphosate using standard "simulation" flask tests with native bacterial populations and coastal seawater from the Great Barrier Reef. The half-life for glyphosate at 25 °C in low-light was 47 days, extending to 267 days in the dark at 25 °C and 315 days in the dark at 31 °C, which is the longest persistence reported for this herbicide. AMPA, the microbial transformation product of glyphosate, was detected under all conditions, confirming that degradation was mediated by the native microbial community. This study demonstrates glyphosate is moderately persistent in the marine water under low light conditions and is highly persistent in the dark. Little degradation would be expected during flood plumes in the tropics, which could potentially deliver dissolved and sediment-bound glyphosate far from shore.

Full article available at http://www.sciencedirect.com/science/article/pii/S0025326X14000228

2.1.2 Glufosinate ammonium -based herbicides

HT plants in connection with the glufosinate ammonium (GA) herbicide has been presented as a flexibility alternative to the control of glyphosate-tolerant weeds. From the toxicological point-of-view, the problems are similar, although the accumulation of information is lower in the case of the later.

The studies below point out problems caused by GAAG to the soil communities and aquatic environments, changes in population

relationships and environmental imbalances, among others.

Ahmad, I.; Malloch, D. 1995. Interaction of soil microflora with the bioherbicide phosphinothricin. *Agriculture, Ecosystems & Environment*, Volume 54, Issue 3, July 1995, Pages 165–174.

Phosphinothricin, a microbial toxin synthesized industrially for chemical weed control and currently under development as a selective weed killer in cultivation of transgenic plants engineered to resist its presence, is investigated for its effects on the distribution of microorganisms in 15 agricultural and non-agricultural soils. In agricultural soils, the presence of 1 mM phosphinothricin reduced the number of fungi isolated by about 20% and bacteria by about 40%. Under these conditions the isolation of bacteria from boreal forest soils was also suppressed by about 20%. Differences in herbicide resistance were confirmed when a random selection of fungi, bacteria and actinomycetes isolated in the absence of herbicide was grown with 1 mM phosphinothricin. Soil isolates growing in the presence of 1 mM phosphinothricin exhibited a wide spectrum of tolerance to increasing herbicide concentration over the range of 0–50 mM phosphinothricin. Of fungal isolates, the plant pathogen *Verticilium alboatrum* was among the most resistant, while the mycoparasitic species *Trichoderma harzianum* and *T. longipilus* were among the most sensitive to the presence of phosphinothricin.

http://www.sciencedirect.com/science/article/pii/016788099500603P

Ahmad, I.; Bissett, J.; Malloch, D. 1995. Effect of phosphinothricin on nitrogen metabolism of *Trichoderma* species and its implications for their control of phytopathogenic fungi. *Pesticide Biochemistry and Physiology*, Volume 53, Issue 1, Pages 49–59.

The antagonistic control of the phytopathogen *Fusarium oxysporum* by *Trichoderma* species is impaired in the presence of phosphinothricin, a microbial toxin commercialized for chemical weed control under trade names Basta, Ignite, and Herbiace. The influence of phosphinothricin on growth parameters and nitrogen metabolism in nine strains of *Trichoderma* and a closely related fungus *Nectria ochroleuca* was investigated. The presence of 1 m*M* phosphinothricin was lethal to the *Trichoderma* anamorph of *Hypocrea gelatinosa* and almost equally inhibitory to *Trichoderma polysporum*. In other species it caused marked reductions in the hyphal protein content, accompanied by lower biomass yields in all except *N. ochroleuca*. Under normal growth conditions, these rapidly growing fungi maintain highly active levels of the two key enzymes of ammonium assimilation, glutamine synthetase, and NADPH-dependent glutamate dehydrogenase. Phosphinothricin, by causing an inhibition in glutamine synthetase activity, diminishes the enzymic potential for the combined operation of these two alternative pathways. The level of NADPH-glutamate dehydrogenase activity was exceptionally high in *N. ochroleuca,* and although declining in the presence of phosphinothricin, remained much higher than those in nine strains of *Trichoderma*. The levels of NADPH-glutamate dehydrogenase in *T. harzianum, T. citrinoviride,* and *T. viride* were higher in the presence than in the absence of phosphinothricin. In the presence of phosphinothricin, the levels of both aspartate aminotransferase and alanine aminotransferase activities were stimulated in *T. harzianum, T. atroviride, T. citrinoviride,* and *T. viride* and that of alanine aminotransferase in *T. koningii*. *Trichoderma harzianum* and *T. atroviride* contain single anionic molecular forms of glutamine synthetase, NADPH-glutamate dehydrogenase, and alanine aminotransferase and two isoforms of aspartate aminotransferase both in the presence and in the absence of phosphinothricin.

http://www.sciencedirect.com/science/article/pii/S0048357585710541

De Snoo, G.; De Jong, F.; Der Poll, R.; Van Der Linden, M. 2001. Effects of glufosinate ammonium on off crop vegetation--interim results. *Meded Rijksuniv Gent Fak Landbouwkd Toegep Biol Wet*, 66(2b): 731-41.

In 2000 a field study was conducted at four different locations concerning the effects of low dosages of glufosinate-ammonium, a leaf acting herbicide, on off crop vegetation. Therefore species rich road verges and ditch banks not adapted to a history of herbicide use were sprayed twice with different dosages of glufosinate-ammonium, simulating drift (0, 2, 4, 16, 32 and 64% of the maximum field dose: 800 g a.i./ha). The parameters studied were short term phytotoxic effects and the effects on biomass, species cover and number of species in autumn (Braun-Blanquêt relevés). The results show significant phytotoxic effects at all dosages of glufosinate-ammonium on the non-target vegetation. The low concentrations (2 and 4%) had most impact when applied early in the season (9% average at the 2% dosage and 22% at 4% dosage, after the first spraying. At high dosages (32 and 64%) a decrease of the biomass of the vegetation was found in August. A comparison between treatments in August shows a small decrease in species number and cover in the 64% compared to the control. In the comparison between the spring and August relevés, the decrease in the mean number of species was significantly stronger in the treated plots than in the untreated ones of 4% and higher. For monocotyledons in all treatments except 16%, a significantly stronger decrease in species number was found compared to the untreated. For dicotyledons only the 64% dosage differed from the untreated. Only at the 64% treatment the total cover of species decreased more than in the untreated plots. Since drift percentages of 2-4% can be expected at 1-2 m from a treated plot it can be concluded that the use of glufosinate-ammonium could result in visible short term phytotoxic effects (max 22%) on off-crop vegetation such as ditch banks and verges. There are also indications that effects on the number and cover of species in autumn can occur. Because in future glufosinate-ammonium could be used on a large scale in herbicide resistant crops like maize, which will be cultivated on the same field for many years, this study will be continued in 2001 in order to investigate if there are--whether or not--sustainable effects on the off crop vegetation.

http://www.ncbi.nlm.nih.gov/pubmed/12425097

Carpenter, D.; Boutin, C. 2010. Sublethal effects of the herbicide glufosinate ammonium on crops and wild plants: short-term effects compared to vegetative recovery and plant reproduction. *Ecotoxicology*, 19: 1322-1326.

Current guidelines for phytotoxicity testing rely heavily on short-term testing of primarily crop species to predict the sensitivity of non-target, wild plants to herbicides. However, little is known on how plants recover following initial growth inhibitions in standard 14-28 day greenhouse tests conducted for pesticide assessment and registration. The objectives of this study were to assess the ability of plant species to recover (biomass and reproduction) when tested at the juvenile stage (routine regulatory testing), comparing crop and wild species and using the herbicide glufosinate ammonium. Ten crops and 10 wild species were tested with a one-time exposure to glufosinate ammonium in a greenhouse. Half the plants of each species (9 doses × 6 replicates) were harvested 3 weeks after being sprayed (short-term). The remaining plants were harvested several weeks later, coinciding with seed set or natural senescence (long-term). Total aboveground biomass and several endpoints related to crop production and plant reproduction were measured. Calculated IC50 values (dosage that results in a 50% decrease in the biomass of a plant as compared to the untreated controls) based solely on aboveground biomass, for species harvested in the long-term were generally higher than those obtained in the short-term (with two exceptions), indicating recovery over time. Crop species did not differ from wild species in terms of sensitivity. However, in seven out of 12 cases where reproduction was measurable, reproductive endpoints were more sensitive than either short or long-term biomass endpoints, indicating the importance of examining these parameters in phytotoxicity testing. Glufosinate ammonium was found to be phytotoxic at low doses (2.64-7.74% g ai/ha of the label rate).

http://www.ncbi.nlm.nih.gov/pubmed/20635139

2.1.3 2,4-D-based herbicides

The emergence of glyphosate-tolerant weeds (and, at a lower degree, to glufosinate ammonium) created market opportunities to new technologies as the same time the efficacy of those GM crops was reduced, which has complexified weed control. The availability of global stocks of herbicide 2,4-D; the prohibition of its use in several countries; the fact of being a dominated technology and under public domain placed it as an interesting alternative for the agribusiness, despite of the alarming risks to the human, animal and environmental health.

By the time this publication was completed, CTNBio authorized the commercial growing ans consumption of the first 2,4-D-tolerant soy variety. Proponents expect that this technology replaces glyphosate- and/or glufosinate ammonium -tolerant varieties. Maize events with the same function are also pending to be voted, being its commercial release also expected. It is a temporary "solution", logical consequence of the HT GMPs expansion. It is possible to state, from now, that the massive use of 2,4-D will determine on the short run an increased emergence of tolerant and resistant weed populations . These already exist worldwide, especially where historic use of 2,4-D has been verified.

In other words, the GM crops tolerant to 2,4-D-based herbicides seems an attempt to delay management crises of agricultural lands occupied by weeds resistant to glyphosate or others. This is verified on a specially acute way in GM maize, cotton and soybean areas. The 2,4-D tolerant crops adoption shall occur by significant part of farmers facing weed resistance problems. It is then possible to predict a drastic increase in the use of 2,4-D herbicide over the next years[47].

47 Events of plants tolerant to 2,4-D herbicides are still uncultivated / marketed, but have since been released for planting in three countries in the world, Canada in 2012, USA in 2014 and Brazil in March 2015.

Part of the environmental implications resulting from such increase in the use of 2,4-D will be discussed below.

Roberts, B.; Dorough, H. 1984. Relative toxicity of chemical to the earthworm *Eisenia foetida*. *Environmental Toxicology and Chemistry*, 3: 67-78.

In the present study, 90 chemicals were tested against *Eisenia foetida* for the purpose of using this organism as the marker species to indicate the relative toxicities of chemicals to earthworms and other soil invertebrates. The worms were exposed to deposits of the chemicals on filter paper for 48 h and the mortality was recorded; concentrations were expressed in $\mu g/cm^2$. Based on the resulting LC_{50} values, the chemicals were classified as supertoxic (< 1.0 $\mu g/cm^2$), extremely toxic (1–10 $\mu g/cm^2$), very toxic (10–100 $\mu g/cm^2$), moderately toxic (100–1,000 $\mu g/cm^2$) or relatively nontoxic (> 1,000 $\mu g/cm^2$). Of the chemicals tested (pesticides, solvents, metals, drugs, carcinogens, etc.), only carbofuran and eserine salicylate, both carbamates, were supertoxic. The remaining chemicals were distributed about equally among the other toxicity categories. The most surprising results were that the phenolic hydrolytic products of parathion, carbaryl, 2,4-D and 2,4,5-T were as toxic, or more toxic, than the parent material, with all of these compounds falling within the extremely toxic and very toxic classifications. Several chemicals, considered only moderately or relatively nontoxic to mammals, were extremely or very toxic to earthworms; among these compounds were carbaryl, malathion, cypermethrin and benomyl. The results of this study further demonstrate the unpredictability of chemical toxicity to different animal species, a fact which complicates the assessment of environmental risk to one or more species based on data attained with another.

http://onlinelibrary.wiley.com/doi/10.1002/etc.5620030109/abstract

Arias, E. 2003. Sister chromatid exchange induction by the herbicide 2,4-dichlorophenoxyacetic acid in chick embryos. *Ecotoxicol Environ Saf*, 55(3): 338-43.

As genetic damage may result from exposure to agricultural chemicals, it seemed appropriate to assess the genotoxic potential of 2,4-dichlorophenoxyacetic acid (2,4-D), a widely used broad-leaf herbicide, using a test system that may provide some indications on the genetic risk to animal species in the wild. In the present study, sister chromatid exchange (SCE) induction and cell cycle kinetics alterations by 2,4-D in 4-day old chick embryos were evaluated. Both a commercial herbicide formulation containing 37% 2,4-D isooctyl ester as active ingredient and pure 2,4-D were tested. Chick embryos were treated with 0, 0.5, 1, 2, or 4 mg 2,4-D. Test solutions were applied to the inner shell membrane on day 0 of incubation. Either commercial formulation or pure 2,4-D induced a dose-related increase in SCE frequency over the concentration range from 0 to 4 mg/embryo. Significantly higher SCE frequency was seen for the 4-mg group of embryos treated with the commercial product. A slightly higher SCE value was observed for the vehicle group (acetone-treated embryos) compared with the negative controls (untreated embryos). Significant inhibition of cell cycle progression was evident in both experimental groups and was generally dose related. The extent of changes in cell kinetics was similar in both groups, although somewhat more marked in the group treated with pure 2,4-D. The present findings corroborate the positive results from recent in vivo rodent studies.

http://www.ncbi.nlm.nih.gov/pubmed/12798768

LaChapelle, A.; Ruygrok, M.; Toomer, M.; Oost, J.; Monnie, M.; Swenson, J.; Compton, A.; Stebbins-Boaz, B. 2007. The hormonal herbicide, 2,4-dichlorophenoxyacetic acid, inhibits *Xenopus oocyte* maturation by targeting translational and posttranslational mechanisms. *Reproductive toxicology*, 23(1):20-31.

The widely used hormonal herbicide, 2,4-dichlorophenoxyacetic acid, blocks meiotic maturation in vitro and is thus a potential environmental endocrine disruptor with early reproductive effects. To test whether maturation inhibition was dependent on protein kinase A, an endogenous maturation inhibitor, oocytes were microinjected with PKI, a specific PKA inhibitor, and exposed to 2,4-D. Oocytes failed to mature, suggesting that 2,4-D is not dependent on PKA activity and likely acts on a downstream target, such as Mos. De novo synthesis of Mos, which is triggered by mRNA poly(A) elongation, was examined. Oocytes were microinjected with radiolabelled in vitro transcripts of Mos RNA and exposed to progesterone and 2,4-D. RNA analysis showed progesterone-induced polyadenylation as expected but none with 2,4-D. 2,4-D-activated MAPK was determined to be cytoplasmic in localization studies but poorly induced Rsk2 phosphorylation and activation. In addition to inhibition of the G2/M transition, 2,4-D caused abrupt reduction of H1 kinase activity in MII phase oocytes. Attempts to rescue maturation in oocytes transiently exposed to 2,4-D failed, suggesting that 2,4-D induces irreversible dysfunction of the meiotic signaling mechanism.

http://www.ncbi.nlm.nih.gov/pubmed/17055699

Correia, F.; Moreira, J. 2010. Effects of glyphosate and 2,4-D on earthworms (*Eisenia foetida*) in laboratory tests. *Bull Environ Contam Toxicol*, 85(3):264-8. doi: 10.1007/s00128-010-0089-7.

Laboratory tests were conducted to compare the effects of various concentrations of glyphosate and 2,4-D on earthworms (Eisenia foetida) cultured in Argissol during 56 days of incubation. The effects on earthworm growth, survival, and reproduction rates were verified for different exposure times. Earthworms kept in glyphosate-treated soil were classified as alive in all evaluations, but showed gradual and significant reduction in mean weight (50%) at all test concentrations. For 2,4-D, 100% mortality was observed in soil treated with 500 and 1,000 mg/kg. At 14 days, 30%-40% mortality levels were observed in all other concentrations. No cocoons or juveniles were found in soil treated with either herbicide. Glyphosate and 2,4-D demonstrated severe effects on the development and reproduction of Eisenia foetida in laboratory tests in the range of test concentrations.

http://www.ncbi.nlm.nih.gov/pubmed/20658223

In case of 2,4-D-based herbicides (and also for glufosinate ammonium, event grown at a lower scale in Brazil) special attention must be given to the fact that such substances form microdrops with high drift potential[48]. In these situations, volatile substances circulate in the air and wind humidity, reaching long distances and "burning" the susceptible plants they may reach. It is expected that agricultural and wild species will be threatened on a dramatic way, with the predictable socioeconomic implications.

48 Negative impacts associated to potential pesticide drift have two main causes: Airborne drift (or drop) - when the product moves out from the target during spraying - and vapor drift - when the product moves out of the target after spraying - and feature a volatile product. Because of its commercial formulation, 2,4-D can be highly volatile, especially when including salts and esters. On its other formulations (acids, for example), most of the impacts caused by the drift is due to airborne drift, and may cause serious damage on neighboring crops. It should be noted that 2,4-D has different risks against the potential impact of drift mainly because of its high phytotoxicity even at extremely low doses.

Sciumbato, A.; Chandler, J.; Senseman, S.; Bovey, R.; Smith, K. 2004. Determining exposure to auxin-like herbicides. I. Quantifying injury to cotton and soybean. *Weed Technology*, 18: 1125–1134.

Volatility and drift are problems commonly associated with auxin-like herbicides. Field and greenhouse studies were conducted at Texas A & M University to develop a method of quantifying volatility and subsequent off-target movement of 2,4-D, dicamba, and triclopyr. Rate–response curves were established by applying reduced rates ranging from 4×10^{-1} to 1×10^{-5} times the normal use rates of the herbicides to cotton and soybean and recording injury for 14 d after treatment (DAT) using a rating scale designed to quantify auxin-like herbicide injury. Injury from herbicide volatility was then produced on additional cotton and soybean plants through exposure to vapors of the dimethylamine salt of 2,4-D, diglycolamine salt of dicamba, and butoxyethyl ester of triclopyr using air chambers inside a greenhouse and volatility plots in the field. Injury resulting from this exposure was evaluated for 14 d using the same injury-evaluation scale that was used to produce the rate–response curves. Volatility-injury data were then applied to the rate–response curves so that herbicide rates corresponding with observed injury could be calculated. Using this method, herbicide volatility rates estimated from greenhouse-cotton injury were determined to be 3.0×10^{-3}, 1.0×10^{-3}, and 4.9×10^{-2} times the use rates of 2,4-D, dicamba, and triclopyr, respectively. Greenhouse-grown soybean developed injury consistent with 1.4×10^{-2}, 1.0×10^{-3}, and 2.5×10^{-2} times the normal use rate of 2,4-D, dicamba, and triclopyr, respectively. Under field conditions, cotton developed injury symptoms that were consistent with 4.0×10^{-3}, 2.0×10^{-3}, and 1.25×10^{-1} times the recommended use rates of 2,4-D, dicamba, and triclopyr, respectively. Field soybean displayed injury symptomology concordant with 1.6×10^{-1}, 1.0×10^{-2}, and 1.1×10^{-1} times the normal use rates of 2,4-D, dicamba, and triclopyr, respectively. This procedure provided herbicide volatility rate estimates that were consistent with rates and injury from the rate–response injury curves. Additional research is needed to ascertain its usefulness in determining long-term effects of drift injury on crop variables such as yield.

http://www.bioone.org/doi/abs/10.1614/WT-03-105R1

2.2 Negative impacts of the HT plants crops on biodiversity

The ecotoxicity of the herbicides associated to the cultures of HT plants indicates that the environmental impacts of such crops are relevant. In addition to the direct toxicity, the changes caused by those products tend to affect trophic and ecosystemic chains (the suppression of great part of the vegetable biomass or the disappearance of vegetable species essential to perform part of the biological cycles of certain animal, insect or microrganisms species) and may generate negative impact on organisms which are not directly sensitive to those products.

In and agricultural system cultivated with HT technology, weeds that do not significantly decrease the productivity of the crop may be considered as non-target organisms (NTOs), as well as the

vegetable species that develop around the crops (sanctuariesand any environment located between the cultivated areas – species known for serving as refuges for the species threatened by conventional agricultural practices). The articles listed below examine such aspects, focusing on glyphosate-tolerant crops.

In addition, soil biota, and especially the fungi and bacteria communities, can be negatively affected by the use of herbicides linked to the HT varieties. This subject is also discussed in some of the references presented below. Other researches, available in item 3.2.1 of Parte 2, examine relationships between the the soil biota degradation in agroecosystems and the associated agricultural (and socioeconomic) problems.

2.2.1 Animals affected by the HT GM technologies

The articles below discuss impacts of the technology on animals, considered here as special cases of non-target organisms, also affected by the HT GM crops.

Watkinson, A.; Freckleton, R.; Robinson, R.; Sutherland, W. 2000. Predictions of biodiversity response to genetically modified herbicide-tolerant crops. *Science*, 289:1554-1557.

We simulated the effects of the introduction of genetically modified herbicide-tolerant (GMHT) crops on weed populations and the consequences for seed-eating birds. We predict that weed populations might be reduced to low levels or practically eradicated, depending on the exact form of management. Consequent effects on the local use of fields by birds might be severe, because such reductions represent a major loss of food resources. The regional impacts of GMHT crops are shown to depend on whether the adoption of GMHT crops by farmers covaries with current weed levels.

http://www.sciencemag.org/content/289/5484/1554

Hawes, C.; Haughton, A.; Osborne, J.; Roy, D.; Clark, S.; Perry, J.; Rothery, P.; Bohan, D.; Brooks, D.; Champion, G.; Dewar, A.; Heard, M.; Woiwod, I.; Daniels, R.; Yound, M.; Parish, A.; Scott, R.; Firbank, L.; Squire, G. 2003. Responses of plants and invertebrate trophic groups to contrasting herbicide regimes in the farm scale evaluations of genetically modified herbicide-tolerant crops. *Philosophical Transactions of the Royal Society of London*, B 358, 1899-1913.

Effects of genetically modified herbicide-tolerant (GMHT) and conventional crop management on invertebrate trophic groups (herbivores, detritivores, pollinators, predators and parasitoids) were compared in beet, maize and spring oilseed rape sites throughout the UK. These trophic groups

were influenced by season, crop species and GMHT management. Many groups increased twofold to fivefold in abundance between early and late summer, and differed up to 10-fold between crop species. GMHT management superimposed relatively small (less than twofold), but consistent, shifts in plant and insect abundance, the extent and direction of these effects being dependent on the relative efficacies of comparable conventional herbicide regimes. In general, the biomass of weeds was reduced under GMHT management in beet and spring oilseed rape and increased in maize compared with conventional treatments. This change in resource availability had knock-on effects on higher trophic levels except in spring oilseed rape where herbivore resource was greatest. Herbivores, pollinators and natural enemies changed in abundance in the same directions as their resources, and detritivores increased in abundance under GMHT management across all crops. The result of the later herbicide application in GMHT treatments was a shift in resource from the herbivore food web to the detritivore food web. The Farm Scale Evaluations have demonstrated over 3 years and throughout the UK that herbivores, detritivores and many of their predators and parasitoids in arable systems are sensitive to the changes in weed communities that result from the introduction of new herbicide regimes.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1693274/pdf/14561321.pdf.

Brooks, D.; Bohan, D.; Champion, G.; Haughton, A.; Hawes, C.; Heard, M.; Clark, S.; Dewar, A.; Firbank, L.; Perry, J.; Rothery, P.; Scott, R.; Woiwod, I.; Birchall, C.; Skellern, M.; Walker, J.; Baker, P.; Bell, D.; Browne, E.; Dewar, A.; Fairfax, C.; Garner, B.; Haylock, L.; Horne, S.; Hulmes, S.; Mason, N.; Norton, L.; Nuttall, P.; Randle, Z.; Rossall, M.; Sands, R.; Singer, E.; Walker, M. 2003. Invertebrate responses to the management of genetically modified herbicide-tolerant and conventional spring crops. I. Soil-surface-active invertebrates. *Philosophical Transactions of the Royal Society of London*, B 358, 1847-1862.

The effects of herbicide management of genetically modified herbicide-tolerant (GMHT) beet, maize and spring oilseed rape on the abundance and diversity of soil-surface-active invertebrates were assessed. Most effects did not differ between years, environmental zones or initial seedbanks or between sugar and fodder beet. This suggests that the results may be treated as generally applicable to agricultural situations throughout the UK for these crops. The direction of the effects was evenly balanced between increases and decreases in counts in the GMHT compared with the conventional treatment. Most effects involving a greater capture in the GMHT treatments occurred in maize, whereas most effects involving a smaller capture were in beet and spring oilseed rape. Differences between GMHT and conventional crop herbicide management had a significant effect on the capture of most surface-active invertebrate species and higher taxa tested in at least one crop, and these differences reflected the phenology and ecology of the invertebrates. Counts of carabids that feed on weed seeds were smaller in GMHT beet and spring oilseed rape but larger in GMHT maize. In contrast, collembolan detritivore counts were significantly larger under GMHT crop management.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1693272/pdf/14561318.pdf

Heard, M.; Hawes, C.; Champion,G.; Clark, S.; Firbank, L.; Haughton, A.; Parish, A.; Perry, J.; Rothery, P.; Scott, R.; Skellern, M.; Squire, G.; Hill, M. 2003. Weeds in fields with contrasting conventional and genetically modified herbicide-tolerant crops. I. Effects on abundance and diversity. *Philos Trans R Soc Lond B Biol Sci*, 29;358 (1439):1819-32.

We compared the seedbanks, seed rains, plant densities and biomasses of weeds under two contrasting systems of management in beet, maize and spring oilseed rape. Weed seedbank and plant density were measured at the same locations in two subsequent seasons. About 60 fields were sown with each crop. Each field was split, one half being sown with a conventional variety managed according to the farmer's normal practice, the other half being sown with a genetically modified herbicide-tolerant (GMHT) variety, with weeds controlled by a broad-spectrum herbicide. In beet

and rape, plant densities shortly after sowing were higher in the GMHT treatment. Following weed control in conventional beet, plant densities were approximately one-fifth of those in GMHT beet. In both beet and rape, this effect was reversed after the first application of broad-spectrum herbicide, so that late-season plant densities were lower in the GMHT treatments. Biomass and seed rain in GMHT crops were between one-third and one-sixth of those in conventional treatments. The effects of differing weed-seed returns in these two crops persisted in the seedbank: densities following the GMHT treatment were about 20% lower than those following the conventional treatment. The effect of growing maize was quite different. Weed density was higher throughout the season in the GMHT treatment. Late-season biomass was 82% higher and seed rain was 87% higher than in the conventional treatment. The difference was not subsequently detectable in the seedbank because the total seed return was low after both treatments. In all three crops, weed diversity was little affected by the treatment, except for transient effects immediately following herbicide application.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1693279/

Bohan, D.; Boffey, C.; Brooks, D.; Clark, S.; Dewar, A.; Firbank, L.; Haughton, A.; Hawes, C.; Heard, M.; May, M.; Osborne, J.; Perry, J.; Rothery, P.; Roy, D.; Scott, R.; Squire, G.; Woiwod, I.; Champion, G. 2005. Effects on weed and invertebrate abundance and diversity of herbicide management in genetically modified herbicide-tolerant winter-sown oilseed rape. *Proc Biol Sci*, B Vol. 272 (1562): 463-474.

We evaluated the effects of the herbicide management associated with genetically modified herbicide-tolerant (GMHT) winter oilseed rape (WOSR) on weed and invertebrate abundance and diversity by testing the null hypotheses that there is no difference between the effects of herbicide management of GMHT WOSR and that of comparable conventional varieties. For total weeds, there were few treatment differences between GMHT and conventional cropping, but large and opposite treatment effects were observed for dicots and monocots. In the GMHT treatment, there were fewer dicots and monocots than in conventional crops. At harvest, dicot biomass and seed rain in the GMHT treatment were one-third of that in the conventional, while monocot biomass was threefold greater and monocot seed rain almost fivefold greater in the GMHT treatment than in the conventional. These differential effects persisted into the following two years of the rotation. Bees and Butterflies that forage and select for dicot weeds were less abundant in GMHT WORS management in July. Year totals for Collembola were greater under GMHT management. There were few other treatment effects on invertebrates, despite the marked effects of herbicide management on the weeds.

http://www.ncbi.nlm.nih.gov/pubmed/15799941

Gibbons, D.; Bohan, D.; Rothery, P.; Stuart, R.; Haughton, A.; Scott, R.; Wilson, J.; Perry, J.; Clark, S.; Dawson, R.; Firbank, L. 2006. Weed seed resources for birds in fields with contrasting conventional and genetically modified herbicide-tolerant crops. *Proc Biol Sci*, 7;273 (1596):1921-8.

The UK Farm Scale Evaluations (FSEs) have shown that the use of broad spectrum herbicides on genetically modified herbicide-tolerant (GMHT) crops can have dramatic effects on weed seed production compared to management of conventional varieties. Here, we use FSE data and information on bird diets to determine how GMHT cropping might change the food resources available to farmland birds. More than 60 fields of each of four crops, spring- and winter-sown oilseed rape, beet and maize, were split, one half being sown with a conventional variety, the other with a GMHT variety. Seed rain from weeds known to be important in the diets of 17 granivorous farmland bird species was measured under the two treatments. In beet and spring oilseed rape, rain of weed seeds important in the diets of 16 bird species was significantly reduced in GMHT compared to conventional halves; for no species did it increase. In winter oilseed

rape, rain of weed seeds important in the diets of 10 species was significantly reduced in GMHT halves; for only one species did it increase significantly. By contrast, in maize, rain of weed seeds important in the diets of seven species was significantly greater in GMHT halves; for no species was it reduced. Treatment effects for the total weed seed energy available to each bird species were very similar to those for seed rain alone. Measuring the effects on individual bird species was outside the scope of this study. Despite this, these results suggest that should beet, spring and winter rape crops in the UK be largely replaced by GMHT varieties and managed as in the FSEs, this would markedly reduce important food resources for farmland birds, many of which declined during the last quarter of the twentieth century. By contrast, GMHT maize would be beneficial to farmland birds.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1634768/

MOD - Brower, L.; Taylor, O.; Williams, E.; Slayback, D.; Zubieta, R.; Ramirez, M. 2012. Decline of monarch butterflies overwintering in Mexico: is the migratory phenomenon at risk? *Insect Conservation and Diversity*, Vol. 5(2): 95-100.

1. During the 2009–2010 overwintering season and following a 15-year downward trend, the total area in Mexico occupied by the eastern North American population of overwintering monarch butterflies reached an all-time low. Despite an increase, it remained low in 2010–2011.
2. Although the data set is small, the decline in abundance is statistically significant using both linear and exponential regression models.
3. Three factors appear to have contributed to reduce monarch abundance: degradation of the forest in the overwintering areas; the loss of breeding habitat in the United States due to the expansion of GM herbicide-resistant crops, with consequent loss of milkweed host plants, as well as continued land development; and severe weather.
4. This decline calls into question the long-term survival of the monarchs' migratory phenomenon.

Full article available at http://onlinelibrary.wiley.com/doi/10.1111/j.1752-4598.2012.00196.x/abstract

Pleasants, J.; Oberhauser, K. 2013. Milkweed loss in agricultural fields because of herbicide use: effect on the monarch butterfly population. *Insect Conservation and Diversity*, Vol. 6(2): 135-144.

1. The size of the Mexican overwintering population of monarch butterflies has decreased over the last decade. Approximately half of these butterflies come from the U.S. Midwest where larvae feed on common milkweed. There has been a large decline in milkweed in agricultural fields in the Midwest over the last decade. This loss is coincident with the increased use of glyphosate herbicide in conjunction with increased planting of genetically modified (GM) glyphosate-tolerant corn (maize) and soybeans (soya).
2. We investigate whether the decline in the size of the overwintering population can be attributed to a decline in monarch production owing to a loss of milkweeds in agricultural fields in the Midwest. We estimate Midwest annual monarch production using data on the number of monarch eggs per milkweed plant for milkweeds in different habitats, the density of milkweeds in different habitats, and the area occupied by those habitats on the landscape.
3. We estimate that there has been a 58% decline in milkweeds on the Midwest landscape and an 81% decline in monarch production in the Midwest from 1999 to 2010. Monarch production in the Midwest each year was positively correlated with the size of the subsequent overwintering population in Mexico. Taken together, these results strongly suggest that a loss of agricultural milkweeds is a major contributor to the decline in the monarch population.
4. The smaller monarch population size that has become the norm will make the species more vulnerable to other conservation threats.

http://onlinelibrary.wiley.com/doi/10.1111/j.1752-4598.2012.00196.x/abstract

Zaller, J.; Heigl, F.; Ruess, L.; Grabmaier, A. 2014. Glyphosate herbicide affects belowground interactions between earthworms and symbiotic mycorrhizal fungi in a model ecosystem. *Scientific Reports*, 9;4:5634.

Herbicides containing glyphosate are widely used in agriculture and private gardens, however, surprisingly little is known on potential side effects on non-target soil organisms. In a greenhouse experiment with white clover we investigated, to what extent a globally-used glyphosate herbicide affects interactions between essential soil organisms such as earthworms and arbuscular mycorrhizal fungi (AMF). We found that herbicides significantly decreased root mycorrhization, soil AMF spore biomass, vesicles and propagules. Herbicide application and earthworms increased soil hyphal biomass and tended to reduce soil water infiltration after a simulated heavy rainfall. Herbicide application in interaction with AMF led to slightly heavier but less active earthworms. Leaching of glyphosate after a simulated rainfall was substantial and altered by earthworms and AMF. These sizeable changes provide impetus for more general attention to side-effects of glyphosate-based herbicides on key soil organisms and their associated ecosystem services.

Full article available at http://www.nature.com/srep/2014/140709/srep05634/pdf/srep05634.pdf

2.2.2 Vegetables (non-target) affected by the HT GM technologies

The articles below analyze impacts of the TH GM crops on non-target vegetables.

REV - Pengue, W. 2005. Transgenic Crops in Argentina: The Ecological and Social Debt. *Bulletin of Science, Technology & Society*, Vol. 25, No. 4, 314-322.

There is no doubt that soybean is the most important crop for Argentina, with a planted surface that rose 11,000,000 hectares and a production of around 35,000,000 metric tons. During the 1990s, there was a significant agriculture transformation in the country, motorize by the adoption of transgenic crops (soy-bean, maize, and cotton) under the no-tillage system. The expansion of this model has been spread not only in the Pampas but also in very rich areas with high biodiversity, opening a new agricultural border to important eco-regions like the Yungas, Great Chaco, and the Mesopotamian Forest. Transgenic cropping is a powerful technology. This produced relevant transformations over the environment and society where it is allowed. Migration, concentration of agribusiness, and loss of food sovereignty are some of the social results. Landscape transformation in the rural sector is evident, and the appearance of tolerance weeds to glyphosate is a reality. Nutrient depletion, soil-structure degradation, potential desertification, and loss of species are other consequences on the environmental level.

http://bst.sagepub.com/content/25/4/314.abstract

Strandberg, B.; Pedersen, M.; Elmegaard, N. 2005. Weed and arthropod populations in conventional and genetically modified herbicide tolerant fodder beet fields. *Agriculture, Ecosystems and Environment*, 105, 243–253.

The introduction of genetically modified herbicide tolerant (GMHT) crops has raised concerns from both scientists and non-governmental organisations about possible effects on arable flora and fauna due to the changes in herbicide application and management that such crops involve. Three

consecutive studies were performed, covering flora and fauna in fields of GMHT and conventional fodder beets over the season, at different locations and under different spraying regimes. At all locations and in the 3 years, a denser and more diverse weed flora and arthropod fauna were found in GMHT beets in early and mid-summer than in conventional beets when glyphosate-treatment occurred at or after label recommendation. Following the herbicide applications the GMHT fields had fewer weed species and seeds and lower weed densities and biomass than conventional fields. However, application of glyphosate earlier than recommended resulted in an extremely low weed diversity, density and biomass during the entire season. Timing of the first glyphosate applications, i.e. the duration of the herbicide free period, was essential in terms of biodiversity improvements. In the long term reduced production of weed seeds in GMHT fields may deplete the weed flora if the GMHT strategy becomes widely adopted.

http://www.sciencedirect.com/science/article/pii/S0167880904001288

Firbank, L.; Rothery, P.; May, M.; Clark, S.; Scott, R.; Stuart, R.; Boffey, C.; Brooks, D.; Champion, G.; Haughton, A.; Hawes, C; Heard, M.; Dewar, A.; Perry, J.; Squire, G. 2006. Effects of genetically modified herbicide-tolerant cropping systems on weed seedbanks in two years of following crops. *Biol Lett*, 2(1): 140–143. doi: 10.1098/rsbl.2005.0390.

The Farm Scale Evaluations (FSEs) showed that genetically modified herbicide-tolerant (GMHT) cropping systems could influence farmland biodiversity because of their effects on weed biomass and seed production. Recently published results for winter oilseed rape showed that a switch to GMHT crops significantly affected weed seedbanks for at least 2 years after the crops were sown, potentially causing longer-term effects on other taxa. Here, we seek evidence for similar medium-term effects on weed seedbanks following spring-sown GMHT crops, using newly available data from the FSEs. Weed seedbanks following GMHT maize were significantly higher than following conventional varieties for both the first and second years, while by contrast, seedbanks following GMHT spring oilseed rape were significantly lower over this period. Seedbanks following GMHT beet were smaller than following conventional crops in the first year after the crops had been sown, but this difference was much reduced by the second year for reasons that are not clear. These new data provide important empirical evidence for longer-term effects of GMHT cropping on farmland biodiversity.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1617187/

Altieri, Miguel. 2009. The ecological impacts of large-scale agrofuel monoculture production systems in the Americas. *Bulletin of Science Technology Society*, vol. 29 no. 3 236-244.

This article examines the expansion of agrofuels in the Americas and the ecological impacts associated with the technologies used in the production of large-scale monocultures of corn and soybeans. In addition to deforestation and displacement of lands devoted to food crops due to expansion of agrofuels, the massive use of transgenic crops and agrochemical inputs, mainly fertilizers and herbicides used in the production of agrofuels, pose grave environmental problems.

http://bst.sagepub.com/content/29/3/236.abstract

REV - Mortensen, D.; Egan, J.; Maxwell, D.; Ryan, M.; Smith, R. 2012. Navigating a critical juncture for sustainable weed management. *BioScience*, Vol. 62, No. 1, pp. 75-84.

Agricultural weed management has become entrenched in a single tactic—herbicide-resistant crops—and needs greater emphasis on integrated practices that are sustainable over the long term.

In response to the outbreak of glyphosate-resistant weeds, the seed and agrichemical industries are developing crops that are genetically modified to have combined resistance to glyphosate and synthetic auxin herbicides. This technology will allow these herbicides to be used over vastly expanded areas and will likely create three interrelated challenges for sustainable weed management. First, crops with stacked herbicide resistance are likely to increase the severity of resistant weeds. Second, these crops will facilitate a significant increase in herbicide use, with potential negative consequences for environmental quality. Finally, the short-term fix provided by the new traits will encourage continued neglect of public research and extension in integrated weed management. Here, we discuss the risks to sustainable agriculture from the new resistant crops and present alternatives for research and policy.

http://bioscience.oxfordjournals.org/content/62/1/75.short

Druille, M.; Cabello, M.; Omacini, M.; Golluscio, R. 2013. Glyphosate reduces spore viability and root colonization of arbuscular mycorrhizal fungi. *Applied Soil Ecology*, 64: 99–103.

Glyphosate is the most widely used herbicide in the world, but its effects on non-target organisms, such as arbuscular mycorrhizal fungi (AMF), are unclear. No studies have been found that made reference to effects of glyphosate on AMF spore viability despite its importance as a source of propagules for the perpetuation and spread of AMF in the system. The objective of this study was to evaluate the effect of glyphosate application on AMF spore viability, and their ability to colonize roots. Soil samples were collected from a grassland area located in the Flooding Pampa region (Argentina). We evaluated three herbicide rates: 0, 0.26 and 1× recommended field rate, 10 and 30 days after application. Part of the soil from each tray was used to estimate the spore viability, and the remainder was used as substrate for growing *Lolium multiflorum* Lam. One month after sowing, total root colonization and percentage of arbuscules and vesicles were determined. The spore viability in herbicide untreated soils was between 5.8- and 7.7-fold higher than in treated soils. This reduction was detected even when the lower rate was applied. Root colonization was significantly lower in plants grown in glyphosate treated soil than in untreated ones. A decrease in arbuscular colonization (but not in vesicles) was found in plants grown in soils treated with the highest herbicide rate. That would indicate that symbiosis functionality was affected, given that arbuscules are the main site for host–fungus nutrient exchange. The results indicate that soil residence time of glyphosate and/or its degradation products was enough to reduce AMF spore viability and their ability to colonize roots. This decrease in propagules viability may affect plant diversity, taking into account the different degrees of mycorrhizal dependency between plant species that may coexist in grassland communities.

http://www.sciencedirect.com/science/article/pii/S0929139312002466

Druille, M.; Omacini, M.; Golluscio, R.; Cabello, M. 2013. Arbuscular mycorrhizal fungi are directly and indirectly affected by glyphosate application. *Applied Soil Ecology*, Volume 72, Pages 143–149.

Glyphosate is a systemic non-selective herbicide, the most widely used in the world. Alongside with its use in agricultural and forestry systems, this herbicide is used in grasslands in late summer with the aim of promoting winter species with the consequent increase in stocking rate. However, its effects on non-target organisms, such as arbuscular mycorrhizal fungi (AMF), are unclear. Arbuscular mycorrhizal fungi (AMF) colonize the root of more than 80% of terrestrial plants, improving their growth and survival, and therefore playing a key role in ecosystem structure and function. The aim of this work was to investigate the possible pathways through which glyphosate application affects AMF spores viability and root colonization in grassland communities. Our hypothesis is that glyphosate application can damage AMF directly (through contact with spores and external hyphae) or indirectly

through the changes it generates on host plants. The experiment had a factorial array with three factors: (1) plant species, at two levels (*Paspalum dilatatum* and*Lotus tenuis*), (2) doses of glyphosate, at three levels (0 l ha^{-1}, 0.8 l ha^{-1} and 3 l ha^{-1}), and (3) application site, at two levels: soil (direct pathway) and plant foliage (indirect pathway). Spore viability was reduced even under the lowest glyphosate rate, but only when it was applied on the soil. Total root colonization for both species was similarly decreased when glyphosate was applied to plant foliage or on soil, with no difference between 0.8 and 3 l ha^{-1}. The number of arbuscules was 20% lower when glyphosate was applied on plant foliage, than when it was applied on the soil. Our findings illustrate that glyphosate application negatively affects AMF functionality in grasslands, due to different causes depending on the herbicide application site. While, under field conditions, the occurrence of direct and/or indirect pathways will depend on the plant cover at the time of glyphosate application, the consequences of this practice on the plant community structure will vary with the mycorrhizal dependence of the species composition regardless of the pathway involved.

http://www.sciencedirect.com/science/article/pii/S0929139313001868

3 Risks of transgenic dissemination/ contamination of non-agricultural speciest[49]

Gene flow represents one of the main routes of contamination of biodiversity with transgenes. The introgression of these transgenes in populations of wild and semi-wild plants (feral populations) may confer adaptative advantage for certain species – which will result in ecological imbalances in the trophic webs where such species are inserted – or, on the contrary, may confer some adaptative disadvantage, weakening these population in certain environments.

When analyzing the ecological consequences of a potential transfer of genes (and transgenes) – through sexual reproduction or horizontal gene transfer –, the character transferred (phenotype) to the receiving species should be interpreted as a key aspect of the risk assessment. With this respect, genes responsible for the synthesis of insecticidal toxins and other biocidal molecules, for resistance to antibiotics, or for some gain/reduction of competitiveness (major or minor resistance to hydric or salt stress, for example), will not be ecologically neutral. Therefore, it may cause disturbances in web connections among the species involved, which deserve to be properly analyzed.

49 References on risks associated with the spread of transgenes in agricultural species are available on item 4 of Part 2.

Finally, many risk scenarios may occur when considering the environment as a potential transgene tank-distributor. For example, the possibility that drug molecules may be found in food chains, by spreading transgenes escaped from biopharmaceutical GM plants (via cross-fertilization with related wild and/or feral plants, or via Horizontal Gene Transfer - HGT - in soil bacteria), represents serious and irreversible risks, well beyond the potential ecological disorders mentioned above.

3.1 Gene flow through pollination[50]

It is worth reminding that hybridization, with or without gene introgression, between native and species manipulated by men, threatens food safety and biodiversity conservation. This subject is discussed in the articles below.

Rhymer, J.; Simberloff, D. 1996. Extinction by hybridization and introgression. *Annual Review of Ecology and Systeatics*, 27:83–109.

Nonindigenous species can bring about a form of extinction of native flora and fauna by hybridization and introgression either through purposeful introduction by humans or through habitat modification, bringing previously isolated species into contact. These phenomena can be especially problematic for rare species coming into contact with more abundant ones. Increased use of molecular techniques focuses attention on the extent of this underappreciated problem that is not always apparent from morphological observations alone. Some degree of gene flow is a normal, evolutionarily constructive process, and all constellations of genes and genotypes cannot be preserved. However, hybridization with or without introgression may, nevertheless, threaten a rare species' existence.

http://www.annualreviews.org/doi/abs/10.1146/annurev.ecolsys.27.1.83?journalCode=ecolsys.1

Allendorf, F.; Leary, R.; Spruell, P.; Wenburg, J. 2001. The problems with hybrids: setting conservation guidelines. *Trends in Ecology & Evolution*, Vol.16 No.11.

Rates of hybridization and introgression are increasing dramatically worldwide because of translocations of organisms and habitat modifications by humans. Hybridization has contributed to the extinction of many species through direct and indirect means. However, recent studies have found that natural hybridization has played an important role in the evolution of many plant and animal taxa. Determining whether hybridization is natural or anthropogenic is crucial for conservation, but is often difficult to achieve. Controversy has surrounded the setting of appropriate conservation policies to deal with hybridization and introgression. Any policy that

50 In Part 2 item 4 there are other references about gene flow between GM and non-GM related species.

deals with hybrids must be flexible and must recognize that nearly every situation involving hybridization is different enough that general rules are not likely to be effective. We provide a categorization of hybridization to help guide management decisions

https://goo.gl/VVV62N

Campbell, L.; Allison, A.; Ridley, C. 2006. Weed evolution after crop gene introgression: greater survival and fecundity of hybrids in a new environment. *Ecology Letters*, Volume 9, Issue 11, Pages: 1198–1209.

Crop-wild hybridization may produce offspring with lower fitness than their wild parents due to deleterious crop traits and outbreeding depression. Over time, however, selection for improved fitness could lead to greater invasiveness of hybrid taxa. To examine evolutionary change in crop-wild hybrids, we established four wild (Raphanus raphanistrum) and four hybrid radish populations (R. raphanistrum x Raphanus sativus) in Michigan (MI), USA. Hybrid and wild populations had similar growth rates over four generations, and pollen fertility of hybrids improved. We then measured hybrid and wild fitness components in two common garden sites within the geographical range of wild radish [MI and California (CA)]. Advanced generation hybrids had slightly lower lifetime fecundity than wild plants in MI but exhibited c. 270% greater lifetime fecundity and c. 22% greater survival than wild plants in CA. Our results support the hypothesis that crop-wild hybridization may create genotypes with the potential to displace parental taxa in new environments.

http://www.ncbi.nlm.nih.gov/pubmed/17040322

Fitzpatrick, B.; Johnson, J.; Kump, D.; Smith, J.; Voss, S.; Shaffer, H. 2010. Rapid spread of invasive genes into a threatened native species. *PNAS*, vol. 107, no. 8, 3606–3610.

When introduced or cultivated plants or animals hybridize with their native relatives, the spread of invasive genes into native populations might have biological, aesthetic, and legal implications. Models suggest that the rate of displacement of native by invasive alleles can be rapid and inevitable if they are favored by natural selection. We document the spread of a few introduced genes 90 km into a threatened native species (the California Tiger Salamander) in 60 years. Meanwhile, a majority of genetic markers (65 of 68) show little evidence of spread beyond the region where introductions occurred. Using computer simulations, we found that such a pattern is unlikely to emerge by chance among selectively neutral markers. Therefore, our results imply that natural selection has favored both the movement and fixation of these exceptional invasive alleles. The legal status of introgressed populations (native populations that are slightly genetically modified) is unresolved by the US Endangered Species Act. Our results illustrate that genetic and ecological factors need to be carefully weighed when considering different criteria for protection, because different rules could result in dramatically different geographic areas and numbers of individuals being protected.

Full article available at http://www.pnas.org/content/107/8/3606.full

Social-environmental damages may be higher when gene flow occurs from GM plants to species, related or not, from the local agrobiodiversity.

Gepts, P.; Papa, R. 2003. Possible effects of (trans)gene flow from crops on the genetic diversity from landraces and wild relatives. *Environ Biosafety Res*, (2): 89–103.

Gene flow is a potential concern associated with the use of transgenic crops because it could affect genetic diversity of related landraces and wild relatives. This concern has taken on added importance with the looming introduction of transgenic crops in centers of crop domestication (Mexico, China) and those producing pharmaceutical compounds. For gene flow to take place among cultivars and their wild relatives, several steps have to be fulfilled, including the presence of cultivars or wild relatives within pollen or seed dispersal range, the ability to produce viable and fertile hybrids, at least partial overlap in flowering time, actual gene flow by pollen or seed, and the establishment of crop genes in the domesticated or wild recipient populations. In contrast with domestication genes, which often make crops less adapted to natural ecosystems, transgenes frequently represent gains of function, which might release wild relatives from constraints that limit their fitness. In most sexually reproducing organisms, the chromosomal region affected by selection of a single gene amounts to a small percentage of the total genome size. Because of gene flow, the level of genetic diversity present in the domesticated gene pool becomes a crucial factor affecting the genetic diversity of the wild gene pool. For some crops, such as cotton and maize, the introduction of transgenic technologies has led to a consolidation of the seed industry and a reduction in the diversity of the elite crop gene pool. Thus, diversity in improved varieties grown by farmers needs to be monitored. Several areas deserve further study, such as the actual magnitude of gene flow and its determinants in different agroecosystems, the long-term effects of gene flow on genetic diversity both across gene pools and within genomes, the expression of transgenes in new genetic backgrounds, and the effects of socio-economic factors on genetic diversity.

http://www.ncbi.nlm.nih.gov/pubmed/15612275

3.2 Ecological imbalances with dissemination of transgenes in wild species[51]

Once a transgenic plant is cultivated in the field, in a commercial or experimental scale, there will be no way or possibility of preventing the escape of transgenes into the environment. Some cases of successful transfer of transgenes to populations of feral species considered weeds were presented in item 3.3 of Part 2.

The articles below show that there may be the introgression of transgenes in genetically related wild species (which have not undergone domestication process), generating the risk of ecological imbalances of different types of natural and semi-natural environments. The potential invasion of these "new species" should become one of the largest concerns of the risk analysis about this subject.

51 It should be noted that the risks associated with the invasiveness potential of species that suffered transgene introgression are larger (and more easily identified) in animal populations. The example of transgenic salmon with higher growth rate leaves no doubt about the negative character of the ecological impacts of a potential escape of such individuals / transgenes on native populations of fish and other aquatic organisms.

REV - Ellstrand, N.; Schierenbeck, K. 2000. Hybridization as a stimulus for the evolution of invasiveness in plants? *PNAS*, vol. 97, no. 13, 7043–7050.

Invasive species are of great interest to evolutionary biologists and ecologists because they represent historical examples of dramatic evolutionary and ecological change. Likewise, they are increasingly important economically and environmentally as pests. Obtaining generalizations about the tiny fraction of immigrant taxa that become successful invaders has been frustrated by two enigmatic phenomena. Many of those species that become successful only do so (*i*) after an unusually long lag time after initial arrival, and/or (*ii*) after multiple introductions. We propose an evolutionary mechanism that may account for these observations. Hybridization between species or between disparate source populations may serve as a stimulus for the evolution of invasiveness. We present and review a remarkable number of cases in which hybridization preceded the emergence of successful invasive populations. Progeny with a history of hybridization may enjoy one or more potential genetic benefits relative to their progenitors. The observed lag times and multiple introductions that seem a prerequisite for certain species to evolve invasiveness may be a correlate of the time necessary for previously isolated populations to come into contact and for hybridization to occur. Our examples demonstrate that invasiveness can evolve. Our model does not represent the *only* evolutionary pathway to invasiveness, but is clearly an underappreciated mechanism worthy of more consideration in explaining the evolution of invasiveness in plants.

Full article available at http://www.pnas.org/content/97/13/7043.full

Warwick, S.; Simard, M.; Légère, A.; Beckie, H.; Braun, L.; Zhu, B.; Mason, P.; Séguin-Swartz, G.; Stewart, C. 2003. Hybridization between transgenic Brassica napus L. and its wild relatives: *Brassica rapa L.*, *Raphanus raphanistrum L.*, *Sinapis arvensis L.*, and *Erucastrum gallicum* (Willd.) O.E. Schulz. *Theor Appl Genet*, 107(3):528-39.

The frequency of gene flow from Brassica napus L. (canola) to four wild relatives, Brassica rapa L., Raphanus raphanistrum L., Sinapis arvensis L. and Erucastrum gallicum (Willd.) O.E. Schulz, was assessed in greenhouse and/or field experiments, and actual rates measured in commercial fields in Canada. Various marker systems were used to detect hybrid individuals: herbicide resistance traits (HR), green fluorescent protein marker (GFP), species-specific amplified fragment length polymorphisms (AFLPs) and ploidy level. Hybridization between B. rapa and B. napus occurred in two field experiments (frequency approximately 7%) and in wild populations in commercial fields (approximately 13.6%). The higher frequency in commercial fields was most likely due to greater distance between B. rapa plants. All F(1) hybrids were morphologically similar to B. rapa, had B. napus- and B. rapa-specific AFLP markers and were triploid (AAC, 2n=29 chromosomes). They had reduced pollen viability (about 55%) and segregated for both self-incompatible and self-compatible individuals (the latter being a B. napus trait). In contrast, gene flow between R. raphanistrum and B. napus was very rare. A single R. raphanistrum x B. napus F1 hybrid was detected in 32,821 seedlings from the HR B. napus field experiment. The hybrid was morphologically similar to R. raphanistrum except for the presence of valves, a B. napus trait, in the distorted seed pods. It had a genomic structure consistent with the fusion of an unreduced gamete of R. raphanistrum and a reduced gamete of B. napus (RrRrAC, 2n=37), both B. napus- and R. raphanistrum-specific AFLP markers, and had <1% pollen viability. No hybrids were detected in the greenhouse experiments (1,534 seedlings), the GFP field experiment (4,059 seedlings) or in commercial fields in Québec and Alberta (22,114 seedlings). No S. arvensis or E. gallicum x B. napus hybrids were detected (42,828 and 21,841 seedlings, respectively) from commercial fields in Saskatchewan. These findings suggest that the probability of gene flow from transgenic B. napus to R. raphanistrum, S. arvensis or E. gallicum is very low (<2-5 x 10(-5)). However, transgenes can disperse in the environment via wild B. rapa in eastern Canada and possibly via commercial B. rapa volunteers in western Canada.

http://www.ncbi.nlm.nih.gov/pubmed/12721639

REV - Ellstrand, N. 2003. Current knowledge of gene flow in plants: implications for transgene flow. *Phil Trans R Soc Lond*, B, 358, 1163-1170.

Plant evolutionary biologists' view of gene flow and hybridization has undergone a revolution. Twenty-five years ago, both were considered rare and largely inconsequential. Now gene flow and hybridization are known to be idiosyncratic, varying with the specific populations involved. Gene flow typically occurs at evolutionarily significant rates and at significant distances. Spontaneous hybridization occasionally has important applied consequences, such as stimulating the evolution of more aggressive invasives and increasing the extinction risk for rare species. The same problems have occurred for spontaneous hybridization between crops and their wild relatives. These new data have implications for transgenic crops: (i) for most crops, gene flow can act to introduce engineered genes into wild populations; (ii) depending on the specific engineered gene(s) and populations involved, gene flow may have the same negative impacts as those observed for traditionally improved crops; (iii) gene flow's idiosyncratic nature may frustrate management and monitoring attempts; and (iv) intercrop transgene flow, although rarely discussed, is equally worthy of study.

Full article available at https://goo.gl/l6kQJZ

Vacher, C.; Weis, A.; Hermann, D.; Kossler, T.; Young, C.; Hochbert, M. 2004. Impact of ecological factors on the initial invasion of Bt transgenes into wild populations of birdseed rape (*Brassica rapa*). *Theor Appl Genet*, 109 (4): 806-14.

The inevitable escape of transgenic pollen from cultivated fields will lead to the emergence of transgenic crop-wild plant hybrids in natural patches of wild plants. The fate of these hybrids and that of the transgene depend on their ability to compete with their wild relatives. Here we study ecological factors that may enhance the fitness of genetically modified hybrids relative to wild plants for a Bacillus thuringiensis (Bt) transgene conferring resistance to insects. Mixed stands of wild plants and first-generation hybrids were grown under different conditions of herbivore pressure and density, with Bt oilseed rape (Brassica napus) as the crop and B. rapa as the wild recipient. Biomass and fitness components were measured from plant germination to the germination of their offspring. The frequency of transgenic seedlings in the offspring generation was estimated using the green fluorescent protein marker. The biomass of F(1) Bt-transgenic hybrids relative to that of wild-type plants was found to be sensitive to both plant density and herbivore pressure, but herbivore pressure appeared as the major factor enhancing their relative fitnesses. In the absence of herbivore pressure, Bt hybrids produced 6.2-fold fewer seeds than their wild neighbors, and Bt plant frequency fell from 50% to 16% within a single generation. Under high herbivore pressure, Bt hybrids produced 1.4-fold more seeds, and Bt plant frequency was 42% in the offspring generation. We conclude that high-density patches of highly damaged wild plants are the most vulnerable to Bt-transgene invasion. They should be monitored early to detect potential transgene spread.

http://www.ncbi.nlm.nih.gov/pubmed/15340690

REV - Chapman, M.; Burke, J. 2006. Letting the gene out of the bottle: the population genetics of genetically modified crops. *New Phytologist*, 170, 429–443.

Genetically modified (GM) plants are rapidly becoming a common feature of modern agriculture. This transition to engineered crops has been driven by a variety of potential benefits, both economic and ecological. The increase in the use of GM crops has, however, been accompanied by growing concerns regarding their potential impact on the environment. Here, we focus on the escape of transgenes from cultivation via crop x wild hybridization. We begin by reviewing the literature on natural hybridization, with particular reference to gene flow between crop plants and their wild relatives. We further show

that natural selection, and not the overall rate of gene flow, is the most important factor governing the spread of favorable alleles. Hence, much of this review focuses on the likely effects of transgenes once they escape. Finally, we consider strategies for transgene containment.

http://www.ncbi.nlm.nih.gov/pubmed/16626466

3.3. Gene flow through horizontal gene transfer (HGT)

Another possibility of escape of transgenic material into the environment, maybe more serious because it reveals regardless of men and their activities, concerns to the Horizontal (trans) Gene Transfer by viruses, transposons and plasmids. As outlined in item 3.1 of Part 1[52] of this document, HGT is a phenomenon that occurs permanently at the evolutionary scale, constituting a major source of impulse to the genetic diversity of the mutational process itself and accounting for changes in the genetic composition of most species present in the biosphere.

There are several publications on this subject. In the case of risk assessment in transgenic plants, these approaches seem to focus on the possibility of introgression of the transgene (or other genomic sequences of the expression cassette, such as the genes of resistance to antibiotics) into other organisms, related or genetically distant from the first. These authors point out the difficulty to observe this phenomenon in experiments, and the complexity associated with dimensioning and mapping the associated risks, despite their frequent occurrence.

Gebhard, F.; Smalla, K. 1998. Transformation of *acinetobacter* sp. strain BD413 by transgenic sugar beet DNA. *Applied and Environmental Microbiology*, p. 1550–1554.

The ability of *Acinetobacter* sp. strain BD413(pFG4Δ*npt*II) to take up and integrate transgenic plant DNA based on homologous recombination was studied under optimized laboratory conditions. Restoration of *npt*II, resulting in kanamycin-resistant transformants, was observed with plasmid DNA, plant DNA, and homogenates carrying the gene *npt*II. Molecular analysis showed that some

52 It is also worth checking Part 1 item 3.3 relating to the specific risks of horizontal gene transfer associated with the use of *Agrobacterium tumefaciens* for the transformation of plants.

transformants not only restored the 317-bp deletion but also obtained additional DNA.

Full article available at http://aem.asm.org/content/64/4/1550.full

Nielsen, K.; Bones, A.; Smalla, K.; van Elsas, J. 1998. Horizontal gene transfer from transgenic plants to terrestrial bacteria a-rare event? *FEMS Microbiology Reviews*, 22: 79-103.

Today, 12 years after the first field release of a genetically modified plant (GMP), over 15,000 field trials at different locations have been performed. As new and unique characteristics are frequently introduced into GMPs, risk assessment has to be performed to assess their ecological impact. The possibilities of horizontal gene transfer (HGT; no parent-to-offspring transfer of genes) from plants to microorganisms are frequently evaluated in such risk assessments of GMPs before release into the field. In this review we indicate why putative HGT from plants to terrestrial (soil and plant associated) bacteria has raised concern in biosafety evaluations. Further, we discuss possible pathways of HGT from plants to bacteria, outline the barriers to HGT in bacteria, describe the strategies used to investigate HGT from plants to bacteria and summarize the results obtained. Only a few cases of HGT from eukaryotes such as plants to bacteria have been reported to date. These cases have been ascertained after comparison of DNA sequences between plants and bacteria. Although experimental approaches in both field and laboratory studies have not been able to confirm the occurrence of such HGT to naturally occurring bacteria, recently two studies have shown transfer of marker genes from plants to bacteria based on homologous recombination. The few examples of HGT indicated by DNA sequence comparisons suggest that the frequencies of evolutionarily successful HGT from plants to bacteria may be extremely low. However, this inference is based on a small number of experimental studies and indications found in the literature. Transfer frequencies should not be confounded with the likelihood of environmental implications, since the frequency of HGT is probably only marginally important compared with the selective force acting on the outcome. Attention should therefore be focused on enhancing the understanding of selection processes in natural environments. Only an accurate understanding of these selective events will allow the prediction of possible consequences of novel genes following their introduction into open environments.

http://www.ncbi.nlm.nih.gov/pubmed/9729765

Gebhard, F.; Smalla, K. 1999. Monitoring field releases of genetically modified sugar beets for persistence of transgenic plant DNA and horizontal gene transfer. *FEMS Microbiology Ecology*, 28 (3): 261-72.

Field releases of transgenic rizomania-resistant sugar beet (*Beta vulgaris*) plants were accompanied by a study of the persistence of DNA from transgenic sugar beet litter in soil and of horizontal gene transfer of plant DNA to bacteria. The transgenic sugar beets contained the marker genes *npt*II and *bar* under the control of the bidirectional TR1/2 promoter conferring kanamycin (Km) and glufosinate ammonium resistance to the plant. Primer systems targeting the construct allowed the specific and sensitive detection of the transgenic DNA in soil. Soil samples were analyzed by cultivation of bacteria on nonselective and Km-selective media to determine the proportion of Km-resistant bacteria and to monitor the culturable fraction for incorporation of transgenic plant DNA. To detect the presence of transgenic DNA independently from cultivation, total soil DNA was extracted and amplified by PCR with three different primer sets specific for the transgenic DNA. Long-term persistence of transgenic DNA could be shown under field conditions (up to 2 years) and also in soil microcosms with introduced transgenic plant DNA. No construct-specific sequences were detected by dot blot hybridizations of bacterial isolates. The experimental limitations of detecting horizontal gene transfer from plants to bacteria under field conditions are discussed.

Full article available at http://onlinelibrary.wiley.com/doi/10.1111/j.1574-6941.1999.tb00581.x/pdf

Nielsen, K.; van Elsas, J.; Smalla, K. 2000. Transformation of *Acinetobacter* sp. Strain BD413(pFG4Δ*nptII*) with Transgenic Plant DNA in Soil Microcosms and Effects of Kanamycin on Selection of Transformants. *Applied and Environmental Microbiology*; 66(3): 1237–1242.

Here we show that horizontal transfer of DNA, extracted from transgenic sugar beets, to bacteria, based on homologous recombination, can occur in soil. Restoration of a 317-bp-deleted *nptII* gene in *Acinetobacter* sp. strain BD413(pFG4) cells incubated in sterile soil microcosms was detected after addition of nutrients and transgenic plant DNA encoding a functional *nptII* gene conferring bacterial kanamycin resistance. Selective effects of the addition of kanamycin on the population dynamics of *Acinetobacter* sp. cells in soil were found, and high concentrations of kanamycin reduced the CFU of *Acinetobacter* sp. cells from 10^9 CFU/g of soil to below detection. In contrast to a chromosomal *nptII*-encoded kanamycin resistance, the pFG4-generated resistance was found to be unstable over a 31-day incubation period in vitro.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC91972/

REV - Heinemann, J.; Travick, T. 2004. Problems in monitoring horizontal gene transfer in field trials of transgenic plants. *Nature Biotechnology*, volume 22 number 9.

Transgenic crops are approved for release in some countries, while many more countries are wrestling with the issue of how to conduct risk assessments. Controls on field trials often include monitoring of horizontal gene transfer (HGT) from crops to surrounding soil microorganisms. Our analysis of antibiotic-resistant bacteria and of the sensitivity of current techniques for monitoring HGT from transgenic plants to soil microorganisms has two major implications for field trial assessments of transgenic crops: first, HGT from transgenic plants to microbes could still have an environmental impact at a frequency approximately a trillion times lower than the current risk assessment literature estimates the frequency to be; and second, current methods of environmental sampling to capture genes or traits in a recombinant are too insensitive for monitoring evolution by HGT. A model for HGT involving iterative short-patch events explains how HGT can occur at high frequencies but be detected at extremely low frequencies.

Full article available at https://goo.gl/MWYJUJ

MOD - Nielsen, K.; Townsend, J. 2004. Monitoring and modeling horizontal gene transfer. *Nature Biotechnology*, Volume 22 number 9.

Monitoring efforts have failed to identify horizontal gene transfer (HGT) events occurring from transgenic plants into bacterial communities in soil or intestinal environments. The lack of such observations is frequently cited in biosafety literature and by regulatory risk assessment. Our analysis of the sensitivity of current monitoring efforts shows that studies to date have examined potential HGT events occurring in less than 2 g of sample material, when combined. Moreover, a population genetic model predicts that rare bacterial transformants acquiring transgenes require years of growth to out-compete wild-type bacteria. Time of sampling is there-fore crucial to the useful implementation of monitoring. A population genetic approach is advocated for elucidating the necessary sample sizes and times of sampling for monitoring HGT into large bacterial populations. Major changes in current monitoring approaches are needed, including explicit consideration of the population size of exposed bacteria, the bacterial generation time, the strength of selection acting on the transgene-carrying bacteria, and the sample size necessary to verify or falsify the HGT hypotheses tested.

http://www.ncbi.nlm.nih.gov/pubmed/15340481

REV - Pontiroli, A.; Simonet, P.; Frostegard, A.; Vogel, T.; Monier, J. 2007. Fate of transgenic plant DNA in the environment. *Environ Biosafety Res*, 6(1-2): 15-35.

This review addresses the possible ecological effects of transgenic plants on micro-organisms in the field, hence, in the phytosphere and in the soil matrix. The important steps involved in the interaction between plant DNA and bacteria and the factors that influence the horizontal gene transfer (HGT) process will be discussed. HGT is a process in which two partners are involved, even if indirectly. In the first section, aspects concerning bacteria, such as their physico-chemical, biological and genetic characteristics, are described. Parameters affecting transgenic DNA fate in the environment are described in the second section. Subsequently, terrestrial habitats are evaluated in terms of their capacity to favor horizontal gene transfer. Finally, we focused on several studies in order to evaluate possible perturbations of soil bacterial community composition due to cultivation of transgenic plants in the field.

Full article available at http://www.ebr-journal.org/articles/ebr/pdf/2007/01/ebr0626.pdf

Ulker, B.; Li, Y.; Rosso, M.; Logemann, E.; Somssich, I.; Weisshaar, B. 2008. T-DNA–mediated transfer of *Agrobacterium tumefaciens* chromosomal DNA into plants. *Nature Biotechnology*, volume 26, number 9.

Besides the well-documented integration of DNA flanked by the transfer DNA borders, occasional insertion of fragments from the tumor-inducing plasmid into plant genomes has also been reported during Agrobacterium tumefaciens-mediated transformation. We demonstrate that large (up to approximately 18 kb) gene-bearing fragments of Agrobacterium chromosomal DNA (AchrDNA) can be integrated into Arabidopsis thaliana genomic DNA during transformation. One in every 250 transgenic plants may carry AchrDNA fragments. This has implications for horizontal gene transfer and indicates a need for greater scrutiny of transgenic plants for undesired bacterial DNA.

http://www.ncbi.nlm.nih.gov/pubmed/18758448

REV - Brigulla, M.; Wackernagel, W. 2010. Molecular aspects of gene transfer and foreign DNA acquisition in prokaryotes with regard to safety issues. *Applied microbiology and biotechnology*, 86(4): 1027-1041.

Horizontal gene transfer (HGT) is part of prokaryotic life style and a major factor in evolution. In principle, any combinations of genetic information can be explored via HGT for effects on prokaryotic fitness. HGT mechanisms including transformation, conjugation, transduction, and variations of these plus the role of mobile genetic elements are summarized with emphasis on their potential to translocate foreign DNA. Complementarily, we discuss how foreign DNA can be integrated in recipient cells through homologous recombination (HR), illegitimate recombination (IR), and combinations of both, site-specific recombination, and the reconstitution of plasmids. Integration of foreign DNA by IR is very low, and combinations of IR with HR provide intermediate levels compared to the high frequency of homologous integration. A survey of studies on potential HGT from various transgenic plants indicates very rare transfer of foreign DNA. At the same time, in prokaryotic habitats, genes introduced into transgenic plants are abundant, and natural HGT frequencies are relatively high providing a greater chance for direct transfer instead of via transgenic plants. It is concluded that potential HGT from transgenic plants to prokaryotes is not expected to influence prokaryotic evolution and to have negative effects on human or animal health and the environment.

http://www.ncbi.nlm.nih.gov/pubmed/20191269

REV - Nielsen, K.; Bøhn, T.; Townsend, J. 2014. Detecting rare gene transfer events in bacterial populations. *Frontiers in Microbiology*, Volume 4, Article 415.

Horizontal gene transfer (HGT) enables bacteria to access, share, and recombine genetic variation, resulting in genetic diversity that cannot be obtained through mutational processes alone. In most cases, the observation of evolutionary successful HGT events relies on the outcome of initially rare events that lead to novel functions in the new host, and that exhibit a positive effect on host fitness. Conversely, the large majority of HGT events occurring in bacterial populations will go undetected due to lack of replication success of transformants. Moreover, other HGT events that would be highly beneficial to new hosts can fail to ensue due to lack of physical proximity to the donor organism, lack of a suitable gene transfer mechanism, genetic compatibility, and stochasticity in tempo-spatial occurrence. Experimental attempts to detect HGT events in bacterial populations have typically focused on the transformed cells or their immediate offspring. However, rare HGT events occurring in large and structured populations are unlikely to reach relative population sizes that will allow their immediate identification; the exception being the unusually strong positive selection conferred by antibiotics. Most HGT events are not expected to alter the likelihood of host survival to such an extreme extent, and will confer only minor changes in host fitness. Due to the large population sizes of bacteria and the time scales involved, the process and outcome of HGT are often not amenable to experimental investigation. Population genetic modeling of the growth dynamics of bacteria with differing HGT rates and resulting fitness changes is therefore necessary to guide sampling design and predict realistic time frames for detection of HGT, as it occurs in laboratory or natural settings. Here we review the key population genetic parameters, consider their complexity and highlight knowledge gaps for further research.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3882822/

The presence of recombinant DNA in the environment, after decomposition of transgenic biological material, represents one of the starting points of the horizontal gene transfer. The articles listed below accuse the presence of transgenes/recombinant DNA in several ecosystems and agricultural systems where transgenic plants are grown, as well as in trophic chains of microrganisms and arthropodes in the surroundings, or in water courses. The studies suggest that this dissemination may be unrestrained.

Lerat, S.; Gulden, R.; Hart, M.; Powell, J.; England, L.; Pauls, K.; Swanton, C.; Klironomos, J.; Trevors, J. 2007. Quantification and persistence of recombinant DNA of Roundup Ready corn and soybean in rotation. *Journal of Agricultural and Food Chemistry*, 55(25): 10226-10231.

