Maize DP4114 x MON89034 x MON87411 x DAS-40278-9 and its sub-combinations

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Organisation: The European GMO-free Citizens (De Gentechvrije Burgers)
Country: The Netherlands
Type: Others...

Comments:

About:

Maize DP4114 x MON89034 x MON87411 x DAS-40278-9 and its sub-combinations

First:

(News from 2021) Mexico to replace 16 million tonnes of gm corn by native variaties and ban the toxic herbicide glyphosate.

“The federal government will go ahead with its plan to stop importing genetically modified (GM) corn and replace it with homegrown maize, according to Deputy Agriculture Minister Suárez.

The official also told the news agency Reuters that the government is sticking to its plan to ban glyphosate, a controversial herbicide.”

https://mexiconewsdaily.com/news/mexico-proceeds-with-plan-to-replace-16mn-tonnes-of-gm-corn-with-homegrown-variety/

In addition to banning Monsanto/Bayer’s cancer-causing Roundup herbicide by 2024, Mexico is now pledging to rid the country of GMO corn by the same date.

To do so, it plans to gradually replace 16 million tons in annual imports of GMO corn from the United States with ancient, indigenous varieties.

https://returntonow.net/2021/11/07/mexico-replaces-16-million-tons-of-gmo-corn-with-native-varieties/

“The Mexican Society of Organic Producers called the move a victory. The group blames GMO crops for contaminating the native, ancient varieties of corn while saying that the widespread use

of dangerous pesticides endangers the health of both producers and consumers while undermining biodiversity."

https://www.agrinews-pubs.com/business/2021/01/18/mexico-bans-gmo-corn-2024-deadline-includes-elimination-of-glyphosate-herbicide/

A question:

How long will it take before this will happen in every country and with every GM crop when Roundup is forbidden?

We, the GMO-free Citizens and Stichting Ekopark in Lelystad, The Netherlands, do not want to eat this genetically modified maize. And we don't want you to market this on the EU. (Under Regulation (EC) No 1829/2003 (application EFSA-GMO-RX-026/2.)

When you approve this, which we will regret, we want every final product to be labelled as GMO, even if you can no longer detect it in a final product.

Our conclusion: Poison stacked with poison.

Below some examples.

We read: "The four-event stack maize was produced by conventional crossing to combine four single maize events: DP4114 expressing Cry1F to confer resistance to lepidopteran pests, Cry34Ab1 and Cry35Ab1 to confer resistance to coleopteran pests, and PAT providing resistance to glufosinate-ammonium containing herbicides; MON 810 expressing Cry1Ab to confer resistance to lepidopteran pests; MIR604 expressing mCry3A to confer resistance to coleopteran pests and PMI as selectable marker; and NK603 expressing CP4 EPSPS and CP4 EPSPS L214P to confer tolerance to glyphosate-containing herbicides." Source: EFSA.

About MON89034

MON89034 (GM maize) causes disease in rats 2018. GMWatch

What's in MON89034 maize?

"MON89034, marketed as YieldGard™ VT Pro™, is a Monsanto GM maize that expresses its own Bt toxin insecticides. MON89034 maize contains a unique mix of insecticidal proteins called Bt toxins. The plants produce a synthetic Bt toxin, Cry1A.105 – a combination of Bt toxins called Cry1Ac/Cry1Ab and Cry1F. There is no natural form of this combined protein, so safety cannot be concluded by comparison with natural Bt toxins used previously."

More

Source GMWatch. Gentech maize approved in the EU causes diseases in rats.

https://www.gmwatch.org/en/106-news/latest-news/18120-gm-maize-approved-in-the-eu-caused-kidney-disease-and-bladder-stones-in-rats

About: DP4114

GMO Bt crops on the chopping block due to insect resistance

Published: 30 September 2020

Quote: “EPA proposes phasing out dozens of Bt corn and cotton products

There is now just ONE Bt trait left on the market without documented insect resistance.

Even the claim that Bt seeds reduced insecticide use pre-resistance was questionable, as Bt seeds are mostly treated with neonicotinoid insecticides – neonic seed treatments rose in parallel with Bt crops.”

https://www.gmwatch.org/en/main-menu/news-menu-title/archive/100-2020/19542-gmo-bt-crops-on-the-chopping-block-due-to-insect-resistance

About MIR604

Coalition demands ban on Bt cowpea in Nigeria and neighbour West African countries

Published: 09 March 2022

Quote: "Cry1Ab has been shown by scientists to be toxic to human and animal liver cells, and also alters the immune system. The use of this transgene was banned in South Africa, where the cultivation of genetically modified maize led to enormous pest resistance and infestation."

https://www.gmwatch.org/en/106-news/latest-news/20003-coalition-demands-ban-on-bt-cowpea-in-nigeria-and-neighbour-west-african-countries

About: NK603

Republished study: long-term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize

• Gilles-Eric Séralini,

• Emilie Clair,

• Robin Mesnage,

• Steeve Gress,

• Nicolas Defarge,

• Manuela Malatesta,

• Didier Hennequin &

• Joël Spiroux de Vendômois

Environmental Sciences Europe volume 26, Article number: 14 (2014)

Abstract, quotes

Background

“The health effects of a Roundup-tolerant NK603 genetically modified (GM) maize.

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.“

GMWatch: “Glyphosate exposure during pregnancy is linked to lower birth weights for babies, a new study of pregnant women has found. Lower birth weights are linked to many health problems later in life, from diabetes to heart problems.”

Glyphosate exposure in pregnancy linked to lower birth weights (gmwatch.org)

Caroline Cox, Northwest Coalition for Alternatives to Pesticides (NCAP) verzamelde een aantal Round-UP problemen:

“Glyphosate can be persistent, can drift, is acutely toxic to humans, has shown a wide spectrum of chronic toxicity in laboratory tests, is hazardous to eurthworms, reduces nitrogen fixation, Roundup contains toxic trade secret ingredients, kills beneficial insects, inhibits mycorrhizal fungi, can increase the spread or severity of plant diseases”.

Also read: GMO_Myths-and-Facts.pdf (gmwatch.org)

DP4114

About GLA. Reprinted with permission.

"Research by Hoechst (Dr. Arno Schulz) on the substrates of Phosphinothricin acetyltransferase(PAT)."

Amsterdam, November 7, 1999. J. van der Meulen, L. Eijsten Quote

GLA and glyphosate.