The presence of the recombinant *cp4 epsps* gene from Roundup Ready (RR) corn and RR soybean was quantified using real-time PCR in soil samples from a field experiment growing RR and conventional corn and soybean in rotation. RR corn and RR soybean *cp4 epsps* persisted in soil

for up to 1 year after seeding. The concentration of recombinant DNA in soil peaked in July and August in RR corn and RR soybean plots, respectively. A small fraction of soil samples from plots seeded with conventional crops contained recombinant DNA, suggesting transgene dispersal by means of natural process or agricultural practices. This research will aid in the understanding of the persistence of recombinant DNA in agricultural cropping systems.

http://pubs.acs.org/doi/abs/10.1021/jf072457z?journalCode=jafcau

REV - Nielsen, K.; Johnsen, P; Bensasson, D.; Daffonchio, D. 2007. Release and persistence of extracellular DNA in the environment. *Environmental Biosafety Research*, 6: 37–53.

The introduction of genetically modified organisms (GMOs) has called for an improved understanding of the fate of DNA in various environments, because extracellular DNA may also be important for transferring genetic information between individuals and species. Accumulating nucleotide sequence data suggest that acquisition of foreign DNA by horizontal gene transfer (HGT) is of considerable importance in bacterial evolution. The uptake of extracellular DNA by natural transformation is one of several ways bacteria can acquire new genetic information given sufficient size, concentration and integrity of the DNA. We review studies on the release, breakdown and persistence of bacterial and plant DNA in soil, sediment and water, with a focus on the accessibility of the extracellular nucleic acids as substrate for competent bacteria. DNA fragments often persist over time in many environments, thereby facilitating their detection and characterization. Nevertheless, the long-term physical persistence of DNA fragments of limited size observed by PCR and Southern hybridization often contrasts with the short-term availability of extracellular DNA to competent bacteria studied in microcosms. The main factors leading to breakdown of extracellular DNA are presented. There is a need for improved methods for accurately determining the degradation routes and the persistence, integrity and potential for horizontal transfer of DNA released from various organisms throughout their lifecycles.

Full article available at http://www.ebr-journal.org/articles/ebr/pdf/2007/01/ebr0608.pdf

Hart, M.; Powell, J.; Gulden, R.; Levy-Booth, D.; Dunfield, K.; Pauls, K.; Swanton, C; Klironomos, J.; Trevors, J. 2009. Detection of transgenic *cp4 epsps* genes in the soil food web. *Agronomy for Sustainable Development*, 29, 497–501.

The persistence and movement of transgenic DNA in agricultural and natural systems is largely unknown. This movement poses a threat of horizontal gene transfer and possible proliferation of genetically modified DNA into the general environment. To assess the persistence of transgenic DNA in a field of Roundup Ready® corn, we quantified the presence of the transgene for glyphosate tolerance within a soil food web. Using quantitative real-time PCR, we identified the *cp4 epsps*transgene in bulk soil microarthropods, nematodes, macroarthropods and earthworms sampled within the corn cropping system. We found evidence of the transgene at all dates and in all animal groups. Transgenic DNA concentration in animal was significantly higher than that of background soil, suggesting the animals were feeding directly on transgenic plant material. It remains to be tested whether this DNA was still within the plant residues, present as free, extracellular DNA or had already undergone genetic transformation into competent bacterial cells. These results are the first to demonstrate the persistence of transgenic crop DNA residues within a food web.

Full article available at https://goo.gl/itXSvc

Chen, J.; Jin, M.; Qiu Z.; Guo, C.; Chen, Z.; Shen, Z.; Wang, X.; Li, J. 2012. A survey of drug resistance bla genes originating from synthetic plasmid vectors in six Chinese rivers. *Environ Sci Technol*, 46(24): 13448-54. doi: 10.1021/es302760s.

Antibiotic resistance poses a significant challenge to human health and its rate continues to rise globally. While antibiotic-selectable synthetic plasmid vectors have proved invaluable tools of genetic engineering, this class of artificial recombinant DNA sequences with high expression of antibiotic resistance genes presents an unknown risk beyond the laboratory setting. Contamination of environmental microbes with synthetic plasmid vector-sourced antibiotic resistance genes may represent a yet unrecognized source of antibiotic resistance. In this study, PCR and real-time quantitative PCR were used to investigate the synthetic plasmid vector-originated ampicillin resistance gene, β-lactam antibiotic (blá), in microbes from six Chinese rivers with significant human interactions. Various levels of blá were detected in all six rivers, with the highest levels in the Pearl and Haihe rivers. To validate the blá pollution, environmental plasmids in the river samples were captured by the E. coli transformants from the community plasmid metagenome. The resultant plasmid library of 205 ampicillin-resistant E. coli (transformants) showed a blá-positive rate of 27.3% by PCR. Sequencing results confirmed the synthetic plasmid vector sources. In addition, results of the Kirby-Bauer disc-diffusion test reinforced the ampicillin-resistant functions of the environmental plasmids. The resistance spectrum of transformants from the Pearl and Haihe rivers, in particular, had expanded to the third- and fourth-generation of cephalosporin drugs, while that of other transformants mainly involved first- and second-generation cephalosporins. This study not only reveals environmental contamination of synthetic plasmid vector-sourced blá drug resistance genes in Chinese rivers, but also suggests that synthetic plasmid vectors may represent a source of antibiotic resistance in humans.

http://www.ncbi.nlm.nih.gov/pubmed/23215020

In the assessment of the environmental impacts of the horizontal (trans)gene transfer, it is important to understand its irreversible nature. Recent research point the possibility that DNA molecules, including those present as free DNA in the environment, maintain their physical structures for thousands of years and thus may be acquired by competent bacteria via HGT.

Willerslev, E.; Hansen, A.; Binladen, J.; Brand, T.; Gilbert, M.; Shapiro, B.; Bunce, M.; Carsten, W.; Gilichinsky, D.; Cooper, A. 2003. Diverse plant and animal genetic records from holocene and pleistocene sediments. *Science*, vol 300.

Genetic analyses of permafrost and temperate sediments reveal that plant and animal DNA may be preserved for long periods, even in the absence of obvious macrofossils. In Siberia, five permafrost cores ranging from 400,000 to 10,000 years old contained at least 19 different plant taxa, including the oldest authenticated ancient DNA sequences known, and megafaunal sequences including mammoth, bison, and horse. The genetic data record a number of dramatic changes in the taxonomic diversity and composition of Beringian vegetation and fauna. Temperate cave sediments in New Zealand also yielded DNA sequences of extinct biota, including two species of ratite moa, and 29 plant taxa characteristic of the prehuman environment. Therefore, many sedimentary deposits may contain unique, and widespread, genetic records of paleoenvironments.

http://www.ncbi.nlm.nih.gov/pubmed/12702808

Overballe-Petersen, S.; Harms, K.; Orlando, L.; Mayar, J.; Rasmussen, S.; Dahl, T.; Rosing, M.; Poole, A.; Sicheritz-Ponten, T.; Brunak, S.; Inselmann, S.; de Vries, J.; Wackernagel, W.; Pybus, O.; Nielsen, R.; Johnsen, P.; Nielsen, K.; Willerslev, E. 2013. Bacterial natural transformation by highly fragmented and damaged DNA. *PNAS*, vol. 110, no. 4919860–19865.

DNA molecules are continuously released through decomposition of organic matter and are ubiquitous in most environments. Such DNA becomes fragmented and damaged (often <100 bp) and may persist in the environment for more than half a million years. Fragmented DNA is recognized as nutrient source for microbes, but not as potential substrate for bacterial evolution. Here, we show that fragmented DNA molecules (≥20 bp) that additionally may contain abasic sites, cross-links, or miscoding lesions are acquired by the environmental bacterium Acinetobacter baylyi through natural transformation. With uptake of DNA from a 43,000-y-old woolly mammoth bone, we further demonstrate that such natural transformation events include ancient DNA molecules. We find that the DNA recombination is RecA recombinase independent and is directly linked to DNA replication. We show that the adjacent nucleotide variations generated by uptake of short DNA fragments escape mismatch repair. Moreover, doublenucleotide polymorphisms appear more common among genomes of transformable than nontransformable bacteria. Our findings reveal that short and damaged, including truly ancient, DNA molecules, which are present in large quantities in the environment, can be acquired by bacteria through natural transformation. Our findings open for the possibility that natural genetic exchange can occur with DNA up to several hundreds of thousands years old.

Full article available at http://www.pnas.org/content/110/49/19860.full

Part 4
Risks to the health associated to the growth and/or use of transgenic plants

After 20 years of controversy regarding the health risks associated with the consumption of transgenic plants, there is no consensus in the scientific community about the possibilities of damages and acceptable limits of consumption, for food composed of genetically modified plants and their parts. As an aggravating factor, most of the studies that point out nutritional and toxicological safety seem to have been held in short-term perspective, ignoring the chronic effects, without considering the associated technological packages (for example, evaluating the consumption of maize tolerant to glyphosate based on grains harvested from a crop where the herbicide has not been used) or even without taking into account the transgenic proteins present in Bt GMP (for example, evaluating the risks of allergenicity with homologous Cry protein, extracted from the bacteria, to attest the safety of a toxin similar to that present in maize). Independent studies that allow questioning those methods establish a worrying picture, which justifies the need for more accurate long-term analysis, supported by robust and consistent methodologies.

When observing all the scientific literature published in specialized journals, it can be realized the importance of the so-called independent studies and the alarming frequency with which they point to significant risks, threatening human and animal health. The problems seem clearly related to low dose effects, causing chronic changes that tend to manifest in the long term. It is suggested, therefore, the need for biosafety data collection, as well as facilitating access to information from studies already completed and ongoing. It is worrying the fact that regulators guide their decisions from publications generated by companies, in which the background information tend to be hidden from the scrutinity of society – in many cases the data are not even available to members of regulatory agencies assessing them.

In the articles listed below, when reviewing the set of toxicological studies published and related to the risks to the health of transgenic plants, some authors consider that the scientific data available do not support the claim of safe consumption of those plants. Other authors are more adamant in stating that physiological changes observed independently in various studies cannot be just fruit of coincidence (especially with regard to biological variables indicator of hepato-renal toxicity), concluding that these products are not biologically safe to human and animal consumption in the long run.

COM - Domingo, J. 2000. Health risks of GM foods: many opinions but few data. *Science*, 288:1748–9.

Without summary.
Full article available at http://stopogm.net/sites/stopogm.net/files/Domingo2000.pdf

REV - Pryme, I.; Lembcke, R. 2003. In vivo studies on possible health consequences of genetically modified food and feed with particular regard to ingredients consisting of genetically modified plant materials. *Nutr Health*, 17: 1-8.

This synopsis reviews published in vivo studies on possible health consequences of genetically modified food and feed where the ingredients in question have consisted of genetically modified plant materials. The following, however, have not been taken into consideration:--ingredients consisting of genetically modified microorganisms or parts of animals/fish--ingredients produced by/from genetically modified organisms but without any DNA present--studies on consequences for the environment or biodiversity--in vitro studies or computer simulations. According to a Norwegian report "Gen-mat" (NOU 2000:29), and a more recent search in Medline and Citations Index, to our knowledge a total of ten studies have been published on the health effects of GM-foods and feeds. In this minireview the data made available in these published studies is discussed.

http://www.ncbi.nlm.nih.gov/pubmed/12803276

REV - Nodari, R.; Guerra, M. 2003. Transgenic plants and their products: effects, risks and food safety (Biosafety of transgenic plants). *Rev Nutr*, Campinas, 16(1):105-116.

This paper provides an overview of the effects of transgenic plants, also known as Genetically Modified Organisms, and food safety. Modern biotechnologies are powerfull tools in reprogramming life. However, a major problem in the risk assessment of the organisms produced by biotechnology is that the outcome of transformations can not be fully foreseen. Potential risks to human health include unpredicted side-effects, allergy, toxicity and intolerance. The main effects on the environment include the gene lateral transfer, genetic pollution, and damage to non-target species. The substantial equivalence principle should be abandoned in favor of more scientific criteria. With the Biosafety Protocol approved January 2000, the precautionary principle was reaffirmed and

the labeling became compulsory. The public perception reached a stage where restrictions on the consumption of genetically modified foods are imposed, compelling enterprises and scientists to a sciencebased approach for the risk assessment analysis.

Full article available at http://www.scielo.br/pdf/rn/v16n1/a10v16n1.pdf

REV - Domingo, J. 2007. Toxicity studies of genetically modified plants: a review of the published literature. *Crit Rev Food Sci Nutr*, 47:721–33.

According to the information reported by the WHO, the genetically modified (GM) products that are currently on the international market have all passed risk assessments conducted by national authorities. These assessments have not indicated any risk to human health. In spite of this clear statement, it is quite amazing to note that the review articles published in international scientific journals during the current decade did not find, or the number was particularly small, references concerning human and animal toxicological/health risks studies on GM foods. In this paper, the scientific information concerning the potential toxicity of GM/transgenic plants using the Medline database is reviewed. Studies about the safety of the potential use of potatoes, corn, soybeans, rice, cucumber, tomatoes, sweet pepper, peas, and canola plants for food and feed were included. The number of references was surprisingly limited. Moreover, most published studies were not performed by the biotechnology companies that produce these products. This review can be concluded raising the following question: where is the scientific evidence showing that GM plants/food are toxicologically safe?

http://www.ncbi.nlm.nih.gov/pubmed/17987446

REV - Dona, A.; Arvanitoyannis, I. 2009. Health risks of genetically modified foods. *Crit Rev Food Sci Nutr*, 49 : 164–75.

As genetically modified (GM) foods are starting to intrude in our diet concerns have been expressed regarding GM food safety. These concerns as well as the limitations of the procedures followed in the evaluation of their safety are presented. Animal toxicity studies with certain GM foods have shown that they may toxically affect several organs and systems. The review of these studies should not be conducted separately for each GM food, but according to the effects exerted on certain organs it may help us create a better picture of the possible health effects on human beings. The results of most studies with GM foods indicate that they may cause some common toxic effects such as hepatic, pancreatic, renal, or reproductive effects and may alter the hematological, biochemical, and immunologic parameters. However, many years of research with animals and clinical trials are required for this assessment. The use of recombinant GH or its expression in animals should be re-examined since it has been shown that it increases IGF-1 which may promote cancer.

http://www.ncbi.nlm.nih.gov/pubmed/18989835

REV - Camara, M.; Marinho, C.; Guilam, M.; Nodari, R. 2009. Transgenic products: a scientific-production evaluation of possible food (in)security. *História, Ciências, Saúde – Manguinhos*, Rio de Janeiro, v.16, n.3, p.669-681.

Based on a bibliographic review, the article identifies and offers a critical analysis of scientific production by the public health field in Brazil on genetically modified organisms and food (in) security. Of the 716 articles found on the portals of the Scientific Electronic Library Online (SciELO) and the Coordinating Agency for the Development of Higher Education (Capes), only

8 address the food security of transgenic products, primarily in terms of risk exposure and the uncertainties about how these products impact health and the environment. The main conclusion involves the fact that the eight analyzed articles do not speak to the question of the security but rather the insecurity of genetically modified foods.

Full article available at https://goo.gl/jrk9dG

REV - Magana-Gomez, J.; de la Barca, A. 2009. Risk assessment of genetically modified crops for nutrition and health. *Nutr Rev*, 67 (1).

The risk assessment of genetically modified (GM) crops for human nutrition and health has not been systematic. Evaluations for each GM crop or trait have been conducted using different feeding periods, animal models, and parameters. The most common result is that GM and conventional sources induce similar nutritional performance and growth in animals. However, adverse microscopic and molecular effects of some GM foods in different organs or tissues have been reported. Diversity among the methods and results of the risk assessments reflects the complexity of the subject. While there are currently no standardized methods to evaluate the safety of GM foods, attempts towards harmonization are on the way. More scientific effort is necessary in order to build confidence in the evaluation and acceptance of GM foods.

http://www.ncbi.nlm.nih.gov/pubmed/19146501

REV - Vendômois, J.; Cellier, D.; Vélot, C.; Clair, E.; Mesnage, R.; Seralini, G. 2010. Debate on GMOs health risks after statistical findings in regulatory tests. *International Journal Biological Science*, 6, 590–598.

We summarize the major points of international debate on health risk studies for the main commercialized edible GMOs. These GMOs are soy, maize and oilseed rape designed to contain new pesticide residues since they have been modified to be herbicide-tolerant (mostly to Roundup) or to produce mutated Bt toxins. The debated alimentary chronic risks may come from unpredictable insertional mutagenesis effects, metabolic effects, or from the new pesticide residues. The most detailed regulatory tests on the GMOs are three-month long feeding trials of laboratory rats, which are biochemically assessed. The tests are not compulsory, and are not independently conducted. The test data and the corresponding results are kept in secret by the companies. Our previous analyses of regulatory raw data at these levels, taking the representative examples of three GM maize NK 603, MON 810, and MON 863 led us to conclude that hepatorenal toxicities were possible, and that longer testing was necessary. Our study was criticized by the company developing the GMOs in question and the regulatory bodies, mainly on the divergent biological interpretations of statistically significant biochemical and physiological effects. We present the scientific reasons for the crucially different biological interpretations and also highlight the shortcomings in the experimental protocols designed by the company. The debate implies an enormous responsibility towards public health and is essential due to nonexistent traceability or epidemiological studies in the GMO-producing countries.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2952409/

REV - Domingo, J.; Bordonaba, J. 2011. A literature review on the safety assessment of genetically modified plants. *Environment International*, 37. 734-42.

In recent years, there has been a notable concern on the safety of genetically modified (GM) foods/plants, an important and complex area of research, which demands rigorous standards. Diverse

groups including consumers and environmental Non Governmental Organizations (NGO) have suggested that all GM foods/plants should be subjected to long-term animal feeding studies before approval for human consumption. In 2000 and 2006, we reviewed the information published in international scientific journals, noting that the number of references concerning human and animal toxicological/health risks studies on GM foods/plants was very limited. The main goal of the present review was to assess the current state-of-the-art regarding the potential adverse effects/safety assessment of GM plants for human consumption. The number of citations found in databases (PubMed and Scopus) has dramatically increased since 2006. However, new information on products such as potatoes, cucumber, peas or tomatoes, among others was not available. Corn/maize, rice, and soybeans were included in the present review. An equilibrium in the number research groups suggesting, on the basis of their studies, that a number of varieties of GM products (mainly maize and soybeans) are as safe and nutritious as the respective conventional non-GM plant, and those raising still serious concerns, was currently observed. Nevertheless, it should be noted that most of these studies have been conducted by biotechnology companies responsible of commercializing these GM plants. These findings suggest a notable advance in comparison with the lack of studies published in recent years in scientific journals by those companies. All this recent information is herein critically reviewed.

http://www.ncbi.nlm.nih.gov/pubmed/21296423

Maghari, B.; Ardekani, A. 2011. Genetically modified foods and social concerns. *Avicenna Journal of Medical Biotechnology*, 3(3): 109–117. PMCID: PMC3558185.

Biotechnology is providing us with a wide range of options for how we can use agricultural and commercial forestry lands. The cultivation of genetically modified (GM) crops on millions of *hectares* of lands and their injection into our food chain is a huge global genetic experiment involving all living beings. Considering the fast pace of new advances in production of genetically modified crops, consumers, farmers and policymakers worldwide are challenged to reach a consensus on a clear vision for the future of world food supply. The current food biotechnology debate illustrates the serious conflict between two groups: 1) Agri-biotech investors and their affiliated scientists who consider agricultural biotechnology as a solution to food shortage, the scarcity of environmental resources and weeds and pests infestations; and 2) independent scientists, environmentalists, farmers and consumers who warn that genetically modified food introduces new risks to food security, the environment and human health such as loss of biodiversity; the emergence of superweeds and superpests; the increase of antibiotic resistance, food allergies and other unintended effects. This article reviews major viewpoints which are currently debated in the food biotechnology sector in the world. It also lays the ground-work for deep debate on benefits and risks of Biotech-crops for human health, ecosystems and biodiversity. In this context, although some regulations exist, there is a need for continuous vigilance for all countries involved in producing genetically engineered food to follow the international scientific bio-safety testing guidelines containing reliable pre-release experiments and post-release track of transgenic plants to protect public health and avoid future environmental harm.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3558185/

REV - Zdziarski, I.; Edwards, J.; Carman, J.; Haynes, J. 2014. GM crops and the rat digestive tract: A critical review. *Environment International*, 73 (2014) 423–433.

The aim of this review is to examine the relationship between genetically modified (GM) crops and health, based on histopathological investigations of the digestive tract in rats. We reviewed published long-term feeding studies of crops containing one or more of three specific traits: herbicide tolerance via the EPSPS gene and insect resistance via cry1Ab or cry3Bb1 genes. These genes are commonly found in commercialised GM crops. Our search found 21 studies for nine (19%) out of the 47 crops approved for human and/or animal consumption. We could find no studies on the other 38 (81%)

approved crops. Fourteen out of the 21 studies (67%) were general health assessments of the GM crop on rat health. Most of these studies (76%) were performed after the crop had been approved for human and/or animal consumption, with half of these being published at least nine years after approval. Our review also discovered an inconsistency in methodology and a lack of defined criteria for outcomes that would be considered toxicologically or pathologically significant. In addition, there was a lack of transparency in the methods and results, which made comparisons between the studies difficult. The evidence reviewed here demonstrates an incomplete picture regarding the toxicity (and safety) of GM products consumed by humans and animals. Therefore, each GM product should be assessed on merit, with appropriate studies performed to indicate the level of safety associated with them. Detailed guidelines should be developed which will allow for the generation of comparable and reproducible studies. This will establish a foundation for evidence-based guidelines, to better determine if GM food is safe for human and animal consumption.

Full article available at http://www.sciencedirect.com/science/article/pii/S0160412014002669

Despite the uncertainties and the deficiencies of information related to risks to consumer health, it is worth mentioning two highly relevant epidemiological studies, also for its uniqueness and apparent originality. These are – to the limit of our knowledge – the only two works of this kind available in the international scientific literature. Both reveal indications of deterioration in the health of the US population over the past two decades, associating this phenomenon to the expansion of GM plant cultivation in that country. Because of its importance, and taking into account the parallels that allow establishing, these studies show the need for detailed assessments, which seek to identify or deny the presence of causal relationships stronger than those identified by correlation methods.

The studies in question directly contradict the arguments most frequently repeated by companies and other promoters of these technologies: "after 20 years consuming GM plants, the world has never noticed any health problems associated with them[53]".

The absence of epidemiological studies that could never be used – by

53 Theoretically, this statement should be based on some kind of epidemiological study, establishing a comparison between the consumption of distinct and diverse populations on the long-term and taking into account broad range of biological variables related to the overall health status of the people. Basically, the test group should primarily feed on GM products, which would be avoided in the control group. This, ideally, could be divided in subgroups being fed with conventional products and others with organic products. Despite its basic and fundamental character, after 20 years of GM commercial growing, health agencies have not set out to perform this type of study. We consider the interests related to the unavailability of this study as a reason for their absence in the set of specialized scientific literature on an international scale.

opinion makers or responsible investigators – as illustrative argument to attest the non-existence of epidemiological damage is unmasked based on the works below.

EPI - Shao, Q.; Chin, K. 2011. Survey of American food trends and the growing obesity epidemic. *Nutr Res Pract*, 5(3): 253-259.

The rapid rise in the incidence of obesity has emerged as one of the most pressing global public health issues in recent years. The underlying etiological causes of obesity, whether behavioral, environmental, genetic, or a combination of several of them, have not been completely elucidated. The obesity epidemic has been attributed to the ready availability, abundance, and overconsumption of high-energy content food. We determined here by Pearson's correlation the relationship between food type consumption and rising obesity using the loss-adjusted food availability data from the United States Department of Agriculture (USDA) Economic Research Services (ERS) as well as the obesity prevalence data from the Behavioral Risk Factor Surveillance System (BRFSS) and the National Health and Nutrition Examination Survey (NHANES) at the Centers for Disease Control and Prevention (CDC). Our analysis showed that total calorie intake and consumption of high fructose corn syrup (HFCS) did not correlate with rising obesity trends. Intake of other major food types, including chicken, dairy fats, salad and cooking oils, and cheese also did not correlate with obesity trends. However, our results surprisingly revealed that consumption of corn products correlated with rising obesity and was independent of gender and race/ethnicity among population dynamics in the U.S. Therefore, we were able to demonstrate a novel link between the consumption of corn products and rising obesity trends that has not been previously attributed to the obesity epidemic. This correlation coincides with the introduction of bioengineered corns into the human food chain, thus raising a new hypothesis that should be tested in molecular and animal models of obesity.

http://www.ncbi.nlm.nih.gov/pubmed/21779530

EPI - Swanson, N.; Leu, A.; Abrahamson, J.; Wallet, B. 2014. Genetically engineered crops, glyphosate and the deterioration of health in the United States of America. *Jornal of Organic Systems*, 9(2).

A huge increase in the incidence and prevalence of chronic diseases has been reported in the United States (US) over the last 20 years. Similar increases have been seen globally. The herbicide glyphosate was introduced in 1974 and its use is accelerating with the advent of herbicide-tolerant genetically engineered (GE) crops. Evidence is mounting that glyphosate interferes with many metabolic processes in plants and animals and glyphosate residues have been detected in both. Glyphosate disrupts the endocrine system and the balance of gut bacteria, it damages DNA and is a driver of mutations that lead to cancer. In the present study, US government databases were searched for GE crop data, glyphosate application data and disease epidemiological data. Correlation analyses were then performed on a total of 22 diseases in these time-series data sets. The Pearson correlation coefficients are highly significant (< 10-5) between glyphosate applications and hypertension (R = 0.923), stroke (R = 0.925), diabetes prevalence (R = 0.971), diabetes incidence (R = 0.935), obesity (R = 0.962), lipoprotein metabolism disorder (R = 0.973), Alzheimer's (R = 0.917), senile dementia (R = 0.994), Parkinson's (R = 0.875), multiple sclerosis (R = 0.828), autism (R = 0.989), inflammatory bowel disease (R = 0.938), intestinal infections (R = 0.974), end stage renal disease (R = 0.975), acute kidney failure (R = 0.978), cancers of the thyroid (R = 0.988), liver (R = 0.960), bladder (R = 0.981), pancreas (R = 0.918), kidney (R = 0.973) and myeloid leukaemia (R = 0.878). The Pearson correlation coefficients are highly significant (< 10-4) between the percentage of GE corn and soy planted in the US and hypertension (R = 0.961), stroke (R = 0.983), diabetes prevalence (R = 0.983), diabetes incidence (R = 0.955), obesity (R = 0.962), lipoprotein metabolism

disorder (R = 0.955), Alzheimer's (R = 0.937), Parkinson's (R = 0.952), multiple sclerosis (R = 0.876), hepatitis C (R = 0.946), end stage renal disease (R = 0.958), acute kidney failure (R = 0.967), cancers of the thyroid (R = 0.938), liver (R = 0.911), bladder (R = 0.945), pancreas (R = 0.841), kidney (R = 0.940) and myeloid leukaemia (R = 0.889). The significance and strength of the correlations show that the effects of glyphosate and GE crops on human health should be further investigated.

Full article available at https://goo.gl/vkWmrt

1 Risks to the health associated to the use of Bt plants

1.1 Interaction possibilities between Bt proteins and mammalian cells

The industry of agricultural biotechnologies argues that the absence of risks to human and animal health, with regard to the consumption of Bt toxins, is due to the fact that these proteins have strict specificity of action. Conditional upon the presence of receptors exclusive of the digestive system of certain insects, and absent in humans and other animals, this characteristic would eliminate risks. They also claim that the proteins would be destroyed by temperature and acidity observed in the digestive system of monogastric and ruminant species.

More recently, these statements are no longer accepted, while still being repeated. In addition to the aforementioned failures of specificity to insects, evidence of biological interactions between those proteins and mammalian cells is accumulated.

In item 1.1 of Part 3, additional references are exposed, pointing out difficulty to accept and even to understand the reasons why some scholars maintain, against evidence, the argument of strict specificity of action of Cry toxins.

Vázquez-Padrón, R.; Gonzáles-Cabrera, J.; García-Tovar, C.; Neri-Bazan, L.; Lopéz- Revilla,

R.; Hernández, M.; Moreno- Fierro, L.; de la Riva, G. 2000. Cry1Ac protoxin from *Bacillus thuringiensis* sp. kurstaki HD73 binds to surface proteins in the mouse small intestine. *Biochem Biophys Res Comm*, 271, 54-58.

Bacillus thuringiensis (Bt), considered a safe insecticide, produces insecticidal proteins named Cry during sporulation, which possess exceptional immunological properties. In this work using an immunohistochemical test we demonstrated that Cry1Ac protoxin (pCry1Ac) binds to the mucosal surface of the mouse small intestine. Ligand blot assay allowed us to detect, under denaturing conditions, six pCry1Ac-binding polypeptides present in brush border membrane vesicles isolated from the small intestine. Moreover, this protein induced in situ temporal changes in the electrophysiological properties of the mouse jejunum. The data obtained indicate a possible interaction in vivo of Cry proteins with the animal bowel which could induce changes in the physiological status of the intestine.

http://www.ncbi.nlm.nih.gov/pubmed/10777680

Mesnage, R.; Clair, E.; Gress, S.; Then, C; Székács, A.; Séralini, G-E. 2012. Cytotoxicity on human cells of Cry1Ab and Cry1Ac Bt insecticidal toxins alone or with a glyphosate-based herbicide. *Journal of Applied Toxicology*, DOI: 10.1002/jat.2712.

The study of combined effects of pesticides represents a challenge for toxicology. In the case of the new growing generation of genetically modified (GM) plants with stacked traits, glyphosate-based herbicides (like Roundup) residues are present in the Roundup-tolerant edible plants (especially corns) and mixed with modified Bt insecticidal toxins that are produced by the GM plants themselves. The potential side effects of these combined pesticides on human cells are investigated in this work. Here we have tested for the very first time Cry1Ab and Cry1Ac Bt toxins (10 ppb to 100 ppm) on the human embryonic kidney cell line 293, as well as their combined actions with Roundup, within 24 h, on three biomarkers of cell death: measurements of mitochondrial succinate dehydrogenase, adenylate kinase release by membrane alterations and caspase 3/7 inductions. Cry1Abcaused cell death from 100 ppm. For Cry1Ac, under such conditions, no effects were detected. The Roundup tested alone from 1 to 20 000 ppm is necrotic and apoptotic from 50 ppm, far below agricultural dilutions (50% lethal concentration 57.5 ppm). The only measured significant combined effect was that Cry1Ab and Cry1Ac reduced caspases 3/7 activations induced by Roundup; this could delay the activation of apoptosis. There was the same tendency for the other markers. In these results, we argue that modified Bt toxins are not inert on nontarget human cells, and that they can present combined side-effects with other residues of pesticides specific to GM plants.

Full article available at http://www.gmoseralini.org/wp-content/uploads/2012/11/mesnage2011.pdf

Mezzomo, B.; Miranda-Vilela, A.; Freire, I.; Barbosa, L.; Portilho, F.; Lavaca, Z.; Grisolia, C. 2013. Hematotoxicity of *Bacillus thuringiensis* as spore-crystal strains Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa in swiss albino mice. *Journal of Hematology & Thromboembolic Diseases*, 1: 104. doi:10.4172/jhtd.1000104.

Formulated and sporulated cultures of Bacillus thuringiensis (Bt) have been widely used against insect pests, but after the advent of genetically modified plants expressing δ-endotoxins, the bioavailability of Cry proteins has been increased. For biosafety reasons their adverse effects should be studied, mainly for non-target organisms. Thus, we evaluated, in Swiss albino mice, the hematotoxicity and genotoxicity of four Bt spore-crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A, administered alone by gavage with a single dose of 27 mg/Kg, 136 mg/Kg or 270 mg/Kg, 24 h, 72 h or 7 days before euthanasia. Binary combinations of these four spore-crystal proteins were also assayed at 270 mg/Kg with a single administration 24 h before euthanasia.

Control mice received filtered water or cyclophosphamide at 27 mg/kg. For hematotoxicity evaluations, blood samples were drawn by cardiac puncture and processed in a multiple automated hematology analyzer; for genotoxicity analyses, micronucleus test was carried out in mice bone marrow cells. Spore-crystal administrations provoked selective hematotoxicity for the 3 exposure times, particularly for erythroid lineage. A significant reduction in bone marrow cell proliferation demonstrated cytotoxic but not genotoxic effects. These effects persisted for all exposure times, becoming more evident at 7 days.Similar results were observed for binary combinations at 24 h, suggesting that further studies are required to clarify the mechanism involved in the hematotoxicity found in mice, and to establish the toxicological risks to non-target organisms, especially mammals, before concluding that these microbiological control agents are safe for mammals.

Full article available at https://goo.gl/ohWP6W

1.2 The destination of Bt proteins (in the organism of beings which ingest them)

The agricultural biotechnology industry, as well as the risk assessment agencies, assumes that the Cry proteins will be completely degraded by the digestive system of animal organisms (they will fail to overcome the gastrointestinal histological barrier). Observations in this direction, obtained based on results of laboratory simulations, have been interpreted as informative enough to allow exempting more comprehensive studies. Thus, there is practically no risk assessment examining the presence and effect of such toxins in the organism of higher animals.

However, several articles have shown that Cry proteins may survive digestion in animals and humans, overcoming the natural barriers and maintaining a significant portion of their biological activities. Even placental barrier is overcome by fragments of these toxins, which have been reported as present in the circulatory system of fetuses and newborns.

References that show the circulation of these toxins into arthropods (insects in particular) and soil microorganisms as well as demonstrate their persistence over trophic chains of agricultural systems, can be found in Part 3 item 1.3 of this book.

Chowdhury, E.; Kuribara, H.; Hino, A.; Sultana, P.; Mikami, O.; Shimada, N.; Guruge, K.; Saito, M.; Nakajima, Y. 2003. Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. *Journal of Animal Science*, 81:2546-2551.

Genetically modified corn has been approved as an animal feed in several countries, but information about the fate of genetically modified DNA and protein in vivo is insufficient. Genetically modified corn Bt11 is developed by inserting a recombinant DNA sequence encoding insecticidal Cry1Abprotein from Bacillus thuringiensis subsp. kurstaki. We examined the presence of corn intrinsic and recombinant cry1Ab gene by PCR, and theCry1Ab protein by immunological tests in the gastrointestinal contents of five genetically modified corn Bt11-fed and five nongenetically modified corn-fed pigs. Fragments of corn zein (242 bp), invertase (226 bp) and of ribulose-1,5-bisphosphate carboxylase/ oxygenase genes (1,028 bp) were detected in the gastrointestinal contents of both Bt11 and nongenetically modified corn-fed pigs. Fragments of recombinant cry1Ab gene (110 bp and 437 bp) were detected in the gastrointestinal contents of the Bt11-fed pigs but not in the control pigs. Neither corn intrinsic nor cry1Ab gene fragments were detected in the peripheral blood by PCR. The gastrointestinal contents were positive for Cry1Ab protein by ELISA, immunochromatography, and immunoblot; however, these methods did not work for blood and precluded conclusions about any potential absorption of the protein. These results suggest that ingested corn DNA and Cry1Ab protein were not totally degraded in the gastrointestinal tract, as shown by their presence in a form detectable by PCR or immunological tests.

http://www.ncbi.nlm.nih.gov/pubmed/14552382

Lutz, B.; Wiedemann, S.; Einspanier, R; Mayer, J.; Albrecht, C. 2005. Degradation of Cry1Ab protein from genetically modified maize in the bovine gastrointestinal tract. *J Agric Food Chem*, 53:1453–1456.

Immunoblotting assays using commercial antibodies were established to investigate the unexpected persistence of the immunoactive Cry1Ab protein in the bovine gastrointestinal tract (GIT) previously suggested by enzyme-linked immunosorbent assay (ELISA). Samples of two different feeding experiments in cattle were analyzed with both ELISA and immunoblotting methods. Whereas results obtained by ELISA suggested that the concentration of the Cry1Ab protein increased during the GIT passage, the immunoblotting assays revealed a significant degradation of the protein in the bovine GIT. Samples showing a positive signal in the ELISA consisted of fragmented Cry1Ab protein of approximately 17 and 34 kDa size. Two independent sets of gastrointestinal samples revealed the apparent discrepancy between the results obtained by ELISA and immunoblotting, suggesting that the antibody used in the ELISA reacts with fragmented yet immunoactive epitopes of the Cry1Ab protein. It was concluded that Cry1Ab protein is degraded during digestion in cattle. To avoid misinterpretation, samples tested positive for Cry1Ab protein by ELISA should be reassessed by another technique.

http://www.ncbi.nlm.nih.gov/pubmed/15740023

Guimaraes, V.; Drumare, M.; Lereclus, D.; Gohar, M.; Lamourette, P.; Nevers, M.; Vaisanen-Tunkelrott, M.; Bernard, H.; Guillon, B.; Créminon, C.; Wal J.; Adel-Patient, K. 2010. *In vitro* digestion of Cry1Ab proteins and analysis of the impact on their immunoreactivity. *J Agric Food Chem*, 58(5): 3222-3231.

A pepsin resistance test performed at pH 1.2 and with high pepsin to protein ratio is one of the steps of the weight-of-evidence approach used for assessment of allergenicity of new proteins. However, the use of other in vitro digestibility tests, performed in more physiologically relevant conditions and in combination with immunological assays so as to increase the value of the information

gained from the studies of stability of a novel protein to digestion for the overall allergenicity assessment, has been proposed. This study then aimed to investigate the stability to digestion of Cry1Abprotoxin and toxin, insecticidal proteins expressed in genetically modified crops, using simulated gastric fluid (SGF) at different pH values and pepsin-to-substrate ratios, in the presence or absence of physiological surfactant phosphatidylcholine (PC). Electrophoresis and immunoblot patterns and residual immunoreactivity of digesta were analyzed. Although Cry1Ab protoxin is extensively degraded at pH 1.2 with high pepsin-to-protein ratio, it is only slightly degraded at pH 2.0 and conserved its immunoreactivity. Furthermore, Cry1Ab proteins were demonstrated to be stable in a more physiologically relevant in vitro digestibility test (pH 2.5, pepsin-to-substrate ratio 1:20 (w/w) with PC). Factors such as pH, SGF composition, and pepsin-to-substrate ratio then greatly influence the digestion of Cry1Ab proteins, confirming that new and more physiologically relevant in vitro digestibility tests should be also considered to study the relationship between the resistance of a protein to digestion and its allergenicity.

http://www.ncbi.nlm.nih.gov/pubmed/20136083

Aris, A; Leblanc, S. 2011. Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec, Canada. *Reprod Toxicol*, 31(4): 528-33. doi: 10.1016/j.reprotox.

Pesticides associated to genetically modified foods (PAGMF), are engineered to tolerate herbicides such as glyphosate (GLYP) and gluphosinate (GLUF) or insecticides such as the bacterial toxin bacillus thuringiensis (Bt). The aim of this study was to evaluate the correlation between maternal and fetal exposure, and to determine exposure levels of GLYP and its metabolite aminomethyl phosphoric acid (AMPA), GLUF and its metabolite 3-methylphosphinicopropionic acid (3-MPPA) and Cry1Ab protein (a Bt toxin) in Eastern Townships of Quebec, Canada. Blood of thirty pregnant women (PW) and thirty-nine nonpregnant women (NPW) were studied. Serum GLYP and GLUF were detected in NPW and not detected in PW. Serum 3-MPPA and CryAb1 toxin were detected in PW, their fetuses and NPW. This is the first study to reveal the presence of circulating PAGMF in women with and without pregnancy, paving the way for a new field in reproductive toxicology including nutrition and utero-placental toxicities.

http://www.ncbi.nlm.nih.gov/pubmed/21338670

1.3 The allergenic potential of Bt plants

The risk of allergy problems caused by contact with Cry toxins requires further concentrated efforts to those accomplished so far. There is no scientific consensus. The specialized literature notes the presence of immune and allergic reactions in studies performed with animal and human beings. The literature also reports that such reactions are associated with the presence of Bt proteins and other components observed (unexpected during the transgene insertion process) in GMPs.

It would also be frequent low-impact immune reactions, incapable of causing – immediately – allergenic reactions. More diffuse

biologically, although noticeable to the consumer, these reactions can bring long-term implications, unknown at this time. It is known that continuous immunological reactions, if kept active by the organism over time, can ultimately result in allergenic and/or inflammatory reactions, among others. In addition, synergy effects and fed back reactions, simple immune responses against certain molecules are likely to turn into complex allergenic reactions against other molecules.

Vázquez-Padrón, R.; Moreno-Fierros, L.; Neri-Bazan, L.; de la Riva, G.; Lopez-Revilla, R. 1999. Intragastric and intra-peritoneal administration of Cry1Ac protoxin from *Bacillus thuringiensis* induces systemic and mucosal antibody responses in mice. *Life Sciences*, 64 (21): 1897-1912.

The spore-forming soil bacterium Bacillus thuringiensis produces parasporal inclusion bodies composed by delta-endotoxins also known as Cry proteins, whose resistance to proteolysis, stability in highly alkaline pH and innocuity to vertebrates make them an interesting candidate to carrier of relevant epitopes in vaccines. The purpose of this study was to determine the mucosal and systemic immunogenicity in mice of Cry1Ac protoxin from B. thuringiensis HD73. Crystalline and soluble forms of the protoxin were administered by intraperitoneal or intragastric route and anti-Cry1Acantibodies of the major isotypes were determined in serum and intestinal fluids. The two forms of Cry1Ac protoxin administered by intraperitoneal route induced a high systemic antibody response, however, only soluble Cry1Ac induced a mucosal response via intragastric. Serum antibody levels were higher than those induced by cholera toxin. Systemic immune responses were attained with doses of soluble Cry1Ac ranging from 0.1 to 100 microg by both routes, and the maximal effect was obtained with the highest doses. High anti-Cry1Ac IgG antibody levels were detected in the large and small intestine fluids from mice receiving the antigen via i.p. These data indicate that Cry1Ac is a potent systemic and mucosal immunogen.

http://www.ncbi.nlm.nih.gov/pubmed/10353588

Bernstein, I.; Bernstein, J.; Miller, M.; Tierzieva, S.; Bernstein, D.; Lummus, Z.; Selgrade, M.; Doerfler, D.; Seligy, V. 1999. Immune responses in farm workers after exposure to *Bacillus thuringiensis* pesticides. *Environmental Health Perspectives*, 107(7): 575-582.

Although health risks to pesticides containing Bacillus thuringiensis (Bt) have been minimal, the potential allergenicity of these organisms has not been evaluated. Therefore, a health survey was conducted in farm workers before and after exposure to Bt pesticides. Farm workers who picked vegetables that required Bt pesticide spraying were evaluated before the initial spraying operation (n = 48) and 1 and 4 months after (n = 32 and 20, respectively). Two groups of low- (n = 44) and medium- (n = 34) exposure workers not directly exposed to Bt spraying were also assessed. The investigation included questionnaires, nasal/mouth lavages, ventilatory function assessment, and skin tests to indigenous aeroallergens and to a variety of Bt spore and vegetative preparations. To authenticate exposure to the organism present in the commercial preparation, isolates from lavage specimens were tested for Bt genes by DNA-DNA hybridization. Humoral immunoglobulin G (IgG) and immunoglobulin E (IgE) antibody responses to spore and vegetative Bt extracts were assayed. There was no evidence of occupationally related respiratory symptoms. Positive skin-prick tests to several spore extracts were seen chiefly in exposed workers. In particular, there was a significant (p <

0.05) increase in the number of positive skin tests to spore extracts 1 and 4 months after exposure to Bt spray. The number of positive skin test responses was also significantly higher in high ($p < 0.05$) than in low- or medium-exposure workers. The majority of nasal lavage cultures from exposed workers was positive for the commercial Bt organism, as demonstrated by specific molecular genetic probes. Specific IgE antibodies were present in more high-exposure workers ($p < 0.05$) than in the low and medium groups. Specific IgG antibodies occurred more in the high ($p < 0.05$) than in the low-exposure group. Specific IgG and IgE antibodies to vegetative organisms were present in all groups of workers. Exposure to Bt sprays may lead to allergic skin sensitization and induction of IgE and IgG antibodies, or both.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1566654/

Vázquez, R.; Moreno-Fierros, L.; Neri-Bazan, L.; De La Riva, G.; Lopez-Revilla, R. 1999. *Bacillus thuringiensis* Cry1Ac protoxin is a potent systemic and mucosal adjuvant. *Scandinavian Journal of Immunology*, 49(6): 578-84.

Recently we demonstrated that recombinant Cry1Ac protoxin from Bacillus thuringiensis is a potent systemic and mucosal immunogen. In this study we compared the adjuvant effects of Cry1Ac and cholera toxin (CT) for the hepatitis B surface antigen (HBsAg) and bovine serum albumin (BSA). The antibody responses of intestinal secretions and serum were determined by ELISA in Balb/c mice immunized through the intragastric (IG) or intraperitoneal (IP) routes. When HBsAg was administered via IG, the anti-HBsAg intestinal response was not enhanced by either Cry1Ac or CT, whereas via IP Cry1Ac increased the anti-HBsAg intestinal immunoglobulin (Ig) G response and CT increased the intestinal IgA and IgM responses. Serum anti-BSA antibodies increased when BSA was co-administered with CT or Cry1Ac by both routes. Cholera toxin and Cry1Ac co-administered via IP increased the IgG anti-BSA response in fluid of the large intestine and CT also increased the IgA and IgM responses slightly. When co-administered via IP, CT and Cry1Ac did not affect the IgG anti-BSA response of the small intestine significantly. We conclude that Cry1Ac is a mucosal and systemic adjuvant as potent as CT which enhances mostly serum and intestinal IgG antibody responses, especially at the large intestine, and its effects depend on the route and antigen used. These features make Cry1Ac of potential use as carrier and/or adjuvant in mucosal and parenteral vaccines.

http://www.ncbi.nlm.nih.gov/pubmed/10354369

Vázquez-Padrón, R.; Moreno-Fierros, l.; Neri-Bazán, l.; Martínez-Gil, A.; de-la-Riva, A.; López-Revilla, R. 2000. Characterization of the mucosal and systemic immune response induced by Cry1Ac protein from *Bacillus thuringiensis* HD 73 in mice. *Brazilian Journal of Medical and Biological Research*, 33, 147-155.

The present paper describes important features of the immune response induced by the Cry1Ac protein from Bacillus thuringiensis in mice. The kinetics of induction of serum and mucosal antibodies showed an immediate production of anti-Cry1Ac IgM and IgG antibodies in serum after the first immunization with the protoxin by either the intraperitoneal or intragastric route. The antibody fraction in serum and intestinal fluids consisted mainly of IgG1. In addition, plasma cells producing anti-Cry1Ac IgG antibodies in Peyer's patches were observed using the solid-phase enzyme-linked immunospot (ELISPOT). Cry1Ac toxin administration induced a strong immune response in serum but in the small intestinal fluids only anti-Cry1AcIgA antibodies were detected. The data obtained in the present study confirm that the Cry1Ac protoxin is a potent immunogen able to induce a specific immune response in the mucosal tissue, which has not been observed in response to most other proteins.

http://www.ncbi.nlm.nih.gov/pubmed/10657055

Moreno-Fierros, l.; Garcíaa, N.; Gutiérrez, R.; López-Revilla, R.; Vázquez-Padrón, R. 2000. Intranasal, rectal and intraperitoneal immunization with protoxin Cry1Ac from *Bacillus thuringiensis* induces compartmentalized serum, intestinal, vaginal and pulmonary immune responses in Balb/c mice. *Microbes and Infection*, 2, 2000, 885–890.

Recently we discovered that the Cry1Ac protoxin of Bacillus thuringiensis administered to Balb/c mice intraperitoneally (i.p.) or intragastrically is a systemic and intestinal immunogen as potent as cholera toxin. To further characterize the mucosal immunogenicity of Cry1Ac we additionally tried the intranasal (i.n.) and rectal routes and used enzyme-linked immunoassays to determine anti-Cry1Ac antibody responses in the serum as well as invaginal and tracheobronchial washes and in the fluids of the large and the small intestine. Immunization by the i.p., i.n. and rectal routes induced IgM, IgG and IgA antibodies in all the mucosal surfaces analyzed, but the magnitude and predominant isotype of each response depended on the route used and the mucosal site analyzed. These data extend our findings on the striking mucosal immunogencity of Cry1Ac and provide additional evidence on the compartmentalization of the mucosal immune system.

http://www.ncbi.nlm.nih.gov/pubmed/10962271

Sagstad, A.; Sanden, M.; Haugland, O.; Hansen A.; Olsvik P.; Hemre G. 2007. Evaluation of stress and immune-response biomarkers in Atlantic salmon, Salmo salar L., fed different levels of genetically modified maize (Bt maize), compared with its near-isogenic parental line and a commercial suprex maize. *Journal of Fish Diseases*, 30: 201–212.

The present study was designed to evaluate if genetically modified (GM) maize (Bt maize, event MON810) compared with the near-isogenic non-modified (nGM) maize variety, added as a starch source at low or high inclusions, affected fish health of post-smolt Atlantic salmon, Salmo salar L. To evaluate the health impact, selected stress- and immune-response biomarkers were quantified at the gene transcript (mRNA) level, and some also at the protein level. The diets with low or high inclusions of GM maize, and its near-isogenic nGM parental line, were compared to a control diet containing GM-free suprex maize (reference diet) as the only starch source. Total superoxide dismutase (SOD) activity in liver and distal intestine was significantly higher in fish fed GM maize compared with fish fed nGM maize and with the reference diet group. Fish fed GM maize showed significantly lower catalase (CAT) activity in liver compared with fish fed nGM maize and to the reference diet group. In contrast, CAT activity in distal intestine was significantly higher for fish fed GM maize compared with fish fed reference diet. Protein level of heat shock protein 70 (HSP70) in liver was significantly higher in fish fed GM maize compared with fish fed the reference diet. No diet-related differences were found in normalized gene expression of SOD, CAT or HSP70 in liver or distal intestine. Normalized gene expression of interleukin-1 beta in spleen and head-kidney did not vary significantly between diet groups. Interestingly, fish fed high GM maize showed a significantly larger proportion of plasma granulocytes, a significantly larger sum of plasma granulocyte and monocyte proportions, but a significantly smaller proportion of plasma lymphocytes, compared with fish fed high nGM maize. In conclusion, Atlantic salmon fed GM maize showed some small changes in stress protein levels and activities, but none of these changes were comparable to the normalized gene expression levels analysed for these stress proteins. GM maize seemed to induce significant changes in white blood cell populations which are associated with an immune response.

http://www.ncbi.nlm.nih.gov/pubmed/17394522

Finamore, A.; Roselli, M.; Britti, S.; Monastra, G.; Ambra, R.; Turrini, A.; Mengheri, E. 2008. Intestinal and peripheral immune response to MON810 maize ingestion in weaning and old mice. *Journal of Agricultural and Food Chemistry*, 56, 11533-11539.

This study evaluated the gut and peripheral immune response to genetically modified (GM) maize in mice in vulnerable conditions. Weaning and old mice were fed a diet containing MON810 or

its parental control maize or a pellet diet containing a GM-free maize for 30 and 90 days. The immunophenotype of intestinal intraepithelial, spleen, and blood lymphocytes of control maize fed mice was similar to that of pellet fed mice. As compared to control maize, MON810 maize induced alterations in the percentage of T and B cells and of CD4(+), CD8(+), gammadeltaT, and alphabetaT subpopulations of weaning and old mice fed for 30 or 90 days, respectively, at the gut and peripheral sites. An increase of serum IL-6, IL-13, IL-12p70, and MIP-1beta after MON810 feeding was also found. These results suggest the importance of the gut and peripheral immune response to GM crop ingestion as well as the age of the consumer in the GMO safety evaluation.

Full article available at https://goo.gl/3aJ5qP

Kroghsbo, S.; Madsen, C.; Poulsen, M.; Schrødera, M.; Kvista, P.; Taylor, M.; Gatehoused, A.; Shu, Q.; Knudsena, I. 2008. Immunotoxicological studies of genetically modified rice expressing PHA-E lectin or Bt toxin in Wistar rats. *Toxicology*, 245(1-2): 24-34.

As part of the SAFOTEST project the immunmodulating effect of Cry1Ab protein from *Bacillus thuringiensis*(Bt) and PHA-E lectin from kidney bean (*Phaseolus vulgaris* erythroagglutinin) was examined in 28- and 90-day feeding studies in Wistar rats. PHA-E lectin was chosen as positive control. Rats were fed control rice, transgenic rice expressing Cry1Ab protein or PHA-E lectin, or transgenic rice spiked with the purified recombinant protein. Total immunoglobulin levels, mitogen-induced cell proliferation, T-dependent antibody response to sheep red blood cells and the antigen-specific antibody response in serum were examined at the end of the studies. A dose-dependent increase in mesenteric lymph node weight and total immunoglobulin A was seen when feeding PHA-E transgenic rice alone or spiked with 0.1% purified PHA-E lectin for 90 days indicating a local effect of PHA-E in the intestine. No adverse effects of Cry1Ab protein were found. An anti-PHA-E and anti-Cry1Ab antibody response was induced both after inhalation (control groups) and after inhalation/ingestion (groups fed recombinant protein alone or together with transgenic rice). In conclusion, only PHA-E lectin was found to have an immunomodulating effect when feeding rats for 90 days with approximately 70 mg PHA-E/kg bodyweight per day. As both PHA-E lectin and Cry1Ab protein were capable of inducing an antigen-specific antibody response it is important to make careful considerations when designing future animal studies to avoid intake of proteins from the other groups by inhalation as well as to examine the sensitization and elicitation potential of 'foreign' proteins before introduction to the world market.

http://www.sciencedirect.com/science/article/pii/S0300483X07008281

Guimaraes, V.; Drumare, M.; Lereclus, D.; Gohar, M.; Lamourette, P.; Nevers, M.; Vaisanen-Tunkelrott, M.; Bernard, H.; Guillon, B.; Créminon, C.; Wal, J.; Adel-Patient, K. 2010. *In vitro* digestion of Cry1Ab proteins and analysis of the impact on their immunoreactivity. *J Agric Food Chem*, 58(5): 3222- 3231.

A pepsin resistance test performed at pH 1.2 and with high pepsin to protein ratio is one of the steps of the weight-of-evidence approach used for assessment of allergenicity of new proteins. However, the use of other in vitro digestibility tests, performed in more physiologically relevant conditions and in combination with immunological assays so as to increase the value of the information gained from the studies of stability of a novel protein to digestion for the overall allergenicity assessment, has been proposed. This study then aimed to investigate the stability to digestion of Cry1Abprotoxin and toxin, insecticidal proteins expressed in genetically modified crops, using simulated gastric fluid (SGF) at different pH values and pepsin-to-substrate ratios, in the presence or absence of physiological surfactant phosphatidylcholine (PC). Electrophoresis and immunoblot patterns and residual immunoreactivity of digesta were analyzed. Although Cry1Ab protoxin is extensively degraded at pH 1.2 with high pepsin-to-protein ratio, it is only slightly degraded at pH

2.0 and conserved its immunoreactivity. Furthermore, Cry1Ab proteins were demonstrated to be stable in a more physiologically relevant in vitro digestibility test (pH 2.5, pepsin-to-substrate ratio 1:20 (w/w) with PC). Factors such as pH, SGF composition, and pepsin-to-substrate ratio then greatly influence the digestion of Cry1Ab proteins, confirming that new and more physiologically relevant in vitro digestibility tests should be also considered to study the relationship between the resistance of a protein to digestion and its allergenicity.

http://www.ncbi.nlm.nih.gov/pubmed/20136083

Walsh, M.; Buzoianu, S.; Gardiner, G.; Rea, M.; Gelencsér, E.; Jánosi, A.; Epstein, M.; Ross, R.; Lawlor, P. 2011. Fate of transgenic DNA from orally administered Bt MON810 maize and effects on immune response and growth in pigs. *PLOS ONE*, 6(11): e27177, doi:10.1371/journal.pone.0027177.

We assessed the effect of short-term feeding of genetically modified (GM: Bt MON810) maize on immune responses and growth in weanling pigs and determined the fate of the transgenic DNA and protein in-vivo. Pigs were fed a diet containing 38.9% GM or non-GM isogenic parent line maize for 31 days. We observed that IL-12 and IFNγ production from mitogenic stimulated peripheral blood mononuclear cells decreased ($P<0.10$) following 31 days of GM maize exposure. While Cry1Ab-specific IgG and IgA were not detected in the plasma of GM maize-fed pigs, the detection of the cry1Ab gene and protein was limited to the gastrointestinal digesta and was not found in the kidneys, liver, spleen, muscle, heart or blood. Feeding GM maize to weanling pigs had no effect on growth performance or body weight. IL-6 and IL-4 production from isolated splenocytes were increased ($P<0.05$) in response to feeding GM maize while the proportion of CD4(+) T cells in the spleen decreased. In the ileum, the proportion of B cells and macrophages decreased while the proportion of CD4(+) T cells increased in GM maize-fed pigs. IL-8 and IL-4 production from isolated intraepithelial and lamina propria lymphocytes were also increased ($P<0.05$) in response to feeding GM maize. In conclusion, there was no evidence of cry1Ab gene or protein translocation to the organs and blood of weaning pigs. The growth of pigs was not affected by feeding GM maize. Alterations in immune responses were detected; however, their biologic relevance is questionable.

Full article available at http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0027177

Gu, J.; Krogdahl, Å.; Sissener, N.; Kortner, T.; Gelencser, E.; Hemre, G.; Bakke, A. 2013. Effects of oral Bt-maize (MON810) exposure on growth and health parameters in normal and sensitised Atlantic salmon, Salmo salar L. *Br J Nutr*, 109(8):1408-23.

Responses to GM maize Bt-maize, MON810) expressing Cry1Ab protein from the soil bacterium Bacillus thuringiensis (Bt) in diets for both normal and immune-sensitised (with soyabean meal (SBM)-induced enteropathy) post-smolt Atlantic salmon were investigated following 33 and 97 d of exposure. Triplicate tanks of salmon were fed one of four diets, all containing 20% whole-kernel meal maize, either Bt-maize or its near-isogenic maternal line, without or with 15% extracted SBM inclusion. The fish fed Bt-maize utilised the feed less efficiently, as revealed by lower protein and mineral digestibilities and lower lipid and energy retention efficiencies. Higher intestinal weight, as well as increased interferon-γ and decreased sodium-glucose co-transporter mRNA expression, and a transient increase in T-helper cell presence, as measured by cluster of differentiation 4 (CD4) protein in the distal intestine (DI), may partly explain the lower nutrient digestibilities and retentions. The Bt-maize seemed to potentiate oxidative cellular stress in the DI of immune-sensitised fish, as indicated by increases in superoxide dismutase and heat shock protein 70 mRNA expression. The data suggest that Cry1Ab protein or other antigens in Bt-maize have local immunogenic effects in salmon DI. No systemic immune responses could be detected, as indicated by haematology, differential leucocyte

counts, plasma clinical chemistry, as well as absence of Cry1Ab-specific antibodies and Cry1Ab protein in plasma. The responses to Bt-maize observed in the present study differed from results from earlier studies in salmon and other animals fed the same event Bt-maize. Longer-term experiments and more in-depth studies on intestinal physiology and immune responses are needed to evaluate health implications.

http://www.ncbi.nlm.nih.gov/pubmed/23182224

1.4 Toxicological risks associated to the consumption of Bt plants

Although inserted in a significant part of the animal and human agricultural-food chains from a decade ago, the debate about toxicological risks associated with consumption of Bt plants remains unfinished. The scientific community differs on the size of potential problems and the validity of evidence pointing their absence.

Recent studies accumulate evidence of toxicity and liver-kidney damage in animals that consume transgenic Bt plants for long term. Other[54] studies, when identifying statistically significant differences, begin to interpret them as biologically irrelevant, dismissing its importance as indicative of risk for consumption. In both cases, a cautious approach would recommend the development of new, more detailed assays, before any conclusion concerning the biosafety of these GMPs.

Séralini, G-E.; Cellier, D.; de Vendomois, J. 2007. New analysis of a rat feeding study with a genetically modified maize reveals signs of hepatorenal toxicity. *Archives Environmental Contamination and Toxicology*, 52, 596-602.

Health risk assessment of genetically modified organisms (GMOs) cultivated for food or feed is under debate throughout the world, and very little data have been published on mid- or long-term toxicological studies with mammals. One of these studies performed under the responsibility

54 Such as Hammond *et al.*, 2006 (Results of a 90-day safety assurance study with rats fed grain from corn borer-protected corn. *Food Chem Toxicol.* 2006, 44:1092-1099); Hammond et al., 2006b (Results of a 90-day safety assurance study with rats fed grain from corn rootworm-protected corn. *Food Chem Toxicol.* 2006, 44:147160); Trabalza-Marinucci *et al.*, 2008 (A three-year longitudinal study on the effects of a diet containing genetically modified Bt176 maize on the health status and performance of sheep. Livestock Science. 2008; 113(2): 178–190); or Buzoianu *et al.,* 2013 (Transgenerational effects of feeding genetically modified maize to nulliparous sows and offspring on offspring growth and health. *J Anim Sci.* 2013 Jan;91(1):318-30).

of Monsanto Company with a transgenic corn MON863 has been subjected to questions from regulatory reviewers in Europe, where it was finally approved in 2005. This necessitated a new assessment of kidney pathological findings, and the results remained controversial. An Appeal Court action in Germany (Münster) allowed public access in June 2005 to all the crude data from this 90-day rat-feeding study. We independently re-analyzed these data. Appropriate statistics were added, such as a multivariate analysis of the growth curves, and for biochemical parameters comparisons between GMO-treated rats and the controls fed with an equivalent normal diet, and separately with six reference diets with different compositions. We observed that after the consumption of MON863, rats showed slight but dose-related significant variations in growth for both sexes, resulting in 3.3% decrease in weight for males and 3.7% increase for females. Chemistry measurements reveal signs of hepatorenal toxicity, marked also by differential sensitivities in males and females. Triglycerides increased by 24-40% in females (either at week 14, dose 11% or at week 5, dose 33%, respectively); urine phosphorus and sodium excretions diminished in males by 31-35% (week 14, dose 33%) for the most important results significantly linked to the treatment in comparison to seven diets tested. Longer experiments are essential in order to indicate the real nature and extent of the possible pathology; with the present data it cannot be concluded that GM corn MON863 is a safe product.

Full article available at https://goo.gl/hHYxyq

Aysun, K.; Akay, M. 2008. A three generation study with genetically modified Bt corn in rats: Biochemical and histopathological investigation. *Food and Chemical Toxicology*, 46, 1164–1170.

For the last ten years, in accordance with the increased use of genetically modified (GM) foods for human and livestock, a large number of feeding studies have been carried out. However, the evidence is still far from proving whether the long-term consumption of GM foods poses a possible danger for human or animal health. Therefore, this study was designed to evaluate the effects of transgenic corn on the rats that were fed through three generations with either GM corn or its conventional counterpart. Tissue samples of stomach, duodenum, liver and kidney were obtained for histopathological examinations. The average diameter of glomeruli, thickness of renal cortex and glomerular volume were calculated and number of affected animals/number of examined animals for liver and kidney histopathology were determined. Amounts of urea, urea nitrogen, creatinine, uric acid, total protein, albumin and globulin were determined; enzyme activities of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyltransferase, creatine kinase and amylase were measured in serum samples. No statistically significant differences were found in relative organ weights of rats within groups but there were some minimal histopathological changes in liver and kidney. Changes in creatinine, total protein and globulin levels were also determined in biochemical analysis.

Full article available at https://goo.gl/uObGMD

Vendômois, J.; Roullier, F.; Cellier, D.; Seralini, G-E. 2009. A comparison of the effects of three GM corn varieties on mammalian health. *International Journal of Biological Sciences*, 5, 706–726.

We present for the first time a comparative analysis of blood and organ system data from trials with rats fed three main commercialized genetically modified (GM) maize (NK 603, MON 810, MON 863), which are present in food and feed in the world. NK 603 has been modified to be tolerant to the broad spectrum herbicide Roundup and thus contains residues of this formulation. MON 810 and MON 863 are engineered to synthesize two different Bt toxins used as insecticides. Approximately 60 different biochemical parameters were classified per organ and measured in serum and urine after 5 and 14 weeks of feeding. GM maize-fed rats were compared first to their respective isogenic or parental non-GM equivalent control

groups. This was followed by comparison to six reference groups, which had consumed various other non-GM maize varieties. We applied nonparametric methods, including multiple pairwise comparisons with a False Discovery Rate approach. Principal Component Analysis allowed the investigation of scattering of different factors (sex, weeks of feeding, diet, dose and group). Our analysis clearly reveals for the 3 GMOs new side effects linked with GM maize consumption, which were sex- and often dose-dependent. Effects were mostly associated with the kidney and liver, the dietary detoxifying organs, although different between the 3 GMOs. Other effects were also noticed in the heart, adrenal glands, spleen and haematopoietic system. We conclude that these data highlight signs of hepatorenal toxicity, possibly due to the new pesticides specific to each GM corn. In addition, unintended direct or indirect metabolic consequences of the genetic modification cannot be excluded.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2793308/

Séralini, G-E.; Vendômois, J.; Cellier, D.; Sultan, C.; Buiatti, M.; Gallagher, L.; Antoniou, M.; Dronamraju, K. 2009. How subchronic and chronic health effects can be neglected for GMOs, pesticides or chemicals. *International Journal of Biological Sciences*, 5:438-443.

Chronic health effects are increasing in the world such as cancers, hormonal, reproductive, nervous, or immune diseases, even in young people. During regulatory toxicological subchronic tests to prevent these on mammalian health, prior commercialization of chemicals, including pesticides and drugs, or GMOs, some statistically significant findings may be revealed. This discussion is about the need to investigate the relevant criteria to consider those as biologically significant. The sex differences and the non linear dose or time related effects should be considered in contrast to the claims of a Monsanto-supported expert panel about a GMO, the MON 863 Bt maize, but also for pesticides or drugs, in particular to reveal hormone-dependent diseases and first signs of toxicities.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2706426/

Adel-Patient, K.; Guimaraes, V.; Paris, A.; Drumare, M.; Ah-Leung, S.; Lamourette, P.; Nevers, M.; Canlet, C.; Molina, J.; Bernard, H.; Creminon, C.; Wal, J. 2011. Immunological and metabolomic impacts of administration of Cry1Ab protein and MON 810 maize in mouse. *PLOS ONE*, 6(1): e16346. doi:10.1371/journal.pone.0016346.

We have investigated the immunological and metabolomic impacts of Cry1Ab administration to mice, either as a purified protein or as the Cry1Ab-expressing genetically modified (GM) MON810 maize. Humoral and cellular specific immune responses induced in BALB/cJ mice after intra-gastric (i.g.) or intra-peritoneal (i.p.) administration of purified Cry1Ab were analyzed and compared with those induced by proteins of various immunogenic and allergic potencies. Possible unintended effects of the genetic modification on the pattern of expression of maize natural allergens were studied using IgE-immunoblot and sera from maize-allergic patients. Mice were experimentally sensitized (i.g. or i.p. route) with protein extracts from GM or non-GM maize, and then anti-maize proteins and anti-Cry1Ab-induced immune responses were analyzed. In parallel, longitudinal metabolomic studies were performed on the urine of mice treated via the i.g. route. Weak immune responses were observed after i.g. administration of the different proteins. Using the i.p. route, a clear Th2 response was observed with the known allergenic proteins, whereas a mixed Th1/Th2 immune response was observed with immunogenic protein not known to be allergenic and with Cry1Ab. This then reflects protein immunogenicity in the BALB/c Th2-biased mouse strain rather than allergenicity. No difference in natural maize allergen profiles was evidenced between MON810 and its non-GM comparator. Immune responses against maize proteins were quantitatively equivalent in mice treated with MON810 vs the non-GM counterpart and no anti-Cry1Ab-specific immune response was detected in mice that received MON810. Metabolomic studies showed a

slight "cultivar" effect, which represented less than 1% of the initial metabolic information. Our results confirm the immunogenicity of purified Cry1Ab without evidence of allergenic potential. Immunological and metabolomic studies revealed slight differences in mouse metabolic profiles after i.g. administration of MON810 vs its non-GM counterpart, but no significant unintended effect of the genetic modification on immune responses was seen.

Full article available at http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0016346

Gab-Alla, A.; El-Shamei, Z.; Shatta, A.; Moussa, E.; Rayan, A. 2012. Morphological and biochemical changes in male rats fed on genetically modified corn (Ajeeb YG). *Journal of American Science*, 8(9) (152).

This study was designed to evaluate the safety of genetically modified (GM) corn (Ajeeb YG). Corn grains from Ajeeb YG or its control (Ajeeb) were incorporated into rodent diets at 30% concentrations administered to rats (n= 10/group) for 45 and 91 days. An additional negative control group of rats (n= 10/group) was fed AIN93G diets. General conditions were observed daily, total body weights were recorded weekly. At the termination of the study periods, some visceral organs (heart, liver, kidneys, testes and spleen) and serum biochemistry were measured. The data showed several statistically significant differences in organs/body weight and serum biochemistry between the rats fed on GM and/or Non-GM corn and the rats fed on AIN93G diets. In general, GM corn sample caused several changes by increase or decrease organs/body weight or serum biochemistry values. This indicates potential adverse health/toxic effects of GM corn and further investigations still needed.

Full article available at https://goo.gl/QL0J7N

El-Shamei, Z.; Gab-Alla, A.; Shatta, A.; Moussa, E.; Rayan, A. 2012. Histopathological changes in some organs of males rats fed on genetically modified corn (Ajeeb YG). *Journal of American Science*, 8(10).

Ajeeb YG is a genetically modified (GM) insect resistant corn produced by incorporated the MON 810 (Monsanto) borer resistance trait in the best corn germplasm Ajeeb. The safety of Ajeeb YG corn was assessed by comparison of toxicology response variables in rats consuming diets containing Ajeeb YG with those containing Ajeeb corn grains. Corn grains from Ajeeb YG or Ajeeb were incorborated into rodent diets at 30% concentrations administered to rats (n= 10/group) for 91 days. An additional negative control group of rats (n= 10/group) were fed AIN93G diets. Rats fed on GM corn showed histopathological changes. Liver displayed cytoplasmic vacuolation of centrolobular hepatocytes and fatty degeneration of hepatocytes. Kidneys showed congestion of renal blood vessels and cystic dilatation of renal tubules. Testes revealed necrosis and desquamation of spermatogoneal germ cells lining seminiferous tubules. Spleen showed slight lymphocytic depletion and splenic congestion. Small intestine showed hyperplasia, hyperactivation of mucous secretory glands and necrosis of intestinal villi were detected. Due to these observations, we suggest that the risk of GM crops cannot be ignored and deserves further investigations in order to identify possible long-term effects, if any, of GM food consumption that might help in the post market surveillance.

Full article available at https://goo.gl/B9hLDb

Carman, J.; Vlieger, H.; Ver Steeg, L.; Sneller, V.; Robinson, G.; Clinch-Jones, C.; Haynes, J.; Edwards, J. 2013. A long-term toxicology study on pigs fed a combined genetically

modified (GM) soy and GM maize diet. *Journal of Organic Systems*, 8 (1): 38-54[55].

A significant number of genetically modified (GM) crops have been approved to enter human food and animal feed since 1996, including crops containing several GM genes 'stacked' into the one plant. We randomised and fed isowean pigs (N=168) either a mixed GM soy and GM corn (maize) diet (N=84) or an equivalent non-GM diet (N=84) in a longterm toxicology study of 22.7 weeks (the normal lifespan of a commercial pig from weaning to slaughter). Equal numbers of male and female pigs were present in each group. The GM corn contained double and triple-stacked varieties. Feed intake, weight gain, mortality and blood biochemistry were measured. Organ weights and pathology were determined post-mortem. There were no differences between pigs fed the GM and non-GM diets for feed intake, weight gain, mortality, and routine blood biochemistry measurements. The GM diet was associated with gastric and uterine differences in pigs. GM-fed pigs had uteri that were 25% heavier than non-GM fed pigs (p=0.025). GM-fed pigs had a higher rate of severe stomach inflammation with a rate of 32% of GM-fed pigs compared to 12% of non-GM-fed pigs (p=0.004). The severe stomach inflammation was worse in GM-fed males compared to non-GM fed males by a factor of 4.0 (p=0.041), and GM-fed females compared to non-GM fed females by a factor of 2.2 (p=0.034).

Full article available at http://www.organic-systems.org/journal/81/8106.pdf

2 Health risks associated with the use of herbicide-tolerant plants

2.1 Specific negative impacts related to the main herbicides associated with the cultivation of transgenic plants of the type HT

Much of the risks to human health, involving planting, consumption or handling of transgenic plants of the type HT, comes from the intrinsic relationship that they have with certain herbicides.

Despite this fact, the health (and environmental) risk assessments validated in Brazil by the National Biosafety Technical Commission (CTNBio) disregard herbicides, do not evaluate the presence of their toxic residues in crops and GM foods.

Articles gathered below demonstrate some types of damage to human health, from the contact with pesticides systematically associated with the cultivation of HT plants (commercial formulations of herbicides to glyphosate, 2,4-D and gluphosinate

55 This study used as the basis of Genetically Modified diet a mix of glyphosate tolerant corn, Bt corn and soybeans tolerant to glyphosate. Therefore, this reference consists of three items of that part, being 1.4, 2.2.1 and 2.2.2.

ammonium, in particular). The focus in this item is the health of rural workers.

Hardell, L.; Eriksson, M.; Lenner, P.; Lundgren, E. 1981. Malignant lymphoma and exposure to chemicals especially organic solvents, chlorophenols and phenoxy acids: A case-control study. *Br J Cancer*, 43:169-176.

A number of men with malignant lymphoma of the histiocytic type and previous exposure to phenoxy acids or chlorophenols were observed and reported in 1979. A matched case-control study has therefore been performed with cases of malignant lymphoma (Hodgkin's disease and non-Hodgkin lymphoma). This study included 169 cases and 338 controls. The results indicate that exposure to phenoxy acids, chlorophenols, and organic solvents may be a causative factor in malignant lymphoma. Combined exposure of these chemicals seemed to increase the risk. Exposure to various other agents was not obviously different in cases and in controls.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2010513/

Hietanen, E.; Linnainmaa, K.; Vainio, H. 1983. Effects of phenoxy herbicides and glyphosate on the hepatic and intestinal biotransformation activities in the rat. *Acta Pharmacol Toxicol*, 53, 103-112.

The effects of phenoxyacid herbicides 2,4-D (2,4-dichlorophenoxyacetic acid) and MCPA (4-chloro-2-methylphenoxyacetic acid), clofibrate, and glyphosate on hepatic and intestinal drug metabolizing enzyme activities were studied in rats intragastrically exposed for 2 weeks. The hepatic ethoxycoumarin O-deethylase activity increased about 2-fold with MCPA. Both 2,4-D and MCPA increased the hepatic epoxide hydrolase activity and decreased the hepatic glutathione S-transferase activity. MCPA also increased the intestinal activities of ethoxycoumarin O-deethylase and epoxide hydrolase. Glyphosate decreased the hepatic level of cytochrome P-450 and monooxygenase activities and the intestinal activity of aryl hydrocarbon hydroxylase. Clofibrate decreased the hepatic activities of UDPglucuronosyltransferase with p-nitrophenol or methylumbelliferone as the substrate. Also 2,4-D decreased the hepatic activity of UDPglucuronosyltransferase with p-nitrophenol as the substrate. MCPA decreased the intestinal activities of UDPglucuronosyltransferase with either p-nitrophenol or methylumbelliferone as the substrate. The results indicate that phenoxyacetic acids, especially MCPA, may have potent effects on the metabolism of xenobiotics. Glyphosate, not chemically related to phenoxyacids, seems to inhibit monooxygenases. Whether these changes are related to the toxicity of these xenobiotics remains to be clarified in further experiments.

http://www.ncbi.nlm.nih.gov/pubmed/6624478

EPI - Savitz, D.; Arbuckle, T.; Kaczor, D.; Curtis, K. 1997. Male pesticide exposure and pregnancy outcome. *Am J Epidemiol*, 146(12): 1025-1036.

Potential health effects of agricultural pesticide use include reproductive outcomes. For the Ontario Farm Family Health Study, the authors sampled Ontario farms from the 1986 Canadian Census of Agriculture, identified farm couples, and obtained questionnaire data concerning farm activities, reproductive health experience, and chemical applications. Male farm activities in the period from 3 months before conception through the month of conception were evaluated in relation to miscarriage, preterm delivery, and small-for-gestational-age births. Among the 1,898 couples

with complete data (64% response), 3,984 eligible pregnancies were identified. Miscarriage was not associated with chemical activities overall but was increased in combination with reported use of thiocarbamates, carbaryl, and unclassified pesticides on the farm. Preterm delivery was also not strongly associated with farm chemical activities overall, except for mixing or applying yard herbicides (odds ratio = 2.1, 95% confidence interval 1.0-4.4). Combinations of activities with a variety of chemicals (atrazine, glyphosate, organophosphates, 4-[2,4-dichlorophenoxy] butyric acid, and insecticides) generated odds ratios of two or greater. No associations were found between farm chemicals and small-for-gestational-age births or altered sex ratio. Based on these data, despite limitations in exposure assessment, the authors encourage continued evaluation of male exposures, particularly in relation to miscarriage and preterm delivery.

http://www.ncbi.nlm.nih.gov/pubmed/9420527

EPI - McDuffie, H.; Pahwa, P.; McLaughlin, J.; Spinelli, J.; Fincham, S.; Dosman, J.; Robson, D.; Skinnider, L.; Choi, N. 2001. Non-Hodgkin's lymphoma and specific pesticide exposures in men: Cross-Canada study of pesticides and health. *Cancer Epidemiol Biomarkers Prev*, 10(11):1155-63.

Our objective in the study was to investigate the putative associations of specific pesticides with non-Hodgkin's Lymphoma [NHL; International Classification of Diseases, version 9 (ICD-9) 200, 202]. We conducted a Canadian multicenter population-based incident, case (n = 517)-control (n = 1506) study among men in a diversity of occupations using an initial postal questionnaire followed by a telephone interview for those reporting pesticide exposure of 10 h/year or more, and a 15% random sample of the remainder. Adjusted odds ratios (ORs) were computed using conditional logistic regression stratified by the matching variables of age and province of residence, and subsequently adjusted for statistically significant medical variables (history of measles, mumps, cancer, allergy desensitization treatment, and a positive history of cancer in first-degree relatives). We found that among major chemical classes of herbicides, the risk of NHL was statistically significantly increased by exposure to phenoxyherbicides [OR, 1.38; 95% confidence interval (CI), 1.06-1.81] and to dicamba (OR, 1.88; 95% CI, 1.32-2.68). Exposure to carbamate (OR, 1.92; 95% CI, 1.22-3.04) and to organophosphorus insecticides (OR, 1.73; 95% CI, 1.27-2.36), amide fungicides, and the fumigant carbon tetrachloride (OR, 2.42; 95% CI, 1.19-5.14) statistically significantly increased risk. Among individual compounds, in multivariate analyses, the risk of NHL was statistically significantly increased by exposure to the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D; OR, 1.32; 95% CI, 1.01-1.73), mecoprop (OR, 2.33; 95% CI, 1.58-3.44), and dicamba (OR, 1.68; 95% CI, 1.00-2.81); to the insecticides malathion (OR, 1.83; 95% CI, 1.31-2.55), 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT), carbaryl (OR, 2.11; 95% CI, 1.21-3.69), aldrin, and lindane; and to the fungicides captan and sulfur compounds. In additional multivariate models, which included exposure to other major chemical classes or individual pesticides, personal antecedent cancer, a history of cancer among first-degree relatives, and exposure to mixtures containing dicamba (OR, 1.96; 95% CI, 1.40-2.75) or to mecoprop (OR, 2.22; 95% CI, 1.49-3.29) and to aldrin (OR, 3.42; 95% CI, 1.18-9.95) were significant independent predictors of an increased risk for NHL, whereas a personal history of measles and of allergy desensitization treatments lowered the risk. We concluded that NHL was associated with specific pesticides after adjustment for other independent predictors.

http://www.ncbi.nlm.nih.gov/pubmed/11700263

EPI - Garry, V.; Harkins, M.; Erickson, L.; Long-Simpson, L.; Holland, S.; Burroughs, B. 2002. Birth defects, season of conception, and sex of children born to pesticide applicators living in the red river Valley of Minnesota, USA. *Environmental Health Perspectives*, Volume 110, Supplement 3.

We previously demonstrated that the frequency of birth defects among children of residents of the Red River Valley (RRV), Minnesota, USA, was significantly higher than in other major agricultural regions of the state during the years 1989-1991, with children born to male pesticide applicators having the highest risk. The present, smaller cross-sectional study of 695 families and 1,532 children, conducted during 1997-1998, provides a more detailed examination of reproductive health outcomes in farm families ascertained from parent-reported birth defects. In the present study, in the first year of life, the birth defect rate was 31.3 births per 1,000, with 83% of the total reported birth defects confirmed by medical records. Inclusion of children identified with birth or developmental disorders within the first 3 years of life and later led to a rate of 47.0 per 1,000 (72 children from 1,532 live births). Conceptions in spring resulted in significantly more children with birth defects than found in any other season (7.6 vs. 3.7%). Twelve families had more than one child with a birth defect (n = 28 children). Forty-two percent of the children from families with recurrent birth defects were conceived in spring, a significantly higher rate than that for any other season. Three families in the kinships defined contributed a first-degree relative other than a sibling with the same or similar birth defect, consistent with a Mendelian inheritance pattern. The remaining nine families did not follow a Mendelian inheritance pattern. The sex ratio of children with birth defects born to applicator families shows a male predominance (1.75 to 1) across specific pesticide class use and exposure categories exclusive of fungicides. In the fungicide exposure category, normal female births significantly exceed male births (1.25 to 1). Similarly, the proportion of male to female children with birth defects is significantly lower (0.57 to 1; p = 0.02). Adverse neurologic and neurobehavioral developmental effects clustered among the children born to applicators of the fumigant phosphine (odds ratio [OR] = 2.48; confidence interval [CI], 1.2-5.1). Use of the herbicide glyphosate yielded an OR of 3.6 (CI, 1.3-9.6) in the neurobehavioral category. Finally, these studies point out that (a) herbicides applied in the spring may be a factor in the birth defects observed and (b) fungicides can be a significant factor in the determination of sex of the children of the families of the RRV. Thus, two distinct classes of pesticides seem to have adverse effects on different reproductive outcomes. Biologically based confirmatory studies are needed.

Full article available at https://goo.gl/GQ4ct0

EPI – Hardell, L.; Eriksson, M.; Nordstrom, M. 2002. Exposure to pesticides as risk factor for non-Hodgkin's lymphoma and hairy cell leukemia: pooled analysis of two Swedish case-control studies. *Leuk Lymphoma*, 43(5): 1043-1049.

Increased risk for non-Hodgkin's lymphoma (NHL) following exposure to certain pesticides has previously been reported. To further elucidate the importance of phenoxyacetic acids and other pesticides in the etiology of NHL a pooled analysis was performed on two case-control studies, one on NHL and another on hairy cell leukemia (HCL), a rare subtype of NHL. The studies were population based with cases identified from cancer registry and controls from population registry. Data assessment was ascertained by questionnaires supplemented over the telephone by specially trained interviewers. The pooled analysis of NHL and HCL was based on 515 cases and 1141 controls. Increased risks in univariate analysis were found for subjects exposed to herbicides (OR 1.75, CI 95% 1.26-2.42), insecticides (OR 1.43, CI 95% 1.08-1.87), fungicides (OR 3.11, CI 95% 1.56-6.27) and impregnating agents (OR 1.48, CI 95% 1.11-1.96). Among herbicides, significant associations were found for glyphosate (OR 3.04, CI 95% 1.08-8.52) and 4-chloro-2-methyl phenoxyacetic acid (MCPA) (OR 2.62, CI 95% 1.40-4.88). For several categories of pesticides the highest risk was found for exposure during the latest decades before diagnosis. However, in multivariate analyses the only significantly increased risk was for a heterogeneous category of other herbicides than above.

http://www.ncbi.nlm.nih.gov/pubmed/12148884

Greenlee, A.; Ellis, T.; Berg, R. 2004. Low-dose agrochemicals and lawn-care pesticides

induce developmental toxicity in murine preimplantation embryos. *Environmental Health Perspectives*, vol. 112 (6), p. 703-709.

Occupational exposures to pesticides may increase parental risk of infertility and adverse pregnancy outcomes such as spontaneous abortion, preterm delivery, and congenital anomalies. Less is known about residential use of pesticides and the risks they pose to reproduction and development. In the present study we evaluate environmentally relevant, low-dose exposures to agrochemicals and lawn-care pesticides for their direct effects on mouse preimplantation embryo development, a period corresponding to the first 5-7 days after human conception. Agents tested were those commonly used in the upper midwestern United States, including six herbicides [atrazine, dicamba, metolachlor, 2,4-dichlorophenoxyacetic acid (2,4-D)], pendimethalin, and mecoprop), three insecticides (chlorpyrifos, terbufos, and permethrin), two fungicides (chlorothalonil and mancozeb), a desiccant (diquat), and a fertilizer (ammonium nitrate). Groups of 20-25 embryos were incubated 96 hr in vitro with either individual chemicals or mixtures of chemicals simulating exposures encountered by handling pesticides, inhaling drift, or ingesting contaminated groundwater. Incubating embryos with individual pesticides increased the percentage of apoptosis (cell death) for 11 of 13 chemicals (p <or= 0.05) and reduced development to blastocyst and mean cell number per embryo for 3 of 13 agents (p <or= 0.05). Mixtures simulating preemergent herbicides, postemergent herbicides, and fungicides increased the percentage of apoptosis in exposed embryos (p <or= 0.05). Mixtures simulating groundwater contaminants, insecticide formulation, and lawn-care herbicides reduced development to blastocyst and mean cell number per embryo (p <or= 0.05). Our data demonstrate that pesticide-induced injury can occur very early in development, with a variety of agents, and at concentrations assumed to be without adverse health consequences for humans.

Full article available at https://goo.gl/DV1eJ8

REV – Kamel, F.; Hoppin, J. 2004. Association of pesticide exposure with neurologic dysfunction and disease. *Environmental Health Perspectives*, Volume 112, number 9.

Poisoning by acute high-level exposure to certain pesticides has well-known neurotoxic effects, but whether chronic exposure to moderate levels of pesticides is also neurotoxic is more controversial. Most studies of moderate pesticide exposure have found increased prevalence of neurologic symptoms and changes in neurobehavioral performance, reflecting cognitive and psychomotor dysfunction. There is less evidence that moderate exposure is related to deficits in sensory or motor function or peripheral nerve conduction, but fewer studies have considered these outcomes. It is possible that the most sensitive manifestation of pesticide neurotoxicity is a general malaise lacking in specificity and related to mild cognitive dysfunction, similar to that described for Gulf War syndrome. Most studies have focused on organophosphate insecticides, but some found neuro-toxic effects from other pesticides, including fungicides, fumigants, and organochlorine and carbamate insecticides. Pesticide exposure may also be associated with increased risk of Parkinson disease; several classes of pesticides, including insecticides, herbicides, and fungicides, have been implicated. Studies of other neurodegenerative diseases are limited and inconclusive. Future studies will need to improve assessment of pesticide exposure in individuals and consider the role of genetic susceptibility. More studies of pesticides other than organophosphates are needed. Major unresolved issues include the relative importance of acute and chronic exposure, the effect of moderate exposure in the absence of poisoning, and the relationship of pesticide-related neurotoxicity to neurodegenerative disease.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1247187/

EPI – Rull, R.; Ritz, B.; Shaw, G. 2006. Neural tube defects and maternal residential proximity to agricultural pesticide applications. *American Journal of Epidemiology*, 163 (8): 743-753.

Residential proximity to applications of agricultural pesticides may be an important source of exposure to agents that have been classified as developmental toxins. Data on two case-control

study populations of infants with neural tube defects (NTDs) and nonmalformed controls delivered in California between 1987 and 1991 were pooled to investigate whether maternal residential proximity to applications of specific pesticides or physicochemical groups of pesticides during early gestation increases the risk of these malformations. Maternal residential proximity within 1,000 m of pesticide applications was ascertained by linking mothers' addresses with agricultural pesticide use reports and crop maps. Odds ratios were computed by using conventional single- and multiple-pesticide and hierarchical multiple-pesticide logistic regression. In single-pesticide models, several pesticides were associated with NTDs after adjustment for study population, maternal ethnicity, educational level, cigarette smoking, and vitamin use. In a hierarchical multiple-pesticide model, effect estimates for only benomyl and methomyl suggested a possible association. Elevated risks of NTDs and anencephaly or spina bifida subtypes were also associated with exposures to chemicals classified as amide, benzimidazole, methyl carbamate, or organophosphorus pesticides and with increasing numbers of pesticides. These results suggest that ambient exposure to certain categories of agricultural pesticides may increase the risk of NTDs.

Full article available at http://aje.oxfordjournals.org/content/163/8/743.long

REV - Benitez-Leite, S.; Macchi, M.; Acosta, M. 2007. Malformaciones congénitas asociadas a agrotóxicos. *Pediatr (Asunción)*, Vol. 34; nº 2; 2007.

Introducción: La exposición a plaguicidas es un riesgo reconocido para la salud humana. Se describe la relación entre la exposición de los padres y malformaciones congénitas en el neonato.
Objetivo: Estudiar la asociación entre la exposición a pesticidas y malformaciones congénitas en neonatos nacidos en el Hospital Regional de Encarnación, Itapúa- Paraguay.
Material y Método: Estudio prospectivo de casos y controles de marzo/2006 a febrero/2007. Se consideró caso a todo neonato con malformación congénita, y control a todo niño sano del mismo sexo que naciera inmediatamente después. No se incluyeron los nacimientos ocurridos fuera del hospital. Se consideró exposición a cualquier contacto con agroquímicos, así como a otros factores de riesgo conocidos para malformación congénita.
Resultados: Se analizaron 52 casos y 87 controles. El promedio de nacimientos por mes fue de 216. Los factores de riesgo asociados significativamente fueron: vivir cerca de campos fumigados (OR 2.46 IC95%1.09- 5.57,p<0,02), vivienda ubicada a <1Km (OR=2,66 IC 95% 1,19 – 5,97), p<0,008), almacenamiento de plaguicidas en el hogar (OR 15,35 IC95%1.96-701,63 p<0.003), contacto en forma directa o accidental con plaguicidas(OR3.19 IC95%0,97-11.4, p<0.04), antecedente de malformación en la familia (OR 6,81 IC5% 1.94-30,56, p<0.001). Los demás factores de riesgo conocidos para malformaciones no tuvieron significancia estadística.
Conclusión: Los resultados muestran una asociación entre exposición a pesticidas y malformaciones congénitas. Se requiere de estudios futuros para confirmar estos hallazgos.

Full article available at https://goo.gl/5G6fK8

REV - Eriksson, M.; Hardell, L.; Carlberg, M.; Akerman, M. 2008. Pesticide exposure as risk factor for non-Hodgkin lymphoma including histopathological subgroup analysis. *International Journal of Cancer*, 123,1657-1663.

We report a population based case-control study of exposure to pesticides as risk factor for non-Hodgkin lymphoma (NHL). Male and female subjects aged 18-74 years living in Sweden were included during December 1, 1999, to April 30, 2002. Controls were selected from the national population registry. Exposure to different agents was assessed by questionnaire. In total 910 (91 %) cases and 1016 (92%) controls participated. Exposure to herbicides gave odds ratio (OR) 1.72, 95% confidence interval (CI) 1.18-2.51. Regarding phenoxyacetic acids highest risk was calculated for MCPA; OR 2.81, 95% CI 1.27-6.22, all these cases had a latency period >10 years. Exposure to glyphosate gave OR 2.02, 95% CI 1.10-3.71 and with >10 years latency period OR 2.26, 95% CI 1.16-4.40. Insecticides overall gave OR 1.28, 95% CI 0.96-1.72 and impregnating agents OR 1.57,

95% CI 1.07-2.30. Results are also presented for different entities of NHL. In conclusion our study confirmed an association between exposure to phenoxyacetic acids and NHL and the association with glyphosate was considerably strengthened.

http://www.ncbi.nlm.nih.gov/pubmed/18623080

Benedetti, D.; Nunes, E.; Sarmento, M.; Porto, C.; Santos, C.; Dias, J.; Silva, J. 2013. Genetic damage in soybean workers exposed to pesticides: evaluation with the comet and buccal micronucleus cytome assays. *Mutat Res*, 752(1-2): 28-33.

Soybean cultivation is widespread in the State of Rio Grande do Sul (RS, Brazil), especially in the city of Espumoso. Soybean workers in this region are increasingly exposed to a wide combination of chemical agents present in formulations of fungicides, herbicides, and insecticides. In the present study, the comet assay in peripheral leukocytes and the buccal micronucleus (MN) cytome assay (BMCyt) in exfoliated buccal cells were used to assess the effects of exposures to pesticides in soybean farm workers from Espumoso. A total of 127 individuals, 81 exposed and 46 non-exposed controls, were evaluated. Comet assay and BMCyt (micronuclei and nuclear buds) data revealed DNA damage in soybean workers. Cell death was also observed (condensed chromatin, karyorhectic, and karyolitic cells). Inhibition of non-specific choline esterase (BchE) was not observed in the workers. The trace element contents of buccal samples were analyzed by Particle-Induced X-ray Emission (PIXE). Higher concentrations of Mg, Al, Si, P, S, and Cl were observed in cells from workers. No associations with use of personal protective equipment, gender, or mode of application of pesticides were observed. Our findings indicate the advisability of monitoring genetic toxicity in soybean farm workers exposed to pesticides.

http://www.ncbi.nlm.nih.gov/pubmed/23347873

Jayasumana, C.; Gunatilake, S.; Senanayake, P. 2014. Glyphosate, hard water and nephrotoxic metals: Are they the culprits behind the epidemic of chronic kidney disease of unknown etiology in Sri Lanka? *Int J Environ Res Public Health*, *11*, 2125-2147; doi:10.3390/ijerph110202125.

The current chronic kidney disease epidemic, the major health issue in the rice paddy farming areas in Sri Lanka has been the subject of many scientific and political debates over the last decade. Although there is no agreement among scientists about the etiology of the disease, a majority of them has concluded that this is a toxic nephropathy. None of the hypotheses put forward so far could explain coherently the totality of clinical, biochemical, histopathological findings, and the unique geographical distribution of the disease and its appearance in the mid-1990s. A strong association between the consumption of hard water and the occurrence of this special kidney disease has been observed, but the relationship has not been explained consistently. Here, we have hypothesized the association of using glyphosate, the most widely used herbicide in the disease endemic area and its unique metal chelating properties. The possible role played by glyphosate-metal complexes in this epidemic has not been given any serious consideration by investigators for the last two decades. Furthermore, it may explain similar kidney disease epidemics observed in Andra Pradesh (India) and Central America. Although glyphosate alone does not cause an epidemic of chronic kidney disease, it seems to have acquired the ability to destroy the renal tissues of thousands of farmers when it forms complexes with a localized geo environmental factor (hardness) and nephrotoxic metals.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3945589/

While rural workers are threatened by direct exposure, when the absorption of poisons also take place by contact and inhalation and

acute poisonings tend to be clearly perceived, in the case of urban consumers the problems tend to be chronic. Contact with low doses, incorporated into the daily consumption of food contaminated by residues of herbicides, tends to be difficult to understand. The relations of cause and effect in such circumstances are shown faint and diffuse, requiring very different analysis protocols from those adopted in expedited reviews.

The following studies show that, by metabolism issues resulting from genetic transformation, herbicides associated with growing GM HT plants tend to accumulate at higher levels in cells of transgenic plants than in conventional plants. The implications in terms of risks for human and animal consumption arise from higher levels of exposure to herbicides, when the supply includes those parts of GMPs.

Duke, S.; Rimando, A.; Pace, P.; Reddy, K.; Smeda, R. 2003. Isoflavone, glyphosate, and aminomethylphosphonic acid levels in seeds of glyphosate-treated, glyphosate-resistant soybean. *J Agric Food Chem*, *51,* 340-344.

The estrogenic isoflavones of soybeans and their glycosides are products of the shikimate pathway, the target pathway of glyphosate. This study tested the hypothesis that nonphytotoxic levels of glyphosate and other herbicides known to affect phenolic compound biosynthesis might influence levels of these nutraceutical compounds in glyphosate-resistant soybeans. The effects of glyphosate and other herbicides were determined on estrogenic isoflavones and shikimate in glyphosate-resistant soybeans from identical experiments conducted on different cultivars in Mississippi and Missouri. Four commonly used herbicide treatments were compared to a hand-weeded control. The herbicide treatments were (1) glyphosate at 1260 g/ha at 3 weeks after planting (WAP), followed by glyphosate at 840 g/ha at 6 WAP; (2) sulfentrazone at 168 g/ha plus chlorimuron at 34 g/ha applied preemergence (PRE), followed by glyphosate at 1260 g/ha at 6 WAP; (3) sulfentrazone at 168 g/ha plus chlorimuron at 34 g/ha applied PRE, followed by glyphosate at 1260 g/ha at full bloom; and (4) sulfentrazone at 168 g/ha plus chlorimuron at 34 g/ha applied PRE, followed by acifluorfen at 280 g/ha plus bentazon at 560 g/ha plus clethodim at 140 g/ha at 6 WAP. Soybeans were harvested at maturity, and seeds were analyzed for daidzein, daidzin, genistein, genistin, glycitin, glycitein, shikimate, glyphosate, and the glyphosate degradation product, aminomethylphosphonic acid (AMPA). There were no remarkable effects of any treatment on the contents of any of the biosynthetic compounds in soybean seed from either test site, indicating that early and later season applications of glyphosate have no effects on phytoestrogen levels in glyphosate-resistant soybeans. Glyphosate and AMPA residues were higher in seeds from treatment 3 than from the other two treatments in which glyphosate was used earlier. Intermediate levels were found in treatments 1 and 2. Low levels of glyphosate and AMPA were found in treatment 4 and a hand-weeded control, apparently due to herbicide drift.

http://www.ncbi.nlm.nih.gov/pubmed/12502430

Arregui, M.; Lenardón, A.; Sanchez, D.; Maitre, M.; Scotta, R.; Enrique, S. 2004. Monitoring glyphosate residues in transgenic glyphosate-resistant soybean. *Pest Manag*

Sci, 60:163-166.

The availability of Roundup Ready (RR) varieties of soybean has increased the use of glyphosate for weed control in Argentina. Glyphosate [(N-phosphonomethyl)glycine] is employed for the eradication of previous crop vegetation and for weed control during the soybean growing cycle. Its action is effective, and low environmental impact has been reported so far. No residues have been observed in soil or water, either of glyphosate or its metabolite, AMPA (aminomethylphosphonic acid). The objective of this work was to monitor glyphosate and AMPA residues in soybean plants and grains in field crops in Santa Fe Province, Argentina. Five sites were monitored in 1997, 1998 and 1999. Individual soybean plants were sampled from emergence to harvest, dried and ground. Analysis consisted in residue extraction with organic solvents and buffers, agitation, centrifugation, clean-up and HPLC with UV detection. In soybean leaves and stems, glyphosate residues ranged from 1.9 to 4.4 mg kg(-1) and from 0.1 to 1.8 mg kg(-1) in grains. Higher concentrations were detected when glyphosate was sprayed several times during the crop cycle, and when treatments approached the flowering stage. AMPA residues were also detected in leaves and in grains, indicating metabolism of the herbicide.

http://www.ncbi.nlm.nih.gov/pubmed/14971683

Bøhn, T.; Cuhra, M.; Traavik, T.; Sanden, M.; Fagan, J.; Primicerio, R. 2014. Compositional differences in soybeans on the market: Glyphosate accumulates in Roundup Ready GM soybeans. *Food Chemistry*, 153, 207–215.

This article describes the nutrient and elemental composition, including residues of herbicides and pesticides, of 31 soybean batches from Iowa, USA. The soy samples were grouped into three different categories: (i) genetically modified, glyphosate-tolerant soy (GM-soy); (ii) unmodified soy cultivated using a conventional "chemical" cultivation regime; and (iii) unmodified soy cultivated using an organic cultivation regime. Organic soybeans showed the healthiest nutritional profile with more sugars, such as glucose, fructose, sucrose and maltose, significantly more total protein, zinc and less fibre than both conventional and GM-soy. Organic soybeans also contained less total saturated fat and total omega-6 fatty acids than both conventional and GM-soy. GM-soy contained high residues of glyphosate and AMPA (mean 3.3 and 5.7mg/kg, respectively). Conventional and organic soybean batches contained none of these agrochemicals. Using 35 different nutritional and elemental variables to characterise each soy sample, we were able to discriminate GM, conventional and organic soybeans without exception, demonstrating "substantial non-equivalence" in compositional characteristics for 'ready-to-market' soybeans.

Full article available at http://www.sciencedirect.com/science/article/pii/S0308814613019201

In 2011, consistent information deficiencies are found, which support risk assessment in HT GMPs. It is scarce, almost zero, the availability of data and systematic studies on degrees of absorption and accumulation of herbicides in HT GM plants and its parts intended for consumption.

Kleter, G.; Unsworth, J.; Harris, C. 2011. The impact of altered herbicide residues in transgenic herbicide-resistant crops on standard setting for herbicide residues. *Pest Management Science*, 67, 10: 1193–1210. DOI 10.1002/ps.2128.

The global area covered with transgenic (genetically modified) crops has rapidly increased since their introduction in the mid-1990s. Most of these crops have been rendered herbicide resistant, for which it can be envisaged that the modification has an impact on the profile and level of herbicide residues within these crops. In this article, the four main categories of herbicide resistance, including resistance to acetolactate-synthase inhibitors, bromoxynil, glufosinate and glyphosate, are reviewed. The topics considered are the molecular mechanism underlying the herbicide resistance, the nature and levels of the residues formed and their impact on the residue definition and maximum residue limits (MRLs) defined by the Codex Alimentarius Commission and national authorities. No general conclusions can be drawn concerning the nature and level of residues, which has to be done on a case-by-case basis. International residue definitions and MRLs are still lacking for some herbicide-crop combinations, and harmonisation is therefore recommended.

http://www.ncbi.nlm.nih.gov/pubmed/21898904

Risk assessments for health, upon consumption of HT plants, should take into account qualitative and quantitative elements. It is known that certain herbicides have a different deterioration metabolism in GM plants compared to what occurs in non-GM isogenic plants from which others originate. It determines that in GM plants there will be different toxic products, resulting from contact with those herbicides. This will naturally involve different risks, which expand on the basis of quantitative aspects related to the presence - in GMPs – of higher residues level (compared to what happens in traditional crops).

Droge-Laser, W.; Siemeling, U.; Pühler, A.; Broer, I. 1994. The metabolites of the herbicide L-Phosphinothricin (GIufosinate) (Identification, stability, and mobility in transgenic, herbicide-resistant, and untransformed plants). *Plant Physiology*, 105: 159-166.

The metabolism of the herbicide L-phosphinothricin (L-Pt) was analyzed in tobacco (Nicotiana tabacum), alfalfa (Medicago sativa), and carrot (Daucus carota). In transgenic, Pt-resistant plants expressing the Pt-N-acetyltransferase gene (pat), L-Pt was acetylated, resulting in two forms of N-acetyl-Pt (ac-Pt). In transgenic plants expressing only low pat-encoded acetylating activity as well as in genetically unmodified plants, three metabolic compounds 4-methylphosphinico-2-oxo-butanoic acid, 3-methylphosphinico-propanoic acid (MPP), and 4-methylphosphinico-2-hydroxy-butanoic acid (MHB) were identified. Hence, the transgene-encoded acetylation of L-Pt competes with a plant-specific degradation. The compounds MPP, MHB, and ac-Pt were found to be the final, stable products of the plant's metabolic pathways. The mobility of these stable compounds in the plant was investigated: L-Pt as well as the derived metabolites were found to be preferentially transported to the upper regions of the plant.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC159341/

Laurent, F.; Debrauwer, L.; Rathahao, E.; Scalla, R. 2000. 2,4-Dichlorophenoxyacetic acid metabolism in transgenic tolerant cotton (*Gossypium hirsutum*). *J Agric Food Chem*, 48, 307–5311.

The metabolic fate of 2,4-dichlorophenoxyacetic acid (2,4-D) was studied in leaves of transgenic 2,4-D-tolerant cotton (Gossypium hirsutum), which is obtained by transfer of the tfdA gene from the bacterium Alcaligenes eutrophus. The tdfA gene codes for a dioxygenase catalyzing the degradation of 2,4-D to 2,4-dichlorophenol (2,4-DCP). [phenyl-(14)C]-2,4-D was administered by petiolar absorption followed by an 18 h water chase or converted to the isopropyl ester and sprayed onto the leaf surface; the leaves were harvested 48 h later. The herbicide was degraded to 2,4-DCP by the bacterial enzyme expressed in the plants. 2,4-DCP was rapidly converted to more polar metabolites and was never found in detectable amounts. Metabolite structures were deduced from enzymatic hydrolysis studies and mass spectrometric analyses. The first metabolite was the glucoside conjugate of 2,4-DCP (2, 4-DCP-beta-O-glucoside). The major terminal metabolites were two more complex glucosides: 2,4-DCP-(6-O-malonyl)glucoside and 2, 4-DCP-(6-O-sulfate) glucoside.

http://www.ncbi.nlm.nih.gov/pubmed/11087477

Müller, B.; Zumdick, A.; Schuphan, I.; Schmidt, B. 2001. Metabolism of the herbicide glufosinate-ammonium in plant cell cultures of transgenic (rhizomania-resistant) and non-transgenic sugarbeet (*Beta vulgaris*), carrot (*Daucus carota*), Purple foxglove (*Digitalis purpurea*) and thorn apple (*Datura stramonium*). *Pest Manag Sci*, 57, 46-56.

The metabolism of the herbicide glufosinate-ammonium was investigated in heterotrophic cell suspension and callus cultures of transgenic (bar-gene) and non-transgenic sugarbeet (Beta vulgaris). Similar studies were performed with suspensions of carrot (Daucus carota), purple foxglove (Digitalis purpurea) and thorn apple (Datura stramonium). 14C-labelled chemicals were the (racemic) glufosinate, L-glufosinate, and D-glufosinate, as well as the metabolites N-acetyl L-glufosinate and 3-(hydroxymethylphosphinyl)propionic acid (MPP). Cellular absorption was generally low, but depended noticeably on plant species, substance and enantiomer. Portions of non-extractable residues ranged from 0.1% to 1.2% of applied 14C. Amounts of soluble metabolites resulting from glufosinate or L-glufosinate were between 0.0% and 26.7% of absorbed 14C in non-transgenic cultures and 28.2% and 59.9% in transgenic sugarbeet. D-Glufosinate, MPP and N-acetyl L-glufosinate proved to be stable. The main metabolite in transgenic sugarbeet was N-acetyl L-glufosinate, besides traces of MPP and 4-(hydroxymethylphosphinyl)butanoic acid (MPB). In non-transgenic sugarbeet, glufosinate was transformed to a limited extent to MPP and trace amounts of MPB. In carrot, D stramonium and D purpurea, MPP was also the main product; MPB was identified as a further trace metabolite in D stramonium and D purpurea.

http://www.ncbi.nlm.nih.gov/pubmed/11455632

Laurent, F.; Debrauwer, L.; Pascal-Lorber, S. 2006. Metabolism of [14C]-2,4-dichlorophenol in edible plants. *Pest Manag Sci*, 62, 558–564.

Several 2,4-dichlorophenoxyacetic acid (2,4-D)-sensitive plants have been modified by genetic engineering with tfdA gene to acquire 2,4-D tolerance. The expression product of this gene degrades 2,4-D to 2,4-dichlorophenol (DCP), which is less phytotoxic but could cause a problem of food safety. After a comparison of 2,4-D and DCP metabolism in transgenic 2,4-D-tolerant and wild cotton (Gossypium hirsutum L.), a direct study of DCP metabolism in edible plants was performed. After petiolar uptake of a [U-phenyl-(14)C]-DCP solution followed by a 48 h water chase, aqueous extracts were analysed by high-performance liquid chromatography. Metabolites were thereafter

isolated and their structural identities were determined by enzymatic and chemical hydrolyses and mass spectrometry analyses. The metabolic fate of DCP was equivalent to 2,4-D metabolism in transgenic 2,4-D-tolerant cotton. In addition, DCP metabolism was similar in transgenic and wild cotton. The major terminal metabolites were DCP-saccharide conjugates in all species, essentially DCP-(6-O-malonyl)-glucoside or its precursor DCP-glucose. The significance of this metabolic pathway with regard to food safety is discussed.

http://www.ncbi.nlm.nih.gov/pubmed/16628540

2.1.1 The active principle glyphosate and its major degradation metabolite (the aminomethylphosphonic acid - AMPA)

Presented as low toxicity and low persistence in the environment, glyphosate has been benefited by a relatively positive public image, which extends to Roundup, commercial formulation responsible for the largest sales volume of that active ingredient. However, recent studies that contradict this image, associating its presence to cancer and cytotoxic reactions, as well as to negative effects on the digestive flora. The aminomethylphosphonic acid (AMPA), the main metabolite of glyphosate degradation, also has toxic and genotoxic properties.

Hietanen, E.; Linnainmaa, K.; Vainio, H. 1983. Effects of phenoxyherbicides and glyphosate on the hepatic and intestinal biotransformation activities in the rat. *Acta Pharmacologica et Toxicologica (Copenh)*, 53(2):103-12.

The effects of phenoxyacid herbicides 2,4-D (2,4-dichlorophenoxyacetic acid) and MCPA (4-chloro-2-methylphenoxyacetic acid), clofibrate, and glyphosate on hepatic and intestinal drug metabolizing enzyme activities were studied in rats intragastrically exposed for 2 weeks. The hepatic ethoxycoumarin O-deethylase activity increased about 2-fold with MCPA. Both 2,4-D and MCPA increased the hepatic epoxide hydrolase activity and decreased the hepatic glutathione S-transferase activity. MCPA also increased the intestinal activities of ethoxycoumarin O-deethylase and epoxide hydrolase. Glyphosate decreased the hepatic level of cytochrome P-450 and monooxygenase activities and the intestinal activity of aryl hydrocarbon hydroxylase. Clofibrate decreased the hepatic activities of UDPglucuronosyltransferase with p-nitrophenol or methylumbelliferone as the substrate. Also 2,4-D decreased the hepatic activity of UDPglucuronosyltransferase with p-nitrophenol as the substrate. MCPA decreased the intestinal activities of UDPglucuronosyltransferase with either p-nitrophenol or methylumbelliferone as the substrate. The results indicate that phenoxyacetic acids, especially MCPA, may have potent effects on the metabolism of xenobiotics. Glyphosate, not chemically related to phenoxyacids, seems to inhibit monooxygenases. Whether these changes are related to the toxicity of these xenobiotics remains to be clarified in further experiments.

http://onlinelibrary.wiley.com/doi/10.1111/j.1600-0773.1983.tb01876.x/abstract

Lioi, M.; Scarf, M.; Santoro, A.; Barbieri, R.; Zeni, O.; Berardino, D.; Ursini, M. 1998. Genotoxicity and oxidative stress induced by pesticide exposure in bovine lymphocyte cultures *in vitro*. *Mutation Research*, 403, 13–20.

The genotoxic activity of the pesticides gliphosate, vinclozolin and DPX-E9636 was studied in in vitro cultures of bovine lymphocytes, using chromosome aberration (CA) and sister chromatid exchange (SCE) frequencies as genetic end-points and a variation of glucose 6-phosphate dehydrogenase (G6PD) enzyme activity as a marker of changes in the normal cell redox state. Results indicated a statistically significant increase of structural aberrations, sister chromatid exchanges and G6PD activity, suggesting that the pesticides tested induce either oxidative stress or a mutagenic effect in this species. The evaluation of both mitotic index and cell viability, after pesticide exposure, demonstrates a high cytotoxic effect which is always associated with the observed genotoxic effect.

http://www.ncbi.nlm.nih.gov/pubmed/9726001

Daruich, J.; Zirulnik, F.; Gimenez, M. 2001. Effect of the herbicide glyphosate on enzymatic activity in pregnant rats and their fetuses. *Environmental Research Section*, A 85**,** 226-231.

To prevent health risk from environmental chemicals, particularly for progeny, we have studied the effects of the herbicide glyphosate on several enzymes of pregnant rats. Glyphosate is an organophosphorated nonselective agrochemical widely used in many countries including Argentina and acts after the sprout in a systemic way. We have studied three cytosolic enzymes: isocitrate dehydrogenase-NADP dependent, glucose-6-phosphate dehydrogenase, and malic dehydrogenase in liver, heart, and brain of pregnant Wistar rats. The treatment was administered during the 21 days of pregnancy, with 1 week as an acclimation period. The results suggest that maternal exposure to agrochemicals during pregnancy induces a variety of functional abnormalities in the specific activity of the enzymes in the studied organs of the pregnant rats and their fetuses.

http://www.ncbi.nlm.nih.gov/pubmed/11237511

Beuret, C.; Zirulnik, F.; Giménez, M. 2005. Effect of the herbicide glyphosate on liver lipoperoxidation in pregnant rats and their fetuses. *Reproductive Toxicology*, 19, 501–504.

Glyphosate is a post-emergence herbicide that acts on the synthesis of amino acids and other endogenous metabolites in plants. It is commonly used in agriculture, forestry, and nurseries for the control or destruction of herbaceous plants. Metabolic processes during development and pregnancy could be sensitive to changes induced by glyphosate such as lipid peroxidation. The present study has investigated the effects that 1% glyphosate oral exposure has on lipoperoxidation and antioxidant enzyme systems in the maternal serum and liver of pregnant rats and their term fetuses at 21 days of gestation. The results suggest that excessive lipid peroxidation induced with glyphosate ingestion leads to an overload of maternal and fetal antioxidant defense systems.

http://www.ncbi.nlm.nih.gov/pubmed/15749264

Hokanson, R.; Fudge, R.; Chowdhary, R; Busbee, D. 2007. Alteration of estrogen regulated gene expression in human cells induced by the agricultural and horticultural herbicide glyphosate. *Hum Exp Toxicol*, 26 (9): 747-52.

Gene expression is altered in mammalian cells (MCF-7 cells), by exposure to a variety of chemicals that mimic steroid hormones or interact with endocrine receptors or their co-factors. Among those populations chronically exposed to these endocrine disruptive chemicals are persons, and their families, who are employed in agriculture or horticulture, or who use agricultural/horticultural chemicals. Among the chemicals most commonly used, both commercially and in the home, is the herbicide glyphosate. Although glyphosate is commonly considered to be relatively non-toxic, we utilized in vitro DNA microarray analysis of this chemical to evaluate its capacity to alter the expression of a variety of genes in human cells. We selected a group of genes, determined by DNA microarray analysis to be dysregulated, and used quantitative real-time PCR to corroborate their altered states of expression. We discussed the reported function of those genes, with emphasis on altered physiological states that are capable of initiating adverse health effects that might be anticipated if gene expression were significantly altered in either adults or embryos exposed in utero.

http://www.ncbi.nlm.nih.gov/pubmed/17984146

Mañas, F.; Peralta, L.; Raviolo, J.; Ovando, H.; Weyers, A.; Ugnia, L.; Cid, M.; Larripa, I.; Gorla, N. 2009. Genotoxicity of AMPA, the environmental metabolite of glyphosate, assessed by the Comet assay and cytogenetic tests. *Ecotoxicology and Environmental Safety*, 72.

Formulations containing glyphosate are the most widely used herbicides in the world. AMPA is the major environmental breakdown product of glyphosate. The purpose of this study is to evaluate the in vitro genotoxicity of AMPA using the Comet assay in Hep-2 cells after 4h of incubation and the chromosome aberration (CA) test in human lymphocytes after 48h of exposition. Potential in vivo genotoxicity was evaluated through the micronucleus test in mice. In the Comet assay, the level of DNA damage in exposed cells at 2.5-7.5mM showed a significant increase compared with the control group. In human lymphocytes we found statistically significant clastogenic effect AMPA at 1.8mM compared with the control group. In vivo, the micronucleus test rendered significant statistical increases at 200-400mg/kg. AMPA was genotoxic in the three performed tests. Very scarce data are available about AMPA potential genotoxicity.

http://www.ncbi.nlm.nih.gov/pubmed/19013644

Mañas, F.; Peralta, L.; Raviolo, J.; Ovando, H.; Weyers, A.; Ugnia, L.; Cid, M.; Larripa, I.; Gorla, N. 2009. Genotoxicity of glyphosate assessed by the Comet assay and cytogenic tests. *Environ Toxicol Pharmacol*, 28: 37–41.

It was evaluated the genotoxicity of glyphosate which up to now has heterogeneous results. The comet assay was performed in Hep-2 cells. The level of DNA damage in the control group (5.42±1.83 arbitrary units) for tail moment (TM) measurements has shown a significant increase ($p<0.01$) with glyphosate at a range concentration from 3.00 to 7.50mM. In the chromosome aberrations (CA) test in human lymphocytes the herbicide (0.20-6.00mM) showed no significant effects in comparison with the control group. In vivo, the micronucleus test (MNT) was evaluated in mice at three doses rendering statistical significant increases at 400mg/kg (13.0±3.08 micronucleated erythrocytes/1000 cells, $p<0.01$). In the present study glyphosate was genotoxic in the comet assay in Hep-2 cells and in the MNT test at 400mg/kg in mice. Thiobarbituric acid reactive substances (TBARs) levels, superoxide dismutase (SOD) and catalase (CAT) activities were quantified in their organs. The results showed an increase in these enzyme activities.

http://www.ncbi.nlm.nih.gov/pubmed/21783980

Thongprakaisang, S.; Thiantanawat, A.; Rangkadilok, N.; Suriyo, T.; Satayavivad, J. 2013. Glyphosate induces human breast cancer cells growth via estrogen receptors. *Food and Chemical Toxicology*, doi: http://dx.doi.org/10.1016/j.fct.2013.05.057.

Glyphosate is an active ingredient of the most widely used herbicide and it is believed to be less toxic than other pesticides. However, several recent studies showed its potential adverse health effects to humans as it may be an endocrine disruptor. This study focuses on the effects of pure glyphosate on estrogen receptors (ERs) mediated transcriptional activity and their expressions. Glyphosate exerted proliferative effects only in human hormone-dependent breast cancer, T47D cells, but not in hormone-independent breast cancer, MDA-MB231 cells, at 10(-12) to 10(-6)M in estrogen withdrawal condition. The proliferative concentrations of glyphosate that induced the activation of estrogen response element (ERE) transcription activity were 5-13 fold of control in T47D-KBluc cells and this activation was inhibited by an estrogen antagonist, ICI 182780, indicating that the estrogenic activity of glyphosate was mediated via ERs. Furthermore, glyphosate also altered both ERα and β expression. These results indicated that low and environmentally relevant concentrations of glyphosate possessed estrogenic activity. Glyphosate-based herbicides are widely used for soybean cultivation, and our results also found that there was an additive estrogenic effect between glyphosate and genistein, a phytoestrogen in soybeans. However, these additive effects of glyphosate contamination in soybeans need further animal study.

http://www.ncbi.nlm.nih.gov/pubmed/23756170

REV – Samsel, A.; Seneff, S. 2013. Glyphosate's suppression of cytochrome P450 enzymes and amino acid biosynthesis by the gut microbiome: Pathways to modern diseases. *Entropy*, 15: 1416–1463.

Glyphosate, the active ingredient in Roundup®, is the most popular herbicide used worldwide. The industry asserts it is minimally toxic to humans, but here we argue otherwise. Residues are found in the main foods of the Western diet, comprised primarily of sugar, corn, soy and wheat. Glyphosate's inhibition of cytochrome P450 (CYP) enzymes is an overlooked component of its toxicity to mammals. CYP enzymes play crucial roles in biology, one of which is to detoxify xenobiotics. Thus, glyphosate enhances the damaging effects of other food borne chemical residues and environmental toxins. Negative impact on the body is insidious and manifests slowly over time as inflammation damages cellular systems throughout the body. Here, we show how interference with CYP enzymes acts synergistically with disruption of the biosynthesis of aromatic amino acids by gut bacteria, as well as impairment in serum sulfate transport. Consequences are most of the diseases and conditions associated with a Western diet, which include gastrointestinal disorders, obesity, diabetes, heart disease, depression, autism, infertility, cancer and Alzheimer's disease. We explain the documented effects of glyphosate and its ability to induce disease, and we show that glyphosate is the "textbook example" of exogenous semiotic entropy: the disruption of homeostasis by environmental toxins.

Full article available at http://www.mdpi.com/1099-4300/15/4/1416

REV – Samsel, A.; Seneff, S. 2013. Glyphosate, pathways to modern diseases II: Celiac sprue and gluten intolerance. *Interdiscip Toxicol*, Vol. 6(4): 159–184. doi: 10.2478/intox-2013-0026.

Celiac disease, and, more generally, gluten intolerance, is a growing problem worldwide, but especially in North America and Europe, where an estimated 5% of the population now suffers from it. Symptoms include nausea, diarrhea, skin rashes, macrocytic anemia and depression. It is a multifactorial disease associated with numerous nutritional deficiencies as well as reproductive issues and increased risk to thyroid disease, kidney failure and cancer. Here, we propose that glyphosate, the active ingredient

in the herbicide, Roundup®, is the most important causal factor in this epidemic. Fish exposed to glyphosate develop digestive problems that are reminiscent of celiac disease. Celiac disease is associated with imbalances in gut bacteria that can be fully explained by the known effects of glyphosate on gut bacteria. Characteristics of celiac disease point to impairment in many cytochrome P450 enzymes, which are involved with detoxifying environmental toxins, activating vitamin D3, catabolizing vitamin A, and maintaining bile acid production and sulfate supplies to the gut. Glyphosate is known to inhibit cytochrome P450 enzymes. Deficiencies in iron, cobalt, molybdenum, copper and other rare metals associated with celiac disease can be attributed to glyphosate's strong ability to chelate these elements. Deficiencies in tryptophan, tyrosine, methionine and selenomethionine associated with celiac disease match glyphosate's known depletion of these amino acids. Celiac disease patients have an increased risk to non-Hodgkin's lymphoma, which has also been implicated in glyphosate exposure. Reproductive issues associated with celiac disease, such as infertility, miscarriages, and birth defects, can also be explained by glyphosate. Glyphosate residues in wheat and other crops are likely increasing recently due to the growing practice of crop desiccation just prior to the harvest. We argue that the practice of "ripening" sugar cane with glyphosate may explain the recent surge in kidney failure among agricultural workers in Central America. We conclude with a plea to governments to reconsider policies regarding the safety of glyphosate residues in foods.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3945755/

2.1.2 Commercial glyphosate-based herbicides formulation

Risk analysis should take into account not only the toxic properties of glyphosate and its major degradation product (the aminomethylphosphonic acid - AMPA) but the whole commercial product sold as "glyphosate-based herbicide" (like Roundup). Indeed, the combined effect of the remaining spray components – known as adjuvant - should also be evaluated.

Indeed, the adjuvants present in commercial formulas carry not only their own toxicity and may also influence the degree of aggressiveness of the active ingredient. This is a sensitive matter, despite the accumulation of evidence. The toxicological classifications applied to pesticides result from studies conducted only with the active ingredients, which are only part of the commercial products. In practice, farmers use composite commercial formulas, which will impact the environment and the people involved not accordingly to what could be estimated through studies focused on possible effects of the only active ingredients. A similar situation occurs in developing acceptable daily intake levels (ADI) or Maximum Residue Limits (MRLs), which also result in low accuracy to assess real risks.

These considerations, which apply to Roundup and a range of other agricultural poisons, reveal the need for more comprehensive and careful studies.

Mesnage, R.; Defarge, N.; Vendômois, J.; Séralini, G-E. 2014. Major pesticides are more toxic to human cells than their declared active principles. *Biomedical Research International*, 2014: 179691.

Pesticides are used throughout the world as mixtures called formulations. They contain adjuvants, which are often kept confidential and are called inerts by the manufacturing companies, plus a declared active principle (AP), which is usually tested alone. This is true even in the longest toxicological regulatory tests performed on mammals . We tested the toxicity of 9 pesticides, comparing active principles and their formulations, on three human cell lines (HepG2, HEK293 and JEG3). We measured mitochondrial activities, membrane degradations, and caspases 3/7 activities. Glyphosate, isoprot uron, fluroxypyr, pirimicarb, imidacloprid, acetamiprid, tebuc onazole, epoxiconazole and prochloraz constitute respectively the active principles of 3 major herbicides, 3 insecticides and 3 fungicides. Fungicides were the most toxic from concentrations 300-600 times lower than agricultural dilutions, followed by herbicides, and then insecticides, with very similar profiles in all cell types. The human placental JEG3 cells appeared to be the most sensitive. Despite its relatively benign reputation, Roundup was by far the most toxic among the herbicides and insecticides tested. Most importantly, 8 formulations out of 9 were several hundred times more toxic than their active principle. Our results challenge the relevance of the Acceptable Daily Intake for pesticides because this norm is calculated from the toxicity of the active principle alone. The study of combinatorial effects of several APs together may be of only secondary importance if the toxicity of the combinations of each AP with its adjuvants is neglected or unknown. Chronic tests on pesticides may not reflect relevant environmental exposures if only one ingredient of these mixtures is tested alone.

Full article available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3955666/

In the case of glyphosate-based formulations, it is known that Polyethoxylated tallow amine (or POEA) has higher toxicity than the active principle itself. This means that the use of herbicides such as Roundup brings higher risks of toxicity than would be found in "pure" applications of the active principle alone, if it were to be used in this way (as would like the scholars who defend their weak aggressiveness).

Sawada, Y.; Nagai, Y.; Ueyama, M.; Yamarnoto, I. 1988. Probable toxicity of surface-active agent in commercial herbicide containing glyphosate. *The Lancet*, 1 (85-80):229.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/2893109

Marc, J.; Mulner-Lorillon, O.; Boulben, S.; Hureau, D.; Durand, G.; Bellé, R. 2002. Pesticide Roundup provokes cell division dysfunction at the level of CDK1/ cyclin B activation. *Chem Res Toxicol*, 15, 32631.

To assess human health risk from environmental chemicals, we have studied the effect on cell cycle regulation of the widely used glyphosate-containing pesticide Roundup. As a model system we have used sea urchin embryonic first divisions following fertilization, which are appropriate for the study of universal cell cycle regulation without interference with transcription. We show that 0.8% Roundup (containing 8 mM glyphosate) induces a delay in the kinetic of the first cell cleavage of sea urchin embryos. The delay is dependent on the concentration of Roundup. The delay in the cell cycle could be induced using increasing glyphosate concentrations (1-10 mM) in the presence of a subthreshold concentration of Roundup 0.2%, while glyphosate alone was ineffective, thus indicating synergy between glyphosate and Roundup formulation products. The effect of Roundup was not lethal and involved a delay in entry into M-phase of the cell cycle, as judged cytologically. Since CDK1/cyclin B regulates universally the M-phase of the cell cycle, we analyzed CDK1/ cyclin B activation during the first division of early development. Roundup delayed the activation of CDK1/cyclin B in vivo. Roundup inhibited also the global protein synthetic rate without preventing the accumulation of cyclin B. In summary, Roundup affects cell cycle regulation by delaying activation of the CDK1/cyclin B complex, by synergic effect of glyphosate and formulation products. Considering the universality among species of the CDK1/cyclin B regulator, our results question the safety of glyphosate and Roundup on human health.

http://www.ncbi.nlm.nih.gov/pubmed/11896679

Richard, S.; Moslemi, S.; Siphautar, H.; Benachour, N.; Séralini, G-E. 2005. Differential effects of glyphosate and Roundup on human placental cells and aromatase. *Environ Health Perspect.* 113 (6), 71620.

Roundup is a glyphosate-based herbicide used worldwide, including on most genetically modified plants that have been designed to tolerate it. Its residues may thus enter the food chain, and glyphosate is found as a contaminant in rivers. Some agricultural workers using glyphosate have pregnancy problems, but its mechanism of action in mammals is questioned. Here we show that glyphosate is toxic to human placental JEG3 cells within 18 hr with concentrations lower than those found with agricultural use, and this effect increases with concentration and time or in the presence of Roundup adjuvants. Surprisingly, Roundup is always more toxic than its active ingredient. We tested the effects of glyphosate and Roundup at lower nontoxic concentrations on aromatase, the enzyme responsible for estrogen synthesis. The glyphosate-based herbicide disrupts aromatase activity and mRNA levels and interacts with the active site of the purified enzyme, but the effects of glyphosate are facilitated by the Roundup formulation in microsomes or in cell culture. We conclude that endocrine and toxic effects of Roundup, not just glyphosate, can be observed in mammals. We suggest that the presence of Roundup adjuvants enhances glyphosate bioavailability and/or bioaccumulation.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1257596/

Martinez, A.; Reyes, I.; Reyes, N. 2007. Cytotoxicity of the herbicide glyphosate in human peripheral blood mononuclear cells. *Biomédica*, 27(4):594-604.

Introduction: Glyphosate is a broad-spectrum, non-selective herbicide and commonly used to eliminate weeds in agricultural and forest settings. Studies evaluating glyphosate toxicity in animals and environment show that commercial formulations of glyphosate are more toxic than the active component itself.

Objectives: Technical grade glyphosate was compared with the commercial formulation Roundup in their respective toxicities on human peripheral blood mononuclear cells.
Materials and Methods: Human peripheral blood mononuclear cells were exposed to different concentrations of glyphosate, either technical grade or in the form of Roundup for 24 h, 48 h, 72 h, and 96 h. Cytotoxicity was assayed by trypan blue dye exclusion method and reduction of (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2Htetrazolium-5-carboxyanilide inner salt)XTT reagent.
Results: Both technical grade glyphosate and Roundup formulation were toxic to human peripheral blood mononuclear cells. Cytotoxicity of Roundup was higher than cytotoxicity of glyphosate, since the LC50 (50% lethal concentration) determined by the trypan blue exclusion method at 24 h was the equivalent of 56.4 microg/ml of glyphosate in the form of Roundup and 1,640 microg/ml (1.64 mg/ml) for technical grade glyphosate.
Conclusions: This in vitro study confirmed the toxic effects on human cells by glyphosate and its commercial preparations. Commercial formulations were more cytotoxic than the active component alone, supporting the concept that additives in commercial formulations play a role in the toxicity attributed to glyphosate-based herbicides

Full article available at https://goo.gl/l5h2HB

Koller, V.; Furhacker, M.; Nersesyan, A.; Mišík, M.; Eisenbauer, M.; Knasmueller, S. 2012. Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells. *Arch Toxicol*, 86 (5): 805-813.

Glyphosate (G) is the largest selling herbicide worldwide; the most common formulations (Roundup, R) contain polyoxyethyleneamine as main surfactant. Recent findings indicate that G exposure may cause DNA damage and cancer in humans. Aim of this investigation was to study the cytotoxic and genotoxic properties of G and R (UltraMax) in a buccal epithelial cell line (TR146), as workers are exposed via inhalation to the herbicide. R induced acute cytotoxic effects at concentrations > 40 mg/l after 20 min, which were due to membrane damage and impairment of mitochondrial functions. With G, increased release of extracellular lactate dehydrogenase indicative for membrane damage was observed at doses > 80 mg/l. Both G and R induced DNA migration in single-cell gel electrophoresis assays at doses > 20 mg/l. Furthermore, an increase of nuclear aberrations that reflect DNA damage was observed. The frequencies of micronuclei and nuclear buds were elevated after 20-min exposure to 10-20 mg/l, while nucleoplasmatic bridges were only enhanced by R at the highest dose (20 mg/l). R was under all conditions more active than its active principle (G). Comparisons with results of earlier studies with lymphocytes and cells from internal organs indicate that epithelial cells are more susceptible to the cytotoxic and DNA-damaging properties of the herbicide and its formulation. Since we found genotoxic effects after short exposure to concentrations that correspond to a 450-fold dilution of spraying used in agriculture, our findings indicate that inhalation may cause DNA damage in exposed individuals.

http://www.ncbi.nlm.nih.gov/pubmed/22331240

Mesnage, R.; Bernay, B.; Séralini, G-E. 2012. Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity. *Toxicology*.

Pesticides are always used in formulations as mixtures of an active principle with adjuvants. Glyphosate, the active ingredient of the major pesticide in the world, is an herbicide supposed to be specific on plant metabolism. Its adjuvants are generally considered as inert diluents. Since side effects for all these compounds have been claimed, we studied potential active principles for toxicity on human cells for 9 glyphosate-based formulations. For this we detailed their compositions and toxicities, and as controls we used a major adjuvant (the polyethoxylated tallowamine POE-15), glyphosate alone, and a total formulation without glyphosate. This was performed after 24h

exposures on hepatic (HepG2), embryonic (HEK293) and placental (JEG3) cell lines. We measured mitochondrial activities, membrane degradations, and caspases 3/7 activities. The compositions in adjuvants were analyzed by mass spectrometry. Here we demonstrate that all formulations are more toxic than glyphosate, and we separated experimentally three groups of formulations differentially toxic according to their concentrations in ethoxylated adjuvants. Among them, POE-15 clearly appears to be the most toxic principle against human cells, even if others are not excluded. It begins to be active with negative dose-dependent effects on cellular respiration and membrane integrity between 1 and 3ppm, at environmental/occupational doses. We demonstrate in addition that POE-15 induces necrosis when its first micellization process occurs, by contrast to glyphosate which is known to promote endocrine disrupting effects after entering cells. Altogether, these results challenge the establishment of guidance values such as the acceptable daily intake of glyphosate, when these are mostly based on a long term in vivo test of glyphosate alone. Since pesticides are always used with adjuvants that could change their toxicity, the necessity to assess their whole formulations as mixtures becomes obvious. This challenges the concept of active principle of pesticides for non-target species.

Full article available at https://goo.gl/EynkQW

The updated scientific literature abounds in studies that record aspects of the toxicity of the glyphosate-based herbicide. Most of them refers to different Roundup formulations, making it more suitable as decision support in terms of risk (commercial product studies using commercial formulation are more accurate / representative than studies based on the active ingredient).

It appears in the articles referenced below these herbicides should be considered cytotoxic, teratogenic, carcinogenic and endocrine disruptors in the medium and long term. These damages are observed at lower doses as those considered “risk-free” by most regulatory agencies (scaled based on residues intake estimates contained in the feed). More recently, antibiotic properties of glyphosate herbicides caught the attention of researchers regarding the caused changes in the symbiotic microbiota of mammals (including humans[56]), with significant impacts on degradation indicators of human / animal health (and environmental degradation).

Walsh, L.; McCormick, C.; Martin, C.; Stocco, D. 2000. Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. *Environmental Health Perspectives*, 108, 769–776.

56 Remember the fundamental biological role of human symbiotic microrganisms on health, such as the quality of the digestive process, skin protection or even neurochemical stability. Indeed, the microbiome is regarded in some fields of biomedical science as an organ of the human body itself, and there are several pathologies that involve microbiome disorders (obesity, autoimmune diseases and cancer probably).

Recent reports demonstrate that many currently used pesticides have the capacity to disrupt reproductive function in animals. Although this reproductive dysfunction is typically characterized by alterations in serum steroid hormone levels, disruptions in spermatogenesis, and loss of fertility, the mechanisms involved in pesticide-induced infertility remain unclear. Because testicular Leydig cells play a crucial role in male reproductive function by producing testosterone, we used the mouse MA-10 Leydig tumor cell line to study the molecular events involved in pesticide-induced alterations in steroid hormone biosynthesis. We previously showed that the organochlorine insecticide lindane and the organophosphate insecticide Dimethoate directly inhibit steroidogenesis in Leydig cells by disrupting expression of the steroidogenic acute regulatory (StAR) protein. StAR protein mediates the rate-limiting and acutely regulated step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane where the cytochrome P450 side chain cleavage (P450scc) enzyme initiates the synthesis of all steroid hormones. In the present study, we screened eight currently used pesticide formulations for their ability to inhibit steroidogenesis, concentrating on their effects on StAR expression in MA-10 cells. In addition, we determined the effects of these compounds on the levels and activities of the P450scc enzyme (which converts cholesterol to pregnenolone) and the 3beta-hydroxysteroid dehydrogenase (3beta-HSD) enzyme (which converts pregnenolone to progesterone). Of the pesticides screened, only the pesticide Roundup inhibited dibutyryl [(Bu)(2)]cAMP-stimulated progesterone production in MA-10 cells without causing cellular toxicity. Roundup inhibited steroidogenesis by disrupting StAR protein expression, further demonstrating the susceptibility of StAR to environmental pollutants.

Full article available at https://goo.gl/zSoAWk

Dallegrave, E.; Mantese, F.; Coelho, R.; Pereira, J.; Dalsenter, P.; Langeloh, A. 2003. The teratogenic potential of the herbicide glyphosate-Roundup in Wistar rats. *Toxicol Lett*, 142, 45-52.

The aim of this study was to assess the teratogenicity of the herbicide glyphosate-Roundup (as commercialized in Brazil) to Wistar rats. Dams were treated orally with water or 500, 750 or 1000 mg/kg glyphosate from day 6 to 15 of pregnancy. Cesarean sections were performed on day 21 of pregnancy, and number of corpora lutea, implantation sites, living and dead fetuses, and resorptions were recorded. Weight and gender of the fetuses were determined, and fetuses were examined for external malformations and skeletal alterations. The organs of the dams were removed and weighed. Results showed a 50%, mortality rate for dams treated with 1000 mg/kg glyphosate. Skeletal alterations were observed in 15.4, 33.1, 42.0 and 57.3% of fetuses from the control, 500, 750 and 1000 mg/kg glyphosate groups, respectively. We may conclude that glyphosate-Roundup is toxic to the dams and induces developmental retardation of the fetal skeleton.

http://www.ncbi.nlm.nih.gov/pubmed/12765238

Marc, J.; Bellé, R.; Morales, J.; Cormier, P.; Mulner-Lorillon, O. 2004. Formulated glyphosate activates the DNA-response checkpoint of the cell cycle leading to the prevention of G2/M transition. *Toxicological Sciences*, 82, 436-442.

A glyphosate containing pesticide impedes at 10 mM glyphosate the G2/M transition as judged from analysis of the first cell cycle of sea urchin development. We show that formulated glyphosate prevented dephosphorylation of Tyr 15 of the cell cycle regulator CDK1/cyclin B in vivo, the end point target of the G2/M cell cycle checkpoint. Formulated glyphosate had no direct effect on the dual specific cdc25 phosphatase activity responsible for Tyr 15 dephosphorylation. At a concentration that efficiently impeded the cell cycle, formulated glyphosate inhibited the synthesis of DNA occurring in S phase of the cell cycle. The extent of the inhibition of DNA synthesis by formulated glyphosate was correlated with the effect on the cell cycle. We conclude that formulated

glyphosate's effect on the cell cycle is exerted at the level of the DNA-response checkpoint of S phase. The resulting inhibition of CDK1/cyclin B Tyr 15 dephosphorylation leads to prevention of the G2/M transition and cell cycle progression.

http://www.ncbi.nlm.nih.gov/pubmed/15375296

Marc, J.; Mulner-Lorillon, O.; Bellé, R. 2004. Glyphosate based pesticides affect cell cycle regulation. *Biology of the Cell*, 96: 245249.

Cell-cycle dysregulation is a hallmark of tumor cells and human cancers. Failure in the cell-cycle checkpoints leads to genomic instability and subsequent development of cancers from the initial affected cell. A worldwide used product Roundup 3plus, based on glyphosate as the active herbicide, was suggested to be of human health concern since it induced cell cycle dysfunction as judged from analysis of the first cell division of sea urchin embryos, a recognized model for cell cycle studies. Several glyphosate-based pesticides from different manufacturers were assayed in comparison with Roundup 3plus for their ability to interfere with the cell cycle regulation. All the tested products, Amega, Cargly, Cosmic, and Roundup Biovert induced cell cycle dysfunction. The threshold concentration for induction of cell cycle dysfunction was evaluated for each product and suggests high risk by inhalation for people in the vicinity of the pesticide handling sprayed at 500 to 4000 times higher dose than the cell-cycle adverse concentration.

http://www.ncbi.nlm.nih.gov/pubmed/15182708

Benedetti, A.; Vituri Cde, L.; Trentin, A; Domingues, M.; Alvarez-Silva, M. 2004. The effects of sub-chronic exposure of Wistar rats to the herbicide Glyphosate-Biocarb. *Toxicol Lett*, 153(2): 227–232.

The object of this study was to analyze the hepatic effects of the herbicide Glyphosate-Biocarb (as commercialized in Brazil) in Wistar rats. Animals were treated orally with water or 4.87, 48.7, or 487 mg/kg of glyphosate each 2 days, during 75 days. Sub-chronic treatment of animals starting from the lowest dose of glyphosate induced the leakage of hepatic intracellular enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), suggesting irreversible damage in hepatocytes. We observed the increase of Kupffer cells in hepatic sinusoid of glyphosate-treated animals. This was followed by large deposition of reticulin fibers, composed mainly of collagen type III. We may conclude that Glyphosate-Biocarb may induce hepatic histological changes as well as AST and ALT leaking from liver to serum in experimental models.

http://www.ncbi.nlm.nih.gov/pubmed/15451553

EPI - Roos, A.; Blair, A.; Rusiecki, J.; Hoppin, J.; Svec, M.; Dosemeci, M.; Sandler, D.; Alavanja, M. 2005. Cancer incidence among glyphosate-exposed pesticide applicators in the agricultural health study. *Environmental Health Perspectives*, Volume 113, Number 1.

Glyphosate is a broad-spectrum herbicide that is one of the most frequently applied pesticides in the world. Although there has been little consistent evidence of genotoxicity or carcinogenicity from in vitro and animal studies, a few epidemiologic reports have indicated potential health effects of glyphosate. We evaluated associations between glyphosate exposure and cancer incidence in the Agricultural Health Study (AHS), a prospective cohort study of 57,311 licensed pesticide applicators in Iowa and North Carolina. Detailed information on pesticide use and other factors was obtained from a self-administered questionnaire completed at time of enrollment (1993-1997).

Among private and commercial applicators, 75.5% reported having ever used glyphosate, of which > 97% were men. In this analysis, glyphosate exposure was defined as a) ever personally mixed or applied products containing glyphosate; b) cumulative lifetime days of use, or "cumulative exposure days" (years of use times days/year); and c) intensity-weighted cumulative exposure days (years of use times days/year times estimated intensity level). Poisson regression was used to estimate exposure-response relations between glyphosate and incidence of all cancers combined and 12 relatively common cancer subtypes. Glyphosate exposure was not associated with cancer incidence overall or with most of the cancer subtypes we studied. There was a suggested association with multiple myeloma incidence that should be followed up as more cases occur in the AHS. Given the widespread use of glyphosate, future analyses of the AHS will allow further examination of long-term health effects, including less common cancers.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1253709/

EPI - Paz-y-Miño, C.; Sánchez, M.; Arévalo, M.; Muñoz, M.; Witte, T.; De-la- Carrera, G.; Leone, P. 2007. Evaluation of DNA damage in an Ecuadorian population exposed to glyphosate. *Genetics and Molecular Biology*, 30, 456-460.

We analyzed the consequences of aerial spraying with glyphosate added to a surfactant solution in the northern part of Ecuador. A total of 24 exposed and 21 unexposed control individuals were investigated using the comet assay. The results showed a higher degree of DNA damage in the exposed group (comet length = 35.5 µm) compared to the control group (comet length = 25.94 µm). These results suggest that in the formulation used during aerial spraying glyphosate had a genotoxic effect on the exposed individuals.

Full article available at https://goo.gl/JFJTEC

Dallegrave, E; Mantese, F; Oliveira, R.; Andrade, A.; Dalsenter, P.; Langeloh, A. 2007. Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. *Arch Toxicol*, 81(9):665-73.

Glyphosate is the active ingredient and polyoxyethyleneamine is the surfactant present in the herbicide Roundup formulation commercialized in Brazil. The aim of this study was to assess the reproductive effects of glyphosate-Roundup on male and female offspring of Wistar rats exposed during pregnancy and lactation. Dams were treated orally with water or 50, 150 or 450 mg/kg glyphosate during pregnancy (21-23 days) and lactation (21 days). These doses do not correspond to human exposure levels. The results showed that glyphosate-Roundup did not induce maternal toxicity but induced adverse reproductive effects on male offspring rats: a decrease in sperm number per epididymis tail and in daily sperm production during adulthood, an increase in the percentage of abnormal sperms and a dose-related decrease in the serum testosterone level at puberty, and signs of individual spermatid degeneration during both periods. There was only a vaginal canal-opening delay in the exposed female offspring. These findings suggest that in utero and lactational exposure to glyphosate-Roundup may induce significant adverse effects on the reproductive system of male Wistar rats at puberty and during adulthood.

http://www.ncbi.nlm.nih.gov/pubmed/17634926

Benachour, N.; Sipahutar, H.; Moslemi, S.; Gasnier, C.; Travert, C.; Séralini, G-E. 2007. Time- and dose- dependent effects of Roundup on human embryonic and placental cells. *Archives of Environmental Contamination and Toxicology*, 53, 126-33.