In 1987, the following article was published: Thomson, C. J. et al., 'Characterisation of the herbicide-resistance gene bar from S.hygroscopicus', EMBO Journal Vol. 6 No 9, pages 2519-23. It described how phosphinothricin-acetyltransferase also has glutamic acid as a substrate, by mixing the two substances and demonstrating the reaction product. Hoechst contested this in a report (93-01) by Dr Arno Schulz: 'L-phosphinothricin N acetyltransferase biochemical characterisation'. Glufosinate had been exposed, TOGETHER with a seriously excessive amount of glutamic acid (and other amino acids) to the effects of the acetyltransferase. Schulz had been unable to demonstrate ANY reaction product with glutamic acid and thus concluded that glutamic acid was not a substrate.

THIS IS INCORRECT AND HIGHLY MISLEADING because • in situations in which the acetyltransferase (present in the modified plant) could have a toxic effect, as in our gastrointestinal tract, large quantities of glufosinate are not simultaneously present (see Thomson). Unbelievable! • it is only logical that, under Schulz's test conditions, the acetyltransferase would acetylate the glufosinate using not only the added acetyl source but also acetylated glutamine acid as an acetyl source (because the transferase has a higher affinity for glufosinate). In a MIXTURE a reaction product will be produced only with the substrate for which it has the highest affinity.

A VERY MISLEADING REPORT. We object to the development of a GMO containing this gene product.

1. According to Hoechst, it is not teratogenic. E. Ebert et al.: 'Summary of safety evaluation toxicity studies of glufosinate ammonium', 1989/1990. Defects found in rabbit progeny were brushed under the carpet by Hoechst, which claimed that they were the result of 'maternal toxicity'!! The toxic effect on the mother was claimed to prevent her giving birth to healthy progeny.

We believe they are playing fast and loose with the words they use. We would put forward instead the research data of Tomoko Fujii et al., from 1996: 'Alterations in the Response to Kainic Acid in Rats Exposed to Glufosinate Ammonium, a Herbicide, during Infantile Period', a study sponsored by the Japanese Ministry of Education, Science, Sports and Culture.

'Exposure to GLA, even in low doses (1 mg/kg) during Infantile Period in the rat, induces alterations in the kainic receptor in the brain'.

T. Watanabe, 1996: 'Apoptose induced by GLA in the neuroepithelium of developing mouse embryos in culture'. Programmed cell death as a result of the secretion of substances which destroy the cell from within; this 'suicide' is regulated by a suicide gene which appears to be activated by GLA. T. Watanabe et al., 1997: 'Developmental and dysmorphogenic effects of GLA in mouse embryos in culture'.

Deformities.

2. It is not considered to be sensitising.

Ms L. Eijsten discovered for herself the exact opposite of GLA's 'non-sensitising properties', something she has reported previously. In 1992, she – and her dog – became sensitised: a parks department employee carried on spraying the edges of the grass in a park, where she was sitting reading on a bench, with Finale SL 14. Nothing apparently amiss.

However, a year later she was walking her dog by grass which had shortly before been sprayed with the same herbicide and promptly, seven hours later, her legs were covered in eczema. She walked the same route the next day, this time in a sleeveless blouse, and within no time her arms and face were also covered in eczema (the dog too had red patches on its stomach).

She has reported on this many times already. The serious thing is, however, that every attempt is made to brush these facts under the carpet, arguing that her symptoms were caused by a food allergy (letter of 10 June 1996 from Mr Top / Ms Terpstra at the Netherlands Ministry of Health, Welfare and Sport (VWS); a very scientific communication.)

The photograph sent showed clearly that the eczema was on unprotected parts of Ms Eijsten's body. And there was no eczema on the back of her hands – logically, because she had washed her hands after the contact. A dermatologist carried out tests involving patches with Vaseline to which the herbicide had been added.

This meant that a hydrophilic substances was being tested using a hydrophobic substance. It was logical that no effect should be visible after the test. The dermatologist carried out tests in the same way three times, despite Ms Eijsten's request that a hydrophilic substances, such as lanolin, be used, or that the herbicide be tested on her skin by itself.

His argument was that he always worked that way, thus making his incompetence clear. He had previously told her that he did not know the herbicide in question and had asked her to bring some with her. That was strange, because Finale had already been in use for some 20 years.

This was also why she collected various articles about Finale and showed the dermatologist an American book describing methods for demonstrating sensitisation. EU LEGISLATION prescribes many methods for demonstrating sensitisation. Ms Eijsten constantly wondered why the dermatologist did not want to carry out any different tests. She found this all very improper. If all dermatologists in the Netherlands took the same approach as 'her dermatologist', no cases of eczema resulting from GLA would ever be found!

Why should the correct tests not be done? We believe that everything possible is being done to cover up the harmful effects of GLA. The annual report of the organisation Consument en Biotechnologie for 1996/1997 reported that Fujii's 1996 report stated that high doses had been found to cause brain damage.

And it should be noted that it was Ms Eijsten who sent the report in question to Consument en Biotechnologie, at their request. The report concerned precisely the fact that the work had been done using very small doses (1 mg/kg). When she complained, they promised to correct the errors.

Recently she was informed that no correction is to be made. No reason was given. This twisting of the truth is an example of false lobbying. We believe that the above information on sensitisation has to be communicated once again, against the background of the dangers which arise when herbicides are sprayed and as a result of drift when herbicide resistant crops are cultivated, be it on a large or a small scale. Murphy's law.

Extract from: Onderzoek van Hoechst (dr. Arno Schulz) betreffende de substraten van Phosphinothricinacetyltransferase(PAT). – Gentechvrij by J. van der Meulen, L. Eijsten

Exposure to GLA, even in low doses (1 mg/kg) during Infantile Period in the rat, induces alterations in the kainic receptor in the brain.

https://www.researchgate.net/publication/244754595_Alterations_in_the_Response_to_Kainic_Acid_in_Rats_Exposed_to_Glufosinate_Ammonium_a_Herbicide_during_Infantile_Period

T. Watanabe. 1996

"Apoptose induced by GLA in the neuroepithelium of developing mouse embryos in culture. Programmed cell death by secretion of substances that destroy the cell internally; this suicide is regulated by a suicide gene, which is apparently clicked on by GLA.