Roundup is the major herbicide used worldwide, in particular on genetically modified plants that have been designed to tolerate it. We have tested the toxicity and endocrine disruption potential of Roundup (Bioforce on human embryonic 293 and placental-derived JEG3 cells, but also on normal human placenta and equine testis. The cell lines have proven to be suitable to estimate hormonal activity and toxicity of pollutants. The median lethal dose (LD(50)) of Roundup with embryonic cells is 0.3% within 1 h in serum-free medium, and it decreases to reach 0.06% (containing among other compounds 1.27 mM glyphosate) after 72 h in the presence of serum. In these conditions, the embryonic cells appear to be 2-4 times more sensitive than the placental ones. In all instances, Roundup (generally used in agriculture at 1-2%, i.e., with 21-42 mM glyphosate) is more efficient than its active ingredient, glyphosate, suggesting a synergistic effect provoked by the adjuvants present in Roundup. We demonstrated that serum-free cultures, even on a short-term basis (1 h), reveal the xenobiotic impacts that are visible 1-2 days later in serum. We also document at lower non-overtly toxic doses, from 0.01% (with 210 microM glyphosate) in 24 h, that Roundup is an aromatase disruptor. The direct inhibition is temperature-dependent and is confirmed in different tissues and species (cell lines from placenta or embryonic kidney, equine testicular, or human fresh placental extracts). Furthermore, glyphosate acts directly as a partial inactivator on microsomal aromatase, independently of its acidity, and in a dose-dependent manner. The cytotoxic, and potentially endocrine-disrupting effects of Roundup are thus amplified with time. Taken together, these data suggest that Roundup exposure may affect human reproduction and fetal development in case of contamination. Chemical mixtures in formulations appear to be underestimated regarding their toxic or hormonal impact.

http://www.ncbi.nlm.nih.gov/pubmed/17486286

Bellé, R.; Le Bouffant, R.; Morales, J.; Cosson, B.; Cormier, P.; Mulner-Lorillon, O. 2007: Sea urchin embryo, DNA-damaged cell cycle checkpoint and the mechanisms initiating cancer development. *J Soc Biol*, 201, 317-327.

Cell division is an essential process for heredity, maintenance and evolution of the whole living kingdom. Sea urchin early development represents an excellent experimental model for the analysis of cell cycle checkpoint mechanisms since embryonic cells contain a functional DNA-damage checkpoint and since the whole sea urchin genome is sequenced. The DNA-damaged checkpoint is responsible for an arrest in the cell cycle when DNA is damaged or incorrectly replicated, for activation of the DNA repair mechanism, and for commitment to cell death by apoptosis in the case of failure to repair. New insights in cancer biology lead to two fundamental concepts about the very first origin of cancerogenesis. Cancers result from dysfunction of DNA-damaged checkpoints and cancers appear as a result of normal stem cell (NCS) transformation into a cancer stem cell (CSC). The second aspect suggests a new definition of "cancer", since CSC can be detected well before any clinical evidence. Since early development starts from the zygote, which is a primary stem cell, sea urchin early development allows analysis of the early steps of the cancerization process. Although sea urchins do not develop cancers, the model is alternative and complementary to stem cells which are not easy to isolate, do not divide in a short time and do not divide synchronously. In the field of toxicology and incidence on human health, the sea urchin experimental model allows assessment of cancer risk from single or combined molecules long before any epidemiologic evidence is available. Sea urchin embryos were used to test the worldwide used pesticide Roundup that contains glyphosate as the active herbicide agent; it was shown to activate the DNA-damage checkpoint of the first cell cycle of development. The model therefore allows considerable increase in risk evaluation of new products in the field of cancer and offers a tool for the discovery of molecular markers for early diagnostic in cancer biology. Prevention and early diagnosis are two decisive elements of human cancer therapy.

http://www.ncbi.nlm.nih.gov/pubmed/18157084

Cavaş, T.; Könen, S. 2007. Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. *Mutagenesis*, vol. 22 no. 4 pp. 263–268.

Glyphosate is a widely used broad-spectrum weed control agent. In the present study, an in vivo study on the genotoxic effects of a technical herbicide (Roundup) containing isopropylamine salt of glyphosate was carried out on freshwater goldfish Carassius auratus. The fish were exposed to three doses of glyphosate formulation (5, 10 and 15 ppm). Cyclophosphamide at a single dose of 5 mg/l was used as positive control. Analysis of micronuclei, nuclear abnormalities and DNA damage were performed on peripheral erythrocytes sampled at intervals of 48, 96 and 144 h posttreatment. Our results revealed significant dose-dependent increases in the frequencies of micronuclei, nuclear abnormalities as well as DNA strand breaks. Our findings also confirmed that the alkaline comet assay and nuclear deformations in addition to micronucleus test on fish erythrocytes in vivo are useful tools in determining the potential genotoxicity of commercial herbicides.

http://www.ncbi.nlm.nih.gov/pubmed/17426049

Malatesta, M.; Perdoni, F.; Santin, G.; Battistelli, S.; Muller, S.; Biggiogerra, M. 2008. Hepatoma tissue culture (HTC) cells as a model for investigating the effects of low concentrations of herbicide on cell structure and function. *Toxicol in Vitro*, 22, 1853-1860.

Previous studies on mice fed genetically modified (GM) soybean demonstrated modifications of the mitochondrial functions and of the transcription/splicing pathways in hepatocytes. The cause(s) of these alterations could not be conclusively established but, since the GM soybean used is tolerant to glyphosate and was treated with the glyphosate-containing herbicide Roundup , the possibility exists that the effects observed may be due to herbicide residues. In order to verify this hypothesis, we treated HTC cells with 1-10mM Roundup and analysed cellular features by flow cytometry, fluorescence and electron microscopy. Under these experimental conditions, the death rate and the general morphology of HTC cells were not affected, as well as most of the cytoplasmic organelles. However, in HTC-treated cells, lysosome density increased and mitochondrial membranes modified indicating a decline in the respiratory activity. Moreover, nuclei underwent morpho-functional modifications suggestive of a decreased transcriptional/splicing activity. Although we cannot exclude that other factors than the presence of the herbicide residues could be responsible for the cellular modifications described in GM-fed mice, the concordance of the effects induced by low concentrations of Roundup on HTC cells suggests that the presence of Roundup residues could be one of the factors interfering with multiple metabolic pathways.

http://www.ncbi.nlm.nih.gov/pubmed/18835430

Prasad, S.; Srivastava, S.; Singh, M.; Shukla, Y. 2009. Clastogenic effects of glyphosate in bone marrow cells of swiss albino mice. *Journal of Toxicology*, Article ID 308985, 6 pages.

Glyphosate (N-(phosphonomethyl) glycine, C(3)H(8)NO(5)P), a herbicide, used to control unwanted annual and perennial plants all over the world. Nevertheless, occupational and environmental exposure to pesticides can pose a threat to nontarget species including human beings. Therefore, in the present study, genotoxic effects of the herbicide glyphosate were analyzed by measuring chromosomal aberrations (CAs) and micronuclei (MN) in bone marrow cells of Swiss albino mice. A single dose of glyphosate was given intraperitoneally (i.p) to the animals at a concentration of 25 and 50 mg/kg b.wt. Animals of positive control group were injected i.p. benzo(a)pyrene (100 mg/kg b.wt., once only), whereas, animals of control (vehicle) group were injected i.p. dimethyl sulfoxide (0.2 mL). Animals from all the groups were sacrificed at sampling times of 24, 48, and 72 hours and their bone marrow was analyzed for cytogenetic and chromosomal damage. Glyphosate treatment significantly increases CAs and MN induction at both treatments

and time compared with the vehicle control ($P < .05$). The cytotoxic effects of glyphosate were also evident, as observed by significant decrease in mitotic index (MI). The present results indicate that glyphosate is clastogenic and cytotoxic to mouse bone marrow.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2809416/

Gasnier, C.; Dumont, C.; Benachour, N.; Clair, E.; Chagnon, M-C.; Séralini, G-E. 2009. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology*, 262, 184191.

Glyphosate-based herbicides are the most widely used across the world; they are commercialized in different formulations. Their residues are frequent pollutants in the environment. In addition, these herbicides are spread on most eaten transgenic plants, modified to tolerate high levels of these compounds in their cells. Up to 400 ppm of their residues are accepted in some feed. We exposed human liver HepG2 cells, a well-known model to study xenobiotic toxicity, to four different formulations and to glyphosate, which is usually tested alone in chronic in vivo regulatory studies. We measured cytotoxicity with three assays (Alamar Blue, MTT, ToxiLight), plus genotoxicity (comet assay), anti-estrogenic (on ERalpha, ERbeta) and anti-androgenic effects (on AR) using gene reporter tests. We also checked androgen to estrogen conversion by aromatase activity and mRNA. All parameters were disrupted at sub-agricultural doses with all formulations within 24h. These effects were more dependent on the formulation than on the glyphosate concentration. First, we observed a human cell endocrine disruption from 0.5 ppm on the androgen receptor in MDA-MB453-kb2 cells for the most active formulation (R400), then from 2 ppm the transcriptional activities on both estrogen receptors were also inhibited on HepG2. Aromatase transcription and activity were disrupted from 10 ppm. Cytotoxic effects started at 10 ppm with Alamar Blue assay (the most sensitive), and DNA damages at 5 ppm. A real cell impact of glyphosate-based herbicides residues in food, feed or in the environment has thus to be considered, and their classifications as carcinogens/mutagens/reprotoxics is discussed.

Full article available at https://goo.gl/9AWBYg

Benachour, N.; Séralini, G-E. 2009. Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. *Chemical Research in Toxicology*, 22(1): 97-105.

We have evaluated the toxicity of four glyphosate (G)-based herbicides in Roundup (R) formulations, from 10^5 times dilutions, on three different human cell types. This dilution level is far below agricultural recommendations and corresponds to low levels of residues in food or feed. The formulations have been compared to G alone and with its main metabolite AMPA or with one known adjuvant of R formulations, POEA. HUVEC primary neonate umbilical cord vein cells have been tested with 293 embryonic kidney and JEG3 placental cell lines. All R formulations cause total cell death within 24 h, through an inhibition of the mitochondrial succinate dehydrogenase activity, and necrosis, by release of cytosolic adenylate kinase measuring membrane damage. They also induce apoptosis via activation of enzymatic caspases 3/7 activity. This is confirmed by characteristic DNA fragmentation, nuclear shrinkage (pyknosis), and nuclear fragmentation (karyorrhexis), which is demonstrated by DAPI in apoptotic round cells. G provokes only apoptosis, and HUVEC are 100 times more sensitive overall at this level. The deleterious effects are not proportional to G concentrations but rather depend on the nature of the adjuvants. AMPA and POEA separately and synergistically damage cell membranes like R but at different concentrations. Their mixtures are generally even more harmful with G. In conclusion, the R adjuvants like POEA change human cell permeability and amplify toxicity induced already by G, through apoptosis and necrosis. The real threshold of G toxicity must take into account the presence of adjuvants but also G metabolism and time-amplified effects or bioaccumulation.

This should be discussed when analyzing the in vivo toxic actions of R. This work clearly confirms that the adjuvants in Roundup formulations are not inert. Moreover, the proprietary mixtures available on the market could cause cell damage and even death around residual levels to be expected, especially in food and feed derived from R formulation-treated crops.

http://pubs.acs.org/doi/abs/10.1021/tx800218n

George, J.; Prasad, S.; Mahmood, Z.; Shukla, Y. 2010. Studies on glyphosate-induced carcinogenicity in mouse skin: A proteomic approach. *J Proteomics*, 73(5), 951-964.

Glyphosate is a widely used broad spectrum herbicide, reported to induce various toxic effects in non-target species, but its carcinogenic potential is still unknown. Here we showed the carcinogenic effects of glyphosate using 2-stage mouse skin carcinogenesis model and proteomic analysis. Carcinogenicity study revealed that glyphosate has tumor promoting activity. Proteomic analysis using 2-dimensional gel electrophoresis and mass spectrometry showed that 22 spots were differentially expressed (>2 fold) on glyphosate, 7, 12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) application over untreated control. Among them, 9 proteins (translation elongation factor eEF-1 alpha chain, carbonic anhydrase III, annexin II, calcyclin, fab fragment anti-VEGF antibody, peroxiredoxin-2, superoxide dismutase [Cu-Zn], stefin A3, and calgranulin-B) were common and showed similar expression pattern in glyphosate and TPA-treated mouse skin. These proteins are known to be involved in several key processes like apoptosis and growth-inhibition, anti-oxidant responses, etc. The up-regulation of calcyclin, calgranulin-B and down-regulation of superoxide dismutase [Cu-Zn] was further confirmed by immunoblotting, indicating that these proteins can be good candidate biomarkers for skin carcinogenesis induced by glyphosate. Altogether, these results suggested that glyphosate has tumor promoting potential in skin carcinogenesis and its mechanism seems to be similar to TPA.

http://www.ncbi.nlm.nih.gov/pubmed/20045496

Paganelli, A.; Gnazzo, V.; Acosta, H.; López, S.; Carrasco, A. 2010. Glyphosate based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signalling. *Chemical Research in Toxicology*.

The broad spectrum herbicide glyphosate is widely used in agriculture worldwide. There has been ongoing controversy regarding the possible adverse effects of glyphosate on the environment and on human health. Reports of neural defects and craniofacial malformations from regions where glyphosate-based herbicides (GBH) are used led us to undertake an embryological approach to explore the effects of low doses of glyphosate in development.*Xenopus laevis* embryos were incubated with 1/5000 dilutions of a commercial GBH. The treated embryos were highly abnormal with marked alterations in cephalic and neural crest development and shortening of the anterior–posterior (A-P) axis. Alterations on neural crest markers were later correlated with deformities in the cranial cartilages at tadpole stages. Embryos injected with pure glyphosate showed very similar phenotypes. Moreover, GBH produced similar effects in chicken embryos, showing a gradual loss of rhombomere domains, reduction of the optic vesicles, and microcephaly. This suggests that glyphosate itself was responsible for the phenotypes observed, rather than a surfactant or other component of the commercial formulation. A reporter gene assay revealed that GBH treatment increased endogenous retinoic acid (RA) activity in *Xenopus* embryos and cotreatment with a RA antagonist rescued the teratogenic effects of the GBH. Therefore, we conclude that the phenotypes produced by GBH are mainly a consequence of the increase of endogenous retinoid activity. This is consistent with the decrease of Sonic hedgehog (Shh) signaling from the embryonic dorsal midline, with the inhibition of *otx2* expression and with the disruption of cephalic neural crest development. The direct effect of glyphosate on early mechanisms of morphogenesis in vertebrate embryos opens

concerns about the clinical findings from human offspring in populations exposed to GBH in agricultural fields.

http://pubs.acs.org/doi/abs/10.1021/tx1001749

Romano, R.; Romano, M.; Bernardi, M.; Furtado, P.; Oliveira, C. 2010. Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology. *Arch Toxicol*, 84 (4), 309–317.

Glyphosate is a herbicide widely used to kill weeds both in agricultural and non-agricultural landscapes. Its reproductive toxicity is related to the inhibition of a StAR protein and an aromatase enzyme, which causes an in vitro reduction in testosterone and estradiol synthesis. Studies in vivo about this herbicide effects in prepubertal Wistar rats reproductive development were not performed at this moment. Evaluations included the progression of puberty, body development, the hormonal production of testosterone, estradiol and corticosterone, and the morphology of the testis. Results showed that the herbicide (1) significantly changed the progression of puberty in a dose-dependent manner; (2) reduced the testosterone production, in semineferous tubules' morphology, decreased significantly the epithelium height ($P < 0.001$; control = 85.8 +/- 2.8 microm; 5 mg/kg = 71.9 +/- 5.3 microm; 50 mg/kg = 69.1 +/- 1.7 microm; 250 mg/kg = 65.2 +/- 1.3 microm) and increased the luminal diameter ($P < 0.01$; control = 94.0 +/- 5.7 microm; 5 mg/kg = 116.6 +/- 6.6 microm; 50 mg/kg = 114.3 +/- 3.1 microm; 250 mg/kg = 130.3 +/- 4.8 microm); (4) no difference in tubular diameter was observed; and (5) relative to the controls, no differences in serum corticosterone or estradiol levels were detected, but the concentrations of testosterone serum were lower in all treated groups ($P < 0.001$; control = 154.5 +/- 12.9 ng/dL; 5 mg/kg = 108.6 +/- 19.6 ng/dL; 50 mg/dL = 84.5 +/- 12.2 ng/dL; 250 mg/kg = 76.9 +/- 14.2 ng/dL). These results suggest that commercial formulation of glyphosate is a potent endocrine disruptor in vivo, causing disturbances in the reproductive development of rats when the exposure was performed during the puberty period.

http://www.ncbi.nlm.nih.gov/pubmed/20012598

Shehata, A.; Schrödl, W.; Aldin, A.; Hafez, H.; Krüger, M. 2012. The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota *In Vitro*. *Curr Microbiol*, DOI 10.1007/s00284-012-0277-2.

The use of glyphosate modifies the environment which stresses the living microorganisms. The aim of the present study was to determine the real impact of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. The presented results evidence that the highly pathogenic bacteria as Salmonella Entritidis, Salmonella Gallinarum, Salmonella Typhimurium, Clostridium perfringens and Clostridium botulinum are highly resistant to glyphosate. However, most of beneficial bacteria as Enterococcus faecalis, Enterococcus faecium, Bacillus badius, Bifidobacterium adolescentis and Lactobacillus spp. were found to be moderate to highly susceptible. Also Campylobacter spp. were found to be susceptible to glyphosate. A reduction of beneficial bacteria in the gastrointestinal tract microbiota by ingestion of glyphosate could disturb the normal gut bacterial community. Also, the toxicity of glyphosate to the most prevalent Enterococcus spp. could be a significant predisposing factor that is associated with the increase in C. botulinum-mediated diseases by suppressing the antagonistic effect of these bacteria on clostridia.

Full article available at https://goo.gl/0QeUdY

Jasper, R.; Locatelli, G.; Pilati, C.; Locatelli, C. 2012. Evaluation of biochemical, hematological and oxidative parameters in mice exposed to the herbicide glyphosate-Roundup®. *Interdisciplinary Toxicology*, 5(3): 133–140, doi: 10.2478/v10102-012-0022-5.

We evaluated the toxicity of hepatic, hematological, and oxidative effects of glyphosate-Roundup® on male and female albino Swiss mice. The animals were treated orally with either 50 or 500 mg/kg body weight of the herbicide, on a daily basis for a period of 15 days. Distilled water was used as control treatment. Samples of blood and hepatic tissue were collected at the end of the treatment. Hepatotoxicity was monitored by quantitative analysis of the serum enzymes ALT, AST, and γ-GT and renal toxicity by urea and creatinine. We also investigated liver tissues histopathologically. Alterations of hematological parameters were monitored by RBC, WBC, hemoglobin, hematocrit, MCV, MCH, and MCHC. TBARS (thiobarbituric acid reactive substances) and NPSH (non-protein thiols) were analyzed in the liver to assess oxidative damage. Significant increases in the levels of hepatic enzymes (ALT, AST, and γ-GT) were observed for both herbicide treatments, but no considerable differences were found by histological analysis. The hematological parameters showed significant alterations (500 mg/kg body weight) with reductions of RBC, hematocrit, and hemoglobin, together with a significant increase of MCV, in both sexes of mice. In males, there was an important increase in lipid peroxidation at both dosage levels, together with an NPSH decrease in the hepatic tissue, whereas in females significant changes in these parameters were observed only at the higher dose rate. The results of this study indicate that glyphosate-Roundup® can promote hematological and hepatic alterations, even at subacute exposure, which could be related to the induction of reactive oxygen species.We evaluated the toxicity of hepatic, hematological, and oxidative effects of glyphosate-Roundup® on male and female albino Swiss mice. The animals were treated orally with either 50 or 500 mg/kg body weight of the herbicide, on a daily basis for a period of 15 days. Distilled water was used as control treatment. Samples of blood and hepatic tissue were collected at the end of the treatment. Hepatotoxicity was monitored by quantitative analysis of the serum enzymes ALT, AST, and γ-GT and renal toxicity by urea and creatinine. We also investigated liver tissues histopathologically. Alterations of hematological parameters were monitored by RBC, WBC, hemoglobin, hematocrit, MCV, MCH, and MCHC. TBARS (thiobarbituric acid reactive substances) and NPSH (non-protein thiols) were analyzed in the liver to assess oxidative damage. Significant increases in the levels of hepatic enzymes (ALT, AST, and γ-GT) were observed for both herbicide treatments, but no considerable differences were found by histological analysis. The hematological parameters showed significant alterations (500 mg/kg body weight) with reductions of RBC, hematocrit, and hemoglobin, together with a significant increase of MCV, in both sexes of mice. In males, there was an important increase in lipid peroxidation at both dosage levels, together with an NPSH decrease in the hepatic tissue, whereas in females significant changes in these parameters were observed only at the higher dose rate. The results of this study indicate that glyphosate-Roundup® can promote hematological and hepatic alterations, even at subacute exposure, which could be related to the induction of reactive oxygen species.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3600513/

REV – Antoniou, M.; Habib, M.; Howard, C.; Jennings, R.; Nodari, R.; Robinson, C.; Fagan, J. 2012. Teratogenic effects of glyphosate-based herbicides: Divergence of regulatory decisions from scientific evidence. *Journal of Environmental & Analytical Toxicology*, S4:006.

The publication of a study in 2010 showing that a glyphosate herbicide formulation and glyphosate alone caused malformations in the embryos of Xenopus laevis and chickens caused a scientific and political controversy. Debate centred on the effects of the production and consumption of genetically modified Roundup® Ready® soy, which is engineered to tolerate applications of glyphosate herbicide. This study, along with others indicating teratogenic and reproductive effects from glyphosate herbicide exposure, was rebutted by the German Federal Office for Consumer Protection and Food Safety, BVL, as well as in industry-sponsored papers. These rebuttals relied partly on unpublished industry-sponsored studies commissioned for regulatory purposes, which, it was claimed, showed that glyphosate is not teratogenic or a reproductive toxin. However, examination of the German authorities' draft assessment report (DAR) on the industry studies, which underlies glyphosate's EU authorisation, revealed further evidence of glyphosate's teratogenicity. Nevertheless, the German and EU authorities

minimized these findings in their assessment and set a potentially unsafe acceptable daily intake (ADI) level for glyphosate. This paper reviews the evidence on the teratogenicity and reproductive toxicity of glyphosate herbicides and concludes that a new and transparent risk assessment needs to be conducted by scientists who are independent of industry and of the regulatory bodies that were involved in the existing authorisation of glyphosate.

Full article available at https://goo.gl/1cSk0g

Romano, M.; Romano, R.; Santos, L.; Wisniewski, P.; Campos, D.A; Souza, P.; Viau, P.; Bernardi, M.; Nunes, M.; Oliveira, C. 2012. Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. *Arch Toxicol*, 86 (4), 663–673.

Sexual differentiation in the brain takes place from late gestation to the early postnatal days. This is dependent on the conversion of circulating testosterone into estradiol by the enzyme aromatase. The glyphosate was shown to alter aromatase activity and decrease serum testosterone concentrations. Thus, the aim of this study was to investigate the effect of gestational maternal glyphosate exposure (50 mg/kg, NOAEL for reproductive toxicity) on the reproductive development of male offspring. Sixty-day-old male rat offspring were evaluated for sexual behavior and partner preference; serum testosterone concentrations, estradiol, FSH and LH; the mRNA and protein content of LH and FSH; sperm production and the morphology of the seminiferous epithelium; and the weight of the testes, epididymis and seminal vesicles. The growth, the weight and age at puberty of the animals were also recorded to evaluate the effect of the treatment. The most important findings were increases in sexual partner preference scores and the latency time to the first mount; testosterone and estradiol serum concentrations; the mRNA expression and protein content in the pituitary gland and the serum concentration of LH; sperm production and reserves; and the height of the germinal epithelium of seminiferous tubules. We also observed an early onset of puberty but no effect on the body growth in these animals. These results suggest that maternal exposure to glyphosate disturbed the masculinization process and promoted behavioral changes and histological and endocrine problems in reproductive parameters. These changes associated with the hypersecretion of androgens increased gonadal activity and sperm production.

http://www.ncbi.nlm.nih.gov/pubmed/22120950

Clair, E.; Mesnage, R.; Travert, C.; Séralini, G-E. 2012. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells *in vitro*, and testosterone decrease at lower levels. *Toxicol In Vitro*, 26 (2), 269–279.

The major herbicide used worldwide, Roundup, is a glyphosate-based pesticide with adjuvants. Glyphosate, its active ingredient in plants and its main metabolite (AMPA) are among the first contaminants of surface waters. Roundup is being used increasingly in particular on genetically modified plants grown for food and feed that contain its residues. Here we tested glyphosate and its formulation on mature rat fresh testicular cells from 1 to 10000ppm, thus from the range in some human urine and in environment to agricultural levels. We show that from 1 to 48h of Roundup exposure Leydig cells are damaged. Within 24-48h this formulation is also toxic on the other cells, mainly by necrosis, by contrast to glyphosate alone which is essentially toxic on Sertoli cells. Later, it also induces apoptosis at higher doses in germ cells and in Sertoli/germ cells co-cultures. At lower non toxic concentrations of Roundup and glyphosate (1ppm), the main endocrine disruption is a testosterone decrease by 35%. The pesticide has thus an endocrine impact at very low environmental doses, but only a high contamination appears to provoke an acute rat testicular toxicity. This does not anticipate the chronic toxicity which is insufficiently tested, and only with glyphosate in regulatory tests.

http://www.ncbi.nlm.nih.gov/pubmed/22200534

Séralini, G-E.; Clair, E.; Mesnage, R.; Gress, S.; Defarge, N.; Malatesta, M.; Hennequin, D.; Vendomois, J. 2012. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. *Food Chem Toxicol*, 50, 4221– 4231.

Retracted.

http://www.ncbi.nlm.nih.gov/pubmed/24490213

COM – Séralini, G-E.; Mesnage, R.; Defarge, N.; Gress, S.; Hennequin, D.; Clair, E.; Malatesta, M.; Vendômois, J. 2013. Answers to critics: Why there is a long term toxicity due to a Roundup-tolerant genetically modified maize and to a Roundup herbicide. *Food and Chemical Toxicology*, 53: 476-83.

Our recent work (Séralini et al., 2012) remains to date the most detailed study involving the life-long consumption of an agricultural genetically modified organism (GMO). This is true especially for NK603 maize for which only a 90-day test for commercial release was previously conducted using the same rat strain (Hammond et al., 2004). It is also the first long term detailed research on mammals exposed to a highly diluted pesticide in its total formulation with adjuvants. This may explain why 75% of our first criticisms arising within a week, among publishing authors, come from plant biologists, some developing patents on GMOs, even if it was a toxicological paper on mammals, and from Monsanto Company who owns both the NK603 GM maize and Roundup herbicide (R). Our study has limits like any one, and here we carefully answer to all criticisms from agencies, consultants and scientists, that were sent to the Editor or to ourselves. At this level, a full debate is biased if the toxicity tests on mammals of NK603 and R obtained by Monsanto Company remain confidential and thus unavailable in an electronic format for the whole scientific community to conduct independent scrutiny of the raw data. In our article, the conclusions of long-term NK603 and Roundup toxicities came from the statistically highly discriminant findings at the biochemical level in treated groups in comparison to controls, because these findings do correspond in an blinded analysis to the pathologies observed in organs, that were in turn linked to the deaths by anatomopathologists. GM NK603 and R cannot be regarded as safe to date.

Full article available at https://goo.gl/HXXUc3

Cavalli, V.; Cattani, D.; Rieg, C.; Pierozan, P.; Zanatta, L.; Parisotto, E.; Filho, D.; Silva, F.; Pessoa-Pureur, R.; Zamoner, A. 2013. Roundup disrupts male reproductive functions by triggering calcium-mediated cell death in rat testis and Sertoli cells. *Free Radical Biology and Medicine*, 65 (2013) 335–346.

Glyphosate is the primary active constituent of the commercial pesticide Roundup. The present results show that acute Roundup exposure at low doses (36 ppm, 0.036 g/L) for 30 min induces oxidative stress and activates multiple stress-response pathways leading to Sertoli cell death in prepubertal rat testis. The pesticide increased intracellular Ca(2+) concentration by opening L-type voltage-dependent Ca(2+) channels as well as endoplasmic reticulum IP3 and ryanodine receptors, leading to Ca(2+) overload within the cells, which set off oxidative stress and necrotic cell death. Similarly, 30 min incubation of testis with glyphosate alone (36 ppm) also increased (45)Ca(2+) uptake. These events were prevented by the antioxidants Trolox and ascorbic acid. Activated protein kinase C, phosphatidylinositol 3-kinase, and the mitogen-activated protein kinases such as ERK1/2 and p38MAPK play a role in eliciting Ca(2+) influx and cell death. Roundup decreased the levels of reduced glutathione (GSH) and increased the amounts of thiobarbituric acid-reactive species (TBARS) and protein carbonyls. Also, exposure to glyphosate-Roundup stimulated the activity of glutathione peroxidase, glutathione reductase, glutathione S-transferase, γ-glutamyltransferase, catalase, superoxide dismutase, and glucose-6-phosphate dehydrogenase, supporting downregulated GSH levels. Glyphosate has been described as an endocrine disruptor affecting the male reproductive system; however, the molecular basis of its toxicity remains to be clarified. We propose that Roundup toxicity, implicated in Ca(2+) overload, cell signaling misregulation, stress response of the endoplasmic reticulum, and/or depleted antioxidant defenses, could contribute to Sertoli cell disruption in spermatogenesis that could have an impact on male fertility.

http://www.ncbi.nlm.nih.gov/pubmed/23820267

George, J.; Shukla, Y. 2013. Emptying of intracellular calcium pool and oxidative stress imbalance are associated with the glyphosate-induced proliferation in human skin keratinocytes HaCaT cells. *ISRN Dermatology*, Volume 2013, Article ID 825180, 12 pages.

We demonstrated that glyphosate possesses tumor promoting potential in mouse skin carcinogenesis and SOD 1, calcyclin (S100A6), and calgranulin B (S100A9) have been associated with this potential, although the mechanism is unclear. We aimed to clarify whether imbalance in between $[Ca^{2+}]_i$ levels and oxidative stress is associated with glyphosate-induced proliferation in human keratinocytes HaCaT cells. The $[Ca^{2+}]_i$ levels, ROS generation, and expressions of G1/S cyclins, IP_3R1, S100A6, S100A9, and SOD 1, and apoptosis-related proteins were investigated upon glyphosate exposure in HaCaT cells. Glyphosate (0.1 mM) significantly induced proliferation, decreases $[Ca^{2+}]_i$, and increases ROS generation in HaCaT cells, whereas antioxidant N-acetyl-L-cysteine (NAC) pretreatment reverts these effects which directly indicated that glyphosate induced cell proliferation by lowering $[Ca^{2+}]_i$ levels via ROS generation. Glyphosate also enhanced the expression of G1/S cyclins associated with a sharp decrease in G0/G1 and a corresponding increase in S-phases. Additionally, glyphosate also triggers S100A6/S100A9 expression and decreases IP_3R1 and SOD 1 expressions in HaCaT cells. Notably, Ca^{2+}suppression also prevented apoptotic related events including Bax/Bcl-2 ratio and caspases activation. This study highlights that glyphosate promotes proliferation in HaCaT cells probably by disrupting the balance in between $[Ca^{2+}]_i$ levels and oxidative stress which in turn facilitated the downregulation of mitochondrial apoptotic signaling pathways.

Full article available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3773425/#!po=65.3846

Séralini, G-E.; Clair, E.; Mesnage, R.; Gress, S.; Defarge, N.; Malatesta, M.; Hennequin, D.; Vendômois, J. 2014**.** Republished study: long-term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. *Environmental Sciences Europe*, 26:14.

Background: The health effects of a Roundup-tolerant NK603 genetically modified (GM) maize (from 11% in the diet), cultivated with or without Roundup application and Roundup alone (from 0.1 ppb of the full pesticide containing glyphosate and adjuvants) in drinking water, were evaluated for 2 years in rats. This study constitutes a follow-up investigation of a 90-day feeding study conducted by Monsanto in order to obtain commercial release of this GMO, employing the same rat strain and analyzing biochemical parameters on the same number of animals per group as our investigation. Our research represents the first chronic study on these substances, in which all observations including tumors are reported chronologically. Thus, it was not designed as a carcinogenicity study. We report the major findings with 34 organs observed and 56 parameters analyzed at 11 time points for most organs.
Results: Biochemical analyses confirmed very significant chronic kidney deficiencies, for all treatments and both sexes; 76% of the altered parameters were kidney-related. In treated males, liver congestions and necrosis were 2.5 to 5.5 times higher. Marked and severe nephropathies were also generally 1.3 to 2.3 times greater. In females, all treatment groups showed a two- to threefold increase in mortality, and deaths were earlier. This difference was also evident in three male groups fed with GM maize. All results were hormone- and sex-dependent, and the pathological profiles were comparable. Females developed large mammary tumors more frequently and before controls; the pituitary was the second most disabled organ; the sex hormonal balance was modified by consumption of GM maize and Roundup treatments. Males presented up to four times more large

palpable tumors starting 600 days earlier than in the control group, in which only one tumor was noted. These results may be explained by not only the non-linear endocrine-disrupting effects of Roundup but also by the overexpression of the EPSPS transgene or other mutational effects in the GM maize and their metabolic consequences.
Conclusion: Our findings imply that long-term (2 year) feeding trials need to be conducted to thoroughly evaluate the safety of GM foods and pesticides in their full commercial formulations.

Full article available at http://www.enveurope.com/content/26/1/14

Koller, V.; Fürhacker, M.; Nersesyan, A.; Mišík, M.; Eisenbauer, M.; Knasmueller, S. 2014. Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells. *Arch Toxicol*, 86(5):805-13. doi: 10.1007/s00204-012-0804-8.

Glyphosate (G) is the largest selling herbicide worldwide; the most common formulations (Roundup, R) contain polyoxyethyleneamine as main surfactant. Recent findings indicate that G exposure may cause DNA damage and cancer in humans. Aim of this investigation was to study the cytotoxic and genotoxic properties of G and R (UltraMax) in a buccal epithelial cell line (TR146), as workers are exposed via inhalation to the herbicide. R induced acute cytotoxic effects at concentrations > 40 mg/l after 20 min, which were due to membrane damage and impairment of mitochondrial functions. With G, increased release of extracellular lactate dehydrogenase indicative for membrane damage was observed at doses > 80 mg/l. Both G and R induced DNA migration in single-cell gel electrophoresis assays at doses > 20 mg/l. Furthermore, an increase of nuclear aberrations that reflect DNA damage was observed. The frequencies of micronuclei and nuclear buds were elevated after 20-min exposure to 10-20 mg/l, while nucleoplasmatic bridges were only enhanced by R at the highest dose (20 mg/l). R was under all conditions more active than its active principle (G). Comparisons with results of earlier studies with lymphocytes and cells from internal organs indicate that epithelial cells are more susceptible to the cytotoxic and DNA-damaging properties of the herbicide and its formulation. Since we found genotoxic effects after short exposure to concentrations that correspond to a 450-fold dilution of spraying used in agriculture, our findings indicate that inhalation may cause DNA damage in exposed individuals.

http://www.ncbi.nlm.nih.gov/pubmed/22331240

EPI - Swanson, N.; Leu, A.; Abrahamson, J.; Wallet, B. 2014. Genetically engineered crops, glyphosate and the deterioration of health in the United States of America. *Journal of Organic Systems*, 9(2).

A huge increase in the incidence and prevalence of chronic diseases has been reported in the United States (US) over the last 20 years. Similar increases have been seen globally. The herbicide glyphosate was introduced in 1974 and its use is accelerating with the advent of herbicide-tolerant genetically engineered (GE) crops. Evidence is mounting that glyphosate interferes with many metabolic processes in plants and animals and glyphosate residues have been detected in both. Glyphosate disrupts the endocrine system and the balance of gut bacteria, it damages DNA and is a driver of mutations that lead to cancer.
In the present study, US government databases were searched for GE crop data, glyphosate application data and disease epidemiological data. Correlation analyses were then performed on a total of 22 diseases in these time-series data sets. The Pearson correlation coefficients are highly significant (< 10-5) between glyphosate applications and hypertension (R = 0.923), stroke (R = 0.925), diabetes prevalence (R = 0.971), diabetes incidence (R = 0.935), obesity (R = 0.962), lipoprotein metabolism disorder (R = 0.973), Alzheimer's (R = 0.917), senile dementia (R = 0.994), Parkinson's (R = 0.875), multiple sclerosis (R = 0.828), autism (R = 0.989), inflammatory bowel disease (R = 0.938), intestinal infections (R = 0.974), end stage renal disease (R = 0.975), acute kidney failure (R = 0.978), cancers of the thyroid (R = 0.988), liver (R = 0.960), bladder (R = 0.981), pancreas (R = 0.918), kidney (R = 0.973) and myeloid leukaemia (R = 0.878).
The Pearson correlation coefficients are highly significant (< 10-4) between the percentage of GE corn and soy planted in the US and hypertension (R = 0.961), stroke (R = 0.983), diabetes prevalence (R = 0.983), diabetes incidence (R = 0.955), obesity (R = 0.962), lipoprotein metabolism disorder (R = 0.955), Alzheimer's (R = 0.937), Parkinson's (R = 0.952), multiple sclerosis (R = 0.876), hepatitis C (R = 0.946), end stage renal disease (R = 0.958), acute kidney failure (R = 0.967), cancers of the thyroid (R = 0.938), liver (R = 0.911), bladder (R = 0.945), pancreas (R = 0.841), kidney (R = 0.940) and myeloid leukaemia (R = 0.889). The significance and strength of the correlations show that the effects of glyphosate and GE crops on human health should be further investigated.

Full article available at https://goo.gl/KFRftb

Because it is a pollutant with high load presence, which can be found in soil, water, air and in various foods (see references contained in Part 3 item 2.1.1), glyphosate is a highly relevant xenobiotic[57], subject in tissues and body fluids of human and animal organisms. Even the placenta shown permeable to glyphosate, facilitating the contamination of fetuses and threatening the stability of characteristics transmitted between generations.

Poulsen, M.; Rytting, E.; Mose, T.; Knudsen, L. 2009. Modeling placental transport: Correlation of *in vitro* BeWo cell permeability and ex vivo human placental perfusion. *Toxicol In Vitro*, 23: 1380–1386.

The placental passage of three compounds with different physicochemical properties was recently investigated in ex vivo human placental perfusion experiments (caffeine, benzoic acid, and glyphosate) [Mose, T., Kjaerstad, M.B., Mathiesen, L., Nielsen, J.B., Edelfors, S., Knudsen, L.E., 2008. Placental passage of benzoic acid, caffeine, and glyphosate in an ex vivo human perfusion system. J. Toxicol. Environ. Health, Part A 71, 984-991]. In this work, the transport of these same three compounds, plus the reference compound antipyrine, was investigated using BeWo (b30) cell monolayers. Transport across the BeWo cells was observed in the rank order of caffeine>antipyrine>benzoic acid>glyphosate in terms of both the apparent permeability coefficient and the initial slope, defined as the linear rate of substance transferred to the fetal compartment as percent per time, a parameter used to compare the two experimental models. The results from the in vitro studies were in excellent agreement with the ex vivo results (caffeine approximately antipyrine>benzoic acid>glyphosate). However the transfer rate was much slower in the BeWo cells compared to the perfusion system. The advantages and limitations of each model are discussed in order to assist in the preparation, prediction, and performance of future studies of maternal-fetal transfer.

http://www.ncbi.nlm.nih.gov/pubmed/19647068

57 Xenobiotics are foreign chemicals to a particular organism or biological system, which can be found even when they are not produced there or expected. The term also indicates substances present in much higher concentrations than the normal level, as has occurred in overdoses of drugs that are not part of the diet of organisms which are observed.

Aris, A.; Leblanc, S. 2011. Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec, Canada. *Reprod Toxicol*, doi:10.1016/j.reprotox.2011.02.004.

Pesticides associated to genetically modified foods (PAGMF), are engineered to tolerate herbicides such as glyphosate (GLYP) and gluphosinate (GLUF) or insecticides such as the bacterial toxin bacillus thuringiensis (Bt). The aim of this study was to evaluate the correlation between maternal and fetal exposure, and to determine exposure levels of GLYP and its metabolite aminomethyl phosphoric acid (AMPA), GLUF and its metabolite 3-methylphosphinicopropionic acid (3-MPPA) and Cry1Ab protein (a Bt toxin) in Eastern Townships of Quebec, Canada. Blood of thirty pregnant women (PW) and thirty-nine nonpregnant women (NPW) were studied. Serum GLYP and GLUF were detected in NPW and not detected in PW. Serum 3-MPPA and CryAb1 toxin were detected in PW, their fetuses and NPW. This is the first study to reveal the presence of circulating PAGMF in women with and without pregnancy, paving the way for a new field in reproductive toxicology including nutrition and utero-placental toxicities.

http://www.ncbi.nlm.nih.gov/pubmed/21338670

Krüger, M.; Schrödl, W.; Neuhaus, J.; Shehata, A. 2013. Field investigations of glyphosate in urine of Danish dairy cows. *J Environ Anal Toxicol*, 3(5): 100186.

In the present study, thirty dairy cows from each of eight Danish dairy farms were investigated for excretion of glyphosate in urine. Blood serum parameters indicative of cytotoxicity as alkaline phosphatase (AP), glutamate dehydrogenase (GLDH), glutamate oxaloacetate transaminase (GOT), creatinine kinase CK), nephrotoxicity, (urea, creatine), cholesterol and the trace elements as manganese (Mn), cobalt (Co), selenium (Se), copper (Cu) and zinc (Zn) were investigated. All cows excreted glyphosate in their urine but in varying concentrations. Increased levels of GLDH, GOT and CK in cows from all farms demonstrate a possible effect of glyphosate on liver and muscle cells. High urea levels in some farms could be due to nephrotoxicity of glyphosate. Also the unexpected very low levels of Mn and Co were observed in all animals which could be explained due to a strong mineral chelating effect of glyphosate. In contrast the mean levels of Cu, Zn and Se were within the normal reference range. In conclusion, this study gives the first documentation to which extent Danish dairy cattle are exposed to Glyphosate and its impact on blood parameters.

Full article available at https://goo.gl/ovE1LI

Krüger, M.; Schledorn, P.; Schrödl, W.; Hoppe, H.; Lutz, W.; Shehata, A. 2014. Detection of glyphosate residues in animals and humans. *Journal Environmental Analytical Toxicology*, 4: 210. doi: 10.4172/2161-0525.1000210.

In the present study glyphosate residues were tested in urine and different organs of dairy cows as well as in urine of hares, rabbits and humans using ELISA and Gas Chromatography-Mass Spectroscopy (GC-MS). The correlation coefficients between ELISA and GC-MS were 0.96, 0.87, 0.97and 0.96 for cattle, human, and rabbit urine and organs, respectively. The recovery rate of glyphosate in spiked meat using ELISA was 91%. Glyphosate excretion in German dairy cows was significantly lower than Danish cows. Cows kept in genetically modified free area had significantly lower glyphosate concentrations in urine than conventional husbandry cows. Also glyphosate was detected in different organs of slaughtered cows as intestine, liver, muscles, spleen and kidney. Fattening rabbits showed significantly higher glyphosate residues in urine than hares. Moreover, glyphosate was significantly higher in urine of humans with conventional feeding. Furthermore, chronically ill humans showed significantly higher glyphosate residues in urine than healthy population. The presence of glyphosate residues in both humans and animals could haul the entire population towards numerous health hazards, studying the impact of glyphosate residues on health

is warranted and the global regulations for the use of glyphosate may have to be re-evaluated.

Full article available at https://goo.gl/JP1Fgt

Krüger, M.; Schrödl, W.; Pedersen, I.; Shehata, A. 2014. Detection of glyphosate in malformed piglets. *Journal Environmental Analytical Toxicology*, 4: 230. doi: 10.4172/2161-0525.1000230.

Glyphosate residues in different organs and tissues as lungs, liver, kidney, brain, gut wall and heart of malformed euthanized one-day-old Danish piglets (N= 38) were tested using ELISA. All organs or tissues had glyphosate in different concentrations. The highest concentrations were seen in the lungs (Range 0.4-80 µg/ml) and hearts (Range 0.15-80 µg/ml). The lowest concentrations were detected in muscles (4.4-6.4 µg/g). The detection of such glyphosate concentrations in these malformed piglets could be an allusion to the cause of these congenital anomalies. Further investigations are urgently needed to prove or exclude the role of glyphosate in malformations in piglets and other animals.

Full article available at https://goo.gl/rCqsvM

2.1.3 Herbicides based on gluphosinate ammonium

The gluphosinate ammonium is another active ingredient of herbicides associated with transgenic plants of the type HT, a fact that ensures its wide dissemination in various agro-ecosystems. The following articles show negative impacts of herbicides formulated based on that active ingredient on human and animal organisms, even in small doses.

Watanabe, T.; Iwase, T. 1996. Development and dymorphogenic effects of glufosinate ammonium on mouse embryos in culture. *Teratogenesis Carcinogenesis and Mutagenesis*, 16, 287-299.

The effects of glufosinate ammonium on embryonic development in mice were examined using whole embryo and micromass cultures of midbrain and limb bud cells. In day 8 embryos cultured for 48 hr, glufosinate caused significant overall embryonic growth retardation and increased embryolethality to 37.5% at 10 micrograms/ml (5.0 x 10(-5) M). All embryos in the treated groups exhibited specific morphological defects including hypoplasia of the prosencephalon (forebrain) (100%) and visceral arches (100%). In day 10 embryos cultured for 24 hr, glufosinate significantly reduced the crown-rump length and the number of somite pairs, and produced a high incidence of morphological defects (84.6%) at 10 micrograms/ml. These embryos were characterized by blister in the lateral head (100%), hypoplasia of prosencephalon (57.1%), and cleft lips (42.9%) at 20 micrograms/ml (10.0 x 10(-5) M). Histological examination of the treated embryos showed numerous cell death (pyknotic debris) present throughout the neuroepithelium in the brain vesicle and neural tube, but did not involve the underlying mesenchyme. In micromass culture, glufosinate inhibited the differentiation of midbrain cells in day 12 embryos with 50% inhibition occurring at 0.55 microgram/ml (2.8 x 10(-6) M). The ratios of 50% inhibition concentration for cell

proliferation to cell differentiation in limb bud cells were 0.76 and 1.52 in day 11 and 12 embryos, respectively. These findings indicate that glufosinate ammonium is embryotoxic in vitro. In addition to causing growth retardation, glufosinate specifically affected the neuroepithelium of the brain vesicle and neural tube, leading to neuroepithelial cell death.

http://www.ncbi.nlm.nih.gov/pubmed/9178451

Fujii, T. 1997. Transgenerational effects of maternal exposure to chemicals on the functional development of the brain in the offspring. *Cancer Causes and Control*, 8, pp. 524-528.

In order to prevent health risk from environmental chemicals, particularly for progeny, we have been performing a risk assessment for various chemicals including therapeutic agents. This paper reports the functional effects of maternal exposure to psychoactive drugs, anticancer drugs, or herbicides on the offspring of rats. Maternal exposure to imipramine in a dose equivalent to the therapeutic dose per unit body weight induced hyperthermic response to chlorpromazine in the male offspring, while normal control rats showed a marked hypothermia. Exposure to ethosuximide resulted in an increase in play fighting behavior in young offspring that was fostered by lactating normal mothers. Single exposures to nimustine or cisplatin, anticancer drugs, at a different gestational stage resulted in an acceleration of growth when exposed at the earlier stage of gestation. Moreover, cisplatin-exposed rats were emotionally unstable, showing a short latent time to the first line-crossing in an open-field during infantile period. The rats exposed to glufosinate ammonium, an herbicide, during the time of neurogenesis in the hippocampus showed a decrease in the wet-dog shakes response to kainic acid at six weeks of age. These results suggest that maternal exposure to chemicals during pregnancy induces a variety of functional abnormalities in the brain of the offspring dependent on the pharmacologic action of chemicals and the stage of gestation even with a single exposure.

http://www.ncbi.nlm.nih.gov/pubmed/9498910

Koyama, K.; Koyama, K.; Goto, K. 1997. Cardiovascular effects of a herbicide containing glufosinate and a surfactant: *In Vitro* and *in Vivo* analyses in rats. *Toxicology and Applied Pharmacology*, 145, 409–414 (1997).

A herbicide, Basta (BASTA), containing glufosinate ammonium (GLA) as the main component and an anionic surfactant, sodium polyoxyethylene alkylether sulfate (AES), causes hemodynamic changes characterized by a decrease in total vascular resistance with an increase or a decrease in cardiac output in human acute oral poisoning. With a motivation based on these clinical observations, we tried to elucidate the exact component and its mode of action that is mostly responsible for the direct cardiovascular effects of this herbicide formulation, investigating the effects of BASTA, GLA, and AES independently on the cardiovascular system in rats in vitro and in vivo. In isolated right atria beating spontaneously in Krebs-Ringer's solution, BASTA and AES produced negative chronotropic responses in a concentration-dependent manner. In electrically driven isolated left atria, BASTA and AES produced positive inotropic responses concentration dependently but negative inotropic responses at extremely high concentrations. In aortic ring segments, BASTA and AES produced no vasoconstrictive effects but exerted significant vasodilative effects when the aortic ring was precontracted with phenylephrine. These in vitro responses caused by BASTA and AES occurred to a similar degree. On the other hand, the main component, GLA, produced no effects in isolated atria and aortas. In anesthetized rats, relatively low doses of BASTA and AES produced a decrease in blood pressure followed by a slight increase in heart rate, which was presumably due to baroreflex caused by the decrease in blood pressure. At an extremely high dose, BASTA and AES produced a decrease in blood pressure with a marked decrease in heart rate. These in vivo responses to BASTA and AES also occurred to a similar degree. In contrast, the main component, GLA, did not produce any effects on heart rate and blood pressure in anesthetized rats. From these results, we concluded that the effects of BASTA in our in vivo experiments were not caused by

the main component, GLA, but was mostly caused by AES through its vasodilative effects plus cardiostimulatory effects at low doses and cardiosuppressiveeffects at high doses.

http://www.ncbi.nlm.nih.gov/pubmed/9266815

Matsumura, N.; Takeuchi, C.; Hishikawa, K.; Fujii, T.; Nakaki, T. 2001. Glufosinate ammonium induces convulsion through N-methyl-d-aspartate receptors in mice. *Neuroscience Letters*, 304, 123-125.

Glufosinate ammonium, a broad-spectrum herbicide, causes convulsion in rodents and humans. Because of the structural similarities betweenglufosinate and glutamate, the convulsion induced by glufosinate ammonium may be ascribed to glutamate receptor activation. Three N-methyl-D-asparate (NMDA) receptor antagonists, dizocilpine, LY235959, and Compound 40, and an alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate receptor antagonist, NBQX, were coadministrated with glufosinate ammonium (80 mg/kg, intraperitoneally) in mice. Statistical analyses showed that the NMDA receptor antagonists markedly inhibited the convulsions, while the AMPA/kainate receptor antagonist had no effect on the convulsion. These results suggest that the convulsion caused by glufosinate ammonium is mediated through NMDA receptors.

http://www.ncbi.nlm.nih.gov/pubmed/11335070

2.1.4 Herbicides based on 2,4-D

Currently, the HT GM plants include in their portfolio an association with herbicides formulated based on 2,4-D. This ensures that its employment will grow and will occupy spaces associated to glyphosate and gluphosinate ammonium, which show decreased efficacy in the control of plants considered undesirable. GM events with this feature have recently been approved for commercial planting in Canada, US and Brazil.

The following studies show that such herbicide, in addition to being carcinogenic (non-Hodgkin lymphoma), causes genotoxic effects, teratogenic effects, endocrine disorders and damage to the reproductive and nervous systems, liver and kidneys. Even at small doses, the impacts are important in cases of studies involving animal models and humans.

Other articles also point out the fact that 2,4-D-based herbicides (especially the cheaper formulations, marketed irregularly and with unclear origin) contain extremely dangerous contaminants, such as

dioxin and other highly carcinogenic substances.

EPI – Hoar, S.; Blair, A.; Holmes, F.; Boysen, C.; Robel, R.; Hoover, R.; Fraumeni, J. 1986. Agricultural herbicide use and risk of lymphoma and soft-tissue sarcoma. *JAMA*, 256:1141-1147.

A population-based case-control study of soft-tissue sarcoma (STS), Hodgkin's disease (HD), and non-Hodgkin's lymphoma (NHL) in Kansas found farm herbicide use to be associated with NHL (odds ratio [OR], 1.6; 95% confidence interval [CI], 0.9, 2.6). Relative risk of NHL increased significantly with number of days of herbicide exposure per year and latency. Men exposed to herbicides more than 20 days per year had a sixfold increased risk of NHL (OR, 6.0; 95% CI, 1.9, 19.5) relative to nonfarmers. Frequent users who mixed or applied the herbicides themselves had an OR of 8.0 (95% CI, 2.3, 27.9) for NHL. Excesses were associated with use of phenoxyacetic acid herbicides, specifically 2,4-dichlorophenoxyacetic acid. Neither STS nor HD was associated with pesticide exposure. This study confirms the reports from Sweden and several US states that NHL is associated with farm herbicide use, especially phenoxyacetic acids. It does not confirm the case-control studies or the cohort studies of pesticide manufacturers and Vietnam veterans linking herbicides to STS or HD.

http://www.ncbi.nlm.nih.gov/pubmed/3801091

EPI – Zahm, S.; Weisenburger, D.; Babbit, P.; Saal, R.; Vaught, J.; Cantor, K.; Blair, A. 1990. A case-control study of non-Hodgkin's lymphoma and the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in Eastern Nebraska. *Epidemiology*, 1:349–356.

To evaluate the role of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in the development of non-Hodgkin's lymphoma (NHL), we conducted a population-based, case-control study in 66 counties in eastern Nebraska. Telephone interviews were conducted with 201 white men diagnosed with NHL between July 1, 1983, and June 30, 1986, and with 725 controls. There was a 50% excess of NHL among men who mixed or applied 2,4-D (odds ratio [OR] = 1.5; 95% confidence interval = 0.9, 2.5). The risk of NHL increased with the average frequency of use to over threefold for those exposed 20 or more days per year (p for trend = 0.051). Adjusting for use of organophosphate insecticides lowered the risk estimate for frequent users (OR = 1.8), but adjustment for fungicide use increased the risk estimate (OR = 4.5). Simultaneous adjustment for organophosphates and fungicides yielded an OR of 3.1 for farmers who mixed or applied 2,4-D more than 20 days per year. Risk also increased with degree of exposure, as indicated by application method and time spent in contaminated clothing, but not with the number of years of 2,4-D use or failure to use protective equipment. Although other pesticides, especially organophosphate insecticides, may be related to NHL, the risk associated with 2,4-D does not appear to be explained completely by these other exposures.

http://www.ncbi.nlm.nih.gov/pubmed/2078610

EPI – Lerda, D.; Rizzi, R. 1991. Study of reproductive function in persons occupationally exposed to 2,4-dichlorophenoxyacetic acid (2,4-D). *Mutat Res Letters*, 262: 47–50.

We studied the reproductive function of 32 male farm sprayers who were exposed to 2,4-D. Sperm analysis was made after 4 days of sexual inactivity. Parameters analyzed were volume, sperm count, mobility and morphology. Exposure level was estimated by measuring the concentration of 2,4-D in the urine. Significant levels of asthenospermia, necrospermia and teratospermia were found in exposed workers compared with unexposed controls. Over time, asthenospermia and necrospermia

diminished but the abnormal spermatozoa (teratospermia) continued.

http://www.ncbi.nlm.nih.gov/pubmed/1986284

EPI – Hayes, H.; Tarone, R.; Cantor, K.; Jessen, C.; McCurnin, D.; Richardson, R. 1991. Case-control study of canine malignant lymphoma: positive association with dog owner's use of 2,4-dichlorophenoxyacetic acid herbicides. *J Natl Cancer Inst*, 83:1226–1231.

A hospital-based case-control study of companion dogs examined the risk of developing canine malignant lymphoma associated with the use of chemicals in and about the home. Information from a self-administered owner questionnaire and/or a telephone interview of about 491 cases, 466 nontumor controls, and 479 tumor controls indicated that owners in households with dogs that developed malignant lymphoma applied 2,4-dichlorophenoxyacetic acid (2,4-D) herbicides to their lawn and/or employed commercial lawn care companies to treat their yard significantly more frequently than control owners (odds ratio = 1.3). In addition, the risk of canine malignant lymphoma rose to a twofold excess with four or more yearly owner applications of 2,4-D. The findings in this study are consistent with occupational studies in humans, which have reported modest associations between agricultural exposure to 2,4-D and increased risk of non-Hodgkin's lymphoma, the histology and epidemiology of which are similar to those of canine malignant lymphoma. The present study suggests that human health implications of 2,4-D exposure in the home environment should receive further investigation

http://www.ncbi.nlm.nih.gov/pubmed/1870148

EPI – Hardell, L.; Eriksson, M. 1999. A case-control study of non-Hodgkin lymphoma and exposure to pesticides. *Cancer*, 85:1353-1360.

Background: The incidence of non-Hodgkin lymphoma (NHL) has increased in most Western countries during the last few decades. Immunodefective conditions are established risk factors. In 1981, the authors reported an increased risk for NHL following exposure to certain pesticides. The current study was designed to further elucidate the importance of phenoxyacetic acids and other pesticides in the etiology of NHL.
Methods: A population-based case-control study in northern and middle Sweden encompassing 442 cases and twice as many controls was performed. Exposure data were ascertained by comprehensive questionnaires, and the questionnaires were supplemented by telephone interviews. In total, 404 cases and 741 controls answered the questionnaire. Univariate and multivariate analyses were performed with the SAS statistical data program.
Results: Increased risk for NHL was found for subjects exposed to herbicides (odds ratio [OR], 1.6; 95% confidence interval [CI], 1.0-2.5) and fungicides (OR, 3.7; 95% CI, 1.1-13.0). Among herbicides, the phenoxyacetic acids dominated (OR, 1.5; 95% CI, 0.9-2.4); and, when subclassified, one of these, 4-chloro-2-methyl phenoxyacetic acid (MCPA), turned out to be significantly associated with NHL (OR, 2.7; 95% CI, 1.0-6.9). For several categories of herbicides, it was noted that only exposure during the most recent decades before diagnosis of NHL was associated with an increased risk of NHL. Exposure to impregnating agents and insecticides was, at most, only weakly related to NHL.
Conclusions: Exposure to herbicides in total, including phenoxyacetic acids, during the decades before NHL diagnosis resulted in increased risk for NHL. Thus, the risk following exposure was related to the latency period. Fungicides also increased the risk for NHL when combined, but this group consisted of several different agents, and few subjects were exposed to each type of fungicide.

Full article available at http://www.beyondpesticides.org/documents/acs-nhlymphoma-1999.pdf

REV – Gandhi, R.; Wandji, S-A.; Snedeker, S. 2000. Critical evaluation of cancer risk from 2,4-D. *Reviews of Environmenta Contamination and Toxicology*, vol. 167, p. 1–33.

2,4-D [(2,4-dichlorophenoxy)-acetic acid] is a widely used postemergent herbicide. It is structurally similar to indoleacetic acid (IAA), a naturally occurring plant hormone (see Fig.1). This similarity allows 2,4-D to mimic IAA and is the basis for its herbicidal action. The synthesis of 2,4-D was first reported in 1941 (ARC 1986I). In 1945, Dow Chemical Co. discovered that a 1:1 mixture of 2,4-D and 2,4,5-T [(2,4,5-trichlorophenoxy)-acetic acid] was a more effective herbicide than either of the two chemicals alone. The mixture was widely used thereafter and referred to as Agent Orange (Lilienfeld and Gallo 1989). Exposure to 2,4-D in the past may have more often been to a mixture of herbicides rather than 2,4-D alone. 2,4-D preparations before 1975 were often contaminated with TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) (IARC 1986;Johnson et al. 1992).

http://link.springer.com/chapter/10.1007%2F978-1-4612-1156-3_1

Kobal, S.; Cebulj-Kadunc, N.; Cestnik, V. 2000. Serum T3 and T4 concentrations in the adult rats treated with herbicide 2,4-dichlorophenoxyacetic acid. *Pflugers Archiv European Journal of Physiology*, vol. 440 (7), R171-2.

The influence of sublethal doses of 2,4-dichlorophenoxyacetic acid (2,4-D) on serum T3 and T4 concentrations in Hsd Cpb: Wistar rats of both sexes was studied. The trial was performed on 24 males and females respectively, each divided into three groups of 8 animals (control, groups 1 and 2). Aqueous solution of the compound (11 mg/kg body weight--group 1 and 110 mg/kg body weight--group 2) or clean tap water (control group) was used. Aliquots of 2.4 ml/kg body weight were administered with a stomach tube from the 1st to 10th day of the experiment. Three days before the first treatment and on the 6th and 13th day of the experiment the serum T3 and T4 concentrations were determined by commercial radioimmunoassay kits (Byk-Sangtec Diagnostica), validated for rats. A significant decrease of serum T4 ($P < 0.01$) and T3 ($P < 0.001$) was determined in males of groups 1 and 2 during the experiment. On the 6th day of experiment serum T4 and T3 values were significantly lower ($P < 0.001$ and 0.01 respectively) in group 2 than in the controls and group 1 of both males and females. During the whole experiment serum T4 levels were lower in females than in males ($P < 0.05$)

http://www.ncbi.nlm.nih.gov/pubmed/11005658

Schreinemachers, D. 2000. Cancer mortality in four northern wheat-producing states. *Environmental Health Perspectives*, 108:873–881.

Chlorophenoxy herbicides are used both in cereal grain agriculture and in nonagricultural settings such as right-of-ways, lawns, and parks. Minnesota, North Dakota, South Dakota, and Montana grow most of the spring and durum wheat produced in the United States. More than 90% of spring and durum wheat is treated with chlorophenoxy herbicides, in contrast to treatment of approximately 30% of winter wheat. In this ecologic study I used wheat acreage as a surrogate for exposure to chlorophenoxy herbicides. I investigated the association of chlorophenoxy herbicides with cancer mortality during 1980-1989 for selected counties based on level of agriculture ([greater and equal to] 20%) and rural population ([greater and equal to] 50%). Age-standardized cancer mortality rates were determined for grouped counties based on tertiles of wheat acreage per county or for individual counties for frequently occurring cancers. The cancer sites that showed positive trends of increasing cancer mortality with increasing wheat acreage were esophagus, stomach, rectum, pancreas, larynx, prostate, kidney and ureter, brain, thyroid, bone, and all cancers (men) and oral cavity and tongue, esophagus, stomach, liver and gall bladder and bile ducts, pancreas, cervix, ovary, bladder, and other urinary organs, and all cancers (women). Rare cancers in men and women and cancers in boys and girls were studied by comparing counties above and below the median of wheat acreage per county. There was increased

mortality for cancer of the nose and eye in both men and women, brain and leukemia in both boys and girls, and all cancers in boys. These results suggest an association between cancer mortality and wheat acreage in counties of these four states.

Full article available at https://goo.gl/GqK8K8

Fofana, D.; Kobae, H.; Oku, S.; Nishi, J-I.; Miyata, K. 2000. Prenatal developmental effects of pure 2,4-dichlorophenoxyacetic acid (2,4-D) on the rat. *Congenital Anomalies*, 40:287–296.

2,4-Dichlorophenoxyacetic acid (2,4-D) a plant growth regulator, has been used worldwide as an herbicide. The phenoxyacetic acid herbicides contain both 2,4-D and 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) along with emulsifiers, solvents and contaminants; these have been recognized as teratogen in the rat and mouse. Although the high teratogenicity of phenoxyacetic herbicides has been attributed to the 2,4,5-T and the contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the possibility that 2,4-D might play a role has not been clearly ruled out We designed this study to evaluate the effects of pure 2,4-D, and herein describe the precise fetal visceral malformations. We randomly distributed pregnant Wistar rats in to three main groups according to organogenesis during which pure 2,4-D was administered at different doses: l)-over all organogenesis (gestational days 6 to 15), 2)-early organogenesis (gestational days 6 to 10), and 3)-late organogenesis (gestational days 11 to 15). We found that the pure 2,4-D is maternally toxic and has a dose-related embryolethality. The visceral malformations induced in the fetuses included ureteric dilatations and hydronephrosis, as reported, in conjunction with the herbicide forms. In addition, we observed an association with renal and urogenital aplasia, which were observed in the early organogenesis period. We performed a histopatho-logical examination and discussed the mechanism of the pathological processes. We conclude that the pure 2,4-D itself is maternally toxic and embryolethal, and potential inducer of kidney and urogenital malformations in the rat. The types of kidney and urogenital malformations seen indicated that the 2,4-D interferes in the early developmental stage of the urogenital system.

http://onlinelibrary.wiley.com/doi/10.1111/j.1741-4520.2000.tb00927.x/abstract

REV – Bradberry, S.; Watt, B.; Proudfoot, A.; Vale, J. 2000. Mechanisms of toxicity, clinical features, and management of acute chlorophenoxy herbicide poisoning: a review. *J Toxicol Clin Toxicol*, vol. 38 (2), p. 111-22.

Introduction: Chlorophenoxy herbicides are used widely for the control of broad-leaved weeds. They exhibit a variety of mechanisms of toxicity including dose-dependent cell membrane damage, uncoupling of oxidative phosphorylation, and disruption of acetylcoenzyme A metabolism. Between January 1962 and January 1999, 66 cases of chlorophenoxy herbicide poisoning following ingestion were reported in the literature.
Features Following Ingestion: Adjuvants in the formulations may have contributed to some of the features observed. Vomiting, abdominal pain, diarrhea, and, occasionally, gastrointestinal hemorrhage were early effects. When present, hypotension was predominantly due to intravascular volume loss, although vasodilation and direct myocardial toxicity may have contributed in some cases. Neurotoxic features included coma, hypertonia, hyperreflexia, ataxia, nystagmus, miosis, hallucinations, convulsions, fasciculation, and paralysis. Hypoventilation occurred not infrequently, usually in association with central nervous system depression, but respiratory muscle weakness was a factor in the development of respiratory failure in some patients. Myopathic symptoms including limb muscle weakness, loss of tendon reflexes, and myotonia were observed and increased creatine kinase activity was noted in some cases. Other clinical features reported included metabolic acidosis, rhabdomyolysis, renal failure, increased aminotransferase activities, pyrexia, and hyperventilation. Twenty-two of 66 patients died.
Features Following Dermal and Inhalational Exposure: Substantial dermal or inhalational 2,4-dichlorophenoxyacetic acid exposure has occasionally led to systemic features but

no such reports have been published in the last 20 years and no fatalities have been reported at any time. Substantial dermal exposure has been reported to cause mild gastrointestinal irritation after a latent period followed by progressive mixed sensory-motor peripheral neuropathy. Mild, transient gastrointestinal and peripheral neuromuscular symptoms have also occurred after occupational inhalation exposure, with or without dermal exposure.
Management: In addition to supportive care, alkaline diuresis to enhance herbicide elimination should be considered in all seriously poisoned patients. Limited clinical data suggest that hemodialysis produces similar herbicide clearance to alkaline diuresis without the need for urine pH manipulation and the administration of substantial amounts of intravenous fluid in an already compromised patient.
Conclusions: While chlorophenoxy herbicide poisoning is uncommon, ingestion of a chlorophenoxy herbicide can result in serious and sometimes fatal sequelae. In severe cases of poisoning, alkaline diuresis or hemodialysis to increase herbicide elimination should be considered.

http://www.ncbi.nlm.nih.gov/pubmed/10778907

EPI – Garry, V.; Tarone, R.; Kirsch, I.; Abdallah, J.; Lombardi, D.; Long, L.; Burroughs, B.; Barr, D.; Kesner, J. 2001. Biomarker correlations of urinary 2,4-D levels in foresters: genomic instability an endocrine disruption. *Environmental Health Perspectives*, vol. 109 (5), p. 495-500.

Forest pesticide applicators constitute a unique pesticide use group. Aerial, mechanical-ground, and focal weed control by application of herbicides, in particular chlorophenoxy herbicides, yield diverse exposure scenarios. In the present work, we analyzed aberrations in G-banded chromosomes, reproductive hormone levels, and polymerase chain reaction-based V(D)J rearrangement frequencies in applicators whose exposures were mostly limited to chlorophenoxy herbicides. Data from appliers where chlorophenoxy use was less frequent were also examined. The biomarker outcome data were compared to urinary levels of 2,4-dichlorophenoxyacetic acid (2,4-D) obtained at the time of maximum 2,4-D use. Further comparisons of outcome data were made to the total volume of herbicides applied during the entire pesticide-use season. Twenty-four applicators and 15 minimally exposed foresters (control) subjects were studied. Categorized by applicator method, men who used a hand-held, backpack sprayer in their applications showed the highest average level (453.6 ppb) of 2,4-D in urine. Serum luteinizing hormone (LH) values were correlated with urinary 2,4-Dlevels, but follicle-stimulating hormone and free and total testosterone were not. At the height of the application season; 6/7 backpack sprayers, 3/4 applicators who used multinozzle mechanical (boom) sprayers, 4/8 aerial applicators, and 2/5 skidder-radiarc (closed cab) appliers had two or more V(D)J region rearrangements per microgram of DNA. Only 5 of 15 minimally exposed (control) foresters had two or more rearrangements, and 3 of these 5 subjects demonstrated detectable levels of 2,4-D in the urine. Only 8/24 DNA samples obtained from the exposed group 10 months or more after their last chlorophenoxy use had two rearrangements per microgram of DNA, suggesting that the exposure-related effects observed were reversible and temporary. Although urinary 2,4-D levels were not correlated with chromosome aberration frequency, chromosome aberration frequencies were correlated with the total volume of herbicides applied, including products other than 2,4-D. In summary, herbicide applicators with high urinary levels of 2,4-D (backpack and boom spray applications) exhibited elevated LH levels. They also exhibited altered genomic stability as measured by V(D)J rearrangement frequency, which appears reversible months after peak exposure. Though highly detailed, the limited sample size warrants cautious interpretation of the data.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1240309/

EPI – Arbuckle, T.; Lin, Z.; Mery, L. 2001. An exploratory analysis of the effect of pesticide

exposure on the risk of spontaneous abortion in an Ontario farm population. *Environ Health Perspect*, 109:851–857.

The toxicity of pesticides on human reproduction is largely unknown--particularly how mixtures of pesticide products might affect fetal toxicity. The Ontario Farm Family Health Study collected data by questionnaire on the identity and timing of pesticide use on the farm, lifestyle factors, and a complete reproductive history from the farm operator and eligible couples living on the farm. A total of 2,110 women provided information on 3,936 pregnancies, including 395 spontaneous abortions. To explore critical windows of exposure and target sites for toxicity, we examined exposures separately for preconception (3 months before and up to month of conception) and postconception (first trimester) windows and for early (< 12 weeks) and late (12-19 weeks) spontaneous abortions. We observed moderate increases in risk of early abortions for preconception exposures to phenoxy acetic acid herbicides [odds ratio (OR) = 1.5; 95% confidence interval (CI), 1.1-2.1], triazines (OR = 1.4; 95% CI, 1.0-2.0), and any herbicide (OR = 1.4; 95% CI, 1.1-1.9). For late abortions, preconception exposure to glyphosate (OR = 1.7; 95% CI, 1.0-2.9), thiocarbamates (OR = 1.8; 95% CI, 1.1-3.0), and the miscellaneous class of pesticides (OR = 1.5; 95% CI, 1.0-2.4) was associated with elevated risks. Postconception exposures were generally associated with late spontaneous abortions. Older maternal age (> 34 years of age) was the strongest risk factor for spontaneous abortions, and we observed several interactions between pesticides in the older age group using Classification and Regression Tree analysis. This study shows that timing of exposure and restricting analyses to more homogeneous endpoints are important in characterizing the reproductive toxicity of pesticides.

Full article available at https://goo.gl/d88pRi

Amer, S.; Aly, F. 2001. Genotoxic effect of 2,4-dichlorophenoxy acetic acid and its metabolite 2,4-dichlorophenol in mouse. *Mutat Res*, 494(1-2):1-12.

The cytogenetic effect of 2,4-dichlorophenoxy acetic acid (2,4-D) and its metabolite 2,4-dichlorophenol (2,4-DCP) was studied in bone-marrow, germ cells and sperm head abnormalities in the treated mice. Swiss mice were treated orally by gavage with 2,4-D at 1.7, 3.3 and 33 mg kg(-1)BW (1/200, 1/100 and 1/10 of LD(50)). 2,4-DCP was intraperitoneally (i.p.) injected at 36, 72 and 180 mg kg(-1)BW (1/10, 1/5, 1/2 of LD(50)). A significant increase in the percentage of chromosome aberrations in bone-marrow and spermatocyte cells was observed after oral administration of 2,4-D at 3.3 mg kg(-1)BW for three and five consecutive days. This percentage increased and reached 10.8+/-0.87 (P<0.01) in bone-marrow and 9.8+/-0.45 (P<0.01) in spermatocyte cells after oral administration of 2,4-D at 33 mg kg(-1)BW for 24 h. This percentage was, however, lower than that induced in bone-marrow and spermatocyte cells by mitomycin C (positive control). 2,4-D induced a dose-dependent increase in the percentage of sperm head abnormalities. The genotoxic effect of 2,4-DCP is weaker than that of 2,4-D, as indicated by the lower percentage of the induced chromosome aberrations (in bone-marrow and spermatocyte cells) and sperm head abnormalities. Only the highest tested concentration of 2,4-DCP (180 mg kg(-1)BW, 1/2 LD(50)) induced a significant percentage of chromosome aberrations and sperm head abnormalities after i.p. injection. The obtained results indicate that 2,4-D is genotoxic in mice in vivo under the conditions tested. Hence, more care should be given to the application of 2,4-D on edible crops since repeated uses may underlie a health hazard.

http://www.ncbi.nlm.nih.gov/pubmed/11423340

Holland, N.; Duramad, P.; Rothman, N.; Figgs, L.; Blair, A.; Hubbard, A.; Smith, M. 2002. Micronucleus frequency and proliferation in human lymphocytes after exposure to herbicide 2,4-dichlorophenoxyacetic acid *in vitro* and *in vivo*. *Mutat Res*, 26;521(1-2):165-78.

Widespread use of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and its association with non-Hodgkin's lymphoma (NHL) and other cancers has raised public concern. Here, micronucleus (MN) formation has been used as a biomarker of genotoxicity, and replicative and mitotic indices (MIs) as biomarkers of cell cycle kinetics in human lymphocytes. Cells were cultured either as whole blood or isolated lymphocytes and treated with pure or commercial forms of 2,4-D at doses between 0.001 and 1 mM for 48 h. Exposure to 2,4-D produced a minimal increase in MN in whole blood and even smaller one in isolated lymphocyte cultures. This induction took place only at levels approaching cytotoxicity and was accompanied by a significant inhibition of replicative index (RI). At a low (0.005 mM) dose of commercial 2,4-D, a small, marginally significant increase in RI (12-15%) was found in two independent sets of experiments (P=0.052). Additionally, we found that lymphocyte RI was more affected by commercial 2,4-D containing 9.4% of the chemically pure 2,4-D, than with an equal concentration of the latter suggesting that other ingredients present in the commercial pesticide may be responsible or may enhance the effect of 2,4-D. Mitotic index, however, did not show any significant change with either commercial or pure 2,4-D. The lymphocytes of 12 male applicators exposed solely to 2,4-D during a 3-month period had a significantly higher RI than the same group prior to exposure and than a control group (P<0.01), in accordance with the in vitro finding of increased RI at low doses.

http://www.ncbi.nlm.nih.gov/pubmed/12438013

Bukowska, B. 2003. Effects of 2,4-D and its metabolite 2,4-dichlorophenol on antioxidant enzymes and level of glutathione in human erythrocytes. *Comp Biochem Physiol C Toxicol Pharmacol*, 135(4): 435-41.

The effects of in vitro exposure of human erythrocytes to different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and its metabolite 2,4-dichlorophenol (2,4-DCP) were studied. The activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and the level of reduced glutathione (GSH) were determined. The activity of erythrocyte superoxide dismutase SOD decreased with increasing dose of 2,4-D and 2,4-DCP, while glutathione peroxidase activity increased. 2,4-D (500 ppm) decreased the level of reduced glutathione in erythrocytes by 18% and 2,4-DCP (250 ppm) by 32%, respectively, in comparison with the controls. These results lead to the conclusion that in vitro administration of herbicide-2,4-D and its metabolite 2,4-DCP causes a decrease in the level of reduced glutathione in erythrocytes and significant changes in antioxidant enzyme activities. Comparison of the toxicity of 2,4-D and 2,4-DCP revealed that the most prominent changes occurred in human erythrocytes incubated with 2,4-DCP.

http://www.ncbi.nlm.nih.gov/pubmed/12965188

Schreinemachers, D. 2003. Birth malformations and other adverse perinatal outcomes in four US wheat-producing states. *Env Health Perspec*, 111(9) 1259-1264.

Chlorophenoxy herbicides are widely used in the United States and Western Europe for broadleaf weed control in grain farming and park maintenance. Most of the spring and durum wheat produced in the United States is grown in Minnesota, Montana, North Dakota, and South Dakota, with more than 85% of the acreage treated with chlorophenoxy herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA). Rates of adverse birth outcomes in rural, agricultural counties of these states during 1995-1997 were studied by comparing counties with a high proportion of wheat acreage and those with a lower

proportion. Information routinely collected and made available by federal agencies was used for this ecologic study. Significant increases in birth malformations were observed for the circulatory/respiratory category for combined sexes [odds ratio (OR) = 1.65; 95% confidence interval (CI), 1.07-2.55]. A stronger effect was observed for the subcategory, which excluded heart malformations (OR = 2.03; 95% CI, 1.14-3.59). In addition, infants conceived during April-June--the time of herbicide application--had an increased chance of being diagnosed with circulatory/respiratory (excluding heart) malformations compared with births conceived during other months of the year (OR = 1.75; 95% CI, 1.09-2.80). Musculoskeletal/integumental anomalies increased for combined sexes in the high-wheat counties (OR = 1.50; 95% CI, 1.06-2.12). Infant death from congenital anomalies significantly increased in high-wheat counties for males (OR = 2.66; 95% CI, 1.52-4.65) but not for females (OR = 0.48; 95% CI, 0.20-1.15). These results are especially of concern because of widespread use of chlorophenoxy herbicides.

Full article available at https://goo.gl/Z9ZTT3

REV – Bradberry, S.; Proudfoot, A.; Vale, J. 2004. Poisoning due to chlorophenoxy herbicides. *Toxicol Rev*, vol. 23 (2), p. 65-73.

Chlorophenoxy herbicides are used widely for the control of broad-leaved weeds. They exhibit a variety of mechanisms of toxicity including dose-dependent cell membrane damage, uncoupling of oxidative phosphorylation and disruption of acetylcoenzyme A metabolism. Following ingestion, vomiting, abdominal pain, diarrhoea and, occasionally, gastrointestinal haemorrhage are early effects. Hypotension, which is common, is due predominantly to intravascular volume loss, although vasodilation and direct myocardial toxicity may also contribute. Coma, hypertonia, hyperreflexia, ataxia, nystagmus, miosis, hallucinations, convulsions, fasciculation and paralysis may then ensue. Hypoventilation is commonly secondary to CNS depression, but respiratory muscle weakness is a factor in the development of respiratory failure in some patients. Myopathic symptoms including limb muscle weakness, loss of tendon reflexes, myotonia and increased creatine kinase activity have been observed. Metabolic acidosis, rhabdomyolysis, renal failure, increased aminotransferase activities, pyrexia and hyperventilation have been reported. Substantial dermal exposure to 2,4-dichlorophenoxy acetic acid (2,4-D) has led occasionally to systemic features including mild gastrointestinal irritation and progressive mixed sensorimotor peripheral neuropathy. Mild, transient gastrointestinal and peripheral neuromuscular symptoms have occurred after occupational inhalation exposure. In addition to supportive care, urine alkalinization with high-flow urine output will enhance herbicide elimination and should be considered in all seriously poisoned patients. Haemodialysis produces similar herbicide clearances to urine alkalinization without the need for urine pH manipulation and the administration of substantial amounts of intravenous fluid in an already compromised patient.

http://www.ncbi.nlm.nih.gov/pubmed/15578861

Sameshima, K.; Kobae, H.; Fofana, D.; Yoshidome, K.; Nishi, J.; Miyata, K. 2004. Effects of pure 2,4-dichlorophenoxyacetic acid on cultured rat embryos. *Congenit Anom (Kyoto)*, 44(2):93-6.

2,4-dichlorophenoxyacetic acid (2,4-D), a plant growth regulator, has been used worldwide as a herbicide. Previously we evaluated the prenataldevelopmental effects of 2,4-D by feeding it to pregnant rats and found that it is maternally toxic and embryolethal, and it induces urogenital malformations in rat fetuses. In the study presented here, we investigated the effects of pure 2,4-D on rat embryos in whole embryo culture. Ratembryos on day 9.5 of gestation were cultured for 48 h at several concentration levels with pure 2,4-D (50-500 microg/mL). 2,4-D caused a concentration-related increase in the incidence of each malformation. Significant decreases in the number of

somites were observed at a concentration of 100 microg/mL or more. At the concentration of 100 microg/mL, there was normal yolk sac circulation. This result suggests that 2,4-D has a detrimental effect on somite development and directly damages developing embryos.

http://www.ncbi.nlm.nih.gov/pubmed/15198722

Bortolozzi, A.; Duffard, A.; Duffard, R.; Antonelli, M. 2004. Effects of 2,4-dichlorophenoxyacetic acid exposure on dopamine D2-like receptors in rat brain. *Neurotoxicol Teratol*, 26(4):599-605.

2,4-Dichlorophenoxyacetic acid (2,4-D), a worldwide-used herbicide, has been associated with a range of adverse health effects on humans and different animal species. Although the mechanism of 2,4-D neurotoxicity remains unknown, we had previously reported changes in various neurotransmitter systems, such as serotonin (5-HT) and dopamine (DA), which were proposed to mediate some of the behavioral effects in rats. In the present work, we examined the impact of 2,4-D exposure on the ontogeny of dopaminergic D2-type receptors in prefrontal cortex (PFc), striatum (CPu), hippocampus (H) and cerebellum (Cer). Pregnant rats were orally exposed to 70 mg/kg/day of 2,4-D from gestation day (GD) 16 to postpartum day 23. After weaning, the pups were assigned to one of the two subgroups: T1 [fed with untreated diet until postnatal day, (PD) 90] and T2 [maintained with 2,4-D diet until PD 90]. Five to eight pups per age and sex were sacrificed at 6, 15, 30, 45 or 90 days of age for membrane receptor binding assays employing [3H] nemonapride. Subchronic 2,4-D exposure (T2 group) increased DA D2-type receptor around 40% in CPu. In addition, DA D2-type receptor levels also increased in PFc (15 and 30 days) and Cer (30 and 90 days). Sex-dependent differences in D2 receptors were observed with T2 female rats being more affected than T2 male rats. When the herbicide treatment was interrupted after weaning (T1 group), DA D2-type receptor density was apparently recovered and stabilized to control level. These findings suggest a reversible vulnerability of D2-type receptors to 2,4-D exposure. Regional increases of D2-type receptor density may explain certain behaviors reported early by us, such as catalepsy and right-turning preference in rats exposed to 2,4-D.

http://www.ncbi.nlm.nih.gov/pubmed/15203183

Bharadwaj, L.; Dhami, K.; Stevens, M.; Renaud, C.; Ali, A. 2005. Altered gene expression in human hepatoma HepG2 cells exposed to low-level 2,4-dichlorophenoxyacetic acid and potassium nitrate. *Toxicol In Vitro*, vol. 19 (5), p. 603-19.

2,4-dichlorophenoxyacetic acid (2,4-D) and nitrate are agricultural contaminants found in rural ground water. It is not known whether levels found in groundwater pose a human or environmental health risk, nor is the mechanism of toxicity at the molecular/cellular level understood. This study focused on determining whether 2,4-D or nitrate at environmentally realistic levels elicit gene expression changes in exposed cells. cDNA microarray technology was used to determine the impact of 2,4-D and nitrate in an in vitro model of exposure. Human hepatoma HepG2 cells were incubated with 2,4-D or nitrate alone for 24 h. Cell viability (neutral red assay) and proliferation (BrdU incorporation) were assessed following exposure. Total RNA from treated and control cells were isolated, reverse transcribed and reciprocal labelled with Cy3 or Cy5 dyes, and hybridized to a human cDNA microarray. The hybridized microarray chips were scanned, quantified and analyzed to identify genes affected by 2,4-D or nitrate exposure based on a two-fold increase or decrease in gene expression and reproducibility (affected in three or more treatments). Following filtering, normalization and hierarchical clustering initial data indicate that numerous genes were found to be commonly expressed in at least three or more treatments of 2,4-D or nitrate tested. The affected genes indicate that HepG2 cells respond to environmental, low-level exposure and produce a cellular

response that is associated with alterations in the expression of many genes. The affected genes were characterized as stress response, cell cycle control, immunological and DNA repair genes. These findings serve to highlight new pathway(s) in which to further probe the effects of environmental levels of 2,4-D and nitrate.

http://www.ncbi.nlm.nih.gov/pubmed/15878651

González, M.; Soloneski, S.; Reigosa, M.; Larramendy, M. 2005. Genotoxicity of the herbicide 2,4-dichlorophenoxyacetic and a commercial formulation, 2,4-dichlorophenoxyacetic acid dimethylamine salt. I. Evaluation of DNA damage and cytogenetic endpoints in Chinese Hamster ovary (CHO) cells. *Toxicol In Vitro*, 19(2): 289-97.

Genotoxicity of the 2,4-dichlorophenoxyacetic acid (2,4-D) and a commercially-used derivative, 2,4-D dimethylamine salt (2,4-D DMA), was evaluated in CHO cells using SCE and single cell gel electrophoresis (SCGE) assays. Log-phase cells were treated with 2.0-10.0 microg/ml of herbicides and harvested 24 and 36 h later for SCE analysis. Both agents induced significant dose-dependent increases in SCE, regardless of the harvesting time (2,4-D: r=0.98 and r=0.88, P<0.01, for 24 and 36 h harvesting times; 2,4-D DMA: r=0.97 and r=0.88, P<0.01, for 24 and 36 h harvesting times). Neither test compound altered cell-cycle progression or proliferative replication index (P>0.05), but the higher doses of both compounds reduced the mitotic index of cultures harvested at 24 and 36 h (P<0.05). A 90-min treatment with 2.0-10.0 microg/ml 2,4-D and 2,4-D DMA produced dose-dependent increases in the frequency of DNA-strand breaks detected in the SCGE assay, both in cultures harvested immediately after treatment and in cultures harvested 36 h later. The doses of 2,4-D and 2,4-D DMA were equally genotoxic in all of the assays. The results indicate that 2,4-D induces SCE and DNA damage in mammalian cells, and should be considered as potentially hazardous to humans.

http://www.ncbi.nlm.nih.gov/pubmed/15649642

LaChapelle, A.; Ruygrok, M.; Toomer, M.; Oost, J.; Monnie, M.; Swenson, J.; Compton, A.; Stebbins-Boaz, B. 2007. The hormonal herbicide, 2,4-dichlorophenoxyacetic acid, inhibits *Xenopus oocyte* maturation by targeting translational and posttranslational mechanisms. *Reproductive toxicology*, 23(1):20-31.

The widely used hormonal herbicide, 2,4-dichlorophenoxyacetic acid, blocks meiotic maturation in vitro and is thus a potential environmental endocrine disruptor with early reproductive effects. To test whether maturation inhibition was dependent on protein kinase A, an endogenous maturation inhibitor, oocytes were microinjected with PKI, a specific PKA inhibitor, and exposed to 2,4-D. Oocytes failed to mature, suggesting that 2,4-D is not dependent on PKA activity and likely acts on a downstream target, such as Mos. De novo synthesis of Mos, which is triggered by mRNA poly(A) elongation, was examined. Oocytes were microinjected with radiolabelled in vitro transcripts of Mos RNA and exposed to progesterone and 2,4-D. RNA analysis showed progesterone-induced polyadenylation as expected but none with 2,4-D. 2,4-D-activated MAPK was determined to be cytoplasmic in localization studies but poorly induced Rsk2 phosphorylation and activation. In addition to inhibition of the G2/M transition, 2,4-D caused abrupt reduction of H1 kinase activity in MII phase oocytes. Attempts to rescue maturation in oocytes transiently exposed to 2,4-D failed, suggesting that 2,4-D induces irreversible dysfunction of the meiotic signaling mechanism.

http://www.ncbi.nlm.nih.gov/pubmed/17055699

Arias, E. 2007. Cytogenetic effects of short- and long-term exposure of chick embryos to the phenoxyherbicide 2,4-D. *Environ Mol Mutagen*, 48(6):462-6.

Before incubation, chick embryos were treated with the herbicide 2,4-dichlorophenoxy acetic acid (2,4-D) by injecting onto the inner shell membrane solutions of 0, 0.5, 1, 2, or 4 mg 2,4-D. A commercial formulation containing 37% 2,4-D iso-octyl ester as active ingredient and pure 2,4-D were tested. Sister chromatid exchange (SCE) and cell cycle kinetics were examined at days 4, 7, and 10 from 22 to 30 embryos per group. After 4 days of exposure to commercial 2,4-D, a small ($P < 0.05$) dose-related increase of SCE was seen for the 4-mg group. An enhanced SCE response upon long-term exposure to 2,4-D was apparent. After 10 days of exposure, SCE frequencies for the 2- and 4-mg commercial 2,4-D, and 4-mg pure 2,4-D groups were significantly higher than for the controls. A significant slowing of cell cycle at concentrations at and above 1 mg was seen. Also observed was a slight, not statistically significant proliferative effect at the lowest dose of 0.5 mg/embryo. Consistent with the results from other test systems, the present findings indicate that 2,4-D has a low to moderate genotoxic activity.

http://www.ncbi.nlm.nih.gov/pubmed/17372986

Stürtz, N.; Deis, R.; Jahn, G.; Duffard, R.; Duffard, A. 2008. Effect of 2,4-dichlorophenoxyacetic acid on rat maternal behaviour. *Toxicology*, 247(2-3): 73-79.

Exposure to 2,4-dichlorophenoxyacetic acid (2,4-D) has several deleterious effects on the nervous system such as alterations in the concentrations of neurotransmitters in the brain and/or behavioral changes, myelination rate, ganglioside pattern [Bortolozzi, A., Duffard, R., Antonelli, M., Evangelista de Duffard, A.M., 2002. Increased sensitivity in dopamine D(2)-like brain receptors from 2,4-dichlorophenoxyacetic acid (2,4-D)-exposed and amphetamine-challenged rats. Ann. N.Y. Acad. Sci. 965, 314-323; Duffard, R., García, G., Rosso, S., Bortolozzi, A., Madariaga, M., DiPaolo, O., Evangelista de Duffard, A.M., 1996. Central nervous system myelin deficit in rats exposed to 2,4-dichlorophenoxyacetic acid throughout lactation. Neurotoxicol. Teratol. 18, 691-696; Evangelista de Duffard, A.M., Orta, C., Duffard, R., 1990. Behavioral changes in rats fed a diet containing 2,4-dichlorophenoxyacetic butyl ester. Neurotoxicology 11, 563-572; Evangelista de Duffard, A.M., Bortolozzi, A., Duffard, R.O., 1995. Altered behavioral responses in 2,4-dichlorophenoxyacetic acid treated and amphetamine challenged rats. Neurotoxicology 16, 479-488; Munro, I.C., Carlo, G.L., Orr, J.C., Sund, K., Wilson, R.M. Kennepohl, E. Lynch, B., Jablinske, M., Lee, N., 1992. A comprehensive, integrated review and evaluation of the scientific evidence relating to the safety of the herbicide 2,4-D. J. Am. Coll. Toxicol. 11, 559-664; Rosso et al., 2000], and its administration to pregnant and lactating rats adversely affects litter growth and milk quality. Since normal growth of the offspring depends on adequate maternal nursing and care, we evaluated the effect of 2,4-D on rat maternal behavior as well as the dam's monoamine levels in arcuate nucleus (AcN) and serum prolactin (PRL) levels. Wistar dams were exposed to the herbicide through the food from post partum day (PPD) 1 to PPD 7. Dams were fed either with a 2,4-D treated diet (15, 25 or 50mg 2,4-D/kg/daybw) or with a control diet. We observed that maternal nesting behavior was not modified by 2,4-D treatment. However, mother-pup interactions, specially the nursing behavior, were altered. Retrieval, crouching and licking of pups were reduced or suspended after 2,4-D treatment. We also observed an increase in the latency of retrieval and crouching in the dams treated with the herbicide. Dams showed movement along cage peripheries, food consumption during the light phase and high self-grooming. In addition of the deficits observed in maternal behavior parameters, increased catecholamine levels and a drastic decrease in indolamine levels in the AcN of treated dams were determined. Serum PRL levels were also diminished by 62%, 68% and 70% with respect to control dams in the 15, 25 and 50mg 2,4-D/kgbw treated dams, respectively. In conclusion, exposure to 2,4-D during the first post partum days produced changes in maternal behavior, serum prolactin and monoamine levels in the AcN of treated dams.

http://www.ncbi.nlm.nih.gov/pubmed/18420331

Maillet, E.; Margolskee, R.; Mosinger, B. 2009. Phenoxy herbicides and fibrates potently inhibit the human chemosensory receptor subunit T1R3. *Journal of Medicinal Chemistry*, 52, 6931–6935 6931. DOI: 10.1021/jm900823s.

We show that phenoxyauxin herbicides and lipid-lowering fibrates inhibit human but not rodent T1R3. T1R3 as a coreceptor in taste cells responds to sweet compounds and amino acids; in endocrine cells of gut and pancreas T1R3 contributes to glucose sensing. Thus, certain effects of fibrates in treating hyperlipidemia and type II diabetes may be via actions on T1R3. Likewise, phenoxy herbicides may have adverse metabolic effects in humans that would have gone undetected in studies on rodents.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2783803/

Stürtz, N.; Jahn, G.; Deis, R.; Rettori, V.; Duffard, R.; Duffard, A. 2010. Effect of 2,4-dichlorophenoxyacetic acid on milk transfer to the litter and prolactin release in lactating rats. *Toxicology*, Volume 271, Issues 1–2, 30, Pages 13-20.

The effects of 2,4-dichlorophenoxyacetic acid (2,4-D) on brain monoamines and the serum level of hormones involved in milk synthesis and on the milk ejection reflex in rats were evaluated. Dams were treated with 2.5, 5, 15, 25, 50 or 70mg 2,4-D/kg bw according to two experimental designs: (a) through food from post partum day 1 (PPD 1) to PPD 16 and the respective control groups or (b) an unique i.p. injection on PPD 11. To measure milk ejection, the litter was separated from the mother at the 11th day of lactation during 8h, returned to their mothers and allowed to suckle for a period of 15min. The procedure was repeated on 3 consecutive days until the end of treatment. The change in litter weight during the suckling period was taken as a measure of the amount of milk ejected during this period. The dams' serum prolactin (PRL), oxytocin (OT) and growth hormone levels were determined by radioimmunoassay. Both treatment regimens produced a dose-dependent decrease in the amount of milk ejected and circulating PRL and OT secreted in response to the suckling stimulus. Administration of OT before returning the pups restored the milk ejection, indicating no impairment in the capacity of the mammary gland to produce and secrete milk. In addition, dopamine levels were increased by the 2,4-D treatments in arcuate nucleus (ArN) and anterior lobe of pituitary gland (AL), while serotonin level was drastically decreased in ArN. 2,4-D treatment increased both calcium-dependent and calcium-independent nitric oxide synthase (NOS) activities in ArN. These results suggest that 2,4-D inhibits the suckling-induced hormone release, milk transfer to the litter at the central level, through a stimulation of hypothalamic NOS and dopamine and by an inhibition of hypothalamic serotonin transmission.

http://www.ncbi.nlm.nih.gov/pubmed/20122984

Another feature of these harmful herbicides is in its quick and easy dispersion, aggravated by its volatilize and drift capacities. This extends - in scope - the public exposed, affecting especially populations living in rural and peri-urban communities in regions where the technology is adopted.

Rudel, R.; Camann, D.; Spengler, J.; Korn, L.; Brody, J. 2003. Phthalates, alkylphenols, pesticides, polybrominated diphenyl ethers, and other endocrine-disrupting compounds in indoor air and dust. *Environmental Science & Technology*, 37(20): 4543-4553. doi:10.1021/es0264596.

Chemicals identified as endocrine-disrupting compounds (EDCs) have widespread consumer uses, yet little is known about indoor exposure. We sampled indoor air and dust in 120 homes, analyzing for 89 organic chemicals identified as EDCs. Fifty-two compounds were detected in air and 66 were detected in dust. These are the first reported measures in residential environments for over 30 of the compounds, including several detected at the highest concentrations. The number of compounds detected per home ranged from 13 to 28 in air and from 6 to 42 in dust. The most abundant compounds in air included phthalates (plasticizers, emulsifiers), o-phenylphenol (disinfectant), 4-nonylphenol (detergent metabolite), and 4-tert-butylphenol (adhesive) with typical concentrations in the range of 50-1500 ng/m3. The penta- and tetrabrominated diphenyl ethers (flame retardants) were frequently detected in dust, and 2,3-dibromo-1-propanol, the carcinogenic intermediate of a flame retardant banned in 1977, was detected in air and dust. Twenty-three pesticides were detected in air and 27 were detected in dust, the most abundant being permethrins and the synergist piperonyl butoxide. The banned pesticides heptachlor, chlordane, methoxychlor, and DDT were also frequently detected, suggesting limited indoor degradation. Detected concentrations exceeded government health-based guidelines for 15 compounds, but no guidelines are available for 28 compounds, and existing guidelines do not consider endocrine effects. This study provides a basis for prioritizing toxicology and exposure research for individual EDCs and mixtures and provides new tools for exposure assessment in health studies.

http://www.ncbi.nlm.nih.gov/pubmed/14594359

EPI - Morgan, M.; Sheldon, L.; Thomas, K.; Egeghy, P.; Croghan, C.; Jones, P.; Chuang, J.; Wilson, N. 2008. Adult and children's exposure to 2,4-D from multiple sources and pathways. *Journal of Exposure Science and Environmental Epidemiology*, 18, 486–494.

In this study, we investigated the 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide exposures of 135 preschool-aged children and their adult caregivers at 135 homes in North Carolina (NC) and Ohio (OH). Participants were randomly recruited from six NC and six OH counties. Monitoring was performed over a 48-h period at the participants' homes. Environmental samples included soil, outdoor air, indoor air, and carpet dust. Personal samples collected by the adult caregivers concerning themselves and their children consisted of solid food, liquid food, hand wipe, and spot urine samples. All samples were analyzed for 2,4-D (free acid form) by gas chromatography/mass spectrometry. 2,4-D was detected in all types of environmental samples but most often in carpet dust samples, with detection frequencies of 83% and 98% in NC and OH, respectively. The median level of 2,4-D in the carpet dust samples was about three times higher in OH homes compared to NC homes (156 vs. 47.5 ng/g, P<0.0002). For personal samples, 2,4-D was more frequently detected in the hand wipe samples from OH participants (>48%) than from NC participants (<9%). Hand wipe levels at the 95th percentile were about five times higher for OH children (0.1 ng/cm(2)) and adults (0.03 ng/cm(2)) than for the NC children (0.02 ng/cm(2)) and adults (<0.005 ng/cm(2)). 2,4-D was detected in more than 85% of the child and adult urine samples in both states. The median urinary2,4-D concentration was more than twice as high for OH children compared to NC children (1.2 vs. 0.5 ng/ml, P<0.0001); however, the median concentration was identical at 0.7 ng/ml for both NC and OH adults. The intraclass correlation coefficient of reliability for an individual's urinary 2,4-Dmeasurements, estimated from the unadjusted (0.31-0.62) and specific gravity-adjusted (0.37-0.73) values, were somewhat low for each group in this study. The variability in urinary 2,4-D measurements over the 48-h period for both children and adults in NC and OH suggests that several spot samples were needed to adequately assess these participants' exposures to 2,4-D in residential settings. Results from this study showed that children and their adult caregivers in NC and OH were likely exposed to 2,4-D through several pathways at their homes. In addition, our findings suggest that the OH children might have been exposed to higher levels of 2,4-D through the dermal and nondietary routes of exposure than the NC children and the NC and OH adults.

http://www.ncbi.nlm.nih.gov/pubmed/18167507

2.2 Various metabolic disorders and/or toxicity aspects related to the consumption of HT plants (compared to non-GM controls)

Despite the fact that HT GMPs cultivation occurs for more than 15 years, the scientific community remains divided regarding the risks that their use can bring to the health and the environment. The concerns are most relevant when considering possible long-term impacts.

As previously mentioned, a significant portion of these risks may arise from overlaps and synergies involving the consumption of plants, residues of herbicides and their main degradation products.

Moreover, toxic effects associated with genetic, epigenetic and/or metabolic disorders possibly present in GMPs cannot be discarded. Uncertainties and randomness related to the genetic modification process - already discussed in Part 1 - should not be disregarded in verifications of potential impacts on the health of consumers.

2.2.1 Soybean tolerant to glyphosate-based herbicides

RR soy, tolerant to glyphosate-based herbicides, is probably the most studied of transgenic events. The scientific community has provided in recent years, several articles pointing toxicological risks to human and animal health associated with its consumption (there are dozens of studies published that may be related to the health impacts of the technology package RR). However, other studies[58] even observing statistically significant differences, tend to interpret them as biologically irrelevant for, consequently, conclude for the absence of risk.

58 Such as Zhu *et al.*, 2004 (Nutritional assessment and fate of DNA of soybean meal from roundup ready or conventional soybeans using rats. *Arch Anim Nutr*. 2004, 58:295-310); Tudisco *et al.,* 2006 (Genetically modified soya bean in rabbit feeding: Detection of DNA fragments and evaluation of metabolic effects by enzymatic analysis. *Animal Science*. 82, 193-199); Tudisco *et al.*, 2010 (Fate of transgenic DNA and evaluation of metabolic effects in goats fed genetically modified soybean and in their offsprings. *Animal.* The Animal Consortium: 1-10; 4:1662-1671).

This divergence is at the center of scientific controversy, being assigned to commitments, methodologies, protocols and ways of reading data. Regardless of the reasons, in both cases the precautionary approach recommends further studies as a necessary step to findings regarding the biosafety of transgenic crops.

Malatesta, M.; Caporaloni, C.; Gavaudan, S.; Rocchi, M.; Serafini, S.; Tiberi, C.; Gazzanelli, G. 2002. Ultrastructural morphometrical and immunocytochemical analyses of hepatocyte nuclei from mice fed on genetically modified soybean. *Cell Struct Funct*, 27, 173180.

No direct evidence that genetically modified (GM) food may represent a possible danger for health has been reported so far; however, the scientific literature in this field is still quite poor. Therefore, we carried out an ultrastructural morphometrical and immunocytochemical study on hepatocytes from mice fed on GM soybean, in order to investigate eventual modifications of nuclear components of these cells involved in multiple metabolic pathways related to food processing. Our observations demonstrate significant modifications of some nuclear features in GM-fed mice. In particular, GM fed-mice show irregularly shaped nuclei, which generally represents an index of high metabolic rate, and a higher number of nuclear pores, suggestive of intense molecular trafficking. Moreover, the roundish nucleoli of control animals change in more irregular nucleoli with numerous small fibrillar centres and abundant dense fibrillar component in GM-fed mice, modifications typical of increased metabolic rate. Accordingly, nucleoplasmic (snRNPs and SC-35) and nucleolar (fibrillarin) splicing factors are more abundant in hepatocyte nuclei of GM-fed than in control mice. In conclusion, our data suggest that GM soybean intake can influence hepatocyte nuclear features in young and adult mice; however, the mechanisms responsible for such alterations remain unknown.

http://www.ncbi.nlm.nih.gov/pubmed/12441651

Malatesta, M.; Caporaloni, C.; Rossi, L.; Battistelli, S.; Rocchi, M.; Tonucci, F.; Gazzanelli, G. 2002. Ultrastructural analysis of pancreatic acinar cells from mice fed on genetically modified soybean. *Journal of Anatomy*, 201:409-415.

No direct evidence that genetically modified (GM) food may represent a possible danger for health has been reported so far; however, the scientific literature in this field is quite poor.Therefore, we investigated the possible effects of a diet containing GM soybean on mouse exocrine pancreas by means of ultrastructural, morphometrical and immunocytochemical analyses. Our observations demonstrate that, although no structural modification occurs in pancreaticacinar cells of mice fed on GM soybean, quantitative changes of some cellular constituents takeplace in comparison to control animals. In particular, a diet containing significant amount of GM food seems to influence the zymogen synthesis and processing.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1570979/

Malatesta, M.; Biggiogera, M.; Manuali, E.; Rocchi, M.; Baldelli, B.; Gazzanelli, G. 2003. Fine structural analysis of pancreatic acinar cell nuclei from mice fed on GM soybean. *Eur J Histochem*, 47, 3858.

We carried out ultrastructural morphometrical and immunocytochemical analyses on pancreatic acinar cell nuclei from mice fed on genetically modified (GM) soybean, in order to investigate possible structural and molecular modifications of nucleoplasmic and nucleolar constituents. We found a significant lowering of nucleoplasmic and nucleolar splicing factors as well as a perichromatin granule accumulation in GM-fed mice, suggestive of reduced post-transcriptional hnRNA processing and/or nuclear export. This is in accordance to already described zymogen synthesis and processing modifications in the same animals.

http://www.ncbi.nlm.nih.gov/pubmed/14706936

Vecchio, L.; Cisterna, B.; Malatesta, M.; Martin, T.; Biggiogera, M. 2004. Ultrastructural analysis of testes from mice fed on genetically modified soybean. *Eur J Histochem*, 48: 448 54.

We have considered the possible effects of a diet containing genetically modified (GM) soybean on mouse testis. This organ, in fact, is a well known bioindicator and it has already been utilized, for instance, to monitor pollution by heavy metals. In this preliminary study, we have focussed our attention on Sertoli cells, spermatogonia and spermatocytes by means of immunoelectron microscopy. Our results point out that the immunolabelling for Sm antigen, hnRNPs, SC35 and RNA Polymerase II is decreased in 2 and 5 month-old GM-fed mice, and is restored to normal at 8 months. In GM-fed mice of all ages considered, the number of perichromatin granules is higher and the nuclear pore density lower. Moreover, we found enlargements in the smooth endoplasmic reticulum in GM-fed mice Sertoli cells. A possible role played by traces of the herbicide to which the soybean is resistant is discussed.

http://www.ncbi.nlm.nih.gov/pubmed/15718213

Cisterna, B.; Flach, F.; Vecchio, L.; Barabino, S.; Battistelli, S.; Martin, T.; Malatesta, M.; Biggiogera, M. 2008. Can a genetically modified organism-containing diet influence embry development? A preliminary study on preimplantation mouse embryos. *European Journal of Histochemistry*, vol. 52 issue 4 (October-December):263-267.

In eukaryotic cells, pre-mRNAs undergo several transformation steps to generate mature mRNAs. Recent studies have demonstrated that a diet containing a genetically modified (GM) soybean can induce modifications of nuclear constituents involved in RNA processing in some tissues of young, adult and old mice. On this basis, we have investigated the ultrastructural and immunocytochemical features of pre-implantation embryos from mice fed either GM or non- GM soybean in order to verify whether the parental diet can affect the morpho-functional development of the embryonic ribonucleoprotein structural constituents involved in pre-mRNA pathways. Morphological observations revealed that the general aspect of embryo nuclear components is similar in the two experimental groups. However, immunocytochemical and in situ hybridization results suggest a temporary decrease of pre-mRNA transcription and splicing in 2-cell embryos and a resumption in 4-8-cell embryos from mice fed GM soybean; moreover, pre-mRNA maturation seems to be less efficient in both 2-cell and 4-8-cell embryos from GM-fed mice than in controls. Although our results are still preliminary and limited to the pre-implantation phases, the results of this study encourage deepening on the effects of food components and/or contaminants on embryo development.

http://www.ncbi.nlm.nih.gov/pubmed/19109102

Malatesta, M.; Boraldi, F.; Annovi, G.; Baldelli, B.; Battistelli, S.; Biggiogera, M.; Quaglino, D. 2008. A long-term study on female mice fed on a genetically modified soybean: effects on liver ageing. *Histochem Cell Biol*, 130, 967-77.

Liver represents a suitable model for monitoring the effects of a diet, due to its key role in controlling the whole metabolism. Although no direct evidence has been reported so far that genetically modified (GM) food may affect health, previous studies on hepatocytes from young female mice fed on GM soybean demonstrated nuclear modifications involving transcription and splicing pathways. In this study, the effects of this diet were studied on liver of old female mice in order to elucidate possible interference with ageing. The morpho-functional characteristics of the liver of 24-month-old mice, fed from weaning on control or GM soybean, were investigated by combining a proteomic approach with ultrastructural, morphometrical and immunoelectron microscopical analyses. Several proteins belonging to hepatocyte metabolism, stress response, calcium signalling and mitochondria were differentially expressed in GM-fed mice, indicating a more marked expression of senescence markers in comparison to controls. Moreover, hepatocytes of GM-fed mice showed mitochondrial and nuclear modifications indicative of reduced metabolic rate. This study demonstrates that GM soybean intake can influence some liver features during ageing and, although the mechanisms remain unknown, underlines the importance to investigate the long-term consequences of GM-diets and the potential synergistic effects with ageing, xenobiotics and/or stress conditions.

http://www.ncbi.nlm.nih.gov/pubmed/18648843

Cirnatu, D.; Jompan, A.; Sin, A.; Zugravu, C. 2011. Multiple organ histopathological changes in broiler chickens fed on genetically modified organism. *Rom J Morphol Embryol*, 52 (1 Suppl): 475-480.

Diet can influence the structural characteristics of internal organs. An experiment involving 130 meat broilers was conducted during 42 days (life term for a meat broiler) to study the effect of feed with protein from genetically modified soy. The 1-day-old birds were randomly allocated to five study groups, fed with soy, sunflower, wheat, fish flour, PC starter. In the diet of each group, an amount of protein from soy was replaced with genetically modified soy (I - 0%, II - 25%, III - 50%, IV - 75%, V - 100% protein from genetically modified soy). The level of protein in soy, either modified, or non-modified, was the same. Organs and carcass weights were measured at about 42 days of age of the birds and histopathology exams were performed during May-June 2009. No statistically significant differences were observed in mortality, growth performance variables or carcass and organ yields between broilers consuming diets produced with genetically modified soybean fractions and those consuming diets produced with near-isoline control soybean fractions. Inflammatory and degenerative liver lesions, muscle hypertrophy, hemorrhagic necrosis of bursa, kidney focal tubular necrosis, necrosis and superficial ulceration of bowel and pancreatic dystrophies were found in tissues from broilers fed on protein from genetically modified soy. Different types of lesions found in our study might be due to other causes (parasites, viral) superimposed but their presence exclusively in groups fed with modified soy raises some serious questions about the consequences of use of this type of feed.

http://www.ncbi.nlm.nih.gov/pubmed/21424096

Carman, J.; Vlieger, H.; Ver Steeg, L.; Sneller, V.; Robinson, G.; Clinch-Jones, C.; Haynes, J.; Edwards, J. 2013. A long-term toxicology study on pigs fed a combined genetically modified (GM) soy and GM maize diet. *Journal of Organic Systems*, 8 (1): 38-54[59].

59 This study used as the basis of Genetically Modified diet a mix of glyphosate tolerant corn, Bt corn and soybeans tolerant to glyphosate. Therefore, this reference consists of three items of that part, being 1.4, 2.2.1 and 2.2.2.

A significant number of genetically modified (GM) crops have been approved to enter human food and animal feed since 1996, including crops containing several GM genes 'stacked' into the one plant. We randomised and fed isowean pigs (N=168) either a mixed GM soy and GM corn (maize) diet (N=84) or an equivalent non-GM diet (N=84) in a longterm toxicology study of 22.7 weeks (the normal lifespan of a commercial pig from weaning to slaughter). Equal numbers of male and female pigs were present in each group. The GM corn contained double and triple-stacked varieties. Feed intake, weight gain, mortality and blood biochemistry were measured. Organ weights and pathology were determined post-mortem. There were no differences between pigs fed the GM and non-GM diets for feed intake, weight gain, mortality, and routine blood biochemistry measurements. The GM diet was associated with gastric and uterine differences in pigs. GM-fed pigs had uteri that were 25% heavier than non-GM fed pigs (p=0.025). GM-fed pigs had a higher rate of severe stomach inflammation with a rate of 32% of GM-fed pigs compared to 12% of non-GM-fed pigs (p=0.004). The severe stomach inflammation was worse in GM-fed males compared to non-GM fed males by a factor of 4.0 (p=0.041), and GM-fed females compared to non-GM fed females by a factor of 2.2 (p=0.034).

Full article available at http://www.organic-systems.org/journal/81/8106.pdf

2.2.2 Corn tolerant to the glyphosate-based herbicides

After the release of RR soy, the cultivation and consumption of GM corn varieties, tolerant to the glyphosate-based herbicide, were allowed. The implications were more significant in that, in corn consumption, there are cases of ingestion with extremely simple processing, involving grain harvest before maturation and therefore more likely to contain pesticide residues. As a result, they accumulated up important studies covering areas not evaluated for events with GM soy.

Among these, perhaps the most controversial was published in late 2012. It is an evaluation of RR corn - NK603, involving long-term study and adopting similar protocols to those used by Monsanto to argue the absence of risks to the PGM consumer. In the center of controversy (for more information on this controversy, see articles in Part 5 item 4.2.3) is the fact that Professor Séralini and his team suggest significant toxicological chronic damage and development of tumors at an alarming rate as a result of continued intake of NK603 for longer periods than those adopted in previous studies.

Hammond[60], B.; Dudekb, R.; Lemen, J.; Nemeth, M. 2004. Results of a 13 week safety assurance study with rats fed grain from glyphosate tolerant corn. *Food and Chemical Toxicology*, 42, 1003–1014.

The current study presents the results of a 13 week feeding study in rats with grain from Roundup Ready corn which is tolerant to the herbicide glyphosate. Herbicide tolerance was accomplished through the introduction of cp4 epsps coding sequences into the corn genome for in planta production of CP4 EPSPS enzymes. Unlike related corn EPSPS enzymes, CP4 EPSPS enzymes are not inhibited by the herbicide glyphosate. Purina TestDiets formulated Roundup Ready corn grain into rodent diets at levels of 11 and 33% (w/w). The responses of rats fed diets containing Roundup Ready corn grain were compared to that of rats fed diets containing non-transgenic grain (controls). All diets were nutritionally balanced and conformed to Purina Mills, Inc. specifications for Certified LabDiet 5002. There were 400 rats in the study divided into 10 groups of 20 rats/sex/group. Overall health, body weight, food consumption, clinical pathology parameters (hematology, blood chemistry, urinalysis), organ weights, gross and microscopic appearance of tissues were comparable between groups fed diets containing Roundup Ready and control corn grain. This study complements extensive agronomic, compositional and farm animal feeding studies with Roundup Ready corn grain, confirming it is as safe and nutritious as existing commercial corn hybrids.

http://www.ncbi.nlm.nih.gov/pubmed/15110110

REV - Vendômois, J.; Roullier, F.; Cellier, D.; Serálini, G-E. 2009. A comparison of the effects of three GM corn varieties on mammalian health. *International Journal of Biological Sciences*, 5, 706–726.

We present for the first time a comparative analysis of blood and organ system data from trials with rats fed three main commercialized genetically modified (GM) maize (NK 603, MON 810, MON 863), which are present in food and feed in the world. NK 603 has been modified to be tolerant to the broad spectrum herbicide Roundup and thus contains residues of this formulation. MON 810 and MON 863 are engineered to synthesize two different Bt toxins used as insecticides. Approximately 60 different biochemical parameters were classified per organ and measured in serum and urine after 5 and 14 weeks of feeding. GM maize-fed rats were compared first to their respective isogenic or parental non-GM equivalent control groups. This was followed by comparison to six reference groups, which had consumed various other non-GM maize varieties. We applied nonparametric methods, including multiple pairwise comparisons with a False Discovery Rate approach. Principal Component Analysis allowed the investigation of scattering of different factors (sex, weeks of feeding, diet, dose and group). Our analysis clearly reveals for the 3 GMOs new side effects linked with GM maize consumption, which were sex- and often dose-dependent. Effects were mostly associated with the kidney and liver, the dietary detoxifying organs, although different between the 3 GMOs. Other effects were also noticed in the heart, adrenal glands, spleen and haematopoietic system. We conclude that these data highlight signs of hepatorenal toxicity, possibly due to the new pesticides specific to each GM corn. In addition, unintended direct or indirect metabolic consequences of the genetic modification cannot be excluded.

Full article available at http://www.ijbs.com/v05p0706.htm

60 What does not appear in the summary of this study but which fully justifies its inclusion in this publication is the fact that it relates statistically significant differences observed during the trialxperiment. As noted by the Center for Research and Independent Information on Genetic Engineering (CRIIGEN, 2007), about 70 (67) statistically significant differences were observed and reported by the company that markets the GM plant, within 12 relating to hematological parameters (hematocrit, platelets, neutrophils, lymphocytes, monocytes, mean corpuscular volume, mean corpuscular hemoglobin concentration); 18 on clinical chemistry parameters (albumin, blood urea nitrogen, creatinine, phosphorus, sodium, chloride, alkaline phosphatase, calcium, potassium); 9 regarding urinary chemical parameters (creatinine, phosphorus, potassium, creatinine turgor, pH, calcium); 6 relative to the weight of the organs (heart, brain, liver); 14 in body weight and weight changes, and 8 concerning levels of food consumption of animals evaluated.

Séralini, G-E.; Clair, E.; Mesnage, R.; Gress, S.; Defarge, N.; Malatesta, M.; Hennequin, D.; Vendomois, J. 2012. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. *Food Chem Toxicol*, 50, 4221– 4231.

Retracted.

http://www.ncbi.nlm.nih.gov/pubmed/24490213

COM – Séralini, G-E.; Mesnage, R.; Defarge, N.; Gress, S.; Hennequin, D.; Clair, E.; Malatesta, M.; Vendômois, J 2013. Answers to critics: Why there is a long term toxicity due to Roundup-tolerant genetically modified maize and to a Roundup herbicide. *Food and Chemical Toxicology*, 53: 476-83.

Our recent work (Séralini et al., 2012) remains to date the most detailed study involving the life-long consumption of an agricultural genetically modified organism (GMO). This is true especially for NK603 maize for which only a 90-day test for commercial release was previously conducted using the same rat strain (Hammond et al., 2004). It is also the first long term detailed research on mammals exposed to a highly diluted pesticide in its total formulation with adjuvants. This may explain why 75% of our first criticisms arising within a week, among publishing authors, come from plant biologists, some developing patents on GMOs, even if it was a toxicological paper on mammals, and from Monsanto Company who owns both the NK603 GM maize and Roundup herbicide (R). Our study has limits like any one, and here we carefully answer to all criticisms from agencies, consultants and scientists, that were sent to the Editor or to ourselves. At this level, a full debate is biased if the toxicity tests on mammals of NK603 and R obtained by Monsanto Company remain confidential and thus unavailable in an electronic format for the whole scientific community to conduct independent scrutiny of the raw data. In our article, the conclusions of long-term NK603 and Roundup toxicities came from the statistically highly discriminant findings at the biochemical level in treated groups in comparison to controls, because these findings do correspond in an blinded analysis to the pathologies observed in organs, that were in turn linked to the deaths by anatomopathologists. GM NK603 and R cannot be regarded as safe to date.

Full article available at https://goo.gl/y1i5iK

Carman, J.; Vlieger, H.; Ver Steeg, L.; Sneller, V.; Robinson, G.; Clinch-Jones, C.; Haynes, J.; Edwards, J. 2013. A long-term toxicology study on pigs fed a combined genetically modified (GM) soy and GM maize diet. *Journal of Organic Systems*, 8 (1): 38-54[61].

A significant number of genetically modified (GM) crops have been approved to enter human food and animal feed since 1996, including crops containing several GM genes 'stacked' into the one plant. We randomised and fed isowean pigs (N=168) either a mixed GM soy and GM corn (maize) diet (N=84) or an equivalent non-GM diet (N=84) in a longterm toxicology study of 22.7 weeks (the normal lifespan of a commercial pig from weaning to slaughter). Equal numbers of male and female pigs were present in each group. The GM corn contained double and triple-stacked varieties. Feed intake, weight gain, mortality and blood biochemistry were measured. Organ weights and pathology were determined post-mortem. There were no differences between pigs fed the GM and non-GM diets for feed intake, weight gain, mortality, and routine blood biochemistry measurements. The GM diet was associated with gastric and uterine differences in pigs. GM-fed pigs had uteri that were 25% heavier than non-GM fed pigs (p=0.025). GM-fed pigs had a higher

61 This study used as the basis of Genetically Modified diet a mix of glyphosate tolerant corn, Bt corn and soybeans tolerant to glyphosate. Therefore, this reference consists of three items of that part, being 1.4, 2.2.1 and 2.2.2.

rate of severe stomach inflammation with a rate of 32% of GM-fed pigs compared to 12% of non-GM-fed pigs (p=0.004). The severe stomach inflammation was worse in GM-fed males compared to non-GM fed males by a factor of 4.0 (p=0.041), and GM-fed females compared to non-GM fed females by a factor of 2.2 (p=0.034).

Full article available at http://www.organic-systems.org/journal/81/8106.pdf

Séralini, G-E.; Clair, E.; Mesnage, R.; Gress, S.; Defarge, N.; Malatesta, M.; Hennequin, D.; Vendômois, J. 2014. Republished study: long-term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. *Environmental Sciences Europe*, 26:14.

Background: The health effects of a Roundup-tolerant NK603 genetically modified (GM) maize (from 11% in the diet), cultivated with or without Roundup application and Roundup alone (from 0.1 ppb of the full pesticide containing glyphosate and adjuvants) in drinking water, were evaluated for 2 years in rats. This study constitutes a follow-up investigation of a 90-day feeding study conducted by Monsanto in order to obtain commercial release of this GMO, employing the same rat strain and analyzing biochemical parameters on the same number of animals per group as our investigation. Our research represents the first chronic study on these substances, in which all observations including tumors are reported chronologically. Thus, it was not designed as a carcinogenicity study. We report the major findings with 34 organs observed and 56 parameters analyzed at 11 time points for most organs.

Results: Biochemical analyses confirmed very significant chronic kidney deficiencies, for all treatments and both sexes; 76% of the altered parameters were kidney-related. In treated males, liver congestions and necrosis were 2.5 to 5.5 times higher. Marked and severe nephropathies were also generally 1.3 to 2.3 times greater. In females, all treatment groups showed a two- to threefold increase in mortality, and deaths were earlier. This difference was also evident in three male groups fed with GM maize. All results were hormone- and sex-dependent, and the pathological profiles were comparable. Females developed large mammary tumors more frequently and before controls; the pituitary was the second most disabled organ; the sex hormonal balance was modified by consumption of GM maize and Roundup treatments. Males presented up to four times more large palpable tumors starting 600 days earlier than in the control group, in which only one tumor was noted. These results may be explained by not only the non-linear endocrine-disrupting effects of Roundup but also by the overexpression of the EPSPS transgene or other mutational effects in the GM maize and their metabolic consequences.

Conclusion: Our findings imply that long-term (2 year) feeding trials need to be conducted to thoroughly evaluate the safety of GM foods and pesticides in their full commercial formulations.

Full article available at http://www.enveurope.com/content/26/1/14

3 Other transgenic plants[62] under evaluation or suspended marketing

Scientific publications applied to risk assessment of transgenic plants cover beyond the dominant species (such as corn and soybeans containing Bt and HT technologies), new events of sparse coverage, already available in some markets, with commercialization process canceled or still undergoing evaluation.

The articles discuss some of these new cases - such as rice and potato - and events in corn and soybean, which have undergone other types of genetic modifications -, indicating risks for human and/or animal health.

The scientific literature also offers articles[63] where the authors consider not relevant from a biological standpoint statistically significant differences in the comparisons between groups of animals fed GMPs and groups of control animals. The authors of this systematization understand how important - and open to different interpretations - these statistical observations, and this contradiction is relevant argument to make further and more

62 At this point it is worth remembering the no history of safe use of transgenic products synthesized in GM microrganisms. In the late 1980s, it was associated with the onset of EMS call disease (*Eosinophilia-Myalgia-Syndrome*) to the use of L-tryptophan produced by a transgenic bacteria marketed by the Japanese company Showa Denko KK, there accounting for approximately 40 fatalities and 1500 people with serious neurological disorders, for the most irreversible. Although industry pointed liability of damage to unwanted chemical constituents involved in L-Tryptophan filtration process (especially IMT EBT), it cannot be ruled out the possibility that the use of transgenic bacteria were involved. This is because some cases of poisoning arose before the filtration process was applied by the Japanese company in focus. In addition, the constituents are revealed similar - but not identical - to L-Tryptophan. The possibility of transgenic organisms produce unwanted substances is explained in item 2 of Part 1. More information on the case of L-Tryptophan can be found at Belongia et al., 1990 (*An investigation of the cause of the eosinophilia-myalgia syndrome associated with tryptophan use*. N Engl J Med. Aug 9 1990; 323(6): 357-365); Kilbourne et al., 1996 (Tryptophan produced by Showa Denko and epidemic eosinophilia-myalgia syndrome. *J Rheumatol Suppl*. 1996 Oct;46:81-8; discussion 89-91) and Müller et al., 1999 (Synthesis and formation of an EMS correlated contaminant in biotechnologically manufactured L-tryptophan. *Adv Exp Med Biol.* 1999;467:481-6).

63 Such as Appenzeller *et al*, 2008 (Subchronic feeding study of herbicide-tolerant soybean DP-356O43-5 in Sprague-Dawley rats. *Food Chem Toxicol.* 2008, 46: 2201-2213) e Appenzeller *et al.*, 2009 (Subchronic feeding study of grain from herbicide-tolerant maize DPO9814O- 6 in prague-Dawley rats. *Food Chem Toxicol.* 2009, 47: 2269-2280).

detailed studies[64].

Nordlee, J.; Taylor, S.; Townsend, J.; Thomas, L.; Bush, R. 1996. Identification of a Brazil-Nut allergen in transgenic soybeans. *The New England Journal of Medicine*, 334: 668-92.

Background: The nutritional quality of soybeans (Glycine max) is compromised by a relative deficiency of methionine in the protein fraction of the seeds. To improve the nutritional quality, methionine-rich 2S albumin from the Brazil nut (Betholletia excelsa) has been introduced into transgenic soybeans. Since the Brazil nut is a known allergenic food, we assessed the allergenicity of the 2S albumin.
Methods: The ability of proteins in transgenic and non-transgenic soybeans, Brazil nuts, and purified 2S albumin to bind to IgE in serum from subjects allergic to Brazil nuts was determined by radioallergosorbent tests (4 subjects) and sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (9 subjects) with immunoblotting and autoradiography. Three subjects also underwent skin-prick testing with extracts of soybean, transgenic soybean, and Brazil nut.
Results: On radioallergosorbent testing of pooled serum from four subjects allergic to Brazil nuts, protein extracts of transgenic soybean inhibited binding of IgE to Brazil-nut proteins. On immunoblotting, serum IgE from eight of nine subjects bound to purified 2S albumin from the Brazil nut and the transgenic soybean. On skin-prick testing, three subjects had positive reactions to extracts of Brazil nut and transgenic soybean and negative reactions to soybean extract.
Conclusions: The 2S albumin is probably a major Brazil-nut allergen, and the transgenic soybeans analyzed in this study contain this protein. Our study show that an allergen from a food known to be allergenic can be transferred into another food by genetic engineering.

Full article available at http://www.nejm.org/doi/full/10.1056/NEJM199603143341103#Top

Ewen, S.; Pusztai, A. 1999. Effect of diets containing genetically modified potatoes expressing Galanthus nivalis lectin on rat small intestine. *Lancet*, 354: 1353-4.

Diets containing genetically modified (GM) potatoes expressing the lectin Galanthus nivalis agglutinin (GNA) had variable effects on different parts of the rat gastrointestinal tract. Some effects, such as the proliferation of the gastric mucosa, were mainly due to the expression of the GNA transgene. However, other parts of the construct or the genetic transformation (or both) could also have contributed to the overall biological effects of the GNA-GM potatoes, particularly on the small intestine and caecum.

http://www.ncbi.nlm.nih.gov/pubmed/10533866

64 Among these plants less studied, it is worth mentioning the case of staked events that add - by conventional breeding - two or more transgenes. It should be noted the paucity of published studies - in the scientific literature - on these GM plants, with the encumbrance that most of these are made by biotech companies. See MacKenzie *et al.*, 2007 (Thirteen week feeding study with transgenic maize grain containing event DAS-O15O7-1 in Sprague-Dawley rats. Food Chem Toxicol 2007, 45:551-562); Malley et al., 2007 (Subchronic feeding study of DAS-59122-7 maize grain in Sprague-Dawley rats. *Food Chem Toxicol* 2007, 45:1277-1292); He *et al.,* 2008 (Comparison of grain from corn rootworm resistant transgenic DAS-59122-7 maize with nontransgenic maize grain in a 90-day feeding study in Sprague-Dawley rats. *Food Chem Toxicol* 2008, 46:1994-2002); Appenzeller et al., 2009 (Subchronic feeding study with genetically modified stacked trait lepidopteran and coleopteran resistant (DAS-O15O7-1xDAS-59122-7) maize grain in Sprague-Dawley rats. *Food Chem Toxicol* 2009, 47:1512-1520). In spite of all these statistically significant differences found it got despised for lack of biohazards.

Schrøder, M.; Wilcks, A.; Jacobsen, H.; Miller, A.; Frenzel, T.; Danier, J.; Rychlik, M.; Shu, Q.; Emami, K.; Sudhakar, D.; Gatehouse, A.; Engel, K-H.; Knudsen, I. 2007. A 90-day safety study in Wistar rats fed genetically modified rice expressing snowdrop lectin *Galanthus nivalis* (GNA). *Food and Chemical Toxicology*, Volume 45, Issue 3, Pages 350-363.

Genetically modified plants expressing insecticidal traits offer a new strategy for crop protection, but at the same time present a challenge in terms of food safety assessment. The present 90-day feeding study was designed to assess the safety of a rice variety expressing the snowdrop Galanthus nivalis lectin (GNA lectin), and forms part of a EU-funded project where the objective has been to develop and validate sensitive and specific methods to assess the safety of genetically modified foods. Male and female Wistar rats were given a purified diet containing either 60% genetically modified or parental rice for 90 days. This corresponds to a mean daily GNA lectin intake of approximately 58 and 67mg/kg body weight for males and females, respectively. Prior to the animal study comprehensive analytical characterization of both rice materials was performed. The chemical analyses showed a number of statistically significant differences, with the majority being within the ranges reported in the literature. In the animal study a range of clinical, biological, immunological, microbiological and pathological parameters were examined. A number of significant differences were seen between groups fed the two diets, but none of them were considered to be adverse. In conclusion, the design of the present animal study did not enable us to conclude on the safety of the GM food. Additional group(s) where the expressed gene products have been spiked to the diet should be included in order to be able to distinguish whether the observed effects were due to the GNA lectin per se or to secondary changes in the GM rice.

Full article available at https://goo.gl/qyOL2u

Krzyzowska, M.; Wincenciak, M.; Winnicka, A.; Baranowski, A.; Jaszczak, K.; Zimny, J.; Niemiałtowski, M. 2010. The effect of multigenerational diet containing genetically modified triticale on immune system in mice. *Polish Journal of Veterinarium Sciences*, 13:423-30.

The safety assessment of genetically modified (GM) food and feed is performed to identify the possible effects upon animal and human health, also the long-term, multigenerational influence upon functioning of different organs and systems, such as the immune system. In this study C57BL/6J mice were fed for five consecutive generations with pellets containing 20% of conventional triticale grain (control) vs. pellets containing 20% of the transgenic triticale grain resistant to BASTA herbicide (experimental). The F5 experimental animals showed enlarged inguinal and axillary lymph nodes, but not spleens, and increased WBC counts in blood (but within the norm for Mus musculus). Immunophenotyped cell suspensions derived from spleens, inguinal and axillaris lymph nodes and PBMCs from blood showed the significant decrease in the percentage of T cells in spleen and lymph nodes and the B cells in lymph nodes and blood of the F5 experimental mice in comparison to the control F5 mice. Immunoblotting analysis of IL-2, IL-4, IL-10, IL-12, IL- 6, IFN-gamma levels in serum showed significantly increased IL-2 levels and decreased IL-6 levels in the F5-experimental mice sera. No significant changes in the levels of IgE in sera in both mice groups were observed. The obtained results indicate that multigenerational use of feeds for rodents containing the GM-triticale leads to expansion of the B cell compartment in the secondary lymphoid organs, but it is not caused by malignant processes or the allergic response.

http://www.ncbi.nlm.nih.gov/pubmed/21033555

4 Risks associated with the use of transgenes and small/non-coding RNA (sRNAs/ncRNA)

In addition to the direct effect of recombinant protein(s) produced by transgenic plants, susceptible to possess allergenic and/or toxic activity, it is necessary to examine the effects of interactions between transgene(s) (or other biological products not protein-coding, such as various types of RNA[65]) and the organisms consuming it.

Of course the concerns tend to grow when the changes involve transgenes with key biological function, able to intervene on other aspects of gene expression in the body consuming it.

4.1 Survival of (trans)gene to digestion and the blood circulation

In general, the industry discards risks associated to transgene interaction with the consumer organism. To do so, it is argued that the DNA is completely degraded during the digestive process.

Contrary to this assumption the following studies note that DNA sequences (gene or transgene) not only survive to digestion, but also circulate in the blood.

The reversibility of the presence of recombinant DNA in the consumer body, after diet periods without GMOs - as highlighted by some of the authors listed below - cannot be concluded by the absence of risks in the long term.

65 The transgenic bean 5.1 event developed by Embrapa and approved for commercialization in 2011 in Brazil, aimed to prevent reproduction in the plant of bean golden mosaic virus. To do so, resorted to RNA interference mechanism, leading to gene silencing of a genetic command related to viral replication (involving ncRNA molecules, notably dsRNA and siRNA).

Schubbert, R.; Rentz, D.; Schmitzx, B.; Doerfler, W. 1997. Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. *PNAS*, 94, 961-6 (1).

Food-ingested foreign DNA is not completely degraded in the gastrointestinal tract of mice. Phage M13mp18 DNA as a test molecule devoid of homology to mouse DNA was pipette-fed to or added to the food supply of mice. The fate of this foreign DNA in the animals was followed by several methods. In 84 animals, fragments of M13mp18 DNA were detected in the contents of the small intestine, the cecum (until 18 h), the large intestine, or the feces. In 254 animals, M13mp18 DNA fragments of up to 976 bp were found in blood 2-8 h after feeding. In buffer-fed control animals, M13mp18 DNA could not be detected. M13mp18 DNA fragments were traced by PCR in peripheral leukocytes and located by fluorescent in situ hybridization in about 1 of 1000 white cells between 2 and 8 h, and in spleen or liver cells up to 24 h after feeding, but not later. M13mp18 DNA could be traced by fluorescent in situ hybridization in the columnar epithelial cells, in the leukocytes in Peyer's patches of the cecum wall, in liver cells, and in B cells, T cells, and macrophages from spleen. These findings suggest transport of foreign DNA through the intestinal wall and Peyer's patches to peripheral blood leukocytes and into several organs. Upon extended feeding, M13mp18 DNA could be recloned from total spleen DNA into a lambda vector. Among about 2.5 x 10(7) lambda plaques, one plaque was isolated that contained a 1299 nucleotide pair fragment (nt 4736-6034) of sequence-identified M13mp18 DNA. This fragment was covalently linked to an 80 nt DNA segment with 70% homology to the mouse IgE receptor gene. The DNA from another lambda plaque also contained mouse DNA, bacterial DNA, and rearranged lambda DNA. Two additional plaques contained M13mp18 DNA fragments of at least 641 (nt 2660-3300) or 794 (nt 4640-5433) nucleotide pairs. The medical and evolutionary implications of these observations may be considerable.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC19622/

Schubbert, R.; Hohlweg, U.; Renz, D.; Doerfler, W. 1998. On the fate of orally ingested foreign DNA in mice: chromosal association and placental transmission to the fetus. *Molecular Genomics and Genetics,* 259 (6): 569-76.

We have previously shown that, when administered orally to mice, bacteriophage M13 DNA, as a paradigm foreign DNA without homology to the mouse genome, can persist in fragmented form in the gastrointestinal tract, penetrate the intestinal wall, and reach the nuclei of leukocytes, spleen and liver cells. Similar results were obtained when a plasmid containing the gene for the green fluorescent protein (pEGFP-C1) was fed to mice. In spleen, the foreign DNA was detected in covalent linkage to DNA with a high degree of homology to mouse genes, perhaps pseudogenes, or to authentic E. coli DNA. We have now extended these studies to the offspring of mice that were fed regularly during pregnancy with a daily dose of 50 microg of M13 or pEGFP-C1 DNA. Using the polymerase chain reaction (PCR) or the fluorescent in situ hybridization (FISH) method, foreign DNA, orally ingested by pregnant mice, can be discovered in various organs of fetuses and of newborn animals. The M13 DNA fragments have a length of about 830 bp. In various organs of the mouse fetus, clusters of cells contain foreign DNA as revealed by FISH. The foreign DNA is invariably located in the nuclei. We have never found all cells of the fetus to be transgenic for the foreign DNA. This distribution pattern argues for a transplacental pathway rather than for germline transmission which might be expected only after long-time feeding regimens. In rare cells of three different fetuses, whose mothers have been fed with M 13 DNA during gestation, the foreign DNA was detected by FISH in association with both chromatids. Is maternally ingested foreign DNA a potential mutagen for the developing fetus?

http://www.ncbi.nlm.nih.gov/pubmed/9819049

Martín-Orúe, S.; O'Donnell, A.; Ariño, J.; Netherwood, T.; Gilbert H.; Mathers, J. 2002. Degradation of transgenic DNA from genetically modified soya and maize in human intestinal simulations. *British Journal of Nutrition*, 87, 533-542.

The inclusion of genetically modified (GM) foods in the human diet has caused considerable debate. There is concern that the transfer of plant-derived transgenes to the resident intestinal microflora could have safety implications. For these gene transfer events to occur, the nucleic acid would need to survive passage through the gastrointestinal tract. The aim of the present study was to evaluate the rate at which transgenes, contained within GM soya and maize, are degraded in gastric and small bowel simulations. The data showed that 80 % of the transgene in naked GM soya DNA was degraded in the gastric simulations, while no degradation of the transgenes contained within GM soya and maize were observed in these acidic conditions. In the small intestinal simulations, transgenes in naked soya DNA were degraded at a similar rate to the material in the soya protein. After incubation for 30 min, the transgenes remaining in soya protein and naked DNA were 52 (sem 13.1) % and 34 (sem 17.5) %, respectively, and at the completion of the experiment (3 h) these values were 5 % and 3 %, respectively. In contrast to the soya transgene, the maize nucleic acid was hydrolysed in the small intestinal simulations in a biphasic process in which approximately 85 % was rapidly degraded, while the rest of the DNA was cleaved at a rate similar to that in the soya material. Guar gum and tannic acid, molecules that are known to inhibit digestive enzymes, did not influence the rate of transgene degradation in soya protein. In contrast guar gum reduced the rate of transgene degradation in naked soya DNA in the initial stages, but the polysaccharide did not influence the amount of nucleic acid remaining at the end of the experiment. Tannic acid reduced the rate of DNA degradation throughout the small bowel simulations, with 21 (sem 5.4) % and 2 (sem 1.8) % of the naked soya DNA remaining in the presence and absence of the phenolic acid, respectively. These data indicate that some transgenes in GM foods may survive passage through the small intestine.

http://www.ncbi.nlm.nih.gov/pubmed/12067423

Netherwood, T.; Martín-Orúe, S.; O'Donnell, A.; Gockling, S.; Graham, J.; Mathers, J.; Gilbert, H. 2004. Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. *Nature Biotechnology*, 22, 204-209.

The inclusion of genetically modified (GM) plants in the human diet has raised concerns about the possible transfer of transgenes from GM plants to intestinal microflora and enterocytes. The persistence in the human gut of DNA from dietary GM plants is unknown. Here we study the survival of the transgene *epsps* from GM soya in the small intestine of human ileostomists (i.e., individuals in which the terminal ileum is resected and digesta are diverted from the body via a stoma to a colostomy bag). The amount of transgene that survived passage through the small bowel varied among individuals, with a maximum of 3.7% recovered at the stoma of one individual. The transgene did not survive passage through the intact gastrointestinal tract of human subjects fed GM soya. Three of seven ileostomists showed evidence of low-frequency gene transfer from GM soya to the microflora of the small bowel before their involvement in these experiments. As this low level of *epsps* in the intestinal microflora did not increase after consumption of the meal containing GM soya, we conclude that gene transfer did not occur during the feeding experiment.

http://www.nature.com/nbt/journal/v22/n2/abs/nbt934.html

Mazza, R.; Soave, M.; Morlacchini, M.; Piva, G.; Marocco, A. 2005. Assessing the transfer of genetically modified DNA from feed to animal tissues. *Transgenic Research*, 14:775–784.

In Europe, public and scientific concerns about the environmental and food safety of GM (Genetically Modified) crops overshadow the potential benefits offered by crop biotechnology to improve food

quality. One of the concerns regarding the use of GM food in human and animal nutrition is the effect that newly introduced sequences may have on the organism. In this paper, we assess the potential transfer of diet-derived DNA to animal tissues after consumption of GM plants. Blood, spleen, liver, kidney and muscle tissues from piglets fed for 35 days with diets containing either GM (MON810) or a conventional maize were investigated for the presence of plant DNA. Only fragments of specific maize genes (Zein, Sh-2) could be detected with different frequencies in all the examined tissues except muscle. A small fragment of the Cry1A(b) transgene was detected in blood, liver, spleen and kidney of the animals raised with the transgenic feed. The intact Cry1A(b) gene or its minimal functional unit were never detected. Statistical analysis of the results showed no difference in recovery of positives for the presence of plant DNA between animals raised with the transgenic feed and animals raised with the conventional feed, indicating that DNA transfer may occur independently from the source and the type of the gene. From the data obtained, we consider it unlikely that the occurrence of genetic transfer associated with GM plants is higher than that from conventional plants.

http://www.ncbi.nlm.nih.gov/pubmed/16245168

Tudisco, R.; Infascelli, F.; Cutrignelli, M.; Bovera, F.; Morcia, C.; Facciolli, P.; Terzi, V. 2006. Fate of feed plant DNA monitored in water buffalo (*Bubalus bubalis*) and rabbit (*Oryctolagus cuniculus*). *Livestock Science*, Vol. 105 (1-3): 12-18.

The effect of the digestion process in the gastro-intestinal tract (GIT) of animal models on the fate and integrity of plant DNA has been widely evaluated since DNA availability and integrity is a key factor for hypothetical horizontal gene transfer of recombinant DNA from GM crop-derived feeds to animal and human gut microflora. In this study, plant DNA sequences from high and low copy number genes were monitored in GIT and tissues of buffaloes and rabbits. Using a real-time PCR approach to track plant DNA in animal samples, we demonstrated the persistence of fragmented plant DNA blood and tissues of buffaloes and rabbits raised with conventional feeding.

https://goo.gl/PTjI1W

Sharma, R.; Damgaard, D.; Alexander, T.; Dugan, M.; Aalhus, J.; Stanford, K.; McAllister, T. 2006. Detection of transgenic and endogenous plant DNA in digesta and tissues of sheep and pigs fed Roundup Ready Canola meal. *J Agric Food Chem*, 54 (5): 1699-1709.

The persistence of plant-derived recombinant DNA in sheep and pigs fed genetically modified (Roundup Ready) canola was assessed by PCR and Southern hybridization analysis of DNA extracted from digesta, gastrointestinal (GI) tract tissues, and visceral organs. Sheep (n = 11) and pigs (n = 36) were fed to slaughter on diets containing 6.5 or 15% Roundup Ready canola. Native plant DNA (high- and low-copy-number gene fragments) and the cp4 epsps transgene that encodes 5-enolpyruvyl shikimate-3-phosphate synthase were tracked in ruminal, abomasal, and large intestinal digesta and in tissue from the esophagus, rumen, abomasum, small and large intestine, liver, and kidney of sheep and in cecal content and tissue from the duodenum, cecum, liver, spleen, and kidney of pigs. High-copy chloroplast-specific DNA (a 520-bp fragment) was detected in all digesta samples, the majority (89-100%) of intestinal tissues, and at least one of each visceral organ sample (frequencies of 3-27%) from sheep and swine. Low-copy rubisco fragments (186- and 540-bp sequences from the small subunit) were present at slightly lower, variable frequencies in digesta (18-82%) and intestinal tissues (9-27% of ovine and 17-25% of porcine samples) and infrequently in visceral organs (1 of 88 ovine samples; 3 of 216 porcine samples). Each of the five cp4 epsps transgene fragments (179-527 bp) surveyed was present in at least 27% of ovine large intestinal content samples (maximum = 64%) and at least 33% of porcine cecal content samples (maximum = 75%). In sheep, transgene fragments were more common in intestinal digesta than in ruminal or abomasal content. Transgene fragments were detected in 0 (esophagus) to 3 (large intestine) GI tract tissues from the 11 sheep and in 0-10 of the duodenal and cecal tissues collected from 36 pigs.

The feed-ingested recombinant DNA was not detected in visceral tissues (liver, kidney) of lambs or in the spleen from pigs. Of note, however, one liver and one kidney sample from the pigs (different animals) were positive for a 278-bp fragment of the transgenic cp4 epsps (denoted F3). Examination of genomic libraries from these tissues yielded no conclusive information regarding integration of the fragment into porcine DNA. This study confirms that feed-ingested DNA fragments (endogenous and transgenic) do survive to the terminal GI tract and that uptake into gut epithelial tissues does occur. A very low frequency of transmittance to visceral tissue was confirmed in pigs, but not in sheep. It is recognized that the low copy number of transgenes in GM feeds is a challenge to their detection in tissues, but there was no evidence to suggest that recombinant DNA would be processed in the gut in any manner different from endogenous feed-ingested genetic material.

http://www.ncbi.nlm.nih.gov/pubmed/16506822

Agodi, A.; Barchitta, M.; Grillo, A.; Sciacca, S. 2006. Detection of genetically modified DNA sequences in milk from the Italian market. *Int J Hyg Environ Health*, 209: 81-88.