T. Watanabe et al. 1997.

"Developmental and Dysmorphogenic Effects of GLA in mouse Embryos in culture". Malformations..

We read: 22 FEBRUARY 2007

Hungary may refuse transgenic maize on its territory.

The EU has decided that Hungary can refuse transgenic maize on its territory.

Specifically, it concerned Monsanto's genetically modified maize (Mon810).

Scientific data, published by Austrian and Hungarian scientists, show that GMO maize does indeed have a negative effect on plants and animals.stop

GMWatch: "GM soy is one of the most widely grown GM crops in the world and accounts for over 90% of US-grown soy. It is engineered to survive being sprayed with toxic glyphosate weedkiller.

GM soy injures the pancreas, rat feeding study shows (gmwatch.org)
https://gmwatch.org/en/106-news/latest-news/20129

The study:

Pancreatic response of rats fed genetically modified soybean.

Javier A. Magaña-Gómez, Guillermo López Cervantes, Gloria Yepiz-Plascencia and Ana M. Calderón de la Barca

J. Appl. Toxicol. 2008;28:217–226

https://www.academia.edu/35754035/Pancreatic_response_of_rats_fed_genetically_modified_soybean

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Maize DP4114 x MON89034 x MON87411 x DAS-40278-9 and its sub-combinations

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Organisation: Testbiotech e.V. - Institute for Independent Impact Assessment of Biotechnology
Country: Germany
Type: Non Profit Organisation

Comments:

Introduction

The GMO Panel assessed the four-event stacked maize DP4114 x MON 89034 × MON87411 × DAS-40278-9, which is derived from crossing four genetically engineered maize events (EFSA, 2022a). The maize contains genes conferring resistance to four herbicides and produces six insecticidal proteins.

• DP4114 expressing Cry1F insecticide with toxicity against lepidoptera, Cry34Ab1 and Cry35Ab1 insecticide with toxicity against coleoptera and PAT protein to confer resistance to glufosinate-ammonium-containing herbicides;

• MON 89034 expressing Cry1A.105 and Cry2Ab2 insecticidal proteins with toxicity against lepidoptera;

• MON 87411 expressing the Cry3Bb1 insecticide with toxicity against coleoptera and the DvSnf7 dsRNA insecticide with toxicity against coleoptera and the CP4 EPSPS protein resistance to glyphosate containing herbicides;

• DAS-40278-9 expressing the aryloxyalkanoate dioxygenase 1 (AAD-1) protein for resistance to 2,4-D and aryloxyphenoxypropionates (AOPP) herbicides such as quizalofop.

Consequently, the stacked maize produces six insecticides (Cry34Ab1 and Cry35Ab1, Cry1A.105, Cry2Ab2, Cry3Bb1 and the DvSnf7 dsRNA) and is resistant to four groups of complementary herbicides (glyphosate, glufosinate, AOPP and 2,4-D-containing herbicides).

1. Systematic literature review

A systematic review as referred to in Regulation (EU) No 503/2013 was not provided by the applicant. Based on preliminary information, the GMO Panel agreed that there is only limited value in undertaking a systematic review (EFSA, 2022a). This is not acceptable. The applicant should have conducted a review including data on each of the toxins produced in the plants, as well as data on all transgenic plants inheriting one or more of the events as combined in the stacked maize.

2. Molecular characterisation

The process of genetic engineering involved several deletions and insertions in the parental maize plants. Whole genome sequencing should have been used to identify unintended genetic changes, such as additional insertions of gene fragments or recombinatorial effects due to the crossings. In order to assess the sequences encoding the newly expressed proteins or any other open reading frames (ORFs) present within the insert and spanning the junction sites, it was assumed that the proteins that might emerge from these DNA sequences would raise no safety issues; and, therefore, no detailed investigations were carried out in this regard. Furthermore, other gene products, such as unintentionally produced ncRNA (non-coding RNA) from additional open reading frames, were not assessed. Thus, uncertainties remain about other biologically active substances resulting from the method of genetic engineering and the newly introduced gene constructs.

Previous research indicated that expression of Cry1A.105, Cry2Ab2 and EPSPS proteins in genetically engineered maize can induce changes in the overall proteome of the respective GM maize line, with impacts on associated endogenous metabolic pathways (Agapito-Tenfen et al., 2014). These transgenes are also present in the stacked maize. Thus, robust data should have been presented to assess whether metabolic changes with relevance to biosafety occur in the stacked maize. Further, Mesnage et al (2016) demonstrated alteration in stress-related metabolic pathways for NK603, which were, amongst others, accompanied by increased levels of polyamines. The authors stated that polyamines can provoke toxicological effect on their own or potentiate adverse effects of histamine.

Environmental stress can cause unexpected patterns of expression in the newly introduced DNA (see, for example, Trtikova et al., 2015). More specifically, Fang et al. (2018) showed that stress responses especially can lead to unexpected changes in plant metabolism, if they inherit additional EPSPS enzymes. However, the expression of the additional enzymes was only measured under field conditions in the US for one year. It is unclear, to which extent specific environmental conditions will influence the overall concentration of the enzymes in the plants. The plants should have been subjected to a much broader range of defined environmental conditions and stressors to gather reliable data on gene expression and functional genetic stability.

Due to increased weed pressure, it has to be expected that these plants can and will be exposed to high and also repeated dosages of glyphosate alone and / or in combination with the other complementary herbicides. Higher applications of herbicides will not only lead to a higher burden of residues in the harvest, but may also influence the expression of the transgenes or other genome activities in the plants. This aspect was completely ignored in risk assessment even though compositional changes were most noticeable after treatment with complementary herbicides; this supports the premise that a potential unexpected aggregation of herbicides increases the impact of genetic modification on plant metabolism (see also comments form the Experts of Member States, EFSA, 2022b).

EFSA should have requested that the applicant submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, also including repeated spraying and the application of each of the relevant herbicides alone and in combination. The material derived from those plants should have been assessed by using 'Omics' techniques to investigate changes in the gene activity of the transgene, as well as the natural genome of the plants.

Data on herbicide application rates and their impact on gene expression

From the available information, it appears that the complementary herbicides were only applied in combination, and only sprayed once. Nevertheless, EFSA is of the opinion that the design of the field trials is in accordance with the expected agricultural practices. To justify this opinion, EFSA should have presented more detailed reasoning. Furthermore, data for both the treated and untreated plants should be presented in the Annex.

Current EFSA practices are such that it is not possible to access the original data submitted by the companies within the period of consultation. Therefore, the opinion has to provide all the data necessary to allow other experts to conclude whether the provisions of GMO regulation are fulfilled.

In light of the information available, we assume that the application and the data provided do not sufficiently represent the agricultural practices, which could include the use of single herbicide applications, higher dosages and repeated spraying.

Therefore, EFSA should have requested the applicant to submit data from field trials that included all the relevant agricultural practices, all active ingredients, all dosages and all

combinations of the complementary herbicides that might be used in the agricultural practice of the GE maize producing countries. Without these data, no reliable conclusion can be drawn as requested in Implementing Regulation 503/2013 (in particular for herbicide tolerant GE plants) to assess whether anticipated agricultural practices influence the expression of the studied endpoints (see also Miyazaki et al., 2019).

Consequently, the GE maize plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are insufficient to conclude on the impact of the herbicide applications on gene expression, plant composition or biological characteristics of the plant as requested in EU Regulation 503/2013.

Impact of genetic backgrounds on gene expression

It is known that the genomic background of the varieties can influence both the expression of the inserted genes and plant metabolism (see, for example, Lohn et al., 2020; Trtikova et al., 2015). However, it appears that the data on gene expression were confined to a single variety. Therefore, EFSA should also have requested additional data from transgenic maize varieties, e.g. those cultivated in South America.

However, EFSA has not taken these issues into consideration. Consequently, the GE maize plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are therefore insufficient to conclude on the impact of the genetic backgrounds on gene expression as requested in EU Regulation 503/2013.

3. Comparative assessment of plant composition and agronomic and phenotypic characteristics

Implementing Regulation 503/2013 requests:

“In the case of herbicide tolerant genetically modified plants and in order to assess whether the expected agricultural practices influence the expression of the studied endpoints, three test materials shall be compared: the genetically modified plant exposed to the intended herbicide; the conventional counterpart treated with conventional herbicide management regimes; and the genetically modified plant treated with the same conventional herbicide management regimes.”

“The different sites selected for the field trials shall reflect the different meteorological and agronomic conditions under which the crop is to be grown; the choice shall be explicitly justified. The choice of non-genetically modified reference varieties shall be appropriate for the chosen sites and shall be justified explicitly.”

The data presented by Pioneer do not meet the requirements of Implementing Regulation 503/2013: (1) the field trials were not conducted in all relevant regions where the GE maize will be cultivated, and not all relevant extreme weather conditions were taken into account (such as drought); (2) the field trials did not take all relevant agricultural management practices into account; (3) not all relevant genetic backgrounds were taken into account.

Data on environmental factors and stress conditions - and their impact on plant composition and phenotype

Field trials to assess plant composition as well as agronomic and phenotypic characteristics of the GE maize were only conducted in the US for one year. Some extreme weather conditions were reported from the field trials. These, however, remain arbitrary and not well defined and do not allow any conclusions to be drawn on how gene expression will be affected by more severe climate stress due to drought, watering or high temperatures. In order to assess changes gene expression, the plants should have been grown in various environmental conditions and exposed to well-defined environmental stress conditions. This requirement is especially relevant in this case, since it is known that the additional epsps gene may show pleiotropic effects, also affecting seed dormancy, growth and stress responses of the plants (see, for example, Fang et al., 2018; Wang et al., 2014; Yang et al., 2017; Beres et al., 2018, Beres, 2019).

It should not be overlooked that, for example, Brazil is among the most important countries for maize imports into the EU: Brazil is a major producer of genetically engineered maize and is one of the largest exporters of maize to the EU (Commission Committee for the Common Organisation of Agricultural Markets, 2021).

Nevertheless, EFSA is of the opinion that the design of the field trials is in accordance with the expected agricultural practices. To justify this opinion, EFSA should have provided a much more detailed reasoning. Due to current EFSA practices, it is not possible to access the original data from the companies within the period of consultation. Therefore, the opinion has to provide all the necessary data to allow other experts to conclude whether the provisions of GMO regulation are fulfilled. In light of the information available, we assume that the application and the data provided do not sufficiently represent the agricultural practices and bio-regional conditions under which these plants are likely to be grown.

No experiments were requested to show to which extent specific environmental conditions influence plant composition and agronomic characteristics. Hence, no data were made available as requested in Implementing regulation 503/2013 to assess whether the expected environmental conditions under which the plants are likely to be cultivated will influence the expression of the studied endpoints.

Data on herbicide application rates and their impact on plant composition as well as agronomic and phenotypic characteristics

Due to the mode of action of the active ingredients in the complementary herbicides, it is plausible that complementary herbicide applications will cause stress responses in the plants, and thus impact gene expression and plant composition. These effects may vary with the amount of herbicide sprayed onto the crop and the various active ingredients which can be used.

From the available information, it looks like that the complementary herbicides were only applied in combination, with only one post-emergent (during the growth of the plants) spraying. Nevertheless, EFSA is of the opinion that the design of the field trials is in accordance with the expected agricultural practices. To justify this opinion, EFSA should have provided a much more

detailed reasoning. Due to current EFSA practices, it is not possible to access the original data from the companies within the period of consultation. Therefore, the opinion has to provide all necessary data to allow other experts to conclude whether the provisions of GMO regulation are fulfilled. In light of the information available, we assume that the application and the data provided do not sufficiently represent the agricultural practices, i.e. single herbicide use, higher dosages and repeated spraying.

EFSA should have requested the applicant to submit data from field trials on all the relevant active ingredients used in agricultural practice, including all dosages and combinations of the complementary herbicides which might be used in agricultural practice in GE maize producing countries. Without these data, no reliable conclusions can be drawn as requested in Implementing Regulation 503/2013 (in particular for herbicide tolerant GE plants) to assess whether anticipated agricultural practices influence the expression of the studied endpoints (see also Miyazaki et al., 2019).

Consequently, the GE maize plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are insufficient to conclude on the impact of the herbicide applications on gene expression, plant composition or biological characteristics of the plant as requested in EU Regulation 503/2013.

Impact of genetic backgrounds on plant composition as well as on agronomic and phenotypic characteristics

Only 8 agronomic and phenotypic endpoints were subjected to statistical analysis, 5 of them were significantly different in plants not sprayed with the complementary herbicides, 6 in plants sprayed with the complementary herbicides. This high number of significant findings should have prompted further investigations.