The possible transfer and accumulation of novel DNA and/or proteins in food for human consumption derived from animals receiving genetically modified (GM) feed is at present the object of scientific dispute. A number of studies failed to identify GM DNA in milk, meat, or eggs derived from livestock receiving GM feed ingredients. The present study was performed in order to: (i) develop a valid protocol by PCR and multicomponent analysis for the detection of specific DNA sequences in milk, focused on GM maize and GM soybean; (ii) assess the stability of transgenic DNA after pasteurization treatment and (iii) determine the presence of GM DNA sequences in milk samples collected from the Italian market. Results from the screening of 60 samples of 12 different milk brands demonstrated the presence of GM maize sequences in 15 (25%) and of GM soybean sequences in 7 samples (11.7%). Our screening methodology shows a very high sensitivity and the use of an automatic identification of the amplified products increases its specificity and reliability. Moreover, we demonstrated that the pasteurization process is not able to degrade the DNA sequences in spiked milk samples. The detection of GM DNA in milk can be interpreted as an indicator of fecal or airborne contamination, respectively, with feed DNA or feed particles, although an alternative source of contamination, possibly recognizable in the natural environment can be suggested. Further studies, performed on a larger number of milk samples, are needed to understand the likely source of contamination of milk collected from the Italian market.

http://www.ncbi.nlm.nih.gov/pubmed/16373205

Chainark, P.; Satoh, S.; Hino, T.; Kiron, V.; Hirono, I.; Aoki, T. 2006. Availability of genetically modified soybean meal in rainbow trout *Oncorhynchus mykiss* diets. *Fisheries Science*, 72: 1072 – 1078.

The use of genetically modified defatted soybean meal (GM SBM) as rainbow trout feed was investigated, in comparison with non-GM SBM. Both SBMs were included at levels of approximately 15 and 30% in four diets (42% protein). The diets were fed to juvenile fish (48.3 g average weight) for 12 weeks. The nutrient use showed that there was no significant difference in growth and feed performance between GM and non-GM SBM groups at both inclusion levels at the end of 12th week. The cauliflower mosaic virus *35S*promoter fragment (220 bp) of the GM SBM was detected in the muscle of fish receiving both levels of GM SBM diet by nested PCR, but the frequency of detection was greater at the higher inclusion level. Additionally, the promoter fragment was not detected by the fifth day after changing the diet to non-GM. Conversely, the promoter fragment was not detected from fish fed with the non-GM SBM diet. The results demonstrated that the availability of protein in GM SBM was similar to that of non-GM SBM, and the promoter fragments found in the muscle of fish were not detectable after changing the diet to non-GM, verifying the availability of the GM SBM in rainbow trout feed.

http://onlinelibrary.wiley.com/doi/10.1111/j.1444-2906.2006.01258.x/abstract

Chainark, P.; Satoh, S.; Hirono, I.; Aoki, T.; Endo, M. 2008. Availability of genetically modified feed ingredient: investigations of ingested foreign DNA in rainbow trout *Oncorhynchus mykiss*. *Fisheries Science*, 74: 380-390.

Foreign DNA fragments from genetically modified defatted soybean meal (GM SEM) in rainbow trout was traced by nested polymerase chain reaction (PCR) and located by *in situ* hybridization. Either a GM or non-GM SBM formulated diet (42% protein) was fed to fish (average weight 50.5 g) for 2 weeks. The degradation results showed that the cauliflower mosaic virus *35S* promoter (220 bp) fragment was detected in the contents of digestive system only in fish fed the GM SBM diet, and it was not detected on the third day after changing the diet to the non-GM SBM diet. For the possible transferal results, the promoter fragment was detected in the leukocyte, head kidney and muscle only of fish fed the GM SBM diet; it was not detected on the fifth day after changing the diet to the non-GM SBM diet. These results suggest that a foreign DNA fragment was not completely degraded and might be taken up into organs through the gastrointestinal tract. However, foreign DNA was not detected after the withdrawal period. Thus, the data show that uptake of DNA from GM SBM might not remain in the tissues of fish fed GM SBM diet

http://link.springer.com/article/10.1111%2Fj.1444-2906.2008.01535.x

Ran, T.; Mei, L.; Lei, W.; Aihua, L.; Ru, H.; Jie, S. 2009. Detection of transgenic DNA in tilapias (*Oreochromis niloticus*, GIFT strain) fed genetically modified soybeans (Roundup Ready). *Aquaculture Research*, 40: 1350-1357.

We used nested-polymerase chain reaction (PCR) to detect Roundup Ready soybean in aquatic feeds and feeding tilapias. A template concentration of 10–10 g μL–1 DNA solution could be detected with a dilute degree of 0.01%. Most (90.6%) of the aquatic feeds containing soybean byproduct included exogenous DNA segments. We also compared genetically modified (GM) soybean with non-GM soybean diets in feeding tilapias (Oreochromis niloticus, GIFT strain) and examined the residual fragments (254 bp) of GM soybeans. Tilapias receiving GM soybean diets had DNA fragments in different tissues and organs, indicating that exogenous GM genes were absorbed systemically and not completely degraded by the tilapia's alimentary canal.

Full article available at https://goo.gl/8W1D3Y

Tudisco, R.; Mastellone, V.; Cutrignelli, M.; Lombardi, P.; Bovera, F.; Mirabella, N.; Piccolo, G.; Calabro, S.; Avallone L.; Infascelli, F. 2010. Fate of transgenic DNA and evaluation of metabolic effects in goats fed genetically modified soybean and in their offsprings. *The Animal Consortium*, 1-10; 4:1662-1671.

The presence of DNA fragments in blood and milk from goats fed conventional (control) or Roundup Ready soybean meal solvent extracted (s.e.; treated) was investigated by using a polymerase chain reaction approach. The same investigation was carried out on blood, skeletal muscle and organs from kids of both groups fed only dams' milk until weaning. Moreover, the possible effects on cell metabolism were evaluated by determination of several specific enzymes in serum, heart, skeletal muscle, liver and kidney. Fragments of the multicopy chloroplast (trnL) gene were found in blood and milk samples from goats of both groups. In kids, the chloroplast fragments were found in samples of both groups. In samples, which proved positive for the presence of chloroplast DNA, fragments of the specific soybean single copy gene (lectin) were detected in several blood and milk samples. The same fragment was also found in control and treated groups of kids. Transgenic fragments were not found in those samples, which were found positive for chloroplast fragments of control groups of either goats or kids. On the contrary, in blood and milk of treated goats, fragments both of the 35S promoter and the CP4 epsps gene were detected. These fragments were also found in treated kids with a significant detection of the 35S promoter in liver, kidney and blood, and of the CP4 epsps gene fragment in liver, kidney, heart and muscle. A significant increase

in lactic dehydrogenase, mainly concerning the lactic dehydrogenase-1 isoenzyme was found in heart, skeletal muscle and kidney of treated kids, thus suggesting a change in the local production of the enzyme. Finally, no significant differences were detected concerning kid body and organ weight.

Full article available at https://goo.gl/r2FRmS

Aris, A.; Leblanc, S. 2011. Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec, Canada. *Reprod Toxicol*, 31(4): 528-33. doi: 10.1016/j.reprotox.2011.02.004.

Pesticides associated to genetically modified foods (PAGMF), are engineered to tolerate herbicides such as glyphosate (GLYP) and gluphosinate (GLUF) or insecticides such as the bacterial toxin bacillus thuringiensis (Bt). The aim of this study was to evaluate the correlation between maternal and fetal exposure, and to determine exposure levels of GLYP and its metabolite aminomethyl phosphoric acid (AMPA), GLUF and its metabolite 3-methylphosphinicopropionic acid (3-MPPA) and Cry1Ab protein (a Bt toxin) in Eastern Townships of Quebec, Canada. Blood of thirty pregnant women (PW) and thirty-nine nonpregnant women (NPW) were studied. Serum GLYP and GLUF were detected in NPW and not detected in PW. Serum 3-MPPA and CryAb1 toxin were detected in PW, their fetuses and NPW. This is the first study to reveal the presence of circulating PAGMF in women with and without pregnancy, paving the way for a new field in reproductive toxicology including nutrition and utero-placental toxicities.

http://www.ncbi.nlm.nih.gov/pubmed/21338670

Grønsberg, I.; Nordgård, L.; Fenton, K.; Hegge, B.; Nielsen, K.; Bardocz, S.; Pusztai, A.; Traavik, T. 2011. Uptake and organ distribution of feed introduced plasmid DNA in growing or pregnant rats. *Food and Nutrition Sciences*, 2, 377-386.

Fragments of DNA present in food and feed are taken up by the gastrointestinal tract (GIT) of mammals. The extent of uptake varies according to organism, study design and DNA source. This study explores the hypothesis that actively growing, as well as pregnant rats, are more likely to take up DNA from the GIT than mature animals due to the high demand for nutrients for tissue and organ development. Plasmid DNA (pDNA) was added to standard feed for growing, and pregnant rats. The young rats received one pDNA (50 µg) containing meal by gavage. Blood, organ and tissue samples were harvested at 2 h to 3 days post feeding (p.f). The pregnant females were fed pellets containing pDNA (100 µg) daily, starting at day 5 after established pregnancy. Females and foeti were killed at days 7 and 14 of gestation, and pups at the time of weaning. Genomic DNA was analyzed by PCR followed by Southern blot and real-time PCR. A 201 bp target sequence was detected in mesenteric lymph nodes, spleen, liver and pancreas from growing rats 2 h p.f. At 6 h, target DNA was detectable in the kidneys, and at three days p.f. in the liver. Target DNA was not detected in samples from pregnant rats, their foeti or pups. In conclusion, low level of feed introduced DNA could be transiently detected in organs of young, growing rats. However, indications of increased DNA uptake levels in the GIT of growing rats were not found.

Full article available at https://goo.gl/4hJY5i

Spisák, S.; Solymosi, N.; Ittzés, P.; Bodor, A.; Kondor, D.; Vattay, G.; Barták, B.; Sipos, F.; Galamb, O.; Tulassay, Z.; Szállási, Z.; Rasmussen, S.; Sicheritz-Ponten, T.; Brunak, S.; Molnár, B.; Csabai, I. 2013. Complete genes may pass from food to human blood. *PLOS*

ONE, 8(7): e69805. doi:10.1371/journal.pone.0069805.

Our bloodstream is considered to be an environment well separated from the outside world and the digestive tract. According to the standard paradigm large macromolecules consumed with food cannot pass directly to the circulatory system. During digestion proteins and DNA are thought to be degraded into small constituents, amino acids and nucleic acids, respectively, and then absorbed by a complex active process and distributed to various parts of the body through the circulation system. Here, based on the analysis of over 1000 human samples from four independent studies, we report evidence that meal-derived DNA fragments which are large enough to carry complete genes can avoid degradation and through an unknown mechanism enter the human circulation system. In one of the blood samples the relative concentration of plant DNA is higher than the human DNA. The plant DNA concentration shows a surprisingly precise log-normal distribution in the plasma samples while non-plasma (cord blood) control sample was found to be free of plant DNA.

Full article available at https://goo.gl/JhuzxG

4.2 Possibility of horizontal transfer of (trans) genes in mammalian cells or symbiotic microorganisms (bacteria of the digestive system and/or the buccal cavity in particular)

A key aspect, in relation to the stability of the transgenic DNA, after subjected to pressures of the digestive system, concerns the subsequent possibility of this genetic material likely to be involved in a horizontal transfer processes in cells and/or symbiotic bacteria, integrated to the body that consumed that DNA. Recently, it has grown significantly the number of authors who consider symbionts bacteria of the human being as essential for different functions related to the welfare of the people, acting as a kind of different "organ". In fact, there are about $10E^{17}$ bacteria in a human body. This is something that beats 10-100 times the estimates of the number of human cells. Only in the digestive system these bacteria comprise over 500 species, of which a significant part is responsible for performing horizontal gene transfer.

The following articles discuss the importance of these biological mechanisms, its complexity and dependence factors, internal

and external, not completely known yet. They also highlight difficulties in identification and detection limits related to such events. The expression "absence of evidence does not mean evidence of absence", created by Carl Sagan, applies clearly to the issue of risks in processes affected by HGT mechanisms.

Grillot-Courvalin, C.; Goussand, S.; Huetz, F.; Ojcius, D.; Courvalin, P. 1998. Functional gene transfer from intracellular bacteria to mammalian cells. *Nature Biotech*, 16: 862-66.

We provide evidence of direct transfer of functional DNA from bacteria to mammalian cells. An Escherichia coli K12 diaminopimelate auxotroph made invasive by cloning the invasin gene from Yersinia pseudotuberculosis transfers DNA after simple co-incubation, into a variety of mammalian cell lines. Transfer efficiency was enhanced in some cells by coexpression of the gene for listeriolysin from Listeria monocytogenes. Expression of the acquired genes occurs in both dividing and quiescent cells. The only requirement for bacteria to transfer genetic material into nonprofessional phagocytic cells and macrophages is the ability to invade the host cell.

http://www.ncbi.nlm.nih.gov/pubmed/9743121

Ho, M-W.; Traavik, T.; Olsvik, O.; Tappeser, B.; Howard, C.; Weizsacker, C.; McGavin, G. 1998. Gene technology and gene ecology of infectious diseases. *Microbial Ecology in Health and Disease*, 10: 33-59.

According to the 1996 WHO Report, the world is heading for a major crisis in public health as outbreaks of new and re-emerging infectious diseases are striking at increasing frequencies within the past 10 to 15 years. The current strains of pathogens are moreover, resistant to known treatments; some strains being resistant to all or nearly all drugs and antibiotics. Horizontal gene transfer is now generally recognized to be responsible for the evolution of virulence and the spread of drug and antibiotic resistances. Many pathogens have crossed species barriers, having acquired genes from phylogenetically distant species that are involved in their ability to cause diseases. Recent findings document the extremely wide scope of horizontal gene transfer and the extensive recombination between genetic material from unrelated species that have contributed to the emergence of virulence and antibiotic resistances. The past 15 years coincide with the development of genetic engineering biotechnology on a commercial scale. Genetic engineering depends on designing vectors for cloning and transferring genes and involves artificially recombining and manipulating genes from unrelated species and their viral pathogens, thereby enhancing the probability for horizontal gene transfer and recombination. The urgent question which needs to be addressed is the extent to which genetic engineering biotechnology, by facilitating horizontal gene transfer and recombination, is contributing to the resurgence of infectious, drug-resistant diseases, and will continue to do so if allowed to proceed unchecked. An enquiry into the possible contribution of genetic engineering biotechnology to the etiology of infectious diseases is all the more pressing in the light of other relevant recent findings indicating that microorganisms genetically engineered for 'contained use' may not be effectively contained. Thus, biologically 'crippled' strains of bacteria can survive in the environment to exchange genes with other species; DNA released from cells is not readily broken down in the environment, thereby retaining the ability to transform organisms; some viral DNA can be more infectious than the virus itself; and routine chemical treatments for inactivating pathogenic microorganisms and viruses, before they are discharged into the environment, may be ineffective, leaving a substantial percentage of pathogens in an active infectious state. The combination of

the different kinds of evidence is sufficiently compelling, especially in view of the precautionary principle, to warrant, at the very least, an independent public enquiry into genetic engineering biotechnology and the etiology of infectious diseases.

Full article available at http://www.microbecolhealthdis.net/index.php/mehd/article/view/7823

Mercer, D.; Scott, K.; Bruce-Johnson, W.; Glover, L.; Flint, H. 1999. Fate of free DNA and transformation of the oral bacterium *Streptococcus gordonii* DL1 by plasmid DNA in human saliva. *Applied and Environmental Microbiology*, p. 6–10.

Competitive PCR was used to monitor the survival of a 520-bp DNA target sequence from a recombinant plasmid, pVACMC1, after admixture of the plasmid with freshly sampled human saliva. The fraction of the target remaining amplifiable ranged from 40 to 65% after 10 min of exposure to saliva samples from five subjects and from 6 to 25% after 60 min of exposure. pVACMC1 plasmid DNA that had been exposed to degradation by fresh saliva was capable of transforming naturally competent Streptococcus gordonii DL1 to erythromycin resistance, although transforming activity decreased rapidly, with a half-life of approximately 50 s. S. gordonii DL1 transformants were obtained in the presence of filter-sterilized saliva and a 1-microg/ml final concentration of pVACMC1 DNA. Addition of filter-sterilized saliva instead of heat-inactivated horse serum to S. gordonii DL1 cells induced competence, although with slightly lower efficiency. These findings indicate that DNA released from bacteria or food sources within the mouth has the potential to transform naturally competent oral bacteria. However, further investigations are needed to establish whether transformation of oral bacteria can occur at significant frequencies in vivo.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC90975/

Duggan, P.; Chambers, P.; Heritage, J.; Forbes, J. 2000. Survival of free DNA encoding antibiotic resistance from transgenic maize and the transformation activity of DNA in ovine saliva, ovine rumen fluid and silage effluent. *FEMS Microbiology Letters*, 191, 71-77.

To assess the likelihood that the bla gene present in a transgenic maize line may transfer from plant material to the microflora associated with animal feeds, we have examined the survival of free DNA in maize silage effluent, ovine rumen fluid and ovine saliva. Plasmid DNA that had previously been exposed to freshly sampled ovine saliva was capable of transforming competent Escherichia coli cells to ampicillin resistance even after 24 h, implying that DNA released from the diet could provide a source of transforming DNA in the oral cavity of sheep. Although target DNA sequences could be amplified by polymerase chain reaction from plasmid DNA after a 30-min incubation in silage effluent and rumen contents, only short term biological activity, lasting less than 1 min, was observed in these environments, as shown by transformation to antibiotic resistance. These experiments were performed under in vitro conditions; therefore further studies are needed to elucidate the biological significance of free DNA in the rumen and oral cavities of sheep and in silage effluent.

http://www.ncbi.nlm.nih.gov/pubmed/11004402

Kunik, T.; Tzfira, T.; Kapulnik, Y.; Gafni, Y.; Dingwall, C.; Citovsky, V. 2001. Genetic transformation of HeLa cells by *Agrobacterium*. *PNAS*, 98 (4): 1871-6.

Agrobacterium tumefaciens is a soil phytopathogen that elicits neoplastic growths on the host plant species. In nature, however, *Agrobacterium* also may encounter organisms belonging to other kingdoms such as insects and animals that feed on the infected plants. Can *Agrobacterium*, then, also infect animal cells? Here, we report that *Agrobacterium* attaches to and genetically transforms several types of human cells. In stably transformed HeLa cells, the integration event occurred at the right border of the tumor-inducing plasmid's transferred-DNA (T-DNA), suggesting bona fide T-DNA transfer and lending support to the notion that *Agrobacterium* transforms human cells by a mechanism similar to that which it uses for transformation of plants cells. Collectively, our results suggest that *Agrobacterium* can transport its T-DNA to human cells and integrate it into their genome.

Full article available at http://www.pnas.org/content/98/4/1871.full.pdf+html

Mercer, D.; Scott, K.; Melville, C.; Glover, L.; Flint, H. 2001. Transformation of an oral bacterium via chromosomal integration of free DNA in the presence of human saliva. *FEMS Microbiol Lett*, 200 (2): 163-7.

Transformation of Streptococcus gordonii DL1 by free DNA was studied in human saliva. Competent S. gordonii could be transformed in vitro with plasmid DNA that had been taken into the human mouth. Transformation also occurred with a plasmid that cannot replicate in S. gordonii, but that has a region of chromosomal homology, by integration into the bacterial chromosome, although linearised plasmid DNA gave no transformants. Linear chromosomal DNA fragments did however transform S. gordonii/Tn916 efficiently in saliva when regions of homology with the recipient chromosome flanked the marker gene. These findings are discussed in relation to the potential for acquisition of DNA sequences, including genetically modifiedDNA, by gut and oral bacteria.

http://www.ncbi.nlm.nih.gov/pubmed/11425469

Duggan, P.; Chambers, P.; Heritage, J.; Forbes, J. 2003. Fate of genetically modified maize DNA in the oral cavity and rumen of sheep. *British Journal of Nutrition*, 89: 159-66.

The polymerase chain reaction (PCR) technique was used to investigate the fate of a transgene in the rumen of sheep fed silage and maize grains from an insect-resistant maize line. A 1914-bp DNA fragment containing the entire coding region of the synthetic cryIA(b) gene was still amplifiable from rumen fluid sampled 5 h after feeding maize grains. The same target sequence, however, could not be amplified from rumen fluid sampled from sheep fed silage prepared from the genetically modified maize line. PCR amplification of a shorter (211-bp), yet still highly specific, target sequence was possible with rumen fluid sampled up to 3 and 24 h after feeding silage and maize grains, respectively. These findings indicate that intact transgenes from silage are unlikely to survive significantly in the rumen since a DNA sequence 211-bp long is very unlikely to transmit genetic information. By contrast, DNA in maize grains persists for a significant time and may, therefore, provide a source of transforming DNA in the rumen. In addition, we have examined the biological activity of plasmid DNA that had previously been exposed to the ovine oral cavity. Plasmid extracted from saliva sampled after incubation for 8 min was still capable of transforming competent Escherichia coli to kanamycin resistance, implying that DNA released from the diet within the mouth may retain sufficient biological activity for the transformation of competent oral bacteria.

Full article available at http://www.mednat.org/alimentazione/Duggan_GMO_Mais.pdf

COM – Heritage, J. 2004. The fate of transgenes in the human gut. *Nat Biotechnol*, 22(2):

170-172.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/14755289

REV – Smets, B.; Barkay, T. 2005. Horizontal gene transfer: perspectives at a crossroads of scientific disciplines. *Nature Reviews Microbiology*, 3: 675-678.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/16145755

Rizzi, A.; Raddadi, N.; Sorlini, C.; Nordgård, L.; Nielsen, K.; Daffonchio, D. 2012. The stability and degradation of dietary DNA in the gastrointestinal tract of mammals: implications for horizontal gene transfer and the biosafety of GMOs. *Critical Reviews in Food Science and Nutrition*, 52 (2) 142-161 (DOI: 10.1080/10408398.2010.499480).

The fate of dietary DNA in the gastrointestinal tract (GIT) of animals has gained renewed interest after the commercial introduction of genetically modified organisms (GMO). Among the concerns regarding GM food, are the possible consequences of horizontal gene transfer (HGT) of recombinant dietary DNA to bacteria or animal cells. The exposure of the GIT to dietary DNA is related to the extent of food processing, food composition, and to the level of intake. Animal feeding studies have demonstrated that a minor amount of fragmented dietary DNA may resist the digestive process. Mammals have been shown to take up dietary DNA from the GIT, but stable integration and expression of internalized DNA has not been demonstrated. Despite the ability of several bacterial species to acquire external DNA by natural transformation, in vivo transfer of dietary DNA to bacteria in the intestine has not been detected in the few experimental studies conducted so far. However, major methodological limitations and knowledge gaps of the mechanistic aspects of HGT calls for methodological improvements and further studies to understand the fate of various types of dietary DNA in the GIT.

http://www.ncbi.nlm.nih.gov/pubmed/22059960

Nordgård, L.; Brusetti, L.; Raddadi, N.; Traavik, T.; Averhoff, B.; Nielsen, K. 2012. An investigation of horizontal transfer of feed introduced DNA to the aerobic microbiota of the gastrointestinal tract of rats. *BMC Research Notes*, 5:170.

Background: Horizontal gene transfer through natural transformation of members of the microbiota of the lower gastrointestinal tract (GIT) of mammals has not yet been described. Insufficient DNA sequence similarity for homologous recombination to occur has been identified as the major barrier to interspecies transfer of chromosomal DNA in bacteria. In this study we determined if regions of high DNA similarity between the genomes of the indigenous bacteria in the GIT of rats and feed introduced DNA could lead to homologous recombination and acquisition of antibiotic resistance genes.
Results: Plasmid DNA with two resistance genes (*npt*I and *aad*A) and regions of high DNA similarity to 16S rRNA and 23S rRNA genes present in a broad range of bacterial species present in the GIT, were constructed and added to standard rat feed. Six rats, with a normal microbiota, were fed DNA containing pellets daily over four days before sampling of the microbiota from the different GI compartments (stomach, small intestine, cecum and colon). In addition, two rats were included as negative controls. Antibiotic resistant colonies growing on selective media were screened

for recombination with feed introduced DNA by PCR targeting unique sites in the putatively recombined regions. No transformants were identified among 441 tested isolates.
Conclusions: The analyses showed that extensive ingestion of DNA (100 µg plasmid) per day did not lead to increased proportions of kanamycin resistant bacteria, nor did it produce detectable transformants among the aerobic microbiota examined for 6 rats (detection limit < 1 transformant per $1,1 \times 10^8$cultured bacteria). The key methodological challenges to HGT detection in animal feedings trials are identified and discussed. This study is consistent with other studies suggesting natural transformation is not detectable in the GIT of mammals.

Full article available at http://www.biomedcentral.com/content/pdf/1756-0500-5-170.pdf

Oraby, H.; Kandil, M.; Hassan, A.; Al-Sharawi, H. 2014. Addressing the issue of horizontal gene transfer from a diet containing genetically modified components into rat tissues. *African Journal of Biotechnology*, 13(48), 4410-4418.

Genetically modified (GM) food crops are considered to have the potential of providing food security especially in developing countries. Scientists have raised concern over the hazards associated with the consumption of genetically modified organisms (GMOs). One of these hazards, which have great controversy reports, is the possible horizontal gene transfer from GM-food or feed to human or animal tissues. Many researches were conducted to investigate the presence of some transgenic sequences in animal tissues fed on GM- crops. Many of the inserted genes in the GM-crops are under the control of the promoter of the Cauliflower mosaic virus (CaMVP35S) and produce insecticidal proteins. Health hazards are suggested to accompany the ingestion of this promoter. CaMVP35S can function in a wide range of organisms (plants and animals). It has also been demonstrated that the CaMV-P35S promoter sequence can convert an adjacent tissue- and organ-specific gene promoter into a globally active promoter. The present work was conducted to evaluate the possibility of horizontal gene transfer from a diet containing DNA segments from Cauliflower mosaic virus -35S promoter (CaMVP-35S) to the cells of different organs of rats fed for three months on diets containing genetically modified components. Analysis of the results revealed that: 1) ingested fragments from the CaMV-35S promoter incorporated into blood, liver, and brain tissues of experimental rats, 2) The total mean of transfer of GM target sequences increased significantly by increasing the feeding durations, and 3) The affinity of different transgenic fragments from the ingested GM-diet, to be incorporated into the different tissues of rats varied from one target sequence to the other.

Full article available at https://goo.gl/XWFN4b

4.3 Unwanted changes of gene expression by means of small non-coding RNA (ncRNA/ sRNAs)

In some GMPs, the inserted transgene does not lead to the production of a protein, but only to a small non-coding RNA transcripts. Through these transcripts, biotech companies seek new products. These include abilities to inactivate/remove the expression of genes involved in crucial physiological pathways

for particular insect pests or to prevent significant replication of pathogens in crops. The risk in this case is associated with the fact that, once synthesized in transgenic plants, these molecules will also be consumed by humans and/or animals intended for the food chain.

This is the case of beans from Embrapa and other events presented as devoid of risks (to human health and the environment) because they do not produce recombinant proteins. Recent findings pointing biological functions for small non-coding RNA, associated with gene regulation, opened new discussion fields.

Indeed, synthesis of some types of ncRNA appears to be accompanied by the formation (unwanted / uncontrolled) of other small RNA molecules (in particular for dsRNA) - which will also be consumed by the target and non-target organisms. The important point is that the dsRNA are biological structures of high stability, tending to remain active in spite of the main mechanisms of gene silencing (RNAi, PTGS and TGS). Furthermore, small non-coding RNAs involved in these mechanisms of gene regulation (in particular siRNA) may have nonspecific actions (off-target) that multiply the possibilities of unwanted and potentially dangerous effects on the organisms.

In parallel, as in most of the effects involving the epigenome, gene silencing obtained by means of dsRNA, will be transmitted from generation to generation (particularly RNAi). It is noteworthy, therefore, the importance of robust and detailed assessments, applied to the potential risk analysis resulting from the consumption of these GMPs.

A significant portion of the scientific community believes that the available knowledge is insufficient for understanding the mode of action of dsRNA molecules and their effects on target and non-

target species[66]. The safe use of these technologies on a commercial scale will require prior advances in basic science, followed by the tests as a condition for the mass technologies. The lack of knowledge about risks associated with the commercial use of non-coding RNA, on public health and the environment[67] is illustrated in the following articles. Selected documents gather uncertainties and knowledge gaps about potential risks and benefits of these technologies and their consequences, notably in the course of gene therapies.

Elbashir, S.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, 411: 494–8.

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. The mediators of sequence-specific messenger RNA degradation are 21- and 22-nucleotide small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer dsRNAs. Here we show that 21-nucleotide siRNA duplexes specifically suppress expression of endogenous and heterologous genes in different mammalian cell lines, including human embryonic kidney (293) and HeLa cells. Therefore, 21-nucleotide siRNA duplexes provide a new tool for studying gene function in mammalian cells and may eventually be used as gene-specific therapeutics.

Full article available at https://goo.gl/oX6qyU

Qiu, S.; Adema, C.; Lane, T. 2005. A computational study of off-target effects of RNA interference. *Nucleic Acids Research*, 33: 1834–1847.

RNA interference (RNAi) is an intracellular mechanism for post-transcriptional gene silencing that is frequently used to study gene function. RNAi is initiated by short interfering RNA (siRNA) of approximately 21 nt in length, either generated from the double-stranded RNA (dsRNA) by using the enzyme Dicer or introduced experimentally. Following association with an RNAi silencing complex, siRNA targets mRNA transcripts that have sequence identity for destruction. A phenotype resulting from this knockdown of expression may inform about the function of the targeted gene. However, 'off-target effects' compromise the specificity of RNAi if sequence identity between siRNA and random mRNA transcripts causes RNAi to knockdown expression of non-targeted genes. The complete off-target effects must be investigated systematically on each gene in a genome by adjusting a group of parameters, which is too expensive to conduct experimentally and motivates a study in silico. This computational study examined the potential for off-target effects of RNAi, employing the genome and transcriptome sequence data of Homo sapiens, Caenorhabditis elegans and Schizosaccharomyces pombe. The chance for RNAi off-target effects proved considerable, ranging from 5 to 80% for each of the organisms, when using as parameter the exact identity between any possible siRNA sequences (arbitrary length ranging from 17 to 28 nt) derived from a dsRNA (range 100-400 nt) representing the coding sequences of target genes

66 More information on the subject of gene silencing by epigenetic mechanisms are available in section 1.2 of Part 1.

67 In Part 2 section 2.3 are complementary information on weaknesses technologies that resort to the use of non-coding RNA, in particular through post-transcriptional gene silencing (PTGS). This is the case of genetically modified plants to resist viruses.

and all other siRNAs within the genome. Remarkably, high-sequence specificity and low probability for off-target reactivity were optimally balanced for siRNA of 21 nt, the length observed mostly in vivo. The chance for off-target RNAi increased (although not always significantly) with greater length of the initial dsRNA sequence, inclusion into the analysis of available untranslated region sequences and allowing for mismatches between siRNA and target sequences. siRNA sequences from within 100 nt of the 5' termini of coding sequences had low chances for off-target reactivity. This may be owing to coding constraints for signal peptide-encoding regions of genes relative to regions that encode for mature proteins. Off-target distribution varied along the chromosomes of C.elegans, apparently owing to the use of more unique sequences in gene-dense regions. Finally, biological and thermodynamical descriptors of effective siRNA reduced the number of potential siRNAs compared with those identified by sequence identity alone, but off-target RNAi remained likely, with an off-target error rate of approximately 10%. These results also suggest a direction for future in vivo studies that could both help in calibrating true off-target rates in living organisms and also in contributing evidence toward the debate of whether siRNA efficacy is correlated with, or independent of, the target molecule. In summary, off-target effects present a real but not prohibitive concern that should be considered for RNAi experiments.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1072799/

Kulkarni, M.; Booker, M.; Silver, S.; Friedman, A.; Hong, P.; Perrimon, N.; Mathey-Prevot, B. 2006. Evidence of off-target effects associated with long dsRNAs in *Drosophila melanogaster* cell-based assays. *Nature Methods*, 3: 833–838.

To evaluate the specificity of long dsRNAs used in high-throughput RNA interference (RNAi) screens performed at the Drosophila RNAi Screening Center (DRSC), we performed a global analysis of their activity in 30 genome-wide screens completed at our facility. Notably, our analysis predicts that dsRNAs containing > or = 19-nucleotide perfect matches identified in silico to unintended targets may contribute to a significant false positive error rate arising from off-target effects. We confirmed experimentally that such sequences in dsRNAs lead to false positives and to efficient knockdown of a cross-hybridizing transcript, raising a cautionary note about interpreting results based on the use of a single dsRNA per gene. Although a full appreciation of all causes of false positive errors remains to be determined, we suggest simple guidelines to help ensure high-quality information from RNAi high-throughput screens.

Full article available at http://www.flyrnai.org/supplement/16964256.pdf

Fedorov, Y.; Anderson, E.; Birmingham, A.; Reynolds, A.; Karpilow, J.; Robinson, K.; Leake, D.; Marshall, W.; Khvorova, A. 2006. Off-target effects by siRNA can induce toxic phenotype. *RNA*, 12(7): 1188–1196.

Although recent microarray studies have provided evidence of RNA interference (RNAi)-mediated off-target gene modulation, little is known about whether these changes induce observable phenotypic outcomes. Here we show that a fraction of randomly selected small inhibitory RNAs (siRNAs) can induce changes in cell viability in a target-independent fashion. The observed toxicity requires an intact RNAi pathway and can be eliminated by the addition of chemical modifications that reduce off-target effects. Furthermore, an analysis of toxic and nontoxic duplexes identifies a strong correlation between the toxicity and the presence of a 4-base-pair motif (UGGC) in the RISC-entering strand of toxic siRNA. This article provides further evidence of siRNA-induced off-target effects generating a measurable phenotype and also provides an example of how such undesirable phenotypes can be mitigated by addition of chemical modifications to the siRNA.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1484448/

COM - Gordon, K.; Waterhouse, P. 2007. RNAi for insect-proof plants. *Nat Biotechnol*, 25:1231–2.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/17989682

Alemán, L.; Doench, J.; Sharp, P. 2007. Comparison of siRNA-induced off-target RNA and protein effects. *RNA*, 13: 385–395.

The downregulation of many mRNAs has been observed through bioinformatic analysis of microarray results following transfection of short interfering RNAs (siRNAs). Many of these mRNA changes are due to the interaction of the siRNA guide strand with partially complementary sites and thus are considered "off-target" effects. To examine the mRNA:siRNA interactions important for off-target effects, we generated a panel of mRNA:siRNA combinations containing single and double mismatches, bulges, and noncanonical base-pairing interactions in the 9th, 10th, and 11th positions of two siRNA binding sites located in the 3' UTR of an integrated reporter gene. Approximately half of the mRNA:siRNA combinations containing mismatches in positions 9-11 result in a twofold or more mRNA decrease with varying degrees of protein knockdown. However, mRNA and protein analysis of the various mRNA:siRNA combinations reveals instances in which mRNA and protein levels do not correlate. Analysis of the resulting degradation products recovered from an imperfectly complementary siRNA interaction with an endogenous gene reveals a small fraction of products that map to the canonical siRNA cleavage site. Furthermore, downregulation of ARGONAUTE 2 (AGO2), the only AGO family protein known to catalyze canonical siRNA-mediated cleavage, did not significantly affect the degree of mRNA knockdown observed for one of the stably expressed reporters after transfection of an imperfectly complementary siRNA. These results indicate that although some degree of canonical siRNA cleavage can take place between a siRNA and an off-target transcript, most off-target mRNA reductions are likely attributable to AGO2-independent degradation processes.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1800510/

Hawkins, P.; Santoso, S.; Adams, C.; Anest, V.; Morris, K. 2009. Promoter targeted small RNAs induce long-term transcriptional gene silencing in human cells. *Nucleic Acids Research*, 37.

Small RNAs targeted to gene promoters in human cells can mediate transcriptional gene silencing (TGS) by directing silent state epigenetic modifications to targeted loci. Many mechanistic details of this process remain poorly defined, and the ability to stably modulate gene expression in this manner has not been explored. Here we describe the mechanisms of establishment and maintenance of long-term transcriptional silencing of the human ubiquitin C gene (*UbC*). Sustained targeting of the *Ub*Cpromoter with a small RNA for a minimum of 3 days resulted in long-term silencing which correlated with an early increase in histone methylation and a later increase in DNA methylation at the targeted locus. Transcriptional silencing of *UbC* required the presence of a promoter-associated RNA. The establishment and maintenance of the TGS were shown to require distinct protein factors. Argonaute 1 (Ago1), DNA methyltransferase 3a (DNMT3a) and histone deacetylase 1 (HDAC1) were required for the initiation of silencing, and DNA methyltransferase 1 (DNMT1) was necessary for maintenance. Taken together the data presented here highlight the cellular pathway with which noncoding RNAs interact to epigenetically regulate gene expression in human cells.

Full article available at https://goo.gl/6GwP6x

REV – Jackson, A.; Linsley, P. 2010. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nature Reviews Drug Discovery*, 9: 57–67 (3).

Small interfering RNAs (siRNAs) are widely used to study gene function owing to the ease with which they silence target genes, and there is considerable interest in their potential for therapeutic applications. In a remarkably short time since their discovery, siRNAs have entered human clinical trials in various disease areas. However, rapid acceptance of the use of siRNAs has been accompanied by recognition of several hurdles for the technology, including a lack of specificity. Off-target activity can complicate the interpretation of phenotypic effects in gene-silencing experiments and can potentially lead to unwanted toxicities. Here, we describe the types of off-target effects of siRNAs and methods to mitigate them, to help enable effective application of this exciting technology.

http://www.ncbi.nlm.nih.gov/pubmed/20043028

REV – Huvenne, H.; Smagghe, G. 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *J Insect Physiol*, 56(3):227-35. doi: 10.1016/j.jinsphys.2009.10.004.

RNA interference already proved its usefulness in functional genomic research on insects, but it also has considerable potential for the control of pest insects. For this purpose, the insect should be able to autonomously take up the dsRNA, for example through feeding and digestion in its midgut. In this review we bring together current knowledge on the uptake mechanisms of dsRNA in insects and the potential of RNAi to affect pest insects. At least two pathways for dsRNA uptake in insects are described: the transmembrane channel-mediated uptake mechanism based on Caenorhabditis elegans' SID-1 protein and an 'alternative' endocytosis-mediated uptake mechanism. In the second part of the review dsRNA feeding experiments on insects are brought together for the first time, highlighting the achievement of implementing RNAi in insect control with the first successful experiments in transgenic plants and the diversity of successfully tested insect orders/species and target genes. We conclude with points of discussion and concerns regarding further research on dsRNA uptake mechanisms and the promising application possibilities for RNAi in insect control.

http://www.ncbi.nlm.nih.gov/pubmed/19837076

REV – Davidson, B.; McCray, P. 2011. Current prospects for RNA interference–based therapies. *Nature Reviews Genetics*, 12: 329–340.

RNA interference (RNAi) is a powerful approach for reducing expression of endogenously expressed proteins. It is widely used for biological applications and is being harnessed to silence mRNAs encoding pathogenic proteins for therapy. Various methods - including delivering RNA oligonucleotides and expressing RNAi triggers from viral vectors - have been developed for successful RNAi in cell culture and in vivo. Recently, RNAi-based gene silencing approaches have been demonstrated in humans, and ongoing clinical trials hold promise for treating fatal disorders or providing alternatives to traditional small molecule therapies. Here we describe the broad range of approaches to achieve targeted gene silencing for therapy, discuss important considerations when developing RNAi triggers for use in humans, and review the current status of clinical trials.

http://www.ncbi.nlm.nih.gov/pubmed/21499294

COM – Hirschi, K. 2012. New foods for thought. *Trends Plant Sci*, 17:123–5.

Recent findings show that genetic material in plant foods may survive digestion, circulate through our bodies and modulate our gene expression. These findings could alter our understanding of nutrition, genetic regulation and open up new vistas for engineering foods.

http://www.ncbi.nlm.nih.gov/pubmed/22265093

Zhang, L.; Hou, D.; Chen, X.; Li, D.; Zhu, l.; Zhang, y.; Li, j.; Bian, Z.; Liang, X.; Cai, X.; Yin, Y.; Wang, C.; Zhang, T.; Zhu, D.; Zhang, D.; Xu, J.; Chen, Q.; Ba, Y.; Liu, J.; Wang, Q.; Chen, J.; Wang, J.; Wang, M.; Zhang, Q.; Zhang, J.; Zen, K.; Zhang, C-Y. 2012. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: Evidence of cross-kingdom regulation by microRNA. *Cell Research*, 22(1):107-26 (Erratum in: Cell Res, 22(1): 273-4).

Our previous studies have demonstrated that stable microRNAs (miRNAs) in mammalian serum and plasma are actively secreted from tissues and cells and can serve as a novel class of biomarkers for diseases, and act as signaling molecules in intercellular communication. Here, we report the surprising finding that exogenous plant miRNAs are present in the sera and tissues of various animals and that these exogenous plant miRNAs are primarily acquired orally, through food intake. MIR168a is abundant in rice and is one of the most highly enriched exogenous plant miRNAs in the sera of Chinese subjects. Functional studies in vitro and in vivo demonstrated that MIR168a could bind to the human/mouse low-density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA, inhibit LDLRAP1 expression in liver, and consequently decrease LDL removal from mouse plasma. These findings demonstrate that exogenous plant miRNAs in food can regulate the expression of target genes in mammals.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3351925/

REV - Heinemann, J.; Agapito-Tenfen, S.; Carman, J. 2013. A comparative evaluation of the regulation of GM crops or products containing dsRNA and suggested improvements to risk assessments. *Environment International*, Vol. 55: 43-55.

Changing the nature, kind and quantity of particular regulatory-RNA molecules through genetic engineering can create biosafety risks. While some genetically modified organisms (GMOs) are intended to produce new regulatory-RNA molecules, these may also arise in other GMOs not intended to express them. To characterise, assess and then mitigate the potential adverse effects arising from changes to RNA requires changing current approaches to food or environmental risk assessments of GMOs. We document risk assessment advice offered to government regulators in Australia, New Zealand and Brazil during official risk evaluations of GM plants for use as human food or for release into the environment (whether for field trials or commercial release), how the regulator considered those risks, and what that experience teaches us about the GMO risk assessment framework. We also suggest improvements to the process.

Full article available at http://www.sciencedirect.com/science/article/pii/S0160412013000494

REV - Lundgren, J.; Duan, J. 2013. RNAi-Based insecticidal crops: Potential effects on nontarget species. *BioScience*, 63(8):657-665.

The potential hazards posed by RNA interference (RNAi)—based pesticides and genetically modified crops to nontarget organisms include off-target gene silencing, silencing the target gene in unintended organisms, immune stimulation, and saturation of the RNAi machinery. Nontarget organisms will vary in their exposure to small RNAs produced by genetically modified crops, but exposure to insecticidal small RNAs will probably occur at a previously unrealized scale for many. Areas that warrant future work include the persistence of insecticidal small RNAs in the environment, describing crop-based food webs to understand those species that are most exposed, sequencing genomes for species to proactively understand those that may be affected by RNAi, and substantiating that laboratory toxicity testing can accurately predict the field-level effects of this technology. The costs and benefits of pesticidal RNA must be considered relative to current pest management options as pesticidal RNAs move from a theoretical approach to being used as a practical tool.

http://www.bioone.org/doi/abs/10.1525/bio.2013.63.8.8

Nunes, F.; Aleixo, A.; Barchuk, A.; Bomtorin, A.; Grozinger, C.; Simões, Z. 2013. Non-Target Effects of green fluorescent protein (GFP)-derived double-stranded RNA (dsRNA-GFP) used in honey bee RNA interference (RNAi) assays. *Insects*, *4*, 90-103; doi:10.3390/insects4010090.

RNA interference has been frequently applied to modulate gene function in organisms where the production and maintenance of mutants is challenging, as in our model of study, the honey bee, Apis mellifera. A green fluorescent protein (GFP)-derived double-stranded RNA (dsRNA-GFP) is currently commonly used as control in honey bee RNAi experiments, since its gene does not exist in the A. mellifera genome. Although dsRNA-GFP is not expected to trigger RNAi responses in treated bees, undesirable effects on gene expression, pigmentation or developmental timing are often observed. Here, we performed three independent experiments using microarrays to examine the effect of dsRNA-GFP treatment (introduced by feeding) on global gene expression patterns in developing worker bees. Our data revealed that the expression of nearly 1,400 genes was altered in response to dsRNA-GFP, representing around 10% of known honey bee genes. Expression changes appear to be the result of both direct off-target effects and indirect downstream secondary effects; indeed, there were several instances of sequence similarity between putative siRNAs generated from the dsRNA-GFP construct and genes whose expression levels were altered. In general, the affected genes are involved in important developmental and metabolic processes associated with RNA processing and transport, hormone metabolism, immunity, response to external stimulus and to stress. These results suggest that multiple dsRNA controls should be employed in RNAi studies in honey bees. Furthermore, any RNAi studies involving these genes affected by dsRNA-GFP in our studies should use a different dsRNA control.

Full article available at http://www.mdpi.com/2075-4450/4/1/90

Some articles examine immunological risks associated with the use of technologies that lead to formation of non-coding RNA molecules (small interfering RNA in particular - siRNA). In fact, some siRNA - belonging to RNA interference machinery - seem to have a key role in regulating the immune system in mammals.

Judge, A.; Sood, V.; Shaw, J.; Fang, D.; McClintock, K.; MacLachlan, I. 2005. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nature Biotechnology*, 23: 457–462.

Short interfering RNAs (siRNAs) that mediate specific gene silencing through RNA interference (RNAi) are widely used to study gene function and are also being developed for therapeutic applications. Many nucleic acids, including double- (dsRNA) and single-stranded RNA (ssRNA), can stimulate innate cytokine responses in mammals. Despite this, few studies have questioned whether siRNA may have a similar effect on the immune system. This could significantly influence the *in vivo* application of siRNA owing to off-target effects and toxicities associated with immune stimulation. Here we report that synthetic siRNAs formulated in nonviral delivery vehicles can be potent inducers of interferons and inflammatory cytokines both *in vivo* in mice and *in vitro* in human blood. The immunostimulatory activity of formulated siRNAs and the associated toxicities are dependent on the nucleotide sequence. We have identified putative immunostimulatory motifs that have allowed the design of siRNAs that can mediate RNAi but induce minimal immune activation.

Full article available at http://www.nature.com/nbt/journal/v23/n4/full/nbt1081.html

REV - Marques, J.; Williams, B. 2005. Activation of the mammalian immune system by siRNAs. *Nature Biotechnology*, 23, 1399-1405 doi:10.1038/nbt1161.

Inhibition of gene expression through RNA interference (RNAi) is emerging as a powerful experimental tool for gene function and target validation studies. The potential uses of this technology seem unlimited, extending to the prevention and therapy of human diseases. However, recent work demonstrating that there are unanticipated, different nonspecific effects associated with the use of small interfering RNAs in mammals has raised concerns about the safe use of RNAi in vivo. These nonspecific effects include activation of the immune system, potentially harming the individual. The application of screening assays for nonspecific activation of both innate and acquired immunity will be necessary for further development of RNAi as a therapeutic tool.

http://www.ncbi.nlm.nih.gov/pubmed/16273073

Robbins, M.; Judge, A.; MacLachlan, I. 2009. siRNA and innate immunity. *Oligonucleotides*, 19: 89–102.

Canonical small interfering RNA (siRNA) duplexes are potent activators of the mammalian innate immune system. The induction of innate immunity by siRNA is dependent on siRNA structure and sequence, method of delivery, and cell type. Synthetic siRNA in delivery vehicles that facilitate cellular uptake can induce high levels of inflammatory cytokines and interferons after systemic administration in mammals and in primary human blood cell cultures. This activation is predominantly mediated by immune cells, normally via a Toll-like receptor (TLR) pathway. The siRNA sequence dependency of these pathways varies with the type and location of the TLR involved. Alternatively nonimmune cell activation may also occur, typically resulting from siRNA interaction with cytoplasmic RNA sensors such as RIG1. As immune activation by siRNA-based drugs represents an undesirable side effect due to the considerable toxicities associated with excessive cytokine release in humans, understanding and abrogating this activity will be a critical component in the development of safe and effective therapeutics. This review describes the intracellular mechanisms of innate immune activation by siRNA, the design of appropriate sequences and chemical modification approaches, and suitable experimental methods for studying their effects, with a view toward reducing siRNA-mediated off-target effects.

http://www.ncbi.nlm.nih.gov/pubmed/19441890

Part 5
Scientific controversies and criticisms of the risk analysis process of transgenic plants

In parallel to the existing criticism on risk analysis procedures on transgenic plants, contradictions accumulate in scientific publications that note, deny or suggest negative impacts of these genetically modified organisms - on the environment and health - regardless of the field of biology considered.

Despite the intensity of the debates, draws attention the fact that authors of studies that indicate problems may have been targeted by defamatory campaigns organized generally by actors (researchers or not) committed to the success of biotechnology and its products.

These attacks, which advance on personal aspects, often come from secondary methodological details, paying little attention to situations where the critical points had already consolidated, providing methodological options accepted since incorporated into studies favorable to the GMPs. In these situations, it is highlighted the adoption of arguments comprised by the distorted logic of "two weights and two measures" by biotech advocates.

1 Criticism of "science-based" risk assessment

Since the introduction of transgenic plants in human food, (1990), the distrust of mechanisms and protocols used by regulatory agencies committed to risk assessment on GMOs grows and consolidates. Among the reasons there is the coincidence of interpretation. In all cases, regulatory agencies use studies produced or financed by biotechnology companies to validate biosafety assumptions claimed by them. More than that, despise studies that point opposite conclusions.

For some years now, several segments of the organized civil society and independent researchers warn the global society about the lack of transparency and scientific rigor present in decision-making

mechanisms that, based on short-term analysis, maintain the nonexistence of long-term risks. Also draw attention to the fragility of methods, insufficient sampling, inconsistency of results and concealment of information necessary for verification of decisions.

In addition, a significant portion of the scientific community has expressed disagreement to insufficient and inadequate protocols and approaches used by regulatory agencies. At this point, stand out almost unusual elements, such as the concept of "substantial equivalence" and its overvaluation, to the detriment of international commitments related to the "precautionary principle".

The following referenced articles show that risk assessments should maintain strict connection with dominant cultural and socioeconomic aspects in the communities that will be affected. Thus, decisions to stimulate or restrain the commercial use of agricultural biotechnologies always possess political overtones, with filter power and own judgments of valuation to be weighted based on the knowledge and the scientific uncertainties, as well as their implications.

COM - Millstone, E.; Brunner, E.; Mayer, S. 1999. Beyond 'substantial equivalence'. *Nature*, Vol 401.

Showing that a genetically modified food is chemically similar to its natural counterpart is not adequate evidence that it is safe for human consumption. Whenever official approval for the introduction of genetically modified (GM) foods has been given in Europe or the United States, regulatory committees have invoked the concept of 'substantial equivalence'. This means that if a GM food can be characterized as substantially equivalent to its 'natural' antecedent, it can be assumed to pose no new health risks and hence to be acceptable for commercial use.

http://www.nature.com/nature/journal/v401/n6753/full/401525a0.html

Nodari, R.; Guerra, M. 2000. Implications of transgenics for environmental and agricultural sustainability. *História, Ciências, Saúde;* Manguinhos, 7(2): 481-491.

The potential risks of GMOs, their impact on human and animal health, and on the environment, as well as their socioeconomic effects, have generated a worldwide discussion which is far from drawing to a close for lack of sufficient scientific information. Part of this information supports risk-

hypotheses previously put forward. Thus the presence of transgenic plant genes in other plants and in other organisms has been confirmed in several occasions. Therefore, gene dissemination to plants of the same species as well as to widely different species is already regarded as an actual risk. The principle of substantial equivalence has opened the way for the liberation of transgenic plants for commercial crops, despite short-term tests, which are quantitatively and qualitatively insufficient to certify that the foods deriving from those plants are healthy and safe. Thus, the adoption of the so-called precautionary principle (PP) has turned out to be the most adequate safety measure to date, or else until scientific data should be able to demonstrate the actual impact of transgenic plants on human and animal health, and on the environment.

Full article available at https://goo.gl/eoo6q6

Zdunczyk, Z. 2001. In vivo experiments on the safety evaluation of GM components of feeds and foods. *Journal of Animal and Feed Sciences*, 10 (Supplement 1): 195-210.

During the last five years, the global area of transgenic crop (GM-genetically modified) cultivation increased 25-fold. About 98% of GM crops are grown in the USA, Argentina and Canada from where they are sent to many importers of soyabean and maize. The results of feeding experiments indicate that soyabean meal obtained from herbicide-tolerant lints and insect-resistant maize are substantially and nutritionally equivalent to their conventional lines. A higher content of insecticidal alpha -amylase inhibitors las well as lectins and alkaloids) may increase plants' resistance to insect attack, as well as the decrease nutritional value of seeds. Evaluation of the concordance of the chemical composition of transgenic and conventional crops (i.e., verification of substantial equivalence) is not sufficient for proving the safety of transgenic food. Sub-chronic in vivo experiments as well as comparison of nutritional equivalence of transgenic and conventional crops are advisable. Such actions are justified not only by the possibility of undesirable transgenic effects, but also by the consumer's right to explicit information on food safety. Without evaluation of nutritional equivalence, information on GM-food safety is much more deficient than existing knowledge on the quality of feeds used in animal nutrition.

http://serials.unibo.it/cgi-ser/start/it/spogli/df-s.tcl?prog_art=8768196&view=articoli

REV – Myhr, A.; Traavik, T. 2002. The precautionary principle: scientific uncertainty and omitted research in the context of GMO use and release. *JAGE (Journal of Agricultural and Environmental Ethics)*, 15: 73-86.

Commercialization of genetically modified organisms (GMOs) have sparked profound controversies concerning adequate approaches to risk regulation. Scientific uncertainty and ambiguity, omitted research areas, and lack of basic knowledge crucial to risk assessments have become apparent. The objective of this article is to discuss the policy and practical implementation of the Precautionary Principle. A major conclusion is that the void in scientific understanding concerning risks posed by secondary effects and the complexity of cause-effect relations warrant further research. Initiatives to approach the acceptance or rejection of a number of risk-associated hypotheses is badly needed. Further, since scientific advice plays a key role in GMO regulations, scientists have a responsibility to address and communicate uncertainty to policy makers and the public. Hence, the acceptance of uncertainty is not only a scientific issue, but is related to public policy and involves an ethical dimension.

Full article available at https://www.cbd.int/doc/articles/2008/A-00637.pdf

Levidow, L.; Murphy, J.; Carr, S. 2007. Recasting "substantial equivalence": Transatlantic governance of GM food. *Science, Technology, and Human Values*; 32(1): 26–64.

When intense public controversy erupted around agricultural biotechnology in the late 1990s, critics found opportunities to challenge risk assessment criteria and test methods for genetically modified (GM) products. In relation to GM food, they criticized the concept of substantial equivalence, which European Union and United States regulators had adopted as the basis for a harmonized, science-based approach to risk assessment. Competing policy agendas framed scientific uncertainty in different ways. Substantial equivalence was contested and eventually recast to accommodate some criticisms. To explain how the concept changed, this article links two analytical perspectives. Regulatory-science perspectives illuminate how the scientification of politics and politicization of science led to shifts in the boundary between science and policy. Governance perspectives illuminate how the collective problem for policy was redefined to provide a new common ground for some stakeholders. Overall, substantial equivalence was recast to govern the social conflict and address legitimacy problems of regulatory procedures.

Full article available at http://oro.open.ac.uk/6751/1/LLJMSC_SubstEquiv_STHV_07corr.pdf

Zolla, L.; Rinalducci, S.; Antonioli, P.; Righetti, P. 2008. Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of genetic modifications. *Journal of Proteome Research*, 7(5): 1850-1861.

To improve the probability of detecting unintended side effects during maize gene manipulations by bombardment, proteomics was used as an analytical tool complementary to the existing safety assessment techniques. Since seed proteome is highly dynamic, depending on the species variability and environmental influence, we analyzed the proteomic profiles of one transgenic maize variety (event MON 810) in two subsequent generations (T05 and T06) with their respective isogenic controls (WT05 and WT06). Thus, by comparing the proteomic profiles of WT05 with WT06 we could determine the environmental effects, while the comparison between WT06 and T06 seeds from plants grown under controlled conditions enabled us to investigate the effects of DNA manipulation. Finally, by comparison of T05 with T06 seed proteomes, it was possible to get some indications about similarities and differences between the adaptations of transgenic and isogenic plants to the same strictly controlled growth environment. Approximately 100 total proteins resulted differentially modulated in the expression level as a consequence of the environmental influence (WT06 vs WT05), whereas 43 proteins resulted up- or down-regulated in transgenic seeds with respect to their controls (T06 vs WT06), which could be specifically related to the insertion of a single gene into a maize genome by particle bombardment. Transgenic seeds responded differentially to the same environment as compared to their respective isogenic controls, as a result of the genome rearrangement derived from gene insertion. To conclude, an exhaustive differential proteomic analysis allows to determine similarities and differences between traditional food and new products (substantial equivalence), and a case-by-case assessment of the new food should be carried out in order to have a wide knowledge of its features.

Full article available at https://goo.gl/fI9xxC

Myhr, A. 2010. The challenge of scientific uncertainty and disunity in risk assessment and management of GM crops. *Environmental Values*, 19: 7-31. Doi: 10.3197/096327110X485365.

The controversy over commercial releases of genetically modified (GM) crops demonstrates that there is a need for new approaches that are more broadly based, transparent and able to acknowledge the uncertainties involved. This article investigates whether new forms of knowledge production as prescribed in the concept of post-normal science can improve risk governance of GM crops.

The GM science review carried out in the UK in 2003 serves as a case study and the focus is on how scientific uncertainty and public concern was taken into account. Some recommendations are advanced for assessing scientific uncertainty, for accommodating scientific disputes and for integrating stakeholders' interests and perspectives in relations to GM crops.

https://goo.gl/l02K67

Myhr, A. 2010. A precautionary approach to genetically modified organisms: Challenges and implications for policy and science. *Journal of Agricultural and Environmental Ethics*. DOI:10.1007/s10806-010-9234-x.

The commercial introduction of genetically modified organisms (GMOs) has revealed a broad range of views among scientists and other stakeholders on perspectives of genetic engineering (GE) and if and how GMOs should be regulated. Within this controversy, the precautionary principle has become a contentious issue with high support from skeptical groups but resisted by GMO advocates. How to handle lack of scientific understanding and scientific disagreement are core issues within these debates. This article examines some of the key issues affecting precaution as a legal standard and as an approach to the use of science in decision-making processes. It is pointed out that there is a need for reflection over the level of scientific evidence required for applying the precautionary principle as well as who should have the burden of proof when there are uncertainties. Further, an awareness of the broader scientific uncertainties found in GMO risk assessment implies that a precautionary approach must be elaborated: both for acknowledging uncertainties and for identification of scientific responses. Since precaution is an important issue within the sustainable development framework, it is suggested that sustainability can provide a normative standard that can help to reveal the influence and negotiate the importance of the various forms of uncertainty. Wise management of uncertainties and inclusion of normative aspects in risk assessment and management may help to ensure sustainable and socially robust GMO innovations at present and in the future.

Full article available at http://bch.cbd.int/database/record.shtml?documentid=101951

REV - Heinemann, J.; Kurenbach, B.; Quist, D. 2011. Molecular profiling — a tool for addressing emerging gaps in the comparative risk assessment of GMOs. *Environment International*, 37, 1285–1293.

Assessing the risks of genetically modified organisms (GMOs) is required by both international agreement and domestic legislation. Many view the use of the "omics" tools for profiling classes of molecules as useful in risk assessment, but no consensus has formed on the need or value of these techniques for assessing the risks of all GMOs. In this and many other cases, experts support case-by-case use of molecular profiling techniques for risk assessment. We review the latest research on the applicability and usefulness of molecular profiling techniques for GMO risk assessment. As more and more kinds of GMOs and traits are developed, broader use of molecular profiling in a risk assessment may be required to supplement the comparative approach to risk assessment. The literature-based discussions on the use of profiling appear to have settled on two findings: 1. profiling techniques are reliable and relevant, at least no less so than other techniques used in risk assessment; and 2. although not required routinely, regulators should be aware of when they are needed. The dismissal of routine molecular profiling may be confusing to regulators who then lack guidance on when molecular profiling might be worthwhile. Molecular profiling is an important way to increase confidence in risk assessments if the profiles are properly designed to address relevant risks and are applied at the correct stage of the assessment.

http://www.ncbi.nlm.nih.gov/pubmed/21624662

Wickson, F.; Wynne, B. 2012. Ethics of science for policy in the environmental governance of biotechnology: MON810 maize in Europe. *Ethics, Policy and Environment*; 15(3): 321-340.

This paper discusses entanglements of science and ethics in the regulation of genetically modified crops. Using the 2009 German ban of genetically modified maize MON810 and debates concerning the quality of science cited to support it, the paper highlights how values are tacitly embedded in science for policy and how ethical questions permeate the way this science is developed, quality-controlled, and given authority in the European regulation of biotechnology. We argue that a lack of recognition and inadequate treatment of such value-commitments influencing science, and through this policy, impinges upon and weakens the ethical standards involved. This has particular significance as Europe debates genetically modified crop legislative reform.

http://philpapers.org/rec/WICEOS

COM - Wickson, F. 2014. Environmental protection goals, policy & publics in the European regulation of GMOs. *Ecological Economics*, 108, 269–273.

One of the most divisive debates in modern agriculture concerns the use of genetically modified organisms (GMOs). In Europe, the policy debate over GMOs has been met with a persistent attempt to retreat into "sound science" as a potential unifying force. However, environmental risk assessment as an aid to regulatory decision-making is inherently entangled with questions of environmental ethics. This is particularly manifested in the setting of environmental protection goals. For the risk assessment of GMOs, the European Food Safety Authority has presented an inconsistent position on environmental protection goals. There is, however, an emerging trend for biodiversity conservation to be enfolded within an ecosystem services frame, and for ecosystem services to be reduced to biological terms. How environmental protection goals are understood, articulated and used to define risk assessment and shape regulatory decision-making is a significant factor in the entrenched debate over the regulation of GMOs in Europe. In negotiating this territory, I suggest that the attempt to enforce a strict divide between nature and culture or social and ecological systems in Europe's risk assessment of GMOs is emphatically counter-productive, for both robust science and considered ethical action.

http://www.sciencedirect.com/science/article/pii/S092180091400295X

After fifteen years of commercialization of Bt plants, basic questions remain, such as the amount of Bt toxin produced in each transgenic event and the reasons for their differentiation / oscillation between parts of the plants and the crop growing period. Certainly this uncertainty creates potential hazards to human and animal health, as well as to the environment.

Saeglitz, C.; Bartsch, D.; Eber, A.; Gathmann, K.; Priesnitz, K.; Schuphan, I. 2006. Monitoring the Cry1Ab susceptibility of European corn borer in Germany. *Journal of Economic Entomology*, 99(5): 1768-1773.

The European corn borer, Ostrinia nubilalis (Hübner), is one of the most important insect pests in corn, Zea mays L. Transgenic corn cultivars expressing Bacillus thuringiensis (Bt) toxin provide a

promising crop protection strategy against European corn borer; however, management is needed to avoid resistance development of the target pest species. The aim of this work was to establish the baseline susceptibility of different European corn borer populations in Germany to be able to forecast a possible development of resistance at an early stage. To standardize test procedures for future resistance management, the efficiency of Cry1Ab toxins from different suppliers and different production was assessed. Furthermore, two different test methods, surface treatment and the incorporation method, were compared with regard to their practicability and efficiency. Neither method provided significant differences in the baseline susceptibility of populations from different German regions. Overall, the data suggested little differentiation among German populations in terms of their susceptibility to Bt toxin and their genetic background. Future monitoring could therefore use a single European corn borer population as a representative for southwestern Germany. However, toxins from different suppliers and different production batches produced a vast range of LC50 values. Changes because of different toxin batches may be mistaken as a change in baseline susceptibility or even as the start of a resistance development. Thus, it is important throughout insect resistance management that the same toxin batches will be available for baseline susceptibility bioassays and for future tests.

Full article available at http://www.biw.kuleuven.be/aee/clo/euwab_files/saeglitz2006.pdf

Székács, A.; Weiss G.; Quist, D.; Takács, E.; Darvas, B.; Meier, M.; Swain, T.; Hilbeck, A. 2011. Interlaboratory comparison of Cry1Ab toxin quantification in MON 810 maize by ezyme-immunoassay. *Food and Agricultural Immunology*, DOI:10.1080/09540105.2011.604773.

A laboratory ring trial was performed in four laboratories for determination of Cry1Ab toxin in leaf material of MON 810 maize using a standardised enzyme-linked immunoassay protocol. Statistical analysis was carried out by the ISO 5725-2 guidelines, sample standard deviation and standard error, within-laboratory and inter-laboratory SD and SE were calculated. Measured inter-laboratory average values were 12.5±4.0, 15.3±4.6 and 72.6±17.8 μg/g for three lyophilised samples, and 27.8±4.3 μg/g for a frozen sample, yet, Cry1Ab concentrations ranged 66.5–160.1% of the corresponding IA. Determined concentrations by in-house protocols were statistically not different in one laboratory and different in two laboratories from the corresponding values by the joint protocol. Results emphasise the importance of a standardised protocol among laboratories for comparable quantitative Cry1Ab toxin determination. However, even when using a standardised protocol, significant differences still occur among toxin concentrations detected in different laboratories, although with a smaller range of variation.

Full article available at http://libra.msra.cn/Publication/57599316/interlaboratory-comparison-of-cry1ab-toxin-quantification-in-mon-810-maize-by-enzyme-immunoassay

2 Lack of scientific rigor in assessing the health risk

The lack of scientific consensus on the absence of health risks and problems associated with daily consumption of GM foods has been discussed in Part 4 of this book.

As a complement, we present the following set of critical studies of the risk analysis processes as conducted by regulatory agencies.

This compilation incorporates weaknesses related to biological, socioeconomic, legal and ethical issues. It also includes monitoring the consumption of these GMPs, as well as the implications of their fragility (monitoring) for public health in the medium and long term.

COM - Schubert, D. 2002. A different perspective on GM food. *Nature Biotechnology*, 20, 969.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/12355105

Freese, W.; Schubert, D. 2004. Safety testing and regulation of genetically engineered foods. *Biotechnology and Genetic Engineering Reviews*, Vol 21. 299-324.

The use of recombinant DNA techniques to engineer food crops with novel traits has aroused tremendous interest and concern throughout the world. Both the public and the scientific community are deeply divided on a host of issues raised by genetically engineered (GE) crops. Do they pose human health or environmental risks? Are they adequately regulated? Should foods containing them be labeled? Should society allow them to be patented? Are they relevant to the developing world? Science alone cannot and will not decide the many disputes that have arisen between and within nations over GE foods. As with the introduction of any powerful new technology, economic, cultural and ethical factors will also come into play. But science can help ground the debate, particularly in the contentious area of regulation.

Full article available at https://goo.gl/8FTHMz

Heinemann, J.; Sparrow, A.; Traavik, T. 2004. Is confidence in the monitoring of GE foods justified? *Trends in Biotechnology*, Vol.22 No.7.

Often the limits of detection of genetically engineered organisms (GEOs, LMOs, GMOs) determine what legislation sets as thresholds of allowable contamination of the human food chain with GEOs. Many countries have legislation that is triggered by certain thresholds of contamination. Importantly, international trade in food and animal feed is becoming increasingly vulnerable to interruptions caused by the ambiguity GEOs can create when shipments are monitored at the border. We examine the tools available for detection. Four key error-generating stages are identified with the aim of prompting a higher uniform standard of routine analysis at export and import points. Contamination of the New Zealand corn crop with GEOs is used as a case study for the application of monitoring tools and vulnerability to errors. These tools fail to meet emerging food safety requirements, but some improvements are in development.

http://www.ncbi.nlm.nih.gov/pubmed/15245904

Seetharam, S. 2010. Should the Bt brinjal controversy concern healthcare professionals and

bio-ethicists? *Indian J Med Ethics*, 7 (1).

The Genetic Engineering Approval Committee's approval of Bt brinjal, the first genetically modified crop for human consumption in India, has sparked off protests across the country. This article questions the so-called benefits of GM crops and highlights some major concerns. These include: inadequately addressed health and environmental risks, inadequate safety guidelines, a lack of transparency in sharing test data, the implications to seed sovereignty of farmers and the lack of informed choice for consumers. Some concerns about field testing by Mahyco, the developer of Bt-brinjal, and the process of evaluation by GEAC remain unresolved. With inadequate information about the crop's long-term safety, a precautionary approach is advocated before national policy allows commercial release of the seeds. A fair process is also needed in the public consultations being proposed by the minister of state for environment and forests. In addition to issues of procedural justice, a basic ethical question remains: do humans have a right to dominate the land and make expendable those creatures that they deem "undesirable"?

http://www.ncbi.nlm.nih.gov/pubmed/20166287

2.1 Criticism to the lack of scientific rigor on the evaluation of the allergenic risk

The following studies discuss the fragility of evaluation protocols of the allergenic risk, which is generally limited to homology comparison in the sequence of amino acids of the recombinant protein with known allergenic molecules, taking into account incomplete databases (due to permanent evolution). They also examine the supposed degradation of the transgenic protein in the simulated gastric environment. Analysis *in silico* of new proteins produced by GMOs and long-term testing in animal models have not been adopted supposedly because they are considered overly burdensome for businesses.

Moreover, the potential share of consumption of transgenic plants (and associated pesticides) in the outburst of cases of new allergies and even in food intolerance processes, achieved by synergistic actions in immunological cross reactions, is not considered by decision makers. Meanwhile, cases of new allergies and intolerances assume pandemic proportions, alongside the expansion of farming and of genetically modified foods.

REV - Hodgson, E. 2001. Genetically modified plants and human health risks: Can additional research reduce uncertainties and increase public confidence? *Toxicological Sciences*, 63, 153–156.

So long as the risks to human health from transgenic plants remain potential rather than actual, and, in any event, appear lower than those from traditional plant breeding, hazard assessment need not be extensive. However, in view of current public attitudes to transgenic plants, it is necessary that those tests that are required, be based on logic, on sound science, and in accordance with the best scientific methodology. This is particularly the case with testing for food allergenicity. Current testing is largely indirect and based on comparisons with other known food allergens. Development of direct tests that involve interaction between the actual transgenic protein in question and the immune system is essential if confidence in the regulatory system is to be restored.

Full article available at http://toxsci.oxfordjournals.org/content/63/2/153.long

Kleter, G.; Peijnenburg, Ad A. 2002. Screening of transgenic proteins expressed in transgenic food crops for the presence of short amino acid sequences identical to potential, IgE – binding linear epitopes of allergens. *BMC Structural Biology*, 2:8.

Background: Transgenic proteins expressed by genetically modified food crops are evaluated for their potential allergenic properties prior to marketing, among others by identification of short identical amino acid sequences that occur both in the transgenic protein and allergenic proteins. A strategy is proposed, in which the positive outcomes of the sequence comparison with a minimal length of six amino acids are further screened for the presence of potential linear IgE-epitopes. This double track approach involves the use of literature data on IgE-epitopes and an antigenicity prediction algorithm.
Results: Thirty-three transgenic proteins have been screened for identities of at least six contiguous amino acids shared with allergenic proteins. Twenty-two transgenic proteins showed positive results of six- or seven-contiguous amino acids length. Only a limited number of identical stretches shared by transgenic proteins (papaya ringspot virus coat protein, acetolactate synthase GH50, and glyphosate oxidoreductase) and allergenic proteins could be identified as (part of) potential linear epitopes.
Conclusion: Many transgenic proteins have identical stretches of six or seven amino acids in common with allergenic proteins. Most identical stretches are likely to be false positives. As shown in this study, identical stretches can be further screened for relevance by comparison with linear IgE-binding epitopes described in literature. In the absence of literature data on epitopes, antigenicity prediction by computer aids to select potential antibody binding sites that will need verification of IgE binding by sera binding tests. Finally, the positive outcomes of this approach warrant further clinical testing for potential allergenicity.

Full article available at http://www.biomedcentral.com/1472-6807/2/8

Bucchini, L.; Goldman, L. 2002. Starlink corn: A risk analysis. *Environmental Health Perspectives*, Volume 110. Nº 1.