It is known that the genomic background of the varieties can influence both the expression of the inserted genes and plant metabolism (see, for example, Lohn et al., 2020; Trtikova et al., 2015). However, it appears that the data on gene expression were confined to a single variety. Therefore, EFSA should also have requested additional data from transgenic maize varieties that are, for example, cultivated in South America.

However, EFSA has not taken these issues into consideration. Consequently, the GE maize plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are therefore insufficient to conclude on the impact of the genetic backgrounds on gene expression as requested in EU Regulation 503/2013.

Data from compositional analysis show the need for further investigations

69 constituents were subjected to statistical analysis (10 in forage and 59 in grain).

• Statistically significant differences between the four-event stacked maize not treated with the complementary herbicide and the non-GM comparator were identified for 35 endpoints (5 in

forage and 30 in grain), some of them in the category III/IV, including the composition of fatty acids (such as lower concentration in oleic acids).

• Statistically significant differences between the four-event stacked maize treated with the complementary herbicide and the non-GM comparator were identified for 21 endpoints (4 in forage and 17 in grain), some of them in the category III/IV, including the composition of fatty acids (such as lower concentration in oleic acids).

Fatty acids are essential to the functioning of many biological processes, including energy supply and signalling. They are, in addition, structural components of cell membranes and also impact the synthesis of secondary metabolites in plants. Therefore, these findings should have prompted a request for further data, but this was, according to EFSA, unnecessary.

Given the above reasoning on the impact of environmental factors, herbicide applications and genetic backgrounds, as well as a higher number of significant findings in fields treated with the complementary herbicides, EFSA should indeed have requested more data: data on agronomic and phenotypic endpoints should be generated from a wider range of clearly defined stress factors, including all relevant agricultural practices and genetic backgrounds. This requirement is especially relevant in this case since it is known that the additional epsps genes may show pleiotropic effects, which also affect seed dormancy, growth and stress responses of the plants (see, for example, Fang et al., 2018; Wang et al., 2014; Yang et al., 2017; Beres et al., 2018, Beres, 2019).

A more detailed analysis would have been necessary to investigate changes in plant composition and phenotype, and also to investigate potential unintended changes in metabolic pathways and the emergence of unintended biologically active gene products.

The material derived from the plants should have been assessed by using ‘Omics’ techniques to investigate changes in the gene activity of the transgene and the plant genome, and also to investigate changes in metabolic pathways and the emergence of unintended biologically active gene products (see Benevenuto et al., 2022). Such in-depth investigations should not depend on findings indicating potential adverse effects, they should always be necessary to draw sufficiently robust conclusions to inform the next steps in risk assessment.

In addition, in awareness of the absence of any independent data on this maize (see literature review, EFSA, 2022a), we strongly recommend establishing a system with independent controls to repeat the trials and double check the data on plant composition and agronomic characteristics.

Conclusion on the comparative assessment of plant composition as well as on phenotypic and agronomic characteristics

The data provided by the applicant and accepted by EFSA are insufficient to conclude on the impact of environmental factors, herbicide applications and genetic backgrounds on gene expression, plant metabolism, plant composition, or on agronomic and phenotypic characteristics.

To gather reliable data on compositional analysis and agronomic characteristics, the plants should have been subjected to a much broader range of defined environmental conditions and stressors. Furthermore, EFSA should have requested the applicant to submit data from field trials which reflect current agricultural practices, including all relevant complementary herbicides and all relevant genetic backgrounds.

However, only samples from field sites located in the US were used to generate the data, and the impact of environmental factors and agricultural practices were not assessed in detail. Herbicide applications in the field trials did not represent all the relevant agricultural practices. Only one transgenic variety was grown in the field trials.

Consequently, the data presented by the applicant and accepted by EFSA are insufficient to conclude on the impact of environmental factors, herbicide applications or different genetic backgrounds on plant composition and agronomic characteristics.

Based on the available data, no final conclusions can be drawn on the safety of the plants. Therefore, the data neither fulfill the requirements of Implementing Regulation 503/2013 nor Regulation 1829/2003. This is also underlined by several statements made by experts from Member States (EFSA, 2022b).

In summary, the GE maize plants tested in the field trials do not sufficiently represent the products intended for import.

4. Toxicity

Implementing Regulation 503/2013 requests:

“Toxicological assessment shall be performed in order to:

(a) demonstrate that the intended effect(s) of the genetic modification has no adverse effects on human and animal health;

(b) demonstrate that unintended effect(s) of the genetic modification(s) identified or assumed to have occurred based on the preceding comparative molecular, compositional or phenotypic analyses, have no adverse effects on human and animal health;”

“In accordance with the requirements of Articles 4 and 16 of Regulation (EC) No 1829/2003, the applicant shall ensure that the final risk characterisation clearly demonstrates that:

(a) the genetically modified food and feed has no adverse effects on human and animal health;”

In addition, Implementing Regulation 503/2013 requests:

“For silencing approaches by RNAi expression, potential ‘off target’ genes should be searched by in silico analysis to assess if the genetic modification could affect the expression of other genes which raise safety concerns.”

Findings from molecular characterisation and comparative approach

As explained above, many significant changes were identified: more than half of the parameters measured for agronomic characteristics and plant composition were significantly different. Even if the changes taken as isolated data might not directly raise safety concerns, the overall number of effects should have been considered as a starting point for much more detailed investigation into their potential health impacts.

However, the data presented by the applicant did not take into account cultivation of the stacked maize under more extreme drought conditions, i.e. neither under realistic agricultural conditions nor considering all relevant countries of cultivation. The range of differences and their significance are likely to be substantially increased in these conditions. Thus, without more data, the true range of unintended effects cannot be determined and safety cannot be demonstrated as requested by EU regulation.

Despite these findings, and in awareness of the lack of more specific data and the resulting major uncertainties, no testing of the whole stacked plant (feeding study) was requested.

Toxicity of Bt toxins

There is new evidence (currently undergoing final peer review) that the toxicity of Cry1A proteins needs to be reassessed: Jneid et al (2022) show that Cry1A toxins induce enterocyte death and intestinal stem cell (ISC) proliferation in the midgut of Drosophila melanogaster, a species that is supposed to be non-susceptible to these Bt toxins.

According to their research, after exposure, a high proportion of the ISC daughter cells differentiated into enteroendocrine cells instead of their initial enterocyte destiny. They show that that Cry1A toxins weaken the Cadherin-dependent adherens junction between the ISC and its immediate daughter progenitor, leading the latter to adopt an enteroendocrine fate. Hence, though not lethal to non-susceptible organisms, Cry toxins can interfere with conserved cell adhesion mechanisms, thereby disrupting intestinal homeostasis and enteroendocrine functions.

As the mechanisms of intestinal progenitor fate choice are conserved in the animal kingdom, it is crucial that the risk assessment of Cry1A toxins investigates whether these proteins can also promote an increased number of enteroendocrine cells (EECs)in other organisms (such as vertebrates and invertebrates). EECs, through the production of neuropeptides and hormones, are involved in the regulation of many physiological functions, such as feeding behavior, metabolism and immune response. Consequences of this increase in EEC number could be, for example, metabolic dysfunctions or inflammatory pathologies. More studies are needed to understand the physiological impacts of this change in intestinal cellular composition on organismal health.

EFSA assumes that the gastrointestinal tract of mammals, including humans, lacks receptors with high specific affinity to Cry proteins. However, for more than a decade they have ignored that there are further mechanisms and processes that may render Bt toxins biologically active in so-called ‘non-target’ organisms. These toxins are produced by bacteria which, in terms of

taxonomy, belong to a group of Bacillus cereus which is known to produce many diseases in humans and animals. Cry1A toxins, in particular, are under discussion as to whether they cause severe effects, including in combination with other stressors or several Bt toxins: Grisolia et al. (2008) report embryo toxicity and developmental delay of mixtures of Cry1Aa + Cry1Ac and Cry 1 Aa + Cry 2A. Similar mixtures are also present in the current application.

Therefore, the safety of the toxin and their combinations can no longer be generally assumed but has to be demonstrated before any further transgenic plants producing these toxins enter the market. It should also be taken into account that interactions with plant constituents, such as proteinase inhibitors, can multiply the toxicity of these toxins (MacIntosh et al., 1990).

It seems that a systematic literature review on the toxicity of Cry toxins as produced in the plants is crucial. Since it is a legal obligation, EFSA and Pioneer cannot escape this crucial step in risk assessment just because they presumed safety of the toxins before the risk assessment was conducted.

In any case, in regard to toxicology and potential synergistic or other combinatorial effects, the negative impacts of Bt toxins on human and animal health cannot be excluded a priori. Bt toxins have several modes of action. They are produced in the plants but their biological characteristics are altered and not identical to their natural templates (Hilbeck & Otto, 2015).

Several publications describe the effects of Bt toxins in mammals: some Cry toxins are known to bind to epithelial cells in the intestines of mice (Vázquez-Padrón et al., 1999, Vásquez-Padrón et al., 2000). As far as potential effects on health are concerned, Thomas and Ellar (1983), Shimada et al. (2003) Huffmann et al. (2004), Ito et al. (2004), Mesnage et al. (2013) and Bondzio et al. (2013) show that Cry proteins could potentially have an impact on the health of mammals. Further publications (de Souza Freire et al., 2014; Mezzomo et al., 2014) confirm hematotoxicity of several Cry toxins, including those being used in genetically engineered plants, such as Cry1Ab and Cry1Ac. These effects seem to occur after high concentrations and tend to become stronger after several days. Such observations call for the study of effects after long-term exposure to various dosages, including in combination with material sprayed with the complementary herbicides. In this context, it is important to consider that the stacked maize is also resistant to the herbicides glyphosate and glufosinate, and the resulting residues should be seen as potential co-stressors at the stage of consumption (see also Then & Bauer-Panskus, 2017).

Relevant findings show that the selectivity and efficacy of Bt toxins produced in GE plants can be influenced by many co-factors (see, for example, Then, 2010; Hilbeck & Otto, 2015). Higher toxicity can also cause lower selectivity (Then, 2010): if synergistic or additive effects occur that increase efficacy of the Bt toxin, its selectivity may be decreased and a wider range of non-target organisms may become susceptible.

One crucial impact factor in this context are protease inhibitors (PI), which show synergistic effects with Bt toxins, strongly enhancing their toxicity. It is likely that PI delay the degradation of Bt proteins and thereby also enhance their toxicity. In many of its comments on EFSA

opinions, Testbiotech has highlighted these effects by referring, for example, to Pardo-López et al. (2009). However, EFSA has never provided a detailed response.

Testbiotech is aware of several publications confirming this gap in risk assessment that EFSA has constantly ignored or denied: as Monsanto already showed in the 1990s, maize, cotton and soybeans produce protease inhibitors (PI), which considerably enhance the toxicity of Bt proteins in plants. In the presence of PIs, Bt toxin will degrade much more slowly than in isolation. This results in a much higher toxicity of the Bt toxin (if it is taken up together with the plant tissue) compared to the isolated toxin (MacIntosh et al., 1990; Zhao et al., 1999; Zhang et al., 2000; Gujar et al., 2004; Zhu et al., 2007; Pardo-López et al., 2009; Ma et al., 2013; Mesén-Porras et al., 2020). The effects described indicate, for example, a 20-fold higher toxicity of Bt proteins if produced in the plants and taken up with PIs (MacIntosh et al., 1990).

It also should be taken into account that the toxicity of Bt toxins can not only be enhanced through interaction with plant enzymes such as PI, but also by Bt toxins (Sharma et al., 2004; Sharma et al., 2010; Tabashnik et al., 2013; Bøhn et al. 2016; Bøhn, 2018), gut bacteria (Broderick et al., 2009), residues from spraying with herbicides (Bøhn et al. 2016; Bøhn, 2018) and other co-stressors (Kramarz et al., 2007; Kramarz et al., 2009; Khalique and Ahmed, 2005; Singh et al., 2007; Zhu et al., 2005; Mason et al., 2011; Reardon et al., 2004; Nawrot-Esposito et al., 2020)

Therefore, any risk assessment that does not take synergistic effects caused by the combination of plant material or other stressors with the Bt toxin into account, is not reliable and systematically underestimates the risks (see also Testbiotech, 2021).

These issues are especially relevant for the stacked events since the overall concentration of Bt toxins is higher and combinatorial effects with other stressors (such as residues from spraying) more likely.