Modern biotechnology has dramatically increased our ability to alter the agronomic traits of plants. Among the novel traits that biotechnology has made available, an important group includes Bacillus thuringiensis-derived insect resistance. This technology has been applied to potatoes, cotton, and corn. Benefits of Bt crops, and biotechnology generally, can be realized only if risks are assessed and managed properly. The case of Starlink corn, a plant modified with a gene that encodes the Bt protein Cry9c, was a severe test of U.S. regulatory agencies. The U.S. Environmental Protection Agency had restricted its use to animal feed due to concern about the potential for allergenicity. However, Starlink corn was later found throughout the human food supply, resulting in food recalls by the Food and Drug Administration and significant disruption of the food supply. Here we examine the regulatory history of Starlink, the assessment framework employed by the U.S. government, assumptions and information gaps, and the key elements of government efforts to

manage the product. We explore the impacts on regulations, science, and society and conclude that only significant advances in our understanding of food allergies and improvements in monitoring and enforcement will avoid similar events in the future. Specifically, we need to develop a stronger fundamental basis for predicting allergic sensitization and reactions if novel proteins are to be introduced in this fashion. Mechanisms are needed to assure that worker and community aeroallergen risks are considered. Requirements are needed for the development of valid assays so that enforcement and post market surveillance activities can be conducted.

Full article available at https://goo.gl/kHVmnM

Sutton, S.; Assa'ad, A.; Steinmetz, C.; Rothenberg, M. 2003. A negative, double-blind, placebo-controlled challenge to genetically modified corn. *J Allergy Clin Immunol*, Volume 112, Number 5.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/14610498

Bernstein, J.; Bernstein, I.; Bucchini, L.; Goldman, L.; Hamilton, R.; Lehrer, S.; Rubin, C.; Sampson, H. 2003. Clinical and laboratory investigation of allergy to genetically modified foods. *Environ Health Perspect*, 111(8): 1114-1121.

Technology has improved the food supply since the first cultivation of crops. Genetic engineering facilitates the transfer of genes among organisms. Generally, only minute amounts of a specific protein need to be expressed to obtain the desired trait. Food allergy affects only individuals with an abnormal immunologic response to food--6% of children and 1.5-2% of adults in the United States. Not all diseases caused by food allergy are mediated by IgE. A number of expert committees have advised the U.S. government and international organizations on risk assessment for allergenicity of food proteins. These committees have created decision trees largely based on assessment of IgE-mediated food allergenicity. Difficulties include the limited availability of allergen-specific IgE antisera from allergic persons as validated source material, the utility of specific IgE assays, limited characterization of food proteins, cross-reactivity between food and other allergens, and modifications of food proteins by processing. StarLink was a corn variety modified to produce a (Italic)Bacillus thuringiensis(/Italic) (Bt) endotoxin, Cry9C. The Centers for Disease Control and Prevention investigated 51 reports of possible adverse reactions to corn that occurred after the announcement that StarLink, allowed for animal feed, was found in the human food supply. Allergic reactions were not confirmed, but tools for postmarket assessment were limited. Workers in agricultural and food preparation facilities have potential inhalation exposure to plant dusts and flours. In 1999, researchers found that migrant health workers can become sensitized to certain Bt spore extracts after exposure to Bt spraying.

Full article available at https://goo.gl/oZGJfF

2.2 Criticism to the lack of scientific rigor on the assessment of the toxicological risk

The scientific literature builds critical documents to the methodologies adopted in the toxicological assessment of transgenic plants. The weaknesses observed allow short-term trials assessing,

for example, a protein extracted from recombinant bacteria (in place of the whole food, obtained from PGM), to sustain the absence of problems which are not observed because the protocols prevent them. Criticism advance demonstrating that the real risk elements, to which consumers are submitted, are not being properly evaluated.

Other articles that highlight key aspects of toxicology, such as representativeness (qualitative and quantitative) of test groups or differentiation of the sexes, are not always addressed properly (and sometimes are totally despised). To search for a dose-response treatment - which ends up hiding endocrine deregulation or neglecting statistically significant differences in key biological variables -, regulatory agencies and biotech companies encourage obtaining conclusions favorable to technology. Distortions arising from these and other methodological flaws end up being relied upon to prove hypothesis of no risk to the health of consumers of GMPs and their parts.

REV - Pusztai, A. 2002. Can science give us the tools for recognizing possible health risks of GM food? *Nutr Health*, 16, 73-84.

Nearly ten years after the introduction of GM foodcrops there are still only a handful of published studies about their safety. Independent studies are even fewer, moreover, no peer-reviewed publications exist in which the results of clinical investigations on the possible effects of GM food on human health are described. Even though the evaluation of the safety or possible toxicity of GM foodstuffs is more difficult than that of drugs or food additives, this scarcity of data and the lack of a scientific database is curious particularly as descriptions of the results of chemical, nutritional and biological testing in some early (unpublished) studies or some more recent publications demonstrate the feasibility of carrying out proper and scientifically valid health risk assessment on GM foods. In this review, after critically examining some of the basic principles, past results and possible novel methods of future health safety assessment of GM foodstuffs, the conclusion appears to be that as the tools for the recognition and indeed for the elimination of the risks GM foods may present for us are available or can be developed, it is the will and the funding for such work that needs to be found.

http://www.ncbi.nlm.nih.gov/pubmed/12102369

Schrøder, M.; Poulsen, M.; Wilcks, A.; Kroghsbo, S.; Miller, A.; Frenzel, T.; Danier, J.;

Rychlik, M.; Emami, K.; Gatehouse, A.; Shu, Q.; Engel, K.; Altosaar, I.; Knudsen, I. 2007. A 90-day safety study of genetically modified rice expressing Cry1Ab protein (*Bacillus thuringiensis* toxin) in Wistar rats. *Food Chem Toxicol*, 45(3): 339-349.

An animal model for safety assessment of genetically modified foods was tested as part of the SAFOTEST project. In a 90-day feeding study on Wistar rats, the transgenic KMD1 rice expressing Cry1Ab protein was compared to its non-transgenic parental wild type, Xiushui 11. The KMD1 rice contained 15mg Bt toxin/kg and based on the average feed consumption the daily intake was 0.54mg Bt toxin/kg body weight. No adverse effects on animal behaviour or weight gain were observed during the study. Blood samples collected one week prior to sacrifice were analyzed and compared for standard haematological and biochemical parameters. A few parameters were significantly different, but all within the normal reference intervals for rats of this breed and age and not in relation to any other findings, thus not considered treatment related. Upon sacrifice a large number of organs were weighed, macroscopic and histopathological examinations were performed with only minor changes to report. The aim of the study was to use a known animal model in performance of safety assessment of a GM crop, in this case KMD1 rice. The results show no adverse or toxic effects of KMD1 rice when tested in the design used in this 90-day study. Nevertheless the experiences from this study lead to the overall conclusion that safety assessment for unintended effects of a GM crop cannot be done without additional test group(s).

http://www.ncbi.nlm.nih.gov/pubmed/17050059

REV - Séralini, G-E.; Vendômois, J.; Cellier, D.; Sultan, C.; Buiatti, M.; Gallagher, L.; Antoniou, M.; Dronamraju, K. 2009. How subchronic and chronic health effects can be neglected for GMOs, pesticides or chemicals. *International Journal of Biological Science*, 5, 438–443.

Chronic health effects are increasing in the world such as cancers, hormonal, reproductive, nervous, or immune diseases, even in young people. During regulatory toxicological subchronic tests to prevent these on mammalian health, prior commercialization of chemicals, including pesticides and drugs, or GMOs, some statistically significant findings may be revealed. This discussion is about the need to investigate the relevant criteria to consider those as biologically significant. The sex differences and the non linear dose or time related effects should be considered in contrast to the claims of a Monsanto-supported expert panel about a GMO, the MON 863 Bt maize, but also for pesticides or drugs, in particular to reveal hormone-dependent diseases and first signs of toxicities.

Full article available at http://www.ijbs.com/v05p0438.htm

REV - Vendômois, J.; Roullier, F.; Cellier, D.; Séralini, G-E. 2009. A comparison of the effects of three GM corn varieties on mammalian health. *International Journal of Biological Sciences*, 5: 706-726.

We present for the first time a comparative analysis of blood and organ system data from trials with rats fed three main commercialized genetically modified (GM) maize (NK 603, MON 810, MON 863), which are present in food and feed in the world. NK 603 has been modified to be tolerant to the broad spectrum herbicide Roundup and thus contains residues of this formulation. MON 810 and MON 863 are engineered to synthesize two different Bt toxins used as insecticides. Approximately 60 different biochemical parameters were classified per organ and measured in serum and urine after 5 and 14 weeks of feeding. GM maize-fed rats were compared first to their respective isogenic or parental non-GM equivalent control groups. This was followed by comparison to six reference groups, which had consumed various other non-GM maize varieties. We applied nonparametric methods, including multiple pairwise comparisons with a False Discovery Rate approach. Principal Component Analysis allowed the

investigation of scattering of different factors (sex, weeks of feeding, diet, dose and group). Our analysis clearly reveals for the 3 GMOs new side effects linked with GM maize consumption, which were sex- and often dose-dependent. Effects were mostly associated with the kidney and liver, the dietary detoxifying organs, although different between the 3 GMOs. Other effects were also noticed in the heart, adrenal glands, spleen and haematopoietic system. We conclude that these data highlight signs of hepatorenal toxicity, possibly due to the new pesticides specific to each GM corn. In addition, unintended direct or indirect metabolic consequences of the genetic modification cannot be excluded.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2793308/

REV - Vendômois, J.; Cellier, D.; Vélot, C.; Clair, E.; Mesnage, R.; Séralini, G-E. 2010. Debate on GMOs health risks after statistical findings in regulatory tests. *International Journal of Biological* Sciences, 6, 590-598.

We summarize the major points of international debate on health risk studies for the main commercialized edible GMOs. These GMOs are soy, maize and oilseed rape designed to contain new pesticide residues since they have been modified to be herbicide-tolerant (mostly to Roundup) or to produce mutated Bt toxins. The debated alimentary chronic risks may come from unpredictable insertional mutagenesis effects, metabolic effects, or from the new pesticide residues. The most detailed regulatory tests on the GMOs are three-month long feeding trials of laboratory rats, which are biochemically assessed. The tests are not compulsory, and are not independently conducted. The test data and the corresponding results are kept in secret by the companies. Our previous analyses of regulatory raw data at these levels, taking the representative examples of three GM maize NK 603, MON 810, and MON 863 led us to conclude that hepatorenal toxicities were possible, and that longer testing was necessary. Our study was criticized by the company developing the GMOs in question and the regulatory bodies, mainly on the divergent biological interpretations of statistically significant biochemical and physiological effects. We present the scientific reasons for the crucially different biological interpretations and also highlight the shortcomings in the experimental protocols designed by the company. The debate implies an enormous responsibility towards public health and is essential due to nonexistent traceability or epidemiological studies in the GMO-producing countries.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2952409/

REV – Séralini, G-E.; Mesnage, R.; Clair, E.; Gress, S.; Vendômois, J.; Cellier, D. 2011. Genetically modified crops safety assessments: present limits and possible improvements. *Environmental Sciences Europe*, 23:10.

Purpose: We reviewed 19 studies of mammals fed with commercialized genetically modified soybean and maize which represent, per trait and plant, more than 80% of all environmental genetically modified organisms (GMOs) cultivated on a large scale, after they were modified to tolerate or produce a pesticide. We have also obtained the raw data of 90-day-long rat tests following court actions or official requests. The data obtained include biochemical blood and urine parameters of mammals eating GMOs with numerous organ weights and histopathology findings.
Methods: We have thoroughly reviewed these tests from a statistical and a biological point of view. Some of these tests used controversial protocols which are discussed and statistically significant results that were considered as not being biologically meaningful by regulatory authorities, thus raising the question of their interpretations.
Results: Several convergent data appear to indicate liver and kidney problems as end points of GMO diet effects in the above-mentioned experiments. This was confirmed by our meta-analysis of all the in vivo studies published, which revealed that the kidneys were particularly affected, concentrating 43.5% of all disrupted parameters in males, whereas the liver was more specifically disrupted in

females (30.8% of all disrupted parameters).
Conclusions: The 90-day-long tests are insufficient to evaluate chronic toxicity, and the signs highlighted in the kidneys and livers could be the onset of chronic diseases. However, no minimal length for the tests is yet obligatory for any of the GMOs cultivated on a large scale, and this is socially unacceptable in terms of consumer health protection. We are suggesting that the studies should be improved and prolonged, as well as being made compulsory, and that the sexual hormones should be assessed too, and moreover, reproductive and multigenerational studies ought to be conducted too.

Full article available at http://www.enveurope.com/content/23/1/10

Snell, C.; Bernheim, A.; Bergé, J.; Kuntz, M.; Pascal, G.; Paris, A.; Ricroch, A. 2012. Assessment of the health impact of GM plant diets in long-term and multigenerational animal feeding trials: A literature review. *Food and Chemical Toxicology*, 50(3-4):1134-48. doi: 10.1016/j.fct.2011.11.048.

The aim of this systematic review was to collect data concerning the effects of diets containing GM maize, potato, soybean, rice, or triticale on animal health. We examined 12 long-term studies (of more than 90 days, up to 2 years in duration) and 12 multigenerational studies (from 2 to 5 generations). We referenced the 90-day studies on GM feed for which long-term or multigenerational study data were available. Many parameters have been examined using biochemical analyses, histological examination of specific organs, hematology and the detection of transgenic DNA. The statistical findings and methods have been considered from each study. Results from all the 24 studies do not suggest any health hazards and, in general, there were no statistically significant differences within parameters observed. However, some small differences were observed, though these fell within the normal variation range of the considered parameter and thus had no biological or toxicological significance. If required, a 90-day feeding study performed in rodents, according to the OECD Test Guideline, is generally considered sufficient in order to evaluate the health effects of GM feed. The studies reviewed present evidence to show that GM plants are nutritionally equivalent to their non-GM counterparts and can be safely used in food and feed.

http://www.ncbi.nlm.nih.gov/pubmed/22155268

REV – Heinemann, J.; Agapito-Tenfen, S.; Carman, J. 2013. A comparative evaluation of the regulation of GM crops or products containing dsRNA and suggested improvements to risk assessments. *Environment International*, Vol. 55: 43-55.

Changing the nature, kind and quantity of particular regulatory-RNA molecules through genetic engineering can create biosafety risks. While some genetically modified organisms (GMOs) are intended to produce new regulatory-RNA molecules, these may also arise in other GMOs not intended to express them. To characterise, assess and then mitigate the potential adverse effects arising from changes to RNA requires changing current approaches to food or environmental risk assessments of GMOs. We document risk assessment advice offered to government regulators in Australia, New Zealand and Brazil during official risk evaluations of GM plants for use as human food or for release into the environment (whether for field trials or commercial release), how the regulator considered those risks, and what that experience teaches us about the GMO risk assessment framework. We also suggest improvements to the process.

Full article available at http://www.sciencedirect.com/science/article/pii/S0160412013000494

Meyer, H.; Hilbeck, A. 2013. Rat feeding studies with genetically modified maize – a comparative evaluation of applied methods and risk assessment standards. *Environmental Sciences Europe*, 25:33 (doi:10.1186/2190-4715-25-33).

A 2-year rat feeding study with genetically modified NK603 maize sparked an international scientific and public debate as well as policy responses by the European Commission. The European Food Safety Authority (EFSA) evaluated the study as defective based on conceptual and methodological shortcomings by retroactive application of the recommendations of its recent guidance on 90-day feeding studies. Our comparative analysis of the three relevant NK603 publications, including a 90-day feeding study of Monsanto, showed that all of them satisfy or fail to satisfy the EFSA evaluation criteria to a comparable extent; the rejection of only one of the papers is, thus, not scientifically justified. We also show that EFSA's criteria are not standard practice in 21 other rat feeding studies lasting at a minimum of 12 months. The review reveals critical double standards in the evaluation of feeding studies submitted as proof of safety for regulatory approval to EFSA. We specifically argue that the current approach to declare statistically significant differences between genetically modified organisms and its parents as 'biologically irrelevant' based on additional reference controls lacks scientific rigor and legal justification in the European Union (EU) system. Only recently, the EU authorities started building up an implementing system based on its own legislation and supportive of the EU approach to risk assessment in the context of technology assessment. Until these issues are resolved, we do not expect that neither the public nor the scientific debate will subside.

Full article available at http://www.enveurope.com/content/25/1/33

COM – Mesnage, R.; Defarge, N.; Vendômois, J.; Séralini, G-E. 2014. Letter to the editor regarding "Delaney et al., 2014": uncontrolled GMOs and their associated pesticides make the conclusions unreliable. *Food Chem Toxicol*, 72:322. doi: 10.1016/j.fct.2014.07.003.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/24997312

It is relevant the fact that the scientific controversy regarding the toxicological risks assessment of pesticides to human health and the environment, assumes the character of almost consensus. There are no doubts about a basic fact: the application of poisons on plants for consumption will bring negative implications for the health of populations and the organisms to which they are intended.

Still, for a significant portion (but not all) of the scientific community and opinion leaders with space in the media, tests and simplified assessments as conducted by regulatory agencies, are sufficient. For another group of researchers, as well as for the general population, analytical methods meet the economic interests and threaten the credibility of the institutions.

Available studies reveal the presence of inappropriate methodologies, unable to effectively represent risks associated with pesticides and their residues in food in general. Widespread use concepts, such as the MRL (Maximum Residue Limit acceptable) and ADI (Acceptable Daily Intake), do not take into account synergies, adjuvants, combinations and superposition of foods made with GMOs or the biological diversity of consumers (age, diseases, hypersensitivity, etc.).

REV - Colborn, T. 2006. A case for revisiting the safety of pesticides: a closer look at neurodevelopment. *Environmental Health Perspectives*, 114(1): doi:10.1289/ehp.7940.

The quality and quantity of the data about the risk posed to humans by individual pesticides vary considerably. Unlike obvious birth defects, most developmental effects cannot be seen at birth or even later in life. Instead, brain and nervous system disturbances are expressed in terms of how an individual behaves and functions, which can vary considerably from birth through adulthood. In this article I challenge the protective value of current pesticide risk assessment strategies in light of the vast numbers of pesticides on the market and the vast number of possible target tissues and end points that often differ depending upon timing of exposure. Using the insecticide chlorpyrifos as a model, I reinforce the need for a new approach to determine the safety of all pesticide classes. Because of the uncertainty that will continue to exist about the safety of pesticides, it is apparent that a new regulatory approach to protect human health is needed.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1332649/

Hardell, L. 2008. Pesticides, soft-tissue sarcoma and non-Hodgkin lymphoma - historical aspects on the precautionary principle in cancer prevention. *Acta Oncologica*, 47: 347-354.

Background. After the 2nd World War a long range of chemical agents have been introduced on the market, both in Sweden and most other countries. From the 1950's several pesticides gained increasing use in agriculture and forestry. In the 1970's public concern increased in Sweden especially regarding use of phenoxy herbicides to combat deciduous wood, although statements from different authorities were reassuring of the safety. *Materials and methods.* At the end of the 1970's the author and his colleagues published the first scientific evidence of an association between exposure to phenoxyacetic acids, chlorophenols and certain malignant tumours, i.e., soft-tissue sarcoma and malignant lymphoma. The study subjects were also exposed to contaminating dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Later studies showed also an association between certain persistent organic pollutants such as polychlorinated biphenyls and non-Hodgkin lymphoma (NHL) with an interaction with titers of antibodies to Epstein-Barr virus early antigen. These results have been corroborated in other studies. *Discussion.* Over the years industry and its allied experts have attacked our studies, but in 1997 IARC classified TCDD as a human carcinogen, Group I. The increasing incidence of NHL in Sweden levelled off about 1990. The author postulated that the regulation or ban of the use of chlorophenols, certain phenoxy herbicides and some persistent organic pollutants in Sweden back in the 1970s has contributed to the now decreasing incidence of NHL. Unfounded criticism from industry experts may prohibit the precautionary principle and early warnings of cancer risk can be ignored. Cancer

risks by certain chlorinated phenols may serve as a model of how the precautionary principle should be used by taking early warnings seriously.

Full article available at http://informahealthcare.com/doi/pdf/10.1080/02841860701753697

Tennekes, H. 2010. The significance of the Druckrey-Küpfmüller equation for risk assessment--the toxicity of neonicotinoid insecticides to arthropods is reinforced by exposure time. *Toxicology*, 276(1): 1-4.

The essence of the Druckrey-Küpfmüller equation dtn = constant (where d = daily dose and t = exposure time-to-effect, with n > 1) for chemical carcinogens is that the total dose required to produce the same effect decreases with decreasing exposure levels, even though the exposure times required to produce the same effect increase with decreasing exposure levels. Druckrey and Küpfmüller inferred that if both receptor binding and the effect are irreversible, exposure time would reinforce the effect. The Druckrey-Küpfmüller equation explains why toxicity may occur after prolonged exposure to very low toxicant levels. Recently, similar dose-response characteristics have been established for the toxicity of the neonicotinoid insecticides imidacloprid and thiacloprid to arthropods. This observation is highly relevant for environmental risk assessment. Traditional approaches that consider toxic effects at fixed exposure times are unable to allow extrapolation from measured endpoints to effects that may occur at other times of exposure. Time-to-effect approaches that provide information on the doses and exposure times needed to produce toxic effects on tested organisms are required for prediction of toxic effects for any combination of concentration and time in the environment.

http://www.ncbi.nlm.nih.gov/pubmed/20803795

Mesnage, R.; Defarge, N.; Vendômois, J.; Séralini, G-E. 2014. Major pesticides are more toxic to human cells than their declared active principles. *BioMed Research International*, vol. 2014, Article ID 179691, 8 pages. doi:10.1155/2014/179691.

Pesticides are used throughout the world as mixtures called formulations. They contain adjuvants, which are often kept confidential and are called inerts by the manufacturing companies, plus a declared active principle (AP), which is usually tested alone. This is true even in the longest toxicological regulatory tests performed on mammals. We tested the toxicity of 9 pesticides, comparing active principles and their formulations, on three human cell lines (HepG2, HEK293 and JEG3). We measured mitochondrial activities, membrane degradations, and caspases 3/7 activities. Glyphosate, isoproturon, fluroxypyr, pirimicarb, imidacloprid, acetamiprid, tebuconazole, epoxiconazole and prochloraz constitute respectively the active principles of 3 major herbicides, 3 insecticides and 3 fungicides. Fungicides were the most toxic from concentrations 300-600 times lower than agricultural dilutions, followed by herbicides, and then insecticides, with very similar profiles in all cell types. The human placental JEG3 cells appeared to be the most sensitive. Despite its relatively benign reputation, Roundup was by far the most toxic among the herbicides and insecticides tested. Most importantly, 8 formulations out of 9 were several hundred times more toxic than their active principle. Our results challenge the relevance of the Acceptable Daily Intake for pesticides because this norm is calculated from the toxicity of the active principle alone. The study of combinatorial effects of several APs together may be of only secondary importance if the toxicity of the combinations of each AP with its adjuvants is neglected or unknown. Chronic tests on pesticides may not reflect relevant environmental exposures if only one ingredient of these mixtures is tested alone.

Full article available at http://www.hindawi.com/journals/bmri/2014/179691/

3 Lack of scientific rigor in the risk assessment for the environment (particularly ONAs)

The environmental risk assessment for transgenic crops - as performed by regulatory agencies - has also received strong criticism by a significant part of the scientific community. Studies show basic mistakes in choosing non-target organisms in agricultural ecosystems coverage, representativeness of traditional technologies and selection of management ways to impact comparisons. Aquatic environments are often neglected, microbial communities are often ignored and even insects nonexistent in biomes where GMPs will be grown are used as local references - simply because they are easy to breed in the laboratory. It should be noted that scientific knowledge about specific protocols of the environmental risk assessment in tropical areas - especially in South America - is almost nil.

In parallel, food webs, ecological functions of the affected organisms, population balances and even natural climate fluctuations are undersized in expedited, short-term reviews and little use with regard to risk assessment. Finally, the very methodologies used in bioassays to assess the subchronic risk in ONA are questionable, as outlined in the following articles.

REV - O'Callaghan, M.; Glare, T.; Burgess, E.; Malone, L. 2005. Effects of plants genetically modified for insect resistance on nontarget organisms. *Annual Review of Entomology*, 50, pp. 271-292.

Insect resistance, based on Bacillus thuringiensis (Bt) endotoxins, is the second most widely used trait (after herbicide resistance) in commercial genetically modified (GM) crops. Other modifications for insect resistance, such as proteinase inhibitors and lectins, are also being used in many experimental crops. The extensive testing on nontarget plant-feeding insects and beneficial species that has accompanied the long-term and wide-scale use of Bt plants has not detected significant adverse effects. GM plants expressing other insect-resistant proteins that have a broader spectrum of activity have been tested on only a limited number of nontarget species. Little is known about the persistence of transgene-derived proteins in soil, with the exception of Bt endotoxins, which can persist in soil for several months. Bt plants appear to have little impact on soil biota

such as earthworms, collembolans, and general soil microflora. Further research is required on the effects of GM plants on soil processes such as decomposition. Assessment of nontarget impacts is an essential part of the risk assessment process for insect-resistant GM plants.

http://www.ncbi.nlm.nih.gov/pubmed/15355241

MOD - Andow, D.; Zwahlen, C. 2006. Assessing environmental risks of transgenic plants. *Ecology Letters*, (2): 196-214.

By the end of the 1980s, a broad consensus had developed that there were potential environmental risks of transgenic plants requiring assessment and that this assessment must be done on a case-by-case basis, taking into account the transgene, recipient organism, intended environment of release, and the frequency and scale of the intended introduction. Since 1990, there have been gradual but substantial changes in the environmental risk assessment process. In this review, we focus on changes in the assessment of risks associated with non-target species and biodiversity, gene flow, and the evolution of resistance. Non-target risk assessment now focuses on risks of transgenic plants to the intended local environment of release. Measurements of gene flow indicate that it occurs at higher rates than believed in the early 1990s, mathematical theory is beginning to clarify expectations of risks associated with gene flow, and management methods are being developed to reduce gene flow and possibly mitigate its effects. Insect pest resistance risks are now managed using a high-dose/refuge or a refuge-only strategy, and the present research focuses on monitoring for resistance and encouraging compliance to requirements. We synthesize previous models for tiering risk assessment and propose a general model for tiering. Future transgenic crops are likely to pose greater challenges for risk assessment, and meeting these challenges will be crucial in developing a scientifically coherent risk assessment framework. Scientific understanding of the factors affecting environmental risk is still nascent, and environmental scientists need to help improve environmental risk assessment.

http://www.ncbi.nlm.nih.gov/pubmed/16958885

Perry, J.; Braak, C.; Dixon, P.; Duan, J.; Hails, R.; Huesken, A.; Lavielle, M.; Marvier, M.; Scardi, M.; Schmidt, K.; Tothmeresz, B.; Schaarschmidt, F.; van der Voet, H. 2009. Statistical aspects of environmental risk assessments of GM plants for effects on non-target organisms. *Environ Biosafety*, Res 8: 65–78.

Previous European guidance for environmental risk assessment of genetically modified plants emphasized the concepts of statistical power but provided no explicit requirements for the provision of statistical power analyses. Similarly, whilst the need for good experimental designs was stressed, no minimum guidelines were set for replication or sample sizes. Furthermore, although substantial equivalence was stressed as central to risk assessment, no means of quantification of this concept was given. This paper suggests several ways in which existing guidance might be revised to address these problems. One approach explored is the ;bioequivalence' test, which has the advantage that the error of most concern to the consumer may be set relatively easily. Also, since the burden of proof is placed on the experimenter, the test promotes high-quality, well-replicated experiments with sufficient statistical power. Other recommendations cover the specification of effect sizes, the choice of appropriate comparators, the use of positive controls, meta-analyses, multivariate analysis and diversity indices. Specific guidance is suggested for experimental designs of field trials and their statistical analyses. A checklist for experimental design is proposed to accompany all environmental risk assessments.

Abstract available at http://www.ncbi.nlm.nih.gov/pubmed/19833074

Liu, W. 2009. Effects of Bt transgenic crops on soil ecosystems: a review of a ten-year research in China. *Frontiers of Agriculture in China*, 3 (2): 190–198.

Bacillus thuringiensis (Bt) transgenic cotton is the unique Bt transgenic crop planted on a large scale in China, and its commercialized varieties and hectareage had increased rapidly in China during the past decade (1997–2006) with broad geographic distribution for the economic, environmental, and health benefits. In 2004, the planting area of Bt transgenic cotton in China ranked first worldwide with up to 370 × 106 hm2. In addition, Bt transgenic rice varieties in field tests have been close to approval for commercialization. However, ecological risks, a complex issue of Bt transgenic crops on soil ecosystem is urgently faced in China due to more than 60 varieties transferred single or bivalent Bt genes grown under diverse geographic regions. Two main pathways, biomass incorporation and root exudates, are involved in the effects of Bt transgenic crops on soil ecosystems. In this paper, the research results in recent years in China involved in the effects of Bt transgenic crops (Bt transgenic cottons and rice) on soil ecosystems were summarized with special attentions paid to the release and persistence of Bt toxins, and the toxicology to microorganisms, as well as the change of soil biochemical properties in soils where Bt transgenic crops were planted or incubated with their biomass. In addition, the complexity and current research defaults of ecological risk evaluation of Bt transgenic crops in China were highlighted.

http://link.springer.com/article/10.1007%2Fs11703-009-0027-9

REV - Lang, A.; Otto, M. 2010. A synthesis of laboratory and field studies on the effects of transgenic *Bacillus thuringiensis* (Bt) maize on non-target *Lepidoptera*. *Entomologia Experimentalis et Applicata*, 135 (2): 121134.

One of the major applications of transgenic crops in agriculture are the so-called Bacillus thuringiensis Berliner (Bt) plants, in particular Bt maizes, which produce insecticidal Cry proteins that target specific orders, such as the Lepidoptera or Coleoptera. We reviewed publications that reported on the direct toxic effects of Bt-maize and/or Cry proteins of current Bt-maize events on larvae of non-target butterflies and moths (Lepidoptera). In total, 20 peer-reviewed publications were identified, of which 16 papers contributed laboratory-based data and seven field-based data. An adverse effect on caterpillars was recorded in 52% of all laboratory-based and in 21% of all field-based observations. The variables most often studied and having the highest occurrence of effects were larval survival, body mass, and developmental time. Parameters of the adult stage were under-represented in the studies. Overall, 11 lepidopteran species were tested. The majority of the studies originated from the USA, with the Monarch butterfly being the most studied, whereas other species and other parts of the world were widely neglected. Laboratory experiments were often run under unrealistic conditions from an ecological point of view. Although the papers we reviewed indicated a potential hazard for Lepidoptera that are exposed to and feed on lepidopteran-specific Bt-maize pollen, a general conclusion on the level of risk for butterflies and moths cannot as yet be drawn. A comprehensive risk characterization would require thorough hazard identification, exposure assessment, and impact assessment. However, our review showed that even the basic level of hazard characterization is as yet incomplete. Reasons for this are the still-limited numbers of publications and concurrent lack of knowledge, the restriction of data to only a few species, the over-representation of North American species, and the identified limitations of both laboratory and field experiments. The findings of this review suggest that more realistic, ecologically meaningful, and detailed experiments and analyses are crucial to improve the present assessment of Bt-maize cultivation effects on Lepidoptera.

Full article available at http://onlinelibrary.wiley.com/doi/10.1111/j.1570-7458.2010.00981.x/pdf

COM - Lang, A., S. Brunzel, M. Dolek, M. Otto and B. Theißen, 2011. Modelling in the light of uncertainty of key parameters: a call to exercise caution in field predictions of Bt-maize effects. *Proceedings of the Royal Society B*.

Without summary.

Full article available at https://goo.gl/rt9cZS

Hilbeck, A.; Meier, M.; Römbke, J.; Jänsch, S.; Teichmann, H.; Tappeser, B. 2011. Environmental risk assessment of genetically modified plants - concepts and controversies. *Environmental Sciences Europe*, 23(13).

Background and purpose: In Europe, the EU Directive 2001/18/EC lays out the main provisions of environmental risk assessment (ERA) of genetically modified (GM) organisms that are interpreted very differently by different stakeholders. The purpose of this paper is to: (a) describe the current implementation of ERA of GM plants in the EU and its scientific shortcomings, (b) present an improved ERA concept through the integration of a previously developed selection procedure for identification of non-target testing organisms into the ERA framework as laid out in the EU Directive 2001/18/EC and its supplement material (Commission Decision 2002/623/EC), (c) describe the activities to be carried out in each component of the ERA and (d) propose a hierarchical testing scheme. Lastly, we illustrate the outcomes for three different crop case examples.
Main features: Implementation of the current ERA concept of GM crops in the EU is based on an interpretation of the EU regulations that focuses almost exclusively on the isolated bacteria-produced novel proteins with little consideration of the whole plant. Therefore, testing procedures for the effect assessment of GM plants on non-target organisms largely follow the ecotoxicological testing strategy developed for pesticides. This presumes that any potential adverse effect of the whole GM plant and the plant-produced novel compound can be extrapolated from testing of the isolated bacteria-produced novel compound or can be detected in agronomic field trials. This has led to persisting scientific criticism.
Results: Based on the EU ERA framework, we present an improved ERA concept that is system oriented with the GM plant at the centre and integrates a procedure for selection of testing organisms that do occur in the receiving environment. We also propose a hierarchical testing scheme from laboratory studies to field trials and we illustrate the outcomes for three different crop case examples.
Conclusions and recommendations: Our proposed concept can alleviate a number of deficits identified in the current approach to ERA of GM plants. It allows the ERA to be tailored to the GM plant case and the receiving environment.

Full article available at http://www.enveurope.com/content/23/1/13

Meyer, H. 2011. Systemic risks of genetically modified crops: the need for new approaches to risk assessment. *Environmental Sciences Europe*, 23:7.

Purpose: Since more than 25 years, public dialogues, expert consultations and scientific publications have concluded that a comprehensive assessment of the implications of genetic engineering in agriculture and food production needs to include health, environmental, social and economical aspects, but only very few legal frameworks allow to assess the two latter aspects. This article aims to explain the divergence between societal debate and biosafety legislation and presents approaches to bring both together.
Main features: The article reviews the development of biosafety regulations in the USA and the EU, focussing on diverging concepts applied for assessing the risks of genetically modified organisms (GMOs).
Results: The dominant environmental risk assessment methodology has been developed to answer basic questions to enable expedient decision making. As a first step, methodologies that take into account complex environmental and landscape aspects should be applied. Expanding the scope of risk assessment, more holistic concepts have been developed, for example the Organisation for Econonomic Co-operation and Development (OECD) concept of systemic risks which includes socio-economic aspects. International bodies as the OECD, the Convention on Biological Diversity

(CBD) and the European Union (EU) have developed the Strategic Environmental Assessment (SEA) as an instrument that includes the additional aspects of risk assessment as demanded by many stakeholders. Interestingly, there had been no attempts yet to link the existing frameworks of GMO risk assessment and SEA.
Conclusions: It is recommended to adapt current models of SEA to assess the systemic risks of GMOs. It is also suggested to revise the EU GMO legislation to promote the inclusion of SEA elements.

Full article available at http://www.enveurope.com/content/23/1/7

COM – Hilbeck, A.; Meier, M.; Trtikova, M. 2012. Underlying reasons of the controversy over adverse effects of Bt toxins on lady beetle and lacewing larvae. *Environmental Sciences Europe*, 24:9.

We outline important underlying reasons that fuel the decades-long controversy over adverse effects of Bt toxins expressed in genetically modified plants on beneficial, nontarget organisms. Inconsistent evaluation standards and asymmetrical levels of scrutiny applied to studies reporting significant adverse effects compared to those finding no adverse effects are described using the examples of the green lacewing (Chrysoperla carnea) and the two-spotted lady beetle (Adalia bipunctata). Additionally, the chosen style and concerted nature of the rather confrontational counter study and responses in the lady beetle cases bear striking similarities to other reported examples in the field of biosafety/risk science of genetically modified plants and to other fields of applied industrial techno-science that suggest deeper issues that go well beyond science. We call for a constructive and respectful scientific discourse where moving the frontiers of our collective knowledge forward takes center stage. Reported phenomena based on robust data must not be rejected or delegitimized on their being surprising and lacking an explained mechanism at the time of their discovery. Exploring mechanisms often requires entirely different expertise and methodologies than those of the discoverers. In particular, in biosafety/risk sciences, plurality of arguments and critical research approaches have to be embraced and actively encouraged rather than discredited or even silenced if we are to learn our 'late lessons' from past technology introductions.

Full article available at http://www.enveurope.com/content/24/1/9

Heinemann, J.; El-Kawy, O. 2012. Observational science in the environmental risk assessment and management of GMOs. *Environment International*, 45 (2012) 68–71.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/22575805

COM - Wickson, F.; Bøhn, T.; Wynne, B.; Hilbeck, A.; Funtowicz, S. 2013. Science-based risk assessment requires careful evaluation of all studies. *Nature Biotechnology*, Volume 31, Number 12, 1077-1078.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/24316639

Gillund, F.; Nordgaard, L.; Bøhn, T.; Wikmark, O.; Konestabo, H.; Hilbeck, A. 2014. Selection of nontarget testing organisms for ERA of GM potato with increased resistance to late blight. *Eur J Potato Research*, 56, (4), pp 293-324 DOI: 10.1007/s11540-013-9245-x.

The deliberate release of any genetically modified (GM) organism in the European Union requires an environmental risk assessment (ERA) prior to commercialisation, including impact assessment on nontarget organisms. We report from two expert workshops where a newly developed selection procedure for identification of ecologically relevant testing organisms was applied to the case of a GM potato with increased resistance to late blight, planned for cultivation in southern Scandinavia. Species known to contribute to important ecological functions in the receiving environment were selected in a stepwise procedure, to arrive at a practical number of ecologically relevant species that are likely to be exposed to the transgene and suitable for experimental testing. Four ecological functional categories were identified: herbivory and disease transmission, natural enemies, ecological soil processes and pollination. Among these, relevant nontarget species were identified for herbivores and soil living pathogens, natural enemies and decomposers/beneficial soil organisms. Out of a total of 16 herbivores, 17 soil-living pathogens, 49 natural enemies and 14 decomposers/beneficial soil organisms in the initial lists, 8 herbivores, 10 soil-living pathogens, 15 natural enemies and 11 decomposers/beneficial soil organisms were identified as possible testing organisms, based on ecological criteria. These findings are highly relevant for determining the scope and structure of an ERA of this type of GM potato. The selection procedure could not be completed because of insufficient information about tissue- and developmental stage-specific expression levels of the transgenic products for this particular GM potato. Thus, the case study illustrates some of the difficulties and knowledge gaps that limit the relevance and quality of ERA of GM plants.

http://link.springer.com/article/10.1007%2Fs11540-013-9245-x

4 Scientific controversies and campaigns that aim to "shoot the messenger"

In recent years, the scientific literature incorporates a rare procedure in periods leading up to the emergence of biotechnology. Alongside the criticism of "official" methodologies for risk assessment, controversies oriented to achieve - if possible demoralize - scholars and research centers who disagree with the prevailing view produced by the companies and/or associated with their interests accumulate.

These controversies are systematically unleashed towards articles that observe or suggest negative impacts of transgenic plants, regardless of the field of biology considered. The following item incorporates extensive documentation about these mechanisms. Its findings contribute to scientific obscurantism, increasing the margin of risks that threatens the health of the environment and consumers of GMPs.

4.1 Discredit campaigns reported in the scientific literature and control of research by biotechnology companies

COM - *The Lancet Editorial*, 1999. Health risks of genetically modified foods. Volume 353, No. 9167, p1811, 29 May 1999.

Without summary.

Full article available at https://goo.gl/TdSx2R

COM - Ewen, S.; Pusztai, A. 1999. Health risks of genetically modified foods. *The Lancet*, Volume 354, No. 9179, p684, 21.

Without summary.

Full article available at https://goo.gl/DNuZTu

Waltz, E. 2009. Under wraps. *Nature Biotechnology*, 27(10): 880-882.

Are the crop industry's strong-arm tactics and close-fisted attitude to sharing seeds holding back independent research and undermining public acceptance of transgenic crops?

http://www.nature.com/nbt/journal/v27/n10/abs/nbt1009-880.html

Waltz, E. 2009. GM crops: Battlefield. *Nature*, 461: 27-32.

Papers suggesting that biotech crops might harm the environment attract a hail of abuse from other scientists. Emily Waltz asks if the critics fight fair.

Full article available at http://www.nature.com/news/2009/090902/full/461027a.html

Lotter, D. 2007. The genetic engineering of food and the failure of science – Part 1: The development of a flawed enterprise. *Int Jrnl of Soc of Agr & Food*, 16(1): 31–49.

A major conflict has developed in science and society between promoters and opponents of transgenic foods. Food, feed, and fiber products derived from transgenic agricultural crops are presented here as a different case from bacterial, industrial, and pharmaceutical crop transgenics and should be parsed from the larger transgenics industry for comprehensive re-evaluation and market roll-back. Reviewed is the development of the crop transgenics industry; the early influence of the biotechnology industry over the US federal regulatory agencies in the context of the development of minimal regulation; the basic technology of plant transgenics; the main transgenic crops, traits, and producing countries; consumer resistance to transgenic foods; industry problems with shrinking investments; the worldwide promotion of transgenic crops; and ecological issues of transgenic crops. Flaws in the one gene–one protein model, the foundation of transgenics, are reviewed in the context of the recent and ongoing restructuring of the science of genetics. Research on the mutational consequences of plant transgenics and its phenotypic ramifications such as allergens and novel proteins is discussed. Major research findings and 'red flag' incidents in the history of transgenic foods and feeds are reviewed that reflect the flaws in the genetic foundations of transgenics.

Full article available at http://ijsaf.org/archive/16/1/lotter1.pdf

Lotter, D. 2008. The genetic engineering of food and the failure of science – Part 2: Academic capitalism and the loss of scientific integrity. *Int Jrnl of Soc of Agr & Food*, 16(1): 50–68.

Factors in the failure of the scientific community to properly oversee agricultural transgenics are presented. The large-scale restructuring of university science programs in the past 25 years from a model based on non-proprietary science for the 'public good' to the 'academic capitalism' model based on the 'knowledge economy' is discussed in the context of the failure of the science community to oversee the transition of transgenic crop technology from the research stage to commercialization. Discussed are increasing science community and university dependence on private industry funding and on development of proprietary technologies; monopolization of the make-up of expert scientific bodies on transgenics by pro-industry scientists with vested interests in transgenics; deficient scientific protocols, bias, and possible fraud in industry-sponsored and industry-conducted research; increasing politically and commercially driven manipulation of science within federal regulatory bodies such as the FDA; and bias in the peer-review process, tolerance by the scientific community of biotechnology industry manipulation of the information environment, and of biased treatment and harassment of non-compliant scientists. Discussed are future food production strategies for developing countries, recently framed in the 2008 UNsponsored International Assessment of Agricultural Knowledge, Science, and Technology, an action plan that emphasizes non-proprietary, agroecology-based approaches to food production and does not include crop transgenics as a central strategy. The under-funding of non-proprietary agroecological approaches to food production is discussed.

Full article available at http://www.ijsaf.org/archive/16/1/lotter2.pdf

4.2 Examples of campaigns aimed at "shooting the messenger"

Several times in the history of research on the impacts of transgenic plants on the environment and human and animal health, articles pointing to risks of GMOs were immediately and severely criticized by certain components of the scientific community. Sometimes as a result of these campaigns, customer reviews and original findings become numb, requiring several years and slow accumulation of new evidence, reiterated in confirming articles, before the anticipated arguments by the first authors are accepted and recognized as scientific grounds. In such cases, the detractors are not remembered, and losses arising from contempt to relevant information on human and environmental health are not assigned to anyone.

4.2.1 Negative effects on ONAs: the case of Monarch butterflies

In 1999, at the beginning of large-scale commercialization of Bt plants, Losey et al anticipated problems for the populations of Monarch butterfly (migratory species of symbolic value in the USA). When developing part of their life cycle in the vicinity of Bt crops, those populations would be at risk. Quickly, the authors suffered a defamatory campaign. Still, after several publications on the subject, negative impacts were confirmed. Currently, the Monarch butterfly is used as a model species for environmental impact testing of those toxins on ONAs.

COM – Losey, J.; Rayor, L.; Carter, M. 1999. Transgenic pollen harms monarch larvae. *Nature*, 399 (6733): 214.

Without summary.

Full article available at http://www.nature.com/scitable/content/transgenic-pollen-harms-monarch-larvae-97961

Jesse, L.; Obrycki, J. 2000. Field deposition of Bt transgenic corn pollen: lethal effects on the monarch butterfly. *Oecologia*, 125:241–248. doi: 10.1007/s004420000502.

We present the first evidence that transgenic Bacillus thuringiensis (Bt) corn pollen naturally deposited on Asclepias syriaca; common milkweed, in a corn field causes significant mortality of Danaus plexippus L. (Lepidoptera: Danaidae) larvae. Larvae feeding for 48 h on A. syriaca plants naturally dusted with pollen from Bt corn plants suffered significantly higher rates of mortality at 48 h (20±3%) compared to larvae feeding on leaves with no pollen (3±3%), or feeding on leaves with non-Bt pollen (0%). Mortality at 120 h of D. plexippus larvae exposed to 135 pollen grains/cm(2) of transgenic pollen for 48 h ranged from 37 to 70%. We found no sub-lethal effects on D. plexippus adults reared from larvae that survived a 48-h exposure to three concentrations of Bt pollen. Based on our quantification of the wind dispersal of this pollen beyond the edges of agricultural fields, we predict that the effects of transgenic pollen on D. plexippus may be observed at least 10 m from transgenic field borders. However, the highest larval mortality will likely occur on A. syriaca plants in corn fields or within 3 m of the edge of a transgenic corn field. We conclude that the ecological effects of transgenic insecticidal crops need to be evaluated more fully before they are planted over extensive areas.

http://www.ncbi.nlm.nih.gov/pubmed/24595835

Pleasants, J.; Hellmich, R.; Dively, G.; Sears, M.; Stanley-Horn, D.; Mattila, H.; Foster, J.; Clark, P.; Jones, G. 2001. Corn pollen deposition on milkweeds in and near cornfields. *PNAS*, vol. 98, no. 21, 11919–11924.

The density of corn pollen on leaves of milkweed plants inside and outside of cornfields was measured in several studies from different localities. The purpose was to obtain a representative picture of naturally occurring pollen densities to provide a perspective for laboratory and field studies of monarch larvae feeding on milkweed leaves with Bt corn pollen. Pollen density was highest (average 170.6 grains per cm2) inside the cornfield and was progressively lower from the field edge outward, falling to 14.2 grains per cm2 at 2 m. Inside the cornfield, and for each distance from the field edge, a frequency distribution is presented showing the proportion of leaf samples with different pollen densities. Inside cornfields, 95% of leaf samples had pollen densities below 600 grains per cm2 and the highest pollen density observed was 1400 grains per cm2, which occurred in a study with a rainless anthesis period. All other studies had rainfall events during the anthesis period. A single rain event can remove 54–86% of the pollen on leaves. Leaves on the upper portion of milkweed plants, where young monarch larvae tend to feed, had only 30–50% of the pollen density levels of middle leaves. In order to accurately interpret results of studies that examine the effects of Bt corn pollen on monarch butterfly larvae it is necessary to know the range and distribution of naturally occurring pollen densities on milkweed leaves. This provides a perspective on both laboratory and field studies in which monarch larvae feed on milkweed leaves with Bt corn pollen (1, 2). It lets us determine how frequently the pollen densities observed in these studies would occur in nature. The studies reported here contribute to the exposure characterization necessary for assessing the risk of Bt corn pollen to monarch butterflies. In particular, this paper describes the densities of corn pollen on milkweed leaves during corn anthesis for a number of geographic locations and under a variety of environmental conditions. We describe the pollen densities (pollen grains per cm2) that were found on leaves of milkweed plants within cornfields as well as near cornfields because corn pollen is wind-dispersed at least 60 m (3) and possibly more than 200 m (4). These data are used in a companion paper (5) on the results of laboratory studies on the responses of monarch larvae fed milkweed leaves with different densities of artificially applied Bt corn pollen. These data are also used in a second companion paper (6) to provide a frame of reference for the Bt pollen densities found in field trials of larvae feeding on milkweed leaves. Finally, these data are used in a summary companion paper (7) that provides a full risk assessment of monarchs and Bt corn pollen. In addition to characterizing naturally occurring pollen densities, we examined several factors that affect pollen deposition on milkweed leaves, including position of a leaf on the plant and rainfall.

Full article available at http://www.pnas.org/content/98/21/11919.full

Stanley-Horn, D.; Dively, G.; Hellmich, R.; Mattila, H.; Sears, M.; Rose, R.; Jesse, L.; Losey, J.; Obrycki, J.; Lewis, L. 2001. Assessing the impact of Cry1Ab-expressing corn pollen on monarch butterfly larvae in field studies. *PNAS*, 98 (21): 11931-11936.

Survival and growth of monarch larvae, Danaus plexippus (L.), after exposure to either Cry1Ab-expressing pollen from three Bacillus thuringiensis (Bt) corn (Zea mays L.) events differing in toxin expression or to the insecticide, λ-cyhalothrin, were examined in field studies. First instars exposed to low doses (≈22 grains per cm2) of event-176 pollen gained 18% less weight than those exposed to Bt11 or Mon810 pollen after a 5-day exposure period. Larvae exposed to 67 pollen grains per cm2 on milkweed leaves from within an event-176 field exhibited 60% lower survivorship and 42% less weight gain compared with those exposed to leaves from outside the field. In contrast, Bt11 pollen had no effect on growth to adulthood or survival of first or third instars exposed for 5 days to ≈55 and 97 pollen grains per cm2, respectively. Similarly, no differences in larval survivorship were observed after a 4-day exposure period to leaves with 504–586 (within fields) or 18–22 (outside the field) pollen grains per cm2 collected from Bt11 and non-Bt sweet-corn fields. However, survivorship and weight gain were drastically reduced in non-Bt fields treated with λ-cyhalothrin. The effects of Bt11 and Mon810 pollen on the survivorship of larvae feeding 14 to 22 days on milkweeds in fields were negligible. Further studies should examine the lifetime and reproductive impact of Bt11 and Mon810 pollen on monarchs after long-term exposure to naturally deposited pollen.

Full article available at http://www.pnas.org/content/98/21/11931.full

Dively, G.; Rose, R.; Sears, M.; Hellmich, R.; Stanley-Horn, D. Calvin, D.; Russo, J.; Anderson, P. 2004. Effects on monarch butterfly larvae (*Lepidoptera*: *Danaidae*) after continuous exposure to Cry1Ab expressing corn during anthesis. *Environmental Entomology*, 33: 1116-1125.

Effects on monarch butterfly, Danaus plexippus L., after continuous exposure of larvae to natural deposits of Bacillus thuringiensis (Bt) and non-Bt pollen on milkweed, were measured in five studies. First instars were exposed at 3–4 and 6–7 d after initial anthesis, either directly on milkweed plants in commercial cornfields or in the laboratory on leaves collected from milkweeds in corn plots. Pollen exposure levels ranging from 122 to 188 grains/cm2/d were similar to within-field levels that monarch butterfly populations might experience in the general population of cornfields. Results indicate that 23.7% fewer larvae exposed to these levels of Bt pollen during anthesis reached the adult stage. A risk assessment procedure used previously was updated with a simulation model estimating the proportion of second-generation monarch butterflies affected. When considered over the entire range of the Corn Belt, which represents only 50% of the breeding population, the risk to monarch butterfly larvae associated with long-term exposure to Bt corn pollen is 0.6% additional mortality. Exposure also prolonged the developmental time of larvae by 1.8 d and reduced the weights of both pupae and adults by 5.5%. The sex ratio and wing length of adults were unaffected. The ecological significance of these sublethal effects is discussed relative to generation mortality and adult performance.

Full article available at https://goo.gl/wDtmww

4.2.2 Spread of transgenic corn in Mexico

In November 2001, American scientists Quist and Chapela published a groundbreaking article, denouncing the presence of transgenes in landrace corn varieties in Mexico (center of origin and diversity of species), while the country was in moratorium stadium for commercial use of transgenic plants. This announcement triggered a series of virulent articles in the scientific literature. Researchers and their methods have been criticized, with claims reverberating through various means of scientific communication, citing the lack of sufficient evidence to support the conclusions cited in that article. After seven years, other teams of researchers have confirmed the original statements with respect to the spread of transgenes in the country, in isolated areas of landrace corn planting, where GMOs were banned. The importance of the original article, to discuss the inevitability of gene contamination, was not rescued and specific losses in terms of transgene flow toward the regions of origin of maize will never be accounted for.

Quist, D.; Chapela, I. 2001. Transgenic DNA introgressed into traditional maize landraces in Oaxaca, Mexico. *Nature*, Vol 414.

Concerns have been raised about the potential effects of transgenic introductions on the genetic diversity of crop landraces and wild relatives in areas of crop origin and diversification, as this diversity is considered essential for global food security. Direct effects on non-target species1, 2, and the possibility of unintentionally transferring traits of ecological relevance onto landraces and wild relatives have also been sources of concern3, 4. The degree of genetic connectivity between industrial crops and their progenitors in landraces and wild relatives is a principal determinant of the evolutionary history of crops and agroecosystems throughout the world5, 6. Recent introductions of transgenic DNA constructs into agricultural fields provide unique markers to measure such connectivity. For these reasons, the detection of transgenic DNA in crop landraces is of critical importance. Here we report the presence of introgressed transgenic DNA constructs in native maize landraces grown in remote mountains in Oaxaca, Mexico, part of the Mesoamerican centre of origin and diversification of this crop.

Full article available at http://stopogm.net/sites/stopogm.net/files/QuistChapela.pdf

COM - Dalton, R. 2001. Transgenic corn found growing in Mexico. *Nature*, Vol 413.

Without summary.

http://www.nature.com/nature/journal/v413/n6854/full/413337b0.html

COM – Mann, C. 2002. Has GM corn 'invaded' Mexico? *Science*, Vol 295.

Without summary.

Full article available at http://www.charlesmann.org/articles/Transgenic-maize-Mexico-Science1.pdf

COM - Quist, D.; Chapela, I. 2002. Quist and Chapela reply. *Nature*, Vol 416.

Without summary.

http://www.nature.com/nature/journal/v416/n6881/full/nature740.html

COM - Dalton, R. 2008. Modified genes spread to local maize. *Nature*, Vol 456.

Without summary.

Full article available at http://www.nature.com/news/2008/081112/full/456149a.html

Piñeyro-Nelson, A.; Heerwaarden, J.; Perales, H.; Serratos-Hernandez, J.; Rangel, A.; Hufford, M.; Gepts, P.; Garay-Arroyo, A.; Rivera-Bustamante, R.; Álvarez-Buylla, E. 2009. Transgenes in Mexican maize: Molecular evidence and methodological considerations for GMO detection in landrace populations. *Molecular Ecology*, 18: 750-61.

A possible consequence of planting genetically modified organisms (GMOs) in centres of crop origin is unintended gene flow into traditional landraces. In 2001, a study reported the presence of

the transgenic 35S promoter in maize landraces sampled in 2000 from the Sierra Juarez of Oaxaca, Mexico. Analysis of a large sample taken from the same region in 2003 and 2004 could not confirm the existence of transgenes, thereby casting doubt on the earlier results. These two studies were based on different sampling and analytical procedures and are thus hard to compare. Here, we present new molecular data for this region that confirm the presence of transgenes in three of 23 localities sampled in 2001. Transgene sequences were not detected in samples taken in 2002 from nine localities, while directed samples taken in 2004 from two of the positive 2001 localities were again found to contain transgenic sequences. These findings suggest the persistence or re-introduction of transgenes up until 2004 in this area. We address variability in recombinant sequence detection by analyzing the consistency of current molecular assays. We also present theoretical results on the limitations of estimating the probability of transgene detection in samples taken from landraces. The inclusion of a limited number of female gametes and, more importantly, aggregated transgene distributions may significantly lower detection probabilities. Our analytical and sampling considerations help explain discrepancies among different detection efforts, including the one presented here, and provide considerations for the establishment of monitoring protocols to detect the presence of transgenes among structured populations of landraces.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3001031/

REV - Snow, A. 2009. Unwanted transgenes re-discovered in Oaxacan maize. *Molecular Ecology*, 18: 569-571.

Transgenic [GM (genetically modified)] maize is shipped all over the world, and its ability to germinate, grow, and hybridize with local landrace s of the crop has generated tremendous scientific, social, and political controversy. The great genetic diversity of farm er-produced landraces represents a vital resource for subsistence farmers, future crop breeding, and cultural heritage preservation. Although the Mexican government banned GM maize cultivation in 1998, vast quantities of living GM grain are imported from the USA and seeds can easily enter the country by other routes. In 2000, Quist & Chapela (2001) discovered transgenes in four ears of landrace maize and in seeds from a government-sponsored Distribution Conasupo Sociedad Anonima (DICONSA) grain distribution centre in Oaxaca. Their controversial paper in Nature set off an explosion of publicity and speculation about how widely these novel genetic elements had proliferated, and what the consequences of ubiquitous gene flow might be. However, a more extensive survey of this region failed to detect transgenes in 2003 and 2004 (Ortiz-García et al. 2005a), suggesting that transgenic plants were rare or absent in the sampled fields. Here, Pineyro-Nelson et al. (2008) provide a valuable counterpoint to that survey, resolving apparent contradictions in the literature and raising the bar for subsequent studies of immigrating transgenes. They show that transgenes were present in Oaxaca in both 2001 and 2004. Their paper explains how sampling methods, statistical analyses, and problems with analytical techniques can lead to inconsistent estimates of transgene frequencies in maize populations. This is a must-read paper for those who follow genetically modified organisms (GMO) biosafety research.

http://www.ncbi.nlm.nih.gov/pubmed/19215581

4.2.3 Toxicity associated with the consumption of NK603 corn

In November 2012, Seralini et al published a critical article on toxicological impacts associated with the consumption of NK603 corn, taking into account their incorporation into medium and long-term diets in rats. The study not only pointed to hepatic

and renal risks and relevant endocrine disruption (responsible for the formation of tumors) as a result of consumption of corn treated with the associated herbicide (Roundup's trade formula), the herbicide itself and GM corn without herbicide (indicative of specific risks of genetic modification), but also validated the importance of conducting studies lasting more than three months - standard currently accepted by regulatory agencies – observing the first severe symptoms from the fourth month.

The study findings sparked violent criticism. The reaction was concentrated in interpreting the results and methodology, extending the personal issues and defamation of the authors and its research institute, the Center for Research and Independent Information on Genetic Engineering (CRIIGEN, French acronym). The articulation created to reduce the impact of the study incorporated pressures on the journal that published the article, leading to changes in its editorial board. The jornal played down the findings, the study was accused of unjustified deficiencies and get retracted. This disclaimer generated new controversy in the scientific community - including by certain authors openly pro-biotechnology. Among the weaknesses was the fact that the criticisms relied upon the presentation of "inconclusive results". Consider, in this point, that this is the condition most often seen in the literature[68]. Finally, the study was republished in another journal, appearing again in the database of validated scientific literature.

Séralini, G-E.; Clair, E.; Mesnage, R.; Gress, S.; Defarge, N.; Malatesta, M.; Hennequin, D.; Vendômois, J. 2012. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. *Food and Chemical* Toxicology, 50, 4221– 4231. Retraction in *Food and Chemical Toxicology*, 2014, 63:244.

Without summary.

Disclaimer announcement available at https://goo.gl/fIF6fS

68 Only four reasons are officially accepted as justification for the retraction of a scientific study: fraud, lack of ethics, plagiarism or results already published. "Inconclusive results" are part of routine incorporated to the method, having significant relevance in the processes of construction of scientific knowledge.

Séralini, G-E.; Mesnage, R.; Defarge, N.; Gress, S.; Hennequin, D.; Clair, E.; Malatesta, M.; Vendômois, J. 2013. Answers to critics: Why there is a long term toxicity due to a Roundup-tolerant genetically modified maize and to a Roundup herbicide. *Food and Chemical Toxicology*, 53: 476-83.

Our recent work (Séralini et al., 2012) remains to date the most detailed study involving the life-long consumption of an agricultural genetically modified organism (GMO). This is true especially for NK603 maize for which only a 90-day test for commercial release was previously conducted using the same rat strain (Hammond et al., 2004). It is also the first long term detailed research on mammals exposed to a highly diluted pesticide in its total formulation with adjuvants. This may explain why 75% of our first criticisms arising within a week, among publishing authors, come from plant biologists, some developing patents on GMOs, even if it was a toxicological paper on mammals, and from Monsanto Company who owns both the NK603 GM maize and Roundup herbicide (R). Our study has limits like any one, and here we carefully answer to all criticisms from agencies, consultants and scientists, that were sent to the Editor or to ourselves. At this level, a full debate is biased if the toxicity tests on mammals of NK603 and R obtained by Monsanto Company remain confidential and thus unavailable in an electronic format for the whole scientific community to conduct independent scrutiny of the raw data. In our article, the conclusions of long-term NK603 and Roundup toxicities came from the statistically highly discriminant findings at the biochemical level in treated groups in comparison to controls, because these findings do correspond in an blinded analysis to the pathologies observed in organs, that were in turn linked to the deaths by anatomopathologists. GM NK603 and R cannot be regarded as safe to date.

Full article available at http://www.sciencedirect.com/science/article/pii/S0278691512008149

Meyer, H.; Hilbeck, A. 2013. Rat feeding studies with genetically modified maize – a comparative evaluation of applied methods and risk assessment standards. *Environmental Sciences Europe*, 25:33 (doi:10.1186/2190-4715-25-33).

A 2-year rat feeding study with genetically modified NK603 maize sparked an international scientific and public debate as well as policy responses by the European Commission. The European Food Safety Authority (EFSA) evaluated the study as defective based on conceptual and methodological shortcomings by retroactive application of the recommendations of its recent guidance on 90-day feeding studies. Our comparative analysis of the three relevant NK603 publications, including a 90-day feeding study of Monsanto, showed that all of them satisfy or fail to satisfy the EFSA evaluation criteria to a comparable extent; the rejection of only one of the papers is, thus, not scientifically justified. We also show that EFSA's criteria are not standard practice in 21 other rat feeding studies lasting at a minimum of 12 months. The review reveals critical double standards in the evaluation of feeding studies submitted as proof of safety for regulatory approval to EFSA. We specifically argue that the current approach to declare statistically significant differences between genetically modified organisms and its parents as 'biologically irrelevant' based on additional reference controls lacks scientific rigor and legal justification in the European Union (EU) system. Only recently, the EU authorities started building up an implementing system based on its own legislation and supportive of the EU approach to risk assessment in the context of technology assessment. Until these issues are resolved, we do not expect that neither the public nor the scientific debate will subside.

Full article available at http://www.enveurope.com/content/25/1/33

Heinemann, J. 2013. Food and Chemical Toxicology. *Food and Chemical Toxicology*, (53), 442.

Without summary.

Full article available at http://www.sciencedirect.com/science/article/pii/S0278691512008009

Séralini, G-E.; Clair, E.; Mesnage, R.; Gress, S.; Defarge, N.; Malatesta, M.; Hennequin, D.; Vendômois, J. 2014. Republished study: long-term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. *Environmental Sciences Europe*, 26:14.

Background: The health effects of a Roundup-tolerant NK603 genetically modified (GM) maize (from 11% in the diet), cultivated with or without Roundup application and Roundup alone (from 0.1 ppb of the full pesticide containing glyphosate and adjuvants) in drinking water, were evaluated for 2 years in rats. This study constitutes a follow-up investigation of a 90-day feeding study conducted by Monsanto in order to obtain commercial release of this GMO, employing the same rat strain and analyzing biochemical parameters on the same number of animals per group as our investigation. Our research represents the first chronic study on these substances, in which all observations including tumors are reported chronologically. Thus, it was not designed as a carcinogenicity study. We report the major findings with 34 organs observed and 56 parameters analyzed at 11 time points for most organs.
Results: Biochemical analyses confirmed very significant chronic kidney deficiencies, for all treatments and both sexes; 76% of the altered parameters were kidney-related. In treated males, liver congestions and necrosis were 2.5 to 5.5 times higher. Marked and severe nephropathies were also generally 1.3 to 2.3 times greater. In females, all treatment groups showed a two- to threefold increase in mortality, and deaths were earlier. This difference was also evident in three male groups fed with GM maize. All results were hormone- and sex-dependent, and the pathological profiles were comparable. Females developed large mammary tumors more frequently and before controls; the pituitary was the second most disabled organ; the sex hormonal balance was modified by consumption of GM maize and Roundup treatments. Males presented up to four times more large palpable tumors starting 600 days earlier than in the control group, in which only one tumor was noted. These results may be explained by not only the non-linear endocrine-disrupting effects of Roundup but also by the overexpression of the EPSPS transgene or other mutational effects in the GM maize and their metabolic consequences.
Conclusion: Our findings imply that long-term (2 year) feeding trials need to be conducted to thoroughly evaluate the safety of GM foods and pesticides in their full commercial formulations.

Full article available at http://www.enveurope.com/content/26/1/14

COM – Casassus, B. 2014. Paper claiming GM link with tumours republished. *Nature*, doi:10.1038/nature.2014.15463.

Without summary.

Full article available at https://goo.gl/mygwAf

COM - Portier, C.; Goldman, L.; Goldstein, B. 2013. Inconclusive findings: Now you see them, now you don't! *Environmental Health Perspectives*, volume 122, number 2.

Without summary.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3915254/

COM - Séralini, G-E.; Mesnage, R.; Defarge, N.; Vendômois, J. 2014. Conclusiveness of toxicity data and double standards. *Food and Chemical Toxicology*, doi: http://dx.doi.org/10.1016/j.fct.2014.04.018.

Without summary.

Full article available at https://goo.gl/luLFmt

COM - Roberfroid, M. 2014. Letter to the editor. *Food and Chemical Toxicology*, 65, 390.

Without summary.

Full article available at http://www.sciencedirect.com/science/article/pii/S0278691514000039

4.3 Conflicts of interest and guidance of Science

In parallel to the above explained discredit campaigns, the issue of conflict of interest has also a strong presence in the problem of the risks (and their ratings) of transgenic plants for the environment and health. In fact, there are several papers that show correlations between studies which point out absence of risks of transgenic plants and funding sources favorable to the development of biotechnology.

On the other hand, independent researchers have difficulties in performing studies on risks of GM plants, since a significant part of biosafety information tends to be confidential. Those researchers also need authorization from the company which owns the technology to use their material in the tests.

COM – Sharpe, V.; Gurian-Sherman, D. 2003. Competing interests. *Nature Biotechnology*, 21, 1131.

Without summary.

http://www.nature.com/nbt/journal/v21/n10/full/nbt1003-1131a.html

Kvakkestad, V.; Gillund, F.; Kjølberg, K.; Vatn, A. 2007. Scientists' perspectives on the deliberate release of GM crops. *Environmental Values*, 16(1): 79–104.

In this paper we analyse scientists' perspectives on the release of genetically modified (GM) crops into the environment, and the relationship between their perspectives and the context that they work within, e.g. their place of employment (university or industry), funding of their research (public or industry) and their disciplinary background (ecology, molecular biology or conventional plant breeding). We employed Q-methodology to examine these issues. Two distinct factors were identified by interviewing 62 scientists. These two factors included 92 per cent of the sample. Scientists in factor 1 had a moderately negative attitude to GM crops and emphasised the uncertainty and ignorance involved, while scientists in factor 2 had a positive attitude to GM crops and emphasised that GM crops are useful and do not represent any unique risks compared to conventional crops. Funding had a significant effect on the perspective held by the scientists in this study. No ecologists were associated with factor 2, while all the scientists employed in the GM-industry were associated with this factor. The strong effects of training and funding might justify certain institutional changes concerning how we organise science and how we make public decisions when new technologies are to be evaluated. Policy makers should encourage more interdisciplinary training and research and they should make sure that representatives of different disciplines are involved in public decisions on new technologies.

http://www.erica.demon.co.uk/EV/EV1605.html

Diels, J.; Cunha, M.; Manaia, C.; Sabugosa-Madeira, B.; Silva, M. 2011. Association of financial or professional conflict of interest to research outcomes on health risks or nutritional assessment studies of genetically modified products. *Food Policy*, 36: 197-203.

Since the first commercial cultivation of genetically modified crops in 1994, the rapidly expanding market of genetically modified seeds has given rise to a multibillion dollar industry. This fast growth, fueled by high expectations towards this new commercial technology and shareholder trust in the involved industry, has provided strong incentives for further research and development of new genetically modified plant varieties. Considering, however, the high financial stakes involved, concerns are raised over the influence that conflicts of interest may place upon articles published in peer-reviewed journals that report on health risks or nutritional value of genetically modified food products. In a study involving 94 articles selected through objective criteria, it was found that the existence of either financial or professional conflict of interest was associated to study outcomes that cast genetically modified products in a favorable light ($p = 0.005$). While financial conflict of interest alone did not correlate with research results ($p = 0.631$), a strong association was found between author affiliation to industry (professional conflict of interest) and study outcome ($p < 0.001$). We discuss these results by comparing them to similar studies on conflicts of interest in other areas, such as biomedical sciences, and hypothesize on dynamics that may help explain such connections.

http://www.sciencedirect.com/science/article/pii/S0306919210001302

REV – Domingo, J.; Bordonaba, J. 2011. A literature review on the safety assessment of genetically modified plants. *Environment International*, 37. 734-42.

In recent years, there has been a notable concern on the safety of genetically modified (GM) foods/plants, an important and complex area of research, which demands rigorous standards. Diverse groups including consumers and environmental Non Governmental Organizations (NGO) have suggested that all GM foods/plants should be subjected to long-term animal feeding studies before approval for human consumption. In 2000 and 2006, we reviewed the information published in international scientific journals, noting that the number of references concerning human and animal toxicological/health risks studies on GM foods/plants was very limited. The main goal of the present review was to assess the current state-of-the-art regarding the potential adverse effects/safety assessment of GM plants for human consumption. The number of citations found in databases (PubMed and Scopus) has dramatically increased since 2006. However, new information on products such as potatoes, cucumber, peas or tomatoes, among others was not available. Corn/maize, rice, and soybeans were included in the present review. An equilibrium in the number research groups suggesting, on the basis of their studies, that a number of varieties of GM products (mainly maize and soybeans) are as safe and nutritious as the respective conventional non-GM plant, and those raising still serious concerns, was currently observed. Nevertheless, it should be noted that most of these studies have been conducted by biotechnology companies responsible of commercializing these GM plants. These findings suggest a notable advance in comparison with the lack of studies published in recent years in scientific journals by those companies. All this recent information is herein critically reviewed.

http://www.ncbi.nlm.nih.gov/pubmed/21296423

Séralini, G-E.; Mesnage, R.; Defarge, N.; Gress, S.; Hennequin, D.; Clair, E.; Malatesta, M.; Vendômois, J. 2013. Answers to critics: Why there is a long term toxicity due to Roundup-tolerant genetically modified maize and to a Roundup herbicide. *Food and Chemical Toxicology*, 53: 476-83.

Our recent work (Séralini et al., 2012) remains to date the most detailed study involving the life-long consumption of an agricultural genetically modified organism (GMO). This is true especially for NK603 maize for which only a 90-day test for commercial release was previously conducted using the same rat strain (Hammond et al., 2004). It is also the first long term detailed research on mammals exposed to a highly diluted pesticide in its total formulation with adjuvants. This may explain why 75% of our first criticisms arising within a week, among publishing authors, come from plant biologists, some developing patents on GMOs, even if it was a toxicological paper on mammals, and from Monsanto Company who owns both the NK603 GM maize and Roundup herbicide (R). Our study has limits like any one, and here we carefully answer to all criticisms from agencies, consultants and scientists, that were sent to the Editor or to ourselves. At this level, a full debate is biased if the toxicity tests on mammals of NK603 and R obtained by Monsanto Company remain confidential and thus unavailable in an electronic format for the whole scientific community to conduct independent scrutiny of the raw data. In our article, the conclusions of long-term NK603 and Roundup toxicities came from the statistically highly discriminant findings at the biochemical level in treated groups in comparison to controls, because these findings do correspond in an blinded analysis to the pathologies observed in organs, that were in turn linked to the deaths by anatomopathologists. GM NK603 and R cannot be regarded as safe to date.

Full article available at http://www.sciencedirect.com/science/article/pii/S0278691512008149

Nielsen, K. 2013. Biosafety data as confidential business information. *PLOS Biology*, 11(3): e1001499.

Confidential business information (CBI) is a necessary tool to protect commercial interests in the rapidly developing field of gene technology. CBI is also often claimed for documentation and materials supporting the biosafety assessments of genetically modified organisms (GMOs) intended for environmental release, food, and feed use. However, such claims oftentimes marginally serve their legitimate purpose to protect commercial interests and unnecessarily limit transparency and public peer review of data submitted to regulatory authorities. CBI and proprietary claims also restrict access to transgene sequence data, transgenic seeds, and other GMO materials, which precludes the development of independent research and monitoring strategies. In the long run, such claims are counterproductive to the safe and responsible commercial development of GM technology as they hinder the accumulation of biosafety data in the open, peer-reviewed literature, which is needed for both public and scientific consensus-building on safety issues and for improvements to the risk-assessment procedure itself. The increasing recognition of conflicts of interest as an invariable part of market-oriented safety-data production, interpretation, and risk communication also calls for transparency and open access to safety-related data and assessments.