In summary, the evidence for enhanced toxicity of Bt proteins produced in maize, cotton and soybeans was published by Monsanto 30 years ago (MacIntosh et al., 1990) and has since then been confirmed in multiple studies. Crucially, EFSA has never assessed this aspect in any of its opinions.

Instead, the toxicity of the Bt toxins was assessed on the basis of feeding studies, using only isolated Bt proteins produced by bacteria for gavage experiments in mice. The data from these experiments were then used to calculate NOAEL (No-Observed-Adverse-Effect Level) and to assess the impact of exposure at the stage of consumption. Therefore, considering the above findings, the basic data for toxicity assessment of the stacked Maize are neither valid nor reliable. In addition, incorrect assumptions were made on the degradation of the Bt toxins at the stage of consumption and similarity to known toxins (see below). Therefore, the Pioneer risk assessment depends entirely on incorrect assumptions in regard to toxicity and exposure.

Immunogenicity of the Bt toxins

There are several studies indicating that immune responses in mammals can be triggered by Bt toxins and have to be considered in this context. Studies with the Cry1Ac toxin (Moreno-Fierros et al., 2000; Vázquez-Padrón et al. 1999; Legorreta-Herrera et al., 2010; Jarillo-Luna et al. 2008; E. González-González et al., 2015; Ibarra-Moreno et al., 2014; Guerrero et al. 2007; Guerrero et al., 2004; Moreno-Fierros et al. 2013; Rubio-Infante et al. 2018) are especially relevant (for review also see Rubio-Infante et al. 2016). Since Cry1Ac is also used as an adjuvant in vaccines, the risks to food consumption can be promoted through synergistic effects, this needs to be addressed and carefully examined.

The synergistic effects described by MacIntosh et al. (1990), Zhao et al. (1999), Zhang et al. (2000) Gujar et al. (2004), Zhu et al. (2007), Pardo-López et al. (2009), Ma et al. (2013), Mesén-Porras et al. (2020) causing higher toxicity of the Bt toxins are also relevant in risk assessment in regard to the immune system: combination with protease inhibitors is likely to be associated with a delay in the degradation of the Bt toxins after consumption. This delay in degradation extends the exposure of the intestinal immune system to Bt toxins and may trigger or enhance chronic inflammation and other immune responses (see also Testbiotech, 2021).

There are also findings from several whole food and feed studies, indicating a risk of inflammatory processes caused by maize producing Bt toxins: A study testing corn with a combination of Bt toxins (Cry1Ab and Cry34Ab1) indicates inflammation in rats (Zdziarski et al., 2018), as well as a study by Carman et al. (2013), feeding a triple stack of NK603, MON863 and MON810 maize. In addition, Ibrahim & Okasha (2016) found indication of inflammatory processes in the jenunum of rats fed with MON810 maize.

In this context, it is relevant that Bt toxins produced by plants can survive digestion to a much higher degree than has been assumed by EFSA and shown by the data of the applicant. Chowdhury et al. (2003) and Walsh et al. (2011) showed that when pigs were fed with Bt maize, Cry1A proteins could frequently and successfully still be found in the colon of pigs at the end of the digestion process. This means that Bt toxins are not degraded quickly in the gut and can persist in larger amounts until digestion is completed; therefore, there is enough time for interaction between various food compounds.

It has to be considered that the concentration of the insecticidal proteins is much higher in gluten meal produced from the maize, and that it can reach a much higher concentrations compared to the kernels.

These issues are especially relevant for the stacked events since the overall concentration of Bt toxins is higher compared to the parental plants.

Not only is the concentration of Bt toxins higher in the stacked Maize, there is also a higher likelihood of combinatorial effects with other stressors (such as residues from spraying). However, neither EFSA nor the applicant considered the potential enhancement of toxic or immunogenic effects caused by interaction with plant components such as PI. In this context, potential impacts on the microbiome also have to be taken into account (see below).

EFSA (2022a), states “To date, there is no evidence for adjuvanticity in the GMOs assessed by the Panel.” However, this statement upends the legal requirements. In accordance with the precautionary principle, EU regulation requests that the safety of GMOs is demonstrated prior to applying for import. Since it cannot generally be denied that Cry toxins have adjuvant effects, it is up to the applicant to provide evidence that these effects are absent in the stacked maize. Therefore, EFSA cannot simply take a different approach and wait for evidence of adjuvant effects to appear.

Effects of residues from spraying with complementary herbicide specific to GE plants and their mixed toxicity

The residues from spraying were considered to be outside the remit of the GMO Panel. However, without detailed assessment of these residues, no conclusion can be drawn on the safety of the imported products: due to specific agricultural management practices in the cultivation of the herbicide-resistant plants, there are, for example, specific patterns of spraying, exposure, occurrence of specific metabolites and emergence of combinatorial effects that require special attention.

EU pesticide regulation and GMO regulation both require a high level of protection for health and the environment. Thus, in regard to herbicide-resistant plants, specific assessment of residues from spraying with complementary herbicides must be considered a prerequisite for granting authorisation.

EU legal provisions, such as Regulation 1829/2003 (and Implementing Regulation 503/2013), state that “any risks which they present for human and animal health and, as the case may be, for the environment” have to be avoided. Therefore, potential adverse effects resulting from combinatorial exposure of various potential stressors need to be tested for mixed toxicity (EFSA, 2019b).

2,4-D, glufosinate and glyphosate have been shown to impact or disturb the microbiome, which can have substantial impact on the long-term toxicity (mixed toxicity) of whole food and feed derived from the stacked event, whereas data on AOPP herbicides, such as quizalofop, seem to be scarce. Dong et al. (2020) show that glufosinate can severely impact the microbiome; Tu et al. (2019) provide evidence on the adverse effects of 2,4-D. This is especially relevant in regard to combinatorial (accumulated) effects caused by the residues from spraying with glyphosate, which is known to cause shifts in the microbial composition and associated microbiomes of plants and animals. Glyphosate has been shown to cause shifts not only in soil organisms (van Bruggen et al., 2018, 2021, Chávez-Ortiz et al., 2022) and rhizosphere microbiome (Cesco et al., 2021) but also in the composition of the intestinal flora of humans (Mesnage et al., 2021a), cattle (Reuter et al., 2007), poultry (Shehata et al., 2013; Ruuskanen et al., 2020), amphibians (Boccioni et al., 2021), earthworms (Owagboriaye et al., 2021) and rodents (Hu et al., 2021; Liu et al., 2022; Mao et al., 2018; Mesnage et al., 2021b, 2021c; Tang et al., 2020) as well as honey bees (Motta et al., 2020) and daphnia (Suppa et al., 2020). Therefore, antibiotic effects caused by chronic exposure to food and feed derived from glyphosate-resistant GE plants, including this GE maize, are not unlikely to trigger significant changes in intestinal bacteria (see also Testbiotech, 2021).

In general, the microbiome can be seen as a common network of life, encompassing and closely interacting with plants, animals and humans. Microbial networks are thought to have co-evolved with their hosts and have developed a mutualistic relationship that benefits both the host and microorganisms. They act at the interphase and communicate between the organisms and their wider environment while at the same time being part of an organism's closer environment. Microbiomes are considered to be vital for the health of higher organisms, i.e. humans, animals and plants.

In regard to food and feed safety, EFSA (2020) considers microbiomes to be highly relevant to the health status of their hosts. Therefore, it is desirable to understand the importance of their role in risk assessment. EFSA expects that gut microbiome research (not only in the case of GE plants) will play a relevant role in regulatory science with potential implications for future risk assessments and predictive risk models. As EFSA states: "considering that the gut microbiome is a biological component directly and indirectly involved in the metabolism of food/feed components and chemicals and in the protection of the host against adverse environmental exposure, it would be useful to establish criteria on how to evaluate the potential adverse impacts of perturbators on this defensive barrier, and consequently, on human/animal health."

A 2019 study commissioned by EFSA on adjuvanticity / immunogenicity assessment of proteins included the role of the microbiome. Parenti et al. (2019) state that "one of the most important drivers of immune response is the gut microbiota and other microbial constituent of the human body which are able to regulate host-pathogen balance and to produce systemic pro-inflammatory stimuli. The lifelong antigenic load represented by foods and bacteria/bacterial products leads to a profound remodeling of the gut microbiota and these changes are emerging as a driving force of the functional homeostasis of the immune system. As a matter of fact, a perturbation of the gut microbiota homeostasis due to irregular lifestyles, stress and age may lead to gut microbiota dysbiosis. This condition may predispose the host to metabolic disorders and inflammation."

These findings are highly relevant for the risk assessment of the GE maize, which inherits combinations of herbicide resistance to glyphosate, AOPP herbicides, such as quizalofop, glufosinate and 2,4-D. These residues may cause gut microbiome perturbation, depending on exposure and combinatorial effects. It has to be considered a plausible hypothesis that the effects on the microbiome can trigger effects on the immune system, food uptake and body weight. This hypothesis and mixed toxicity need to be tested before any conclusion can be drawn on the health safety of food and feed. Since no such data can be derived from pesticide risk assessment, experimental data on mixed toxicity of the stacked maize have to be requested from the applicant.

In general, antibiotic effects and other adverse health effects might occur from exposure to a diet containing these plants that were not assessed under pesticide regulation. These adverse effects on health might be triggered by the residues from spraying with the complementary herbicide (see also van Bruggen et al., 2018). Further attention should be paid to the specific toxicity of the metabolites of the pesticide active ingredients that might occur specifically in the stacked event.

However, no attempts have been made to integrate the microbiome into the risk assessment of food and feed derived from the GE maize. This is in direct contradiction to Regulation 1829/2003 which requests “genetically modified food and feed should only be authorised for placing on the Community market after a scientific evaluation of the highest possible standard, to be undertaken under the responsibility of the European Food Safety Authority (Authority), of any risks which they present for human and animal health and, as the case may be, for the environment.” (Recital 9).

EU legal provisions such as Regulation 1829/2003 (as well as Implementing Regulation 503/2013) state that “any risks which they present for human and animal health and, as the case may be, for the environment” have to be avoided. Therefore, potential adverse effects that result from combinatorial exposure of various potential stressors need specification, and their assessment needs to be prioritised. We conclude that the health risk assessment currently performed by EFSA for the stacked maize is unacceptable. We propose testing these plants following the whole mixture approach, considering them to be “insufficiently chemically defined to apply a component-based approach” (EFSA, 2019).

Despite all these open questions regarding potential health impacts, we are not aware of a single sub-chronic or chronic feeding study performed with whole food and feed derived from the stacked maize. This observation is supported by the literature review carried out by the company which did not yield any peer reviewed publication. In this context, it is relevant to consider that the outcome of the feeding studies with the parental plants raised several questions concerning their results, methodology and reliability.

For this purpose, EFSA should have requested the company to submit data from field trials with the highest dosage of complementary herbicides that can be tolerated by the plants, including repeated spraying. The material derived from the plants should have been assessed in regard to organ toxicity, immune responses and reproductive toxicity, also taking combinatorial effects with other plants components into account.

As a result, the toxicological assessment carried out by EFSA is not acceptable.

Toxicity of ncsRNA DvSnf7

A report commissioned and published by EFSA in 2019 (Davalos et al., 2019) considers the role of ncsRNA in the risk assessment of GE plants. Davalos et al. summarise current findings on ncsRNAs produced by plants; they discuss to which extent they can be taken up via food or feed consumption and show cross kingdom activity due to unintentional interaction with human or animal gene regulation.

Potential off target genes in mammals

As Davalos et al. (2019) show, there are many matches between the ncsRNA produced in food and medical plants and regulatory pathways in human and animals. There is no doubt that in cases where relevant plant molecules are transmitted into the cells of humans and animals, RNAi

effects, such as gene silencing, can occur and, for example, genes in animals can be downregulated by plant nscRNA.

Therapeutic effects from the uptake of ncsRNA from the gut have been evidenced in several publications. Some of the research shows that biological effects can be achieved with very low dosages (for references see Davalos et al., 2019).

Stability of ncsRNA

It appears that some findings depend on the specific type of ncsRNA. For example, naked synthetic ncsRNA used by some researchers, is degraded very quickly compared to ncsRNA produced by plants (for references see Davalos et al., 2019).

The Davalos et al. (2019) study found strong indications that plant miRNAs are more stable than previously anticipated. This is due to structural properties influencing their stability and turnover "However, when assessing the stability of plant ncRNAs outside the plant, compelling evidence exists that plant miRNAs are highly