Full article available at https://goo.gl/KBaQEl

5 Social conflicts and food security

The absence of scientific consensus goes beyond the issue of biological risks related to the expansion of GMPs, on the environment and human health. Issues such as costs and benefits, opportunity and convenience for society and their populations are also dispute objects. The issue of economic profitability for different profiles of farmers and at the advantages promised by industries in their contrast with real world data feed the controversy. Among the most repeated assertions, at the center of disputes, there are the hypothesis of essentiality of GMPs for the global fight against hunger (incorporating political developments relating to food security and sovereignty), and the relevance of developing biofortified plants through genetic engineering.

The following articles indicate the opposite reality to that promised by the biotechnology sector. Softening and relativizing the supposed benefits of these technologies on all these dimensions, the literature highlights the superiority of conventional breeding and agroecology-based systems to address the problem of world hunger.

To address these issues, it is necessary to debate on the role of farmers in building demands and the orientation of agricultural research. The official system of research, sponsored by resources of society, seems to move away from its purpose, serving interests that do not respond to the issue of national development, but rather the strategic objectives of biotechnology and its associated industries.

Cavalli, S. 2001. Segurança alimentar: a abordagem dos alimentos transgênicos. *Revista de Nutrição da Puccamp*, 14 (suplemento): 41-46.

O objetivo desta comunicação é discutir a relação entre a segurança alimentar e os alimentos geneticamente modificados. A biotecnologia e a engenharia genética têm sido encaradas como parte da segunda revolução verde, justificando-se, entre outras prerrogativas, o uso de alimentos transgênicos como solução do problema da fome no mundo, sem risco à saúde da população e ao meio ambiente. Face a essa premissa, discute-se a segurança alimentar sob os enfoques qualitativos e quantitativos, destacando as atribuições dos órgãos responsáveis e suas interfaces com alimentos geneticamente modificados. Acredita-se que os alimentos transgênicos não sejam a solução para o problema da fome no mundo.

Full article available at https://goo.gl/ebcsa1

Tucker, G. 2003. Nutritional enhancement of plants. *Current Opinion in Biotechnology*, 14:221–225.

Plants can provide most of the nutrients required in the human diet; however, the major staple crops are often deficient in some of these nutrients. Thus, malnutrition, with respect to micronutrients like vitamin A, iron and zinc, affects >40% of the world's population. Advances in molecular biology are being exploited to produce crops enhanced in these key nutrients. Other nutritional targets include the modification of fatty acid composition and the enhancement of antioxidant levels, particularly carotenoids, such as lycopene, and flavonoids. However, the benefit of these 'biofortified' crops to human nutrition remains to be elucidated.

Full article available at http://www.ncbi.nlm.nih.gov/pubmed/12732325

Lacey, H. 2007. Há alternativas ao uso dos transgênicos? *Novos estudos – CEBRAP*, no.78, São Paulo.

A existência ou não de alternativa ao uso de transgênicos capaz de satisfazer a demanda mundial por alimento e nutrientes é uma questão que permanece aberta à investigação científica. A importância dos transgênicos ainda não está bem fundamentada no conhecimento científico disponível, em parte porque as conquistas e o potencial da agroecologia não foram objeto de atenção científica suficiente.

Full article available at https://goo.gl/5vyv8z

COM - Herren, H. 2008. Food fault lines. *Nature*, 456, 21.

Without summary.

Full article available at http://www.nature.com/nature/journal/v456/n1s/pdf/twas08.21a.pdf

Richards, P.; Bruin-Hoekzema, M.; Hughes, S.; Kudadjie-Freeman, C.; Offei, S.; Struik, P.; Zannou, A. 2009. Seed systems for African food security: linking molecular genetic analysis and cultivator knowledge in West Africa. *Int J Technol Manage*, 45:196–214.

A challenge for African countries is how to integrate new sources of knowledge on plant genetics with knowledge from farmer practice to help improve food security. This paper considers the knowledge content of farmer seed systems in the light of a distinction drawn in artificial intelligence research between supervised and unsupervised learning. Supervised learning applied to seed systems performance has a poor record in Africa. The paper discusses an alternative – unsupervised learning supported by functional genomic analysis. Recent work in West Africa on sorghum, African rice and white yam is described. Requirements for laboratory-based analytical support are outlined. A science-backed 'farmer first' approach – while feasible – will require a shift in policy and funding by major investors.

http://www.inderscience.com/info/inarticle.php?artid=21528

Vanloqueren, G.; Baret, P. 2009. How agricultural research systems shape a technological regime that develops genetic engineering but locks out agroecological innovations. *Research Policy*, 39: 971–83.

Agricultural science and technology (S&T) is under great scrutiny. Reorientation towards more holistic approaches, including agroecology, has recently been backed by a global international assessment of agriculture S&T for development (IAASTD). Understanding the past and current trends of agricultural S&T is crucial if such recommendations are to be implemented. This paper shows how the concepts of technological paradigms and trajectories can help analyse the agricultural S&T landscape and dynamics. Genetic engineering and agroecology can be usefully analysed as two different technological paradigms, even though they have not been equally successful in influencing agricultural research. We used a Systems of Innovation (SI) approach to identify the determinants of innovation (the factors that influence research choices) within agricultural research systems. The influence of each determinant is systematically described (e.g. funding priorities, scientists' cognitive and cultural routines etc.). As a result of their interactions, these determinants construct a technological regime and a lock-in situation that hinders the development of agroecological engineering. Issues linked to breaking out of this lock-in situation are finally discussed.

http://www.sciencedirect.com/science/article/pii/S0048733309000614

Howard, P. 2009. Visualizing consolidation in the global seed industry: 1996–2008. *Sustainability*, 1, 1266-1287; doi:10.3390/su1041266.

The commercial seed industry has undergone tremendous consolidation in the last 40 years as transnational corporations entered this agricultural sector, and acquired or merged with competing firms. This trend is associated with impacts that constrain the opportunities for renewable agriculture, such as reductions in seed lines and a declining prevalence of seed saving. To better characterize the current structure of the industry, ownership changes from 1996 to 2008 are represented visually

with information graphics. Since the commercialization of transgenic crops in the mid-1990s, the sale of seeds has become dominated globally by Monsanto, DuPont and Syngenta. In addition, the largest firms are increasingly networked through agreements to cross-license transgenic seed traits.

Full article available at http://www.mdpi.com/2071-1050/1/4/1266

REV - Stone, G. 2010. The anthropology of genetically modified crops. *Annual Review of Anthropology*, 39: 381– 400.

By late in the twentieth century, scientists had succeeded in manipulating organisms at the genetic level, mainly by gene transfer. The major impact of this technology has been seen in the spread of genetically modified (GM) crops, which has occurred with little controversy in some areas and with fierce controversy elsewhere. GM crops raise a very wide range of questions, and I address three areas of particular interest for anthropology and its allied fields. First are the political economic aspects of GM, which include patenting of life forms and new relationships among agriculture, industry, and the academy. Second is the wide diversity in response and resistance to the technology. Third is the much-debated question of GM crops for the developing world. This analysis is approached first by determining what controls research agendas and then by evaluating actual impacts of crops to date.

http://www.annualreviews.org/doi/abs/10.1146/annurev.anthro.012809.105058

COM – Tirado, R.; Johnston, P. 2010. Food security: GM crops threaten biodiversity. *Science*, 328(5975):170-1. doi: 10.1126/science.328.5975.170-a.

Without summary.

http://www.ncbi.nlm.nih.gov/pubmed/20378798

Stone, G.; Glover, D. 2011. Genetically modified crops and the 'food crisis': discourse and material impacts. *Development in Practice*, 21:4-5, 509-516.

Uma onda de reportagens na mídia e argumentações retóricas apontaram as culturas geneticamente modificadas como uma solução para a "crise alimentar global" que se manifestou com o aumento repentino nos preços mundiais dos alimentos durante 2007–08. Argumentava-se em geral sobre o potencial das tecnologias GM de lidar com a crise, mesmo embora as culturas e características úteis tipicamente invocadas tivessem ainda de ser desenvolvidas e apesar do fato do progresso real ter sido alcançado na verdade utilizando-se cultivos convencionais. O caso ilustra vividamente o uso instrumental da retórica da crise alimentar para promover culturas GM.

https://goo.gl/5xOic1

Henn, M. 2011. The speculator's bread: What is behind rising food prices? *EMBO Reports*, 12(4): 296–301.

Speculators increasingly invest into food markets for financial gain, with potentially devastating consequences for millions of poor people who cannot afford food at inflated prices.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3077254/

Birch, A.; Begg, G.; Squire, G. 2011. How agro-ecological research helps to address food security issues under new IPM and pesticide reduction policies for global crop production systems. *Journal of Experimental Botany*, 62, *3251-3261.*

Drivers behind food security and crop protection issues are discussed in relation to food losses caused by pests. Pests globally consume food estimated to feed an additional one billion people. Key drivers include rapid human population increase, climate change, loss of beneficial on-farm biodiversity, reduction in per capita cropped land, water shortages, and EU pesticide withdrawals under policies relating to 91/414 EEC. IPM (Integrated Pest Management) will be compulsory for all EU agriculture by 2014 and is also being widely adopted globally. IPM offers a 'toolbox' of complementary crop- and region-specific crop protection solutions to address these rising pressures. IPM aims for more sustainable solutions by using complementary technologies. The applied research challenge now is to reduce selection pressure on single solution strategies, by creating additive/synergistic interactions between IPM components. IPM is compatible with organic, conventional, and GM cropping systems and is flexible, allowing regional fine-tuning. It reduces pests below economic thresholds utilizing key 'ecological services', particularly biocontrol. A recent global review demonstrates that IPM can reduce pesticide use and increase yields of most of the major crops studied. Landscape scale 'ecological engineering', together with genetic improvement of new crop varieties, will enhance the durability of pest-resistant cultivars (conventional and GM). IPM will also promote compatibility with semiochemicals, biopesticides, precision pest monitoring tools, and rapid diagnostics. These combined strategies are urgently needed and are best achieved via multi-disciplinary research, including complex spatio-temporal modelling at farm and landscape scales. Integrative and synergistic use of existing and new IPM technologies will help meet future food production needs more sustainably in developed and developing countries, in an era of reduced pesticide availability. Current IPM research gaps are identified and discussed.

Full article available at https://goo.gl/ygtNqo

Jacobsen, S-E.; Sørensen, M.; Pedersen, S.; Weiner, J. 2013. Feeding the world: genetically modified crops versus agricultural biodiversity. *Agronomy for Sustainable Development*, Volume 33, Issue 4, pp 651-662.

The growing demand for food poses major challenges to humankind. We have to safeguard both biodiversity and arable land for future agricultural food production, and we need to protect genetic diversity to safeguard ecosystem resilience. We must produce more food with less input, while deploying every effort to minimize risk. Agricultural sustainability is no longer optional but mandatory. There is still an on-going debate among researchers and in the media on the best strategy to keep pace with global population growth and increasing food demand. One strategy favors the use of genetically modified (GM) crops, while another strategy focuses on agricultural biodiversity. Here, we discuss two obstacles to sustainable agriculture solutions. The first obstacle is the claim that genetically modified crops are necessary if we are to secure food production within the next decades. This claim has no scientific support, but is rather a reflection of corporate interests. The second obstacle is the resultant shortage of research funds for agrobiodiversity solutions in comparison with funding for research in genetic modification of crops. Favoring biodiversity does not exclude any future biotechnological contributions, but favoring biotechnology threatens future biodiversity resources. An objective review of current knowledge places GM crops far down the list of potential solutions in the coming decades. We conclude that much of the research funding currently available for the development of GM crops would be much better spent in other research areas of plant science, e.g., nutrition, policy research, governance, and solutions close to local market conditions if the goal is to provide sufficient food for the world's growing population in a sustainable way.

Full article available at http://www.ensser.org/fileadmin/files/2013_Jacobsen-etal.pdf

Far from pretending to provide an exhaustive list of the studies and the results obtained through conventional breeding, to generate biofortified plants and new varieties adapted to the agronomic and environmental challenges in these accelerated times of climate change (notably in case of agroecological-based systems), we mention below some concrete cases and relevant discussions on the topic.

REV - Pretty, J.; Morison, J.; Hine, R. 2003. Reducing food poverty by increasing agricultural sustainability in developing countries. *Agriculture, Ecosystems & Environment*; Volume 95, Issue 1, Pages 217–234.

We examined the extent to which farmers have improved food production in recent years with low cost, locally available and environmentally sensitive practices and technologies. We analysed by survey during 1999–2000 208 projects in 52 developing countries, in which 8.98 million farmers have adopted these practices and technologies on 28.92 million hectares, representing 3.0% of the 960 million hectares of arable and permanent crops in Africa, Asia and Latin America. We found improvements in food production occurring through one or more of four mechanisms: (i) intensification of a single component of farm system; (ii) addition of a new productive element to a farm system; (iii) better use of water and land, so increasing cropping intensity; (iv) improvements in per hectare yields of staples through introduction of new regenerative elements into farm systems and new locally appropriate crop varieties and animal breeds. The 89 projects with reliable yield data show an average per project increase in per hectare food production of 93%. The weighted average increases across these projects were 37% per farm and 48% per hectare. In the 80 projects with small (<5 ha) farms where cereals were the main staples, the 4.42 million farms on 3.58 million hectares increased household food production by 1.71 t per year. We report on the practices and technologies that have led to these increases: increased water use efficiency, improvements to soil health and fertility, and pest control with minimal or zero-pesticide use. This research reveals promising advances in the adoption of practices and technologies that are likely to be more sustainable, with substantial benefits for the rural poor. With further explicit support, particularly through national policy reforms and better markets, these improvements in food security could spread to much larger numbers of farmers and rural people in the coming decades.

http://www.sciencedirect.com/science/article/pii/S0167880902000877

Zhu, Y.; Chen, H.; Fan, J.; Wang, Y.; Li, Y.; Chen, J.; Fan, J.; Yang, S.; Hu, L.; Leung, H.; Mew, T.; Teng, P.; Wang, Z.; Mundt, C. 2000. Genetic diversity and disease control in rice. *Nature*, 17, 406: 718–722.

Crop heterogeneity is a possible solution to the vulnerability of monocultured crops to disease. Both theory and observation indicate that genetic heterogeneity provides greater disease suppression when used over large areas, though experimental data are lacking. Here we report a unique cooperation among farmers, researchers and extension personnel in Yunnan Province, China—genetically diversified rice crops were planted in all the rice fields in five townships in 1998 and ten townships in 1999. Control plots of monocultured crops allowed us to calculate the effect of diversity on the severity of rice blast, the major disease of rice. Disease-susceptible rice varieties planted in mixtures with resistant varieties had 89% greater yield and blast was 94% less severe than when they were grown in monoculture. The experiment was so successful that fungicidal sprays were no longer

applied by the end of the two-year programme. Our results support the view that intraspecific crop diversification provides an ecological approach to disease control that can be highly effective over a large area and contribute to the sustainability of crop production.

http://www.ncbi.nlm.nih.gov/pubmed/10963595

COM – Xu, K.; Xu, X.; Fukao, T.; Canlas, P.; Maghirang-Rodriguez, R.; Heuer, S.; Ismail, A.; Bailey-Serres, J.; Ronald, P.; Mackill, D. 2006. *Sub1A* is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature*, 442(7103): 705-708.

Without summary.

http://www.nature.com/nature/journal/v442/n7103/full/nature04920.html

Virk, D.; Steele, K.; Witcomb, J. 2007. Mass and line selection can produce equally uniform rice varieties. *Field Crops Research*, 100 (2–3): 341–7.

We compared intra-varietal variation of two rice varieties (Ashoka 200F and Ashoka 900F) produced by a very simple bulk breeding method (mass selection with no line selection at any stage) with one line-selected variety (Ashoka 228), all derived from the same cross. Their parents, the upland variety Kalinga III and the irrigated transplanted medium-lowland variety IR64, both originated through line selection and were used as control varieties. Panicle-to-row progenies of all the varieties were evaluated in eastern India under irrigation in the dry season and in the rainy season using two water regimes; entirely rainfed or with supplementary irrigation. Intra-varietal variation for quantitative traits, irrespective of the method of breeding, was low. Only Ashoka 200F had significant variation for grain yield, days to flowering and plant height but only in the dry season. This season was not the target of the breeding programme and selection would be unlikely to produce a worthwhile response for the rainy season. At 43 SSR loci there was more genetic variation between lines within the bulk-selected varieties than within Ashoka 228. Kalinga III was the least variable variety while IR64 had less heterozygosity but greater heterogeneity than the two bulk-selected varieties. Despite the greater simplicity of the method, mass selection in bulk populations produced varieties that met the distinctness, uniformity and stability (DUS) criteria for seed certification in India. Such uniformity was achieved because of the high selection pressures applied to the bulk. We conclude that this very simple bulk-population breeding approach is highly cost-effective and produces sufficient seed for wide testing earlier than any alternative method.

http://www.sciencedirect.com/science/article/pii/S0378429006001821

Collard, B.; Mackill, D. 2008. Marker-assisted selection: An approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363(1491): 557-572.

DNA markers have enormous potential to improve the efficiency and precision of conventional plant breeding via marker-assisted selection (MAS). The large number of quantitative trait loci (QTLs) mapping studies for diverse crops species have provided an abundance of DNA marker-trait associations. In this review, we present an overview of the advantages of MAS and its most widely used applications in plant breeding, providing examples from cereal crops. We also consider reasons why MAS has had only a small impact on plant breeding so far and suggest ways in which the potential of MAS can be realized. Finally, we discuss reasons why the greater adoption of MAS in the future is inevitable, although the extent of its use will depend on available resources, especially for orphan crops, and may be delayed in less-developed countries. Achieving a substantial impact on crop improvement by MAS represents the great challenge for agricultural scientists in the next few decades.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2610170/

Singh, A.; Ansari, M.; Pareek, A.; Singla-Pareek, S. 2008. Raising salinity tolerant rice: Recent progress and future perspectives. *Physiology and Molecular Biology of Plants*, 14: 23 – 32.

With the rapid growth in population consuming rice as staple food and the deteriorating soil and water quality around the globe, there is an urgent need to understand the response of this important crop towards these environmental abuses. With the ultimate goal to raise rice plant with better suitability towards rapidly changing environmental inputs, intensive efforts are on worldwide employing physiological, biochemical and molecular tools to perform this task. In this regard, efforts of plant breeders need to be duly acknowledged as several salinity tolerant varieties have reached the farmers field. Parallel efforts from molecular biologists have yielded relevant knowledge related to perturbations in gene expression and proteins during stress. Employing transgenic technology, functional validation of various target genes involved in diverse processes such as signaling, transcription, ion homeostasis, antioxidant defense etc for enhanced salinity stress tolerance has been attempted in various model systems and some of them have been extended to crop plant rice too. However, the fact remains that these transgenic plants showing improved performance towards salinity stress are yet to move from 'lab to the land'. Pondering this, we propose that future efforts should be channelized more towards multigene engineering that may enable the taming of this multigene controlled trait. Recent technological achievements such as the whole genome sequencing of rice is leading to a shift from single gene based studies to genome wide analysis that may prove to be a boon in re-defining salt stress responsive targets.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3550660/

Hattori, Y.; Nagai, K.; Furukawa, S.; Song, X-J.; Kawano, R.; Sakakibara, H.; Wu, J.; Matsumoto, T.; Yoshimura, A.; Kitano, H.; Matsuoka, M.; Mori, H.; Ashikari, M. 2009. The ethylene response factors *SNORKEL1* and *SNORKEL2* allow rice to adapt to deep water. *Nature*, 460: 1026–1030.

Living organisms must acquire new biological functions to adapt to changing and hostile environments. Deepwater rice has evolved and adapted to flooding by acquiring the ability to significantly elongate its internodes, which have hollow structures and function as snorkels to allow gas exchange with the atmosphere, and thus prevent drowning1, 2, 3. Many physiological studies have shown that the phytohormones ethylene, gibberellin and abscisic acid are involved in this response4, 5, 6, 7, 8, but the gene(s) responsible for this trait has not been identified. Here we show the molecular mechanism of deepwater response through the identification of the genes SNORKEL1 and SNORKEL2, which trigger deepwater response by encoding ethylene response factors involved in ethylene signalling. Under deepwater conditions, ethylene accumulates in the plant and induces expression of these two genes. The products of SNORKEL1 and SNORKEL2 then trigger remarkable internode elongation via gibberellin. We also demonstrate that the introduction of three quantitative trait loci from deepwater rice into non-deepwater rice enabled the latter to become deepwater rice. This discovery will contribute to rice breeding in lowland areas that are frequently flooded during the rainy season.

http://www.nature.com/nature/journal/v460/n7258/abs/nature08258.html

Li, S.; Nugroho, A.; Rocheford, T.; White, W. 2010. Vitamin A equivalence of the β-carotene in β-carotene–biofortified maize porridge consumed by women. A*merican Journal of Clinical Nutrition*.

Background: β-Carotene-biofortified maize is being developed through plant breeding as a sustainable agronomic approach to alleviate vitamin A deficiency.
Objective: Our objective was to quantify the vitamin A equivalence of the β-carotene in β-carotene-biofortified maize based on consumption of a single serving of maize porridge.

Design: Six healthy women each consumed three 250-g portions of maize porridge as follows: 1) β-carotene-biofortified maize porridge containing 527 μg (0.98 μmol) total β-carotene, 2) white maize porridge with a β-carotene reference dose containing 595 μg (1.11 μmol) added β-carotene, and 3) white maize porridge with a vitamin A reference dose containing 286 μg retinol activity equivelent (1.00 μmol) added retinyl palmitate. Each portion contained 8.0 g added sunflower oil. The porridges were consumed in random order separated by ≥2 wk. Blood samples were collected over 9 h. Retinyl palmitate was analyzed in plasma triacylglycerol-rich lipoprotein (TRL) fractions by HPLC with coulometric array electrochemical detection.
Results: Mean (± SD) areas under the curve for retinyl palmitate in the TRL fractions (nmol · h) were 24.0 ± 9.4, 89.7 ± 34.7, and 80.1 ± 24.8 after ingestion of the β-carotene-biofortified maize porridge, the white maize porridge with the β-carotene reference dose, and the white maize porridge with the vitamin A reference dose, respectively. On average, 6.48 ± 3.51 μg (mean ± SD) of the β-carotene in β-carotene-biofortified maize porridge and 2.34 ± 1.61 μg of the β-carotene in the reference dose were each equivalent to 1 μg retinol.
Conclusion: β-Carotene in biofortified maize has good bioavailability as a plant source of vitamin A.

Full article available at http://ajcn.nutrition.org/content/92/5/1105.long

COM - Gilbert, N. 2014. Cross-bred crops get fit faster. *Nature*, 292, Vol 513.

Without summary.

Full article available at http://www.nature.com/news/cross-bred-crops-get-fit-faster-1.15940.

Final Considerations

This review of studies in the scientific literature that point to risks and uncertainties concerning the use of transgenic plants on a commercial scale does not claim to be exhaustive. Still, bringing together around 750 published articles that contradict certain aspects of the dominant view in the media and in the regulatory agencies, provides irrefutable evidence of lack of consensus in the scientific community on the subject.

It should be noted the fact that this publication is not intended to support or reaffirm the findings and conclusions presented in these articles. The hypothesis of risks, even if reiterated, do not attest to the existence of real problems, and at the other end, repeated claims of absence of risk based on inconclusive studies do not provide effective security to the consumer.

The purpose of this document is to prove one simple fact: extensive review of scientific studies published in specialized journals allows us to affirm that, within the limits of current scientific knowledge, we cannot conclude to the absence of risks in farming and feeding transgenic plants and parts. Or, in short, it is not possible to say, with seriousness and probity, that the technology involved does not imply significant risks to the health and the environment.

Moreover, this book emphasizes the need of a similar work applied to the systematization of studies published in the scientific literature that conclude the absence of risks and uncertainties associated with the use of transgenic plants on a commercial scale. Such research would qualify and quantify the controversy that occurs in the scientific community on issues related to biosafety of such plants. At this point, it stands out that the literature used by regulatory agencies in the risks assessment of transgenic plants, are predominantly produced, financed or supported by interested companies or researchers who are or have been linked to biotech industry.

In parallel, the analysis of these studies publication dates also shows that the controversy continues unabated and current, refuting fallacious claims that it is a resolved and surpassed debate.

Indeed, since the commercial release of the first GM plant for cultivation and consumption - tomato Flav / Savr in 1994 - the debates have not cooled off. On the contrary, they became more fierce, expanding to all areas of biosafety and affecting the most fundamental assumptions of genetic engineering. Currently, as paradoxical as it may seem, geneticists reject attempts to define what is a gene. It is known with certainty that the current scientific knowledge is not robust enough to define with precision, what is a gene.

In addition to expanding the realm of uncertainty, as can be seen in this work, records of negative, concrete impacts, resulting from the use of this technology, accumulate. They tend to be more severe in countries which adopted transgenic plants earlier on a scale significant space-time, but are present in all locations. This statement can be illustrated, among other facts, by the increase in pesticide use - due notably to the development of populations of plants and insects genetically resistant to technology and to management problems of agricultural systems - due to the spread of transgenes in agricultural and ruderal species.

It is still not possible to clearly predict or rule out the negative impacts associated with the consumption of GMOs, although over ten studies that conclusively show toxicity effects - especially liver-kidney damage associated with the consumption of transgenic plants already commercially released - are already available. These results appear in the medium and long term where these plants come to represent a significant part of the daily diet of organisms under test.

The information contained in this publication and therefore in all

scientific articles summarized here contrast with the positioning of the assessment bodies of the risk of such plants. Since, in Brazil (CTNBio) as in most countries where this technology thrives (FSANZ, GTR, EFSA, and others), those regulatory organizations should consider the updated scientific literature, surprisingly, about 750 articles cited herein remain in an invisibility limbo.

When making decisions or issuing opinions supposedly unbiased, but denying space to the doubts raised here, regulatory agencies allow questions that go beyond the scientific issue. It is not irrelevant that, by a majority of its members, these agencies systematically take a position favorable to the use of transgenic plants for cultivation and human and animal consumption.

Such a size of collection of studies incorporated into the scientific literature - representing the views of about THOUSANDS of researchers with studies accepted by peer-reviewed journals - highlights the importance of political bias and the underlying economic pressures to the positioning of the different countries when they decide to commercially release such plants. In fact, the science as a whole, and biogenetic in particular, would not have by itself the competence - or legitimacy - to decide whether the risks illustrated in this document should be considered acceptable or negligible by society as a whole. In this sense, a call for social action becomes necessary, also involving the scientific knowledge accumulated in other areas of current knowledge.

There should also be a review of the decision-making regulation where commissions or agencies comprised only by scientists have a say with finalistic character on the spread of transgenes (and/or its expression products - knowingly or unknowingly) on the environment and human and animal food chains. Of course, this is the case of Brazil where, through the CTNBio, 27 people decide about risks that threaten the future of national biodiversity.

In the view of the authors of this document, the information contained herein, taken from the collection published by the international scientific community, justify political decision by the moratorium on the cultivation and consumption of transgenic plants, especially Bt and HT. Simple application of the precautionary principle, taking into account this collection, would not allow the opposite conclusion.

In this context, it is worth remembering that this option has been adopted by about 20 nations - temporarily or definitively - and that GM crops are now grown on a significant scale only in less than ten countries worldwide.

The finding of the studies referenced in this publication strongly contrast with repetitive assertions of agricultural biotechnology and agrochemical industries, often literally built into favorable opinions presented in CTNBio, reporting on the alleged absence of risks to the environment and public health of their products, so as to create the illusion of unanimous position of the scientific community on the subject.

With regard specifically to the issue of health risks associated with consumption of transgenic plants, it is worth highlighting in this conclusion the systematic argument used by those parties when confronted with articles that link such biorisks: "GM foods are already consumed since over 15 years around the world and we have never seen public health problems." In the limit of knowledge of these authors, there is no single epidemiological study in the literature to back up such claim. However, this document referenced at least three articles[69] to conclude the opposite, that the long term consumption of genetically modified products may cause a general degradation of public health, and in particular consumers' health.

69 The two other articles not made explicit here comprise the study of Swamson et al. (2014), available in Part 4, and Seneff et al. (2015), available in the afterword.

One aimed to characterize dietary factors involved in the obesity epidemic of the USA population (Shao & Chin, 2011[70]). These authors state that:

> Therefore, we were able to demonstrate a novel link between the consumption of corn products and rising obesity trends that has not been previously attributed to the obesity epidemic. This correlation coincides with the introduction of bioengineered corns into the human food chain, thus raising a new hypothesis that should be tested in molecular and animal models of obesity" (SHAO; CHIN, 2011, p. 253)

Coincidentally with the closing of this publication, a new study was published in the scientific literature with the title "*No scientific consensus on GMOs safety*". Endorsed by more than 300 independent researchers from several countries, Hilbeck et al. (2015)[71] claim that the available scientific literature published in specialized magazines does not support the so widespread hypothesis of safety of GMOs. Conducting a similar research line to the one adopted in this book, those authors state that the scarcity and contradictory results available in the scientific literature prevent any affirmative responsibly supported by research evidence. Thus, efforts to certify or convey the idea of security (or indeed insecurity) of GM would be scientifically incorrect in that they would not rely upon objective analysis of updated scientific literature.

Finally, it remains to thank all the scientists who research and publish on the biosafety of transgenic plants. Despite the political and ideological situation hamper studies that could contradict the dominant agro-industrial model, these authors have built the knowledge disclosed herein, highlighting differences and reducing the obscurity imposed by the interests of a few.

In parallel, we dedicate this work to all scientists, opinion makers and political actors that contribute significantly to the advancement

70 Qin Shao and Khew-Voon Chin (2011): Survey of American food trends and the growing obesity epidemic. Nutr Res Pract. 5(3): 253-259.

71 Angelika Hilbeck, Rosa Binimelis, Nicolas Defarge, Ricarda Steinbrecher, András Székács, Fern Wickson, Michael Antoniou, Philip L. Bereano, Ethel Ann Clark, Michael Hansen, Eva Novotny, Jack Heinemann, Hartmut Meyer, Vandana Shiva and Brian Wynne, 2015. No scientific consensus on GMO safety. Environmental Sciences Europe 2015, 27:4. Gives afterword.

of science and its democratization and lead the debate for those who are directly involved in the biosafety issues related to the use of transgenic plants in commercial scale: farmers and consumers.

Finally, one must recognize and honor the existence of serious and committed scientists and researchers, committed to protect public health, environment and recognition of the socio-cultural role of agriculture that hold positions in regulatory biosafety agencies and that guide its operations by the precautionary principle. These citizens are a minority in these spaces of confrontation, where scientific knowledge tends to be minimized by political and economic interests of certain sectors of society. They often suffer psychological harassment, are professionally disqualified or sometimes simply ignored. Nevertheless, some remain in those spaces, aware of their social responsibility. This work should serve them comfort, when pointing out that, despite maintaining dissenting voices composing the meager set of dissenting opinions in those decision-making spaces, their positions are supported by at least 750 studies published in the scientific literature.

Afterword

Security-related discussions of transgenic products worsened in 2015, with some great publications of international impact. In the first three months of the year - after completion of the research that based this book - 19 more articles pointing out concerns about transgenic plants were systematized (an average equivalent of about six per month!), as made available in the international literature. As stated earlier, the systematization of scientific studies pointing to risks and uncertainties of transgenic plants is a task that is still in its beginning. In fact, the scientific controversy about it remains intense and present, and its permanence over the last 25 years should be interpreted as a warning for the need to adopt a precautionary approach in decision making.

About half of these articles point out risks to human and animal health and to the environment associated with the use of glyphosate. Among these it is worth mentioning the study published by the International Agency for Research on Cancer[72] (IARC), linked to the World Health Organization (WHO), that classifies glyphosate as "possibly carcinogenic", which is the last step before classification as "confirmed carcinogen." Although the opinions of this agency do not have binding character with the policies of member countries, Anvisa said recently that the product will be re-evaluated in Brazil. This makes it the cornerstone of the biotechnology industry that is being targeted.

Furthermore, in addition to the clear correlation between the risks of glyphosate to health and those associated with the consumption of transgenic plants tolerant to the herbicide (which contains high glyphosate residues), four other studies point directly to health risks associated with consumption Bt transgenic plants and/or HT.

72 The study sanctioned by IARC formed the basis for a warning about pesticide use impacts in Brazil, released the following month by the National Cancer Institute (Inca). Available at: http://www1.inca.gov.br/inca/Arquivos/comunicacao/posicionamento_do_inca_ sobre_os_agrotoxicos_06_abr_15.pdf.

Another article worth mentioning in this afterword is presented by Crisp et al. (2015). Analyzing and comparing the genomes of dozens of animal species (flies, nematodes and vertebrates, including primates), the authors note that the horizontal transfer of genes between species during evolution resulted in the acquisition of tens or hundreds of "exogenous" genes, now widely involved in the metabolism of receptor organisms. In the case of humans, the research identified at least 33 examples of genes acquired through HGT. These findings challenge the obscurantism imposed by regulatory agencies which, like CTNBio, systematically refuse to assess risks associated with HGT between transgenic plants and environmental organisms. The refusal takes place on the pretext that the likelihood of HGT occur would be null, very remote and/or scientifically unproven.

On the other hand, with regard to the efficiency of the technology and its flaws, two new papers show theoretical weaknesses in the assumptions that underlie the use of agricultural biotechnologies of Bt type (synthesizing insecticidal toxins) and HT (aimed at "clean" crops with herbicides baths). Indeed, Jaludin et al. (2015) observed populations of ruderal plants (*Eleusine indica*) which evolved to acquire a genetic / metabolomic profile simultaneously insensitive to the glyphosate-based herbicide, gluphosinate ammonium, paraquat herbicides and ACCase inhibitors. The presence of such populations in field is a clear sign of predictable failure of technology. The commercialization of transgenic plants tolerant to several herbicides will not solve the weed management problems already associated to the populations of ruderal plants resistant to herbicides. In parallel, Carriere et al. (2015) reinforce the relative inefficiency of pyramiding insecticide Bt transgenes in plants. Indeed, insect populations acquire a cross-resistance profile against various types of Bt proteins, even when they have different molecular mechanisms of action.

Anyway, even on issues relating to the commercial use of transgenic plants, two other articles reinforce evidence of failures of technology. They reveal that some transgenes cannot express themselves correctly (from a quantitative point of view) and impose new functions to modified organisms, without changing other characteristics. In coffee plant, the researchers observed a negative correlation between the synthesis of Cry1Ac and plant growth rate. In another study, which focused on the Mon810 corn, the authors observed modification of the expression profile of Bt proteins under pressure from environmental stress conditions, resulting in significant changes in the amount of toxin actually produced in the tested range. Now, it should be noted that fluctuations in the amount of Cry proteins synthesized represent a risk factor for the development / strengthening of insect populations resistant to Bt, negatively impacting the success of the technology in its claim to control "insect pests."

Andreassen, M.; Rocca, E.; Bøhn, T.; Wikmark, O-G.; Berg, J.; Løvik, M.; Traavik, T.; Nygaard, U. 2015. Humoral and cellular immune responses in mice after airway administration of *Bacillus thuringiensis* Cry1Ab and MON810 cry1Ab-transgenic maize. *Food and Agricultural Immunology*, 26:4, 521-537, DOI:10.1080/09540105.2014.988128.

Genetically modified (GM) crops may bring new proteins with immunogenic and allergenic properties into the food and feed chains. The most commonly grown GM maize, MON810, expresses a modified version of the insecticidal Cry1Ab protein originating in the soil bacterium*Bacillus thuringiensis* (Bt). Immune reactions following inhalation of pollen and debris from such plants have been scarcely studied. We exposed BALB/c mice to purified Cry1Ab proteins and Cry1Ab-containing MON810 plant materials by intranasal installation. No anti-Cry1Ab antibodies were detected following exposure to the plant materials. Exposure to purified Cry1Ab resulted in specific anti-Cry1Ab IgG1 and IgE production, indicating inherent immunogenicity and allergenicity. Mice exposed to leaf extracts from both MON810 and unmodified maize demonstrated influx of lymphocytes and eosinophils in the broncho-alveolar lavage, and increased cytokine release in mediastinal lymph node cells. The results indicate that the airway exposure to Cry1Ab proteins may be a route of practical relevance.

Full article available at http://www.tandfonline.com/doi/pdf/10.1080/09540105.2014.988128

Carrière, Y.; Crickmore, N.; Tabashnik, B. 2015. Optimizing pyramided transgenic Bt crops for sustainable pest management. *Nature Biotechnology*, ISSN 1087-0156.

Transgenic crop pyramids producing two or more Bacillus thuringiensis (Bt) toxins that kill the same insect pest have been widely used to delay evolution of pest resistance. To assess the potential of pyramids to achieve this goal, we analyze data from 38 studies that report effects of ten Bt toxins used in transgenic crops against 15 insect pests. We find that compared with optimal low levels of insect survival, survival on currently used pyramids is often higher for both susceptible insects and insects resistant to one of the toxins in the pyramid. Furthermore, we find that cross-resistance and antagonism between toxins used in pyramids are common, and that these problems are associated with the similarity of the amino acid sequences of domains II and III of the toxins, respectively. This analysis should assist in future pyramid design and the development of sustainable resistance management strategies.

http://www.nature.com/nbt/journal/v33/n2/full/nbt.3099.html#close

Crisp, A.; Boschetti, C.; Perry, M.; Tunnacliffe, A.; Micklem, G. 2015. Expression of multiple horizontally acquired genes is a hallmark of both vertebrate and invertebrate genomes. *Genome Biology*, 16(1): 50, doi: 10.1186/s13059-015-0607-3.

Background: A fundamental concept in biology is that heritable material, DNA, is passed from parent to offspring, a process called vertical gene transfer. An alternative mechanism of gene acquisition is through horizontal gene transfer (HGT), which involves movement of genetic material between different species. HGT is well-known in single-celled organisms such as bacteria, but its existence in higher organisms, including animals, is less well established, and is controversial in humans.
Results: We have taken advantage of the recent availability of a sufficient number of high-quality genomes and associated transcriptomes to carry out a detailed examination of HGT in 26 animal species (10 primates, 12 flies and four nematodes) and a simplified analysis in a further 14 vertebrates. Genome-wide comparative and phylogenetic analyses show that HGT in animals typically gives rise to tens or hundreds of active 'foreign' genes, largely concerned with metabolism. Our analyses suggest that while fruit flies and nematodes have continued to acquire foreign genes throughout their evolution, humans and other primates have gained relatively few since their common ancestor. We also resolve the controversy surrounding previous evidence of HGT in humans and provide at least 33 new examples of horizontally acquired genes.
Conclusions: We argue that HGT has occurred, and continues to occur, on a previously unsuspected scale in metazoans and is likely to have contributed to biochemical diversification during animal evolution.

Full article available at http://genomebiology.com/2015/16/1/50

Cuhra, M.; Traavik, T.; Dando, M.; Primicerio, R.; Holderbaum, D.; Bøhn, T. 2015. Glyphosate-residues in Roundup-Ready soybean impair *Daphnia magna* life-cycle. *Journal of Agricultural Chemistry and Environment*, 4, 24-36. doi: 10.4236/jacen.2015.41003.

Herbicide tolerant plants such as Roundup-Ready soybean contain residues of glyphosate herbicide. These residues are considered safe and previous animal-feeding-studies have failed to find negative effects related to such chemical residues. The present study tests 8 experimental soy- meal diets as feed in groups (each containing 20 individuals) of test-animals (D. magna). The diets have different levels of glyphosate residues and we show that animal growth, reproductive maturity and number of offspring are correlated with these chemicals. The tested soybeans are from ordinary agriculture in Iowa USA and the residues are below the regulatory limits. Despite this, clear negative effects are seen in life-long feeding. The work enhances the need for including analysis of herbicide residues in future assessment of GMO.

Full article available at https://goo.gl/m3PpDz

Guyton, K.; Loomis, D.; Grosse, Y.; El Ghissassi, F.; Benbrahim-Tallaa, L.; Guha, N.; Scoccianti, C.; Mattock, H.; Straif, K.; International Agency for Research on Cancer Monograph Working Group - IARC (Lyon, France). 2015. Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. *The Lancet Oncology*, pii: S1470-2045(15)70134-8. doi: 10.1016/S1470-2045(15)70134-8.

In March, 2015, 17 experts from 11 countries met at the International Agency for Research on Cancer (IARC; Lyon, France) to assess the carcinogenicity of the organophosphate pesticides tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate (table). These assessments will be published as volume 112 of the IARC Monographs.

http://www.thelancet.com/journals/lanonc/article/PIIS1470-2045(15)70134-8/fulltext

REV - Hilbeck, A.; Binimelis, R.; Defarge, N.; Steinbrecher, R.; Székács, A.; Wickson, F.; Antoniou, M.; Bereano, P.; Clark, E.; Hansen, M.; Novotny, E.; Heinemann, J.; Meyer, H.; Shiva, V.; Wynne, B. 2015. No scientific consensus on GMO safety. *Environmental Sciences Europe*, 27:4.

A broad community of independent scientific researchers and scholars challenges recent claims of a consensus over the safety of genetically modified organisms (GMOs). In the following joint statement, the claimed consensus is shown to be an artificial construct that has been falsely perpetuated through diverse fora. Irrespective of contradictory evidence in the refereed literature, as documented below, the claim that there is now a consensus on the safety of GMOs continues to be widely and often uncritically aired. For decades, the safety of GMOs has been a hotly controversial topic that has been much debated around the world. Published results are contradictory, in part due to the range of different research methods employed, an inadequacy of available procedures, and differences in the analysis and interpretation of data. Such a lack of consensus on safety is also evidenced by the agreement of policymakers from over 160 countries - in the UN?s Cartagena Biosafety Protocol and the Guidelines of the Codex Alimentarius - to authorize careful case-by-case assessment of each GMO by national authorities to determine whether the particular construct satisfies the national criteria for `safe?. Rigorous assessment of GMO safety has been hampered by the lack of funding independent of proprietary interests. Research for the public good has been further constrained by property rights issues, and by denial of access to research material for researchers unwilling to sign contractual agreements with the developers, which confer unacceptable control over publication to the proprietary interests.The joint statement developed and signed by over 300 independent researchers, and reproduced and published below, does not assert that GMOs are unsafe or safe. Rather, the statement concludes that the scarcity and contradictory nature of the scientific evidence published to date prevents conclusive claims of safety, or of lack of safety, of GMOs. Claims of consensus on the safety of GMOs are not supported by an objective analysis of the refereed literature.

Full article available at http://www.enveurope.com/content/27/1/4/abstract

Jalaludin, A.; Yu, Q.; Powles, S. 2015. Multiple resistance across glufosinate, glyphosate, paraquat and ACCase-inhibiting herbicides in an *Eleusine indica* population. *Weed Research*, 55, 82–8.

An *Eleusine indica* population was previously reported as the first global case of field-evolved glufosinate resistance. This study re-examines glufosinate resistance and investigates multiple resistance to other herbicides in the population. Dose–response experiments with glufosinate showed that the resistant population is 5-fold and 14-fold resistant relative to the susceptible population,

based on GR_{50} and $LD_{50\ R}/S$ ratio respectively. The selected glufosinate-resistant subpopulation also displayed a high-level resistance to glyphosate, with the respective GR_{50} and $LD_{50\ R}/S$ ratios being 12- and 144-fold. In addition, the subpopulation also displayed a level of resistance to paraquat and ACCase-inhibiting herbicides fluazifop-P-butyl, haloxyfop-P-methyl and butroxydim. ACCase gene sequencing revealed that the Trp-2027-Cys mutation is likely responsible for resistance to the ACCase inhibitors examined. Here, we confirm glufosinate resistance and importantly, we find very high-level glyphosate resistance, as well as resistance to paraquat and ACCase-inhibiting herbicides. This is the first confirmed report of a weed species that evolved multiple resistance across all the three non-selective global herbicides, glufosinate, glyphosate and paraquat.

Full article available at http://onlinelibrary.wiley.com/doi/10.1111/wre.12118/full

Jayasumana, C.; Paranagama, P.; Agampodi, S.; Wijewardane, C.; Gunatilake, S.; Siribaddana, S. 2015. Drinking well water and occupational exposure to herbicides is associated with chronic kidney disease, in Padavi-Sripura, Sri Lanka. *Environmental Health*, 14(1):6.

Background: The chronic kidney disease of unknown etiology (CKDu) among paddy farmers in was first reported in 1994 and has now become most important public health issue in dry zone of Sri Lanka. The objective was to identify risk factors associated with the epidemic in an area with high prevalence.
Methods: A case control study was carried out in Padavi-Sripura hospital in Trincomalee district. CKDu patients were defined using health ministry criteria. All confirmed cases (N = 125) fulfilling the entry criteria were recruited to the study. Control selection (N = 180) was done from people visiting the hospital for CKDu screening. Socio-demographic and data related to usage of applying pesticides and fertilizers were studied. Drinking water was also analyzed using ICP-MS and ELISA to determine the levels of metals and glyphosate.
Results: Majority of patients were farmers (N = 107, 85.6%) and were educated up to 'Ordinary Level' (N = 92, 73.6%). We specifically analyzed for the effect modification of, farming by sex, which showed a significantly higher risk for male farmers with OR 4.69 (95% CI 1.06-20.69) in comparison to their female counterparts. In the multivariable analysis the highest risk for CKDu was observed among participants who drank well water (OR 2.52, 95% CI 1.12-5.70) and had history of drinking water from an abandoned well (OR 5.43, 95% CI 2.88-10.26) and spray glyphosate (OR 5.12, 95% CI 2.33-11.26) as a pesticide. Water analysis showed significantly higher amount of hardness, electrical conductivity and glyphosate levels in abandoned wells. In addition Ca, Mg, Ba, Sr, Fe, Ti, V and Sr were high in abandoned wells. Surface water from reservoirs in the endemic area also showed contamination with glyphosate but at a much lower level. Glyphosate was not seen in water samples in the Colombo district.
Conclusion: The current study strongly favors the hypothesis that CKDu epidemic among farmers in dry zone of Sri Lanka is associated with, history of drinking water from a well that was abandoned. In addition, it is associated with spraying glyphosate and other pesticides in paddy fields. Farmers do not use personnel protective equipments and wears scanty clothing due to heat when spraying pesticides.

Full article available at http://www.ehjournal.net/content/pdf/1476-069X-14-6.pdf

COM - Kloor, K. 2015. Agricultural researchers rattled by demands for documents from group opposed to GM foods. *Science*, Vol 347, ISSUE 6223, p.699.

Without summary.

Full article available at https://goo.gl/Bk0p4Q

Kurenbach, B.; Marjoshi, D.; Amábile-Cuevas, C.; Ferguson, G.; Godsoe, W.; Gibson, P.; Heinemann, J. 2015. Sublethal exposure to commercial formulations of the herbicides dicamba, 2,4-dichlorophenoxyacetic acid, and glyphosate cause changes in antibiotic susceptibility in *Escherichia coli* and Salmonella enterica serovar Typhimurium. *mBio*, 6(2):e00009-15. Doi:10.1128/mBio.00009-15.

Biocides, such as herbicides, are routinely tested for toxicity but not for sublethal effects on microbes. Many biocides are known to induce an adaptive multiple-antibiotic resistance phenotype. This can be due to either an increase in the expression of efflux pumps, a reduced synthesis of outer membrane porins, or both. Exposures of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to commercial formulations of three herbicides—dicamba (Kamba), 2,4-dichlorophenoxyacetic acid (2,4-D), and glyphosate (Roundup)—were found to induce a changed response to antibiotics. Killing curves in the presence and absence of sublethal herbicide concentrations showed that the directions and the magnitudes of responses varied by herbicide, antibiotic, and species. When induced, MICs of antibiotics of five different classes changed up to 6-fold. In some cases the MIC increased, and in others it decreased. Herbicide concentrations needed to invoke the maximal response were above current food maximum residue levels but within application levels for all herbicides. Compounds that could cause induction had additive effects in combination. The role of *soxS*, an inducer of the AcrAB efflux pump, was tested in β-galactosidase assays with *soxS-lacZ* fusion strains of *E. coli*. Dicamba was a moderate inducer of the *sox* regulon. Growth assays with Phe-Arg β-naphtylamide (PAβN), an efflux pump inhibitor, confirmed a significant role of efflux in the increased tolerance of*E. coli* to chloramphenicol in the presence of dicamba and to kanamycin in the presence of glyphosate. Pathways of exposure with relevance to the health of humans, domestic animals, and critical insects are discussed.

Full article available at http://mbio.asm.org/content/6/2/e00009-15.full

Moore, H.; Chivers, D.; Ferrari, M. 2015. Sub-lethal effects of Roundup™ on tadpole anti-predator responses. *Ecotoxicology and Environmental Safety*, 111, 281-285.

Roundup™ is a commonly used pesticide applied to agriculture and forest habitats. These areas are generally ideal for amphibians due to the presence of small, ephemeral water bodies. While Roundup™ has been shown to have lethal effects on many species of amphibians, effects on behaviour and sensory perception have yet to be considered. Here, we exposed wood frog tadpoles to a sub-lethal concentration of Roundup™ and showed that the ability of tadpoles to respond to injured conspecific cues, an important source of information regarding local predation risk, was impaired. Subsequent experiments revealed that impaired responses likely result from a chemical reaction between the Roundup™ and the cues and that tadpoles chronically exposed to Roundup™ had reduced basal movement rates compared with unexposed tadpoles. Our data demonstrate that environmentally-relevant concentrations of Roundup™ can drastically alter movement and anti-predator responses of tadpoles, with potential negative consequences for the population.

http://www.sciencedirect.com/science/article/pii/S0147651314004801

Oraby, H.; Kandil, M.; Shaffie, N.; Ghaly, I. 2015. Biological impact of feeding rats with a genetically modified-based diet. *Turkish Journal of Biology*, 39.

This work was conducted in the context of postmarketing biosafety assessment of genetically modified products. It presents a systematic approach based on a chronic toxicity study on Wistar albino rats, with a range of combined parameters including biochemical, histopathological, and cytogenetic to evaluate the negative impact of a genetically modified (GM) diet on animal health. Histopathological and biochemical analysis procedures were performed in the liver, kidney, and testis. Cytogenetic analysis was evaluated in germ cells and the liver. The results revealed that the

laboratory diet used in our investigation was proved experimentally, using the PCR assay, to contain genetically modified components without being labeled as such. The results of all parameters evaluated in our investigation were consistent and confirm that the GM diet fed to rats for 30, 60, or 90 days has deleterious histopathological and histochemical impacts. Biochemical alterations in alanine aminotransferase, aspartate aminotransferase, creatinine, uric acid, and malondialdehyde concentrations were also observed. Genotoxicity of the GM diet was also demonstrated in germ cells as increased numbers of cells with chromosomal aberrations and in liver cells as increased ratios of DNA fragmentation. In conclusion, the results of the present work indicate that there are health hazards linked to the ingestion of diets containing genetically modified components.

Full article available at https://goo.gl/WNppTB

Perthuis, B.; Vassal, J-M.; Fenouillet, C.; Leroy, T. 2015. Cry1Ac insecticidal protein levels in genetically modified *Coffea canephora* Pierre coffee plants were negatively correlated with the growth speed in a field experiment. *Euphytica*, Volume 202, Issue 3, pp 373-383.

Genetically-modified coffee clones (GMCs) were presented in a previous work. They were created from a single commercial clone of *Coffea canephora* Pierre (clone 126). Therefore they all have the same genotype, except for the localization of the transgenic insertions. They synthesize the *Bacillus thuringiensis* endotoxin Cry1Ac against *Leucoptera coffeella* Guérin-Méneville, a secondary pest of*C. canephora* and one of the main pest of *C. arabica* in South America. The synthetic *Cry1Ac* gene is regulated by the *EF1*α-*A1* promoter of *Arabidopsis thaliana* (L.) Heynh. They were tested in an experimental field for their resistance against the pest insect in a previous work. In the present work, levels of Cry1Ac were measured in the leaves. The insecticidal protein was evenly distributed in all the leaves. Cry1Ac levels were measured once in the coffee plants of a sensitive GMC and of 14 resistant ones grown in the experimental field and in plants grown in a greenhouse. Some resistant GMCs contained higher levels but it is not possible to confirm that it would be enough for a sustainable resistance to the pest. Growth speed was variable in the plot. The correlation with plant height and other indicators of plant growth was examined. Cry1Ac levels in the GMCs were negatively correlated with growth speed. The latter was not statistically influenced neither by Cry1Ac synthesis nor by the genetic modification in itself as seen by comparing the GMCs and the unmodified control clone 126. Hence the conclusion is that the growth conferred to field-grown plants by environmental factors and especially the soil was probably the underlying cause of the negative correlation. Other field experiments would be necessary in order to confirm this result. It would be important that the genetic construct inserted in these GMCs and mainly the *EF1*α-*A1* promoter of the *Cry1Ac* gene be reconsidered since Cry1Ac levels might be too low to provide efficient and sustainable protection against *L. coffeella* in a highly favourable environment for coffee plants. Alternatives are discussed.

http://link.springer.com/article/10.1007%2Fs10681-014-1258-2

Reisig, D.; Reay-Jones, F. 2015. Inhibition of *Helicoverpa zea* (*Lepidoptera*: *Noctuidae*) growth by transgenic corn expressing Bt toxins and development of resistance to Cry1Ab. *Environmental Entomology*, 1–11; DOI: 10.1093/ee/nvv076.

Transgenic corn, Zea mays L., that expresses the Bacillus thuringiensis (Bt) toxin Cry1Ab is only moderately toxic to Helicoverpa zea (Boddie) and has been planted commercially since 1996. Growth and development of H. zea was monitored to determine potential changes in susceptibility to this toxin over time. Small plots of corn hybrids expressing Cry1F, Cry1FCry1Ab, Cry1AbCry3Bb1, Cry1A.105Cry2Ab2Cry3Bb1, Cry1A.105Cry2Ab2, and Vip3Aa20Cry1AbmCry3A were planted in both 2012 and 2013 in North and South Carolina with paired non-Bt hybrids from the same genetic background. H. zea larvae were sampled on three time periods from ears and the following factors were measured: kernel area injured (cm2) by H. zea larvae, larval number per ear, larval

weight, larval length, and larval head width. Pupae were sampled on a single time period and the following factors recorded: number per ear, weight, time to eclosion [the emergence of an insect from the pupa case], and the number that eclosed. There was no reduction in larval weight, number of insect entering the pupal stadium, pupal weight, time to eclosion, and number of pupae able to successfully eclose to adulthood in the hybrid expressing Cry1Ab compared with a non-Bt paired hybrid. As Cry1Ab affected these in 1996, H. zea may be developing resistance to Cry1Ab in corn, although these results are not comprehensive, given the limited sampling period, size, and geography. We also found that the negative impacts on larval growth and development were greater in corn hybrids with pyramided traits compared with single traits.

http://ee.oxfordjournals.org/content/early/2015/05/20/ee.nvv076

Rótolo, G.; Francis, C.; Craviotto, R.; Ulgiati, S. 2015. Environmental assessment of maize production alternatives: Traditional, intensive and GMO-based cropping patterns. *Ecological Indicators*, Volume 57, Pages 48–60.

The evolution of maize production patterns in Argentina is evaluated over the last 25 years to compare costs, benefits, environmental performance and sustainability as well as to identify the main driving sources and improvement potential. Results from Argentina cropping systems are compared to other systems worldwide in order to put the Argentina results in a broader context. The study focuses on three farming categories: (1) traditional, low-intensity systems, (2) conventional, high-intensity systems, and (3) GMO-based cropping systems. Low input intensity systems include traditional cropping patterns with seed selection by farmers and conventional hybrid seed coupled to plowing and crop-animal rotation techniques; high input intensity systems use conventional hybrid seeds and recommended chemicals, irrigation and machinery with important soil erosion consequences; and GMO-based cropping systems use herbicide resistant transgenic hybrids, pesticides, higher fertilizer rates, and no-till practices. In each of the three cases, input flows are compared to the achieved yield (in mass and income terms) to better understand relative efficiencies and options for improvement. The study of GMO systems required a preliminary investigation of GMO seed production by seed companies, where a large investment in terms of prior knowledge and high-tech laboratory research is required. The assessments used the Emergy Accounting (EMA) approach. EMA includes material, energy, labor, money, and knowledge flows into the assessment and expands its focus over larger time and spatial scales than conventional economic and cumulative energy demand methods. Emergy-based environmental indicators of grain production for high-intensity hybrid and GMO systems both show a lower performance than low-intensity, traditional patterns in terms of resource return, renewability and sustainability. The fraction of renewability in low-intensity systems is between 28% and 63%, while it is between 8% and 26% for high-intensity hybrid and GMO systems. Calculated indicators also show that GMO-based maize production patterns do not guarantee the expected improvement over conventional high-intensity cropping systems or low-intensity systems in terms of performance and sustainability. Strong reliance on nonrenewable resources and technology, as well as role of direct and indirect labor costs are important factors in determining long-term sustainability and environmental stability of maize production systems.

http://www.sciencedirect.com/science/article/pii/S1470160X15001661

Seneff, S.; Swanson, N.; Li, C. 2015. Aluminum and glyphosate can synergistically induce pineal gland pathology: Connection to gut dysbiosis and neurological disease. *Agricultural Sciences*, 6, 42-70.

Many neurological diseases, including autism, depression, dementia, anxiety disorder and Parkinson's disease, are associated with abnormal sleep patterns, which are directly linked to pineal gland dysfunction. The pineal gland is highly susceptible to environmental toxicants. Two pervasive

substances in modern industrialized nations are aluminum and glyphosate, the active ingredient in the herbicide, Roundup?. In this paper, we show how these two toxicants work synergistically to induce neurological damage. Glyphosate disrupts gut bacteria, leading to an overgrowth of Clostridium difficile. Its toxic product, p-cresol, is linked to autism in both human and mouse models. p-Cresol enhances uptake of aluminum via transferrin. Anemia, a result of both aluminum disruption of heme and impaired heme synthesis by glyphosate, leads to hypoxia, which induces increased pineal gland transferrin synthesis. Premature birth is associated with hypoxic stress and with substantial increased risk to the subsequent development of autism, linking hypoxia to autism. Glyphosate chelates aluminum, allowing ingested aluminum to bypass the gut barrier. This leads to anemia-induced hypoxia, promoting neurotoxicity and damaging the pineal gland. Both glyphosate and aluminum disrupt cytochrome P450 enzymes, which are involved in melatonin metabolism. Furthermore, melatonin is derived from tryptophan, whose synthesis in plants and microbes is blocked by glyphosate. We also demonstrate a plausible role for vitamin D3 dysbiosis in impaired gut function and impaired serotonin synthesis. This paper proposes that impaired sulfate supply to the brain mediates the damage induced by the synergistic action of aluminum and glyphosate on the pineal gland and related midbrain nuclei.

Full article available at https://goo.gl/iTDpY0

Lima, I.; Baumeier, N.; Rosa, R.; Campelo, P.; Rosa, E. 2015. Influence of glyphosate in planktonic and biofilm growth of *Pseudomonas aeruginosa. Brazilian Journal of Microbiology*, 45(3): 971–975.

This study evaluated the impact of different concentrations of glyphosate (Rondup®) on planktonic and biofilm growth of *P. aeruginosa*. Aerobic and anaerobic cultures of *P. aeruginosa* ATCC®15442 inoculated in MHB + glyphosate (0.845 ppm, 1.690 ppm, 8.45 ppm, 16.90 ppm, 84.50 ppm, 169 ppm, 845 ppm, and 1690 ppm) and cultured in normoxia and anoxia, following their OD_{560nm} every hour for 24 h. Biofilms of adapted cells were formed in the presence of glyphosate (0.845 to 1690 ppm) in normoxia and anoxia for 36 h. Glyphosate at concentrations higher than 84.5 ppm reduces the cell density of planktonic aerobic cultures ($p < 0.05$). However, these same concentrations favor the planktonic anaerobic growth ($p < 0.05$). On the other hand, the herbicide favors a slight growth of biofilms in a concentration-dependent manner up to 84.5 ppm ($p > 0.05$), and more pronounced over 169 ppm. Anaerobic biofilms have their growth more readily favored ($p < 0.05$), regardless of concentration. In a concentration-dependent manner, glyphosate interferes with the growth ability of *P. aeruginosa* ATCC®15442.

Full article available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4204984/

Webster, T.; Santos, E. 2015. Global transcriptomic profiling demonstrates induction of oxidative stress and of compensatory cellular stress responses in brown trout exposed to glyphosate and Roundup. *BMC Genomics*, 16.

Background: Glyphosate, the active ingredient in Roundup formulations, is the most widely used herbicide worldwide, and as a result contaminates surface waters and has been detected in food residues, drinking water and human urine, raising concerns for potential environmental and human health impacts. Research has shown that glyphosate and Roundup can induce a broad range of biological effects in exposed organisms, particularly via generation of oxidative stress. However, there has been no comprehensive investigation of the global molecular mechanisms of toxicity of glyphosate and Roundup for any species. We aimed to characterise and compare the global mechanisms of toxicity of glyphosate and Roundup in the liver of brown trout (*Salmo trutta*), an ecologically and economically important vertebrate species, using RNA-seq on an Illumina HiSeq 2500 platform. To do this, we exposed juvenile female brown trout to 0, 0.01, 0.5 and 10 mg/L of glyphosate and Roundup (glyphosate acid equivalent) for 14 days, and sequenced 6 replicate liver

samples from each treatment.
Results: We assembled the brown trout transcriptome using an optimised *de novo* approach, and subsequent differential expression analysis identified a total of 1020 differentially-regulated transcripts across all treatments. These included transcripts encoding components of the antioxidant system, a number of stress-response proteins and pro-apoptotic signalling molecules. Functional analysis also revealed over-representation of pathways involved in regulating of cell-proliferation and turnover, and up-regulation of energy metabolism and other metabolic processes.
Conclusions: These transcriptional changes are consistent with generation of oxidative stress and the widespread induction of compensatory cellular stress response pathways. The mechanisms of toxicity identified were similar across both glyphosate and Roundup treatments, including for environmentally relevant concentrations. The significant alterations in transcript expression observed at the lowest concentrations tested raises concerns for the potential toxicity of this herbicide to fish populations inhabiting contaminated rivers.

http://www.biomedcentral.com/1471-2164/16/32

Trtikova, M.; Wikmark, O.; Zemp, N.; Widmer, A.; Hilbeck, A. 2015. Transgene expression and Bt protein content in transgenic Bt maize (MON810) under optimal and stressful environmental conditions. *PLOS ONE*, 10(4): e0123011. doi:10.1371/journal.pone.0123011.

Bt protein content in transgenic insect resistant (Bt) maize may vary between tissues within plants and between plants growing under different environmental conditions. However, it is unknown whether and how Bt protein content correlates with transgene expression, and whether this relationship is influenced by stressful environmental conditions. Two Bt maize varieties containing the same transgene cassette (MON 810) were grown under optimal and stressful conditions. Before and during stress exposure, the upper leaves were analysed for transgene expression using quantitative RT-PCR and for Bt content using ELISA. Under optimal conditions there was no significant difference in the transgene expression between the two investigated Bt maize varieties whereas Bt protein content differed significantly. Transgene expression was correlated with Bt protein content in only one of the varieties. Under stressful environmental conditions we found similar transgene expressions as under optimal conditions but Bt content responded differently. These results suggest that Bt content is not only controlled by the transgene expression but is also dependent on the genetic background of the maize variety. Under stressful conditions the concentration of Bt protein is even more difficult to predict.

Full article available at http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0123011

Tudisco, R.; Calabrò, S.; Cutrignelli, M.; Moniello, G.; Grossi, M.; Mastellone, V.; Lombardi, P.; Pero, M.; Infascelli, F. 2015. Genetically modified soybean in a goat diet: Influence on kid performance. *Small Ruminant Research*, Pages 67–74.

The in vivo and *post mortem* performance and serum immunoglobulin G (IgG) concentration in kids born from goats fed conventional (group C) or genetically modified (group T) soybean meal were evaluated. The goat colostrum quality, in terms of chemical composition, as well as immunoglobulin concentration, and the presence of feed DNA fragments were also investigated. Kid birth weights were similar, while significantly ($P < 0.05$) higher in those born from goats in group C at day 30 and at slaughtering. In addition, kids from mothers fed conventional soybean recorded significant ($P < 0.05$) higher height at the withers and chest width. Concerning the post mortem measurements, only carcass weights were significantly affected by the treatment resulting in lighter T kids ($P < 0.05$). Colostrum from the treated groups recorded a significantly ($P < 0.01$) lower percentage of protein and fat. Similarly, both chemical parameters significantly differed in milk collected 15 days after kidding, although these differences disappeared in the successive samplings. Both colostrum and kids serum IgG concentration were significantly ($P < 0.01$) lower in the treated

groups. Transgenic target DNA sequences (35S and CP4 EPSPS) were not detected in colostrum from goats that received a diet containing conventional soybean meal. By contrast, transgenic DNA fragments were amplified ($P < 0.05$) in samples from goats that received the transgenic soy bean.

http://www.sciencedirect.com/science/article/pii/S0921448815000528

Young, F.; Ho, D.; Glynn, D.; Edwards, V. 2015. Endocrine disruption and cytotoxicity of glyphosate and roundup in human JAr cells *in vitro*. *Integrative Pharmacology, Toxicology and Genotoxicology*; doi: 10.15761/IPTG.1000104, Volume 1(1): 12-19.

The toxicity of the active molecule in herbicides has been used to determine safe concentrations, because other components are considered inert. Roundup, which contains the active molecule Glyphosate, was described as an endocrine disrupter because non-cytotoxic concentrations inhibited progesterone synthesis in vitro. Human chorioplacental JAr cells synthesise progesterone, and increase synthesis when stimulated by chorionic gonadotrophin (hCG), or the transduction molecule cAMP. JAr cells were exposed to two Roundup formulations, and compared with the same concentrations of glyphosate ± cAMP, or ± hCG for 1, 4, 24, 48 or 72h. The surviving viable cells were quantified using an MTT assay, and progesterone was measured in an ELISA. hCG and cAMP stimulated progesterone synthesis by cells in vitro as expected. In contrast to previous reports, JAr cell death preceded decreased progesterone synthesis, and steroidogenesis was unaffected by low, non-cytotoxic concentrations of Roundup or glyphosate. Roundup was more cytotoxic than glyphosate alone; the 24h EC50 was 16mM for glyphosate, but 0.008mM when glyphosate was in a 7.2g/L Roundup formulation. Significant cytotoxicity was caused by glyphosate in Roundup ($p<0.01$) after 24h, and cytotoxicity was observed in vitro after exposure toa range of concentrations comparable to the Australian Drinking Water Guidelines.Endocrine disruption effects were secondary to cytotoxicity. Roundup was more cytotoxic than the same concentration of glyphosate alone, indicating that the other constituents of the herbicide are not inert. There is a compelling need to conduct in vivo studies to characterise the toxicity of glyphosate in a Roundup formulation, to facilitate re-evaluation of existing public health guidelines.

Full article available at http://www.gmoevidence.com/wp-content/uploads/2015/03/IPTG-1-104.pdf

Glossary

Allele (of a gene): Member of a portion (or range) of different forms of a gene. The genotype of an individual for that gene is the set of alleles he has. As an example, we mention the case of the gene encoding the color of flowers of many kinds. A single gene controls the color of petals, but there may be several forms (alleles) of such different gene. One way can give red color to the petals, while the other will give the white color. Thus, the flower color will depend on which of these two alleles the organism has for this gene and how they interact.

Conjugation: transfer of genetic material between bacteria through direct contact between cells.

Genetic drift (drift or allelic): mechanism, acting in accordance with natural selection, modifies the characteristics of the species over time. It is a stochastic process that acts on populations by modifying the frequency of alleles and the predominance of certain characteristics of the population. Genetic drift can lead to the disappearance of some alleles, especially in small populations.

Dominant (character): in genetics, a dominant character, that if transmitted to the offspring will automatically be expressed by the offspring (offspring will synthesize the Cry protein, for example).

Exudate: refers to, in this case, a physiologic fluid that seeps into the soil by the roots.

Phenotype (or phenotypic characteristics): the phenotype of an organism is concretized by any visually observable characteristic, such as its morphology, or physiological properties. The phenotype results from the expression of genes of an organism (genotype), and the influence of environmental factors and possible interactions between them.

Fitness: animals that leave more offspring compared to other individuals of the same species are considered to have greater fitness. The combination of genotype and environment defining thus the fitness. If some changes do occur in the body genotype, then the fitness will be affected, and the frequency of this genotype will vary in succeeding generations, especially the genotype with the highest fitness.

Antibiotic resistance marker gene: the techniques used to transfer a new gene within the genome of a plant are quite inefficient. Few cells actually integrate the gene of interest (transgene or HT Bt, for example). In order to determine which cells have integrated the transgene, certain types of markers are needed. In some cases, antibiotic resistance marker genes are used because they are easy to use. All cells that have actually integrated the marker gene into its genome also integrated the interest gene. Furthermore, these cells are resistant to the antibiotic. So, simply put the transformed cells into contact with the antibiotic and select the ones that survive. Even if these marker genes have no other functions after this selection process, they stay as part of transgenic plants and represent important risk factors.

Genotype: the genotype of an organism is its genetic information. All organisms that have the same genotype may not look or act the same way, especially because the appearance and behavior are also related to environmental and developmental conditions. Moreover, all the organisms which have an identical appearance (phenotype even) do not necessarily have the same genotype.

Phylogenetic group: in biology, phylogeny is the study of evolutionary relationships among various groups of organisms (e.g., species or populations) that are discovered by molecular sequencing and morphological data matrices. The taxonomy was strongly influenced by phylogeny.

Taxonomic group: classifying organisms as biological and morphological similarities.

Hemocoel: name given to a series of spaces (sinuses) between organs of some insects and other organisms. A mixture of blood, lymph and interstitial fluid, called hemolymph, circulates through hemocoel.

Introgression: moving a gene of a species within the gene pool from another species.

Invasiveness: an invasive species refers to a non-native species that negatively affects the habitat where it develops in relation to economic, environmental or ecological aspects.

Leghemoglobin: protein found in nitrogen-fixing nodules of the roots of leguminous plants. It is produced by plants in response to infection of the roots by nitrogen-fixing bacteria as part of a symbiotic interaction between plants and bacteria. The roots not infected by Rhizobium do not synthesize leghemoglobin.

Nosocomial: adverse effect caused by medical treatment. A nosocomial disease is contracted in hospitals.

Order: in the taxonomy of insects, species are grouped into genres, and these are grouped into families. The Order groups Families. Ex .: Lepidoptera (butterflies), Coleoptera (beetles), Hemiptera (Barber).

Replication origin: A DNA sequence located on a chromosome (or a plasmid), which initiates replication.

Plasmid: extracromossomal DNA molecule, distinct from chromosomal DNA, which is able to replicate independently of it. In most cases, it is circular and relatively small. The plasmids are often observed in bacteria, but may also exist in eukaryotic organisms such as fungi.

Pleiotropy: the influence of a single gene on multiple phenotypic characteristics. Consequently, a mutation in the gene will affect all these characteristics simultaneously.

Selection pressure: can be brought about by the intensity with which an environmental factor tends to eliminate an organism or to give you adaptive advantages.

Prokaryote: group of organisms that lack a cell nucleus (= karyon) or any membrane-bound organelle. They differ from eukaryotes, which have a cell nucleus. Most prokaryotes are unicellular.

Promoter: in biology, the promoter is a DNA region that facilitates transcription of a particular gene. Promoters are often located near the genes they regulate, but this rule suffers numerous exceptions.

Proteolysis: direct degradation (digestion) of protein by cellular enzymes called proteases.

Receptor: in biochemistry, a receptor is a protein molecule to which another molecule can bind (peptide, hormone, toxin, etc.). When binding occurs (signal), this usually leads to a cellular response.

Recessive (character): In genetics, a recessive character may be expressed only in the case of reproduction with an individual who also has the same gene allele. Otherwise the gene allele may be transmitted in the offspring, but will not be expressed.

Backcross: crossing a hybrid with one of their parents (or a genetically similar to the individual parent), in order to obtain offspring with closest genetic identity of their parents.

Retrotransposons: genetic elements that can multiply in a genome and are ubiquitous in the DNA of many eukaryotic organisms, such as plants.

Terminator: part of a gene sequence that indicates the end of a gene during the transcription of chromosomal DNA.

Transduction: the transfer of bacterial genes between bacteria through bacterial or bacteriophage virus.

Transformation: uptake of DNA free of the environment, its integration into the host genome of competent bacteria and subsequent expression of the new genetic information acquired.

Ubiquitous: that are present (or seem to present) everywhere at once; ubiquitous.

Selective advantage: it can be achieved by a genetic advantage of an organism over its competitors, which will increase their capacity for survival and reproduction over time in a given environment.

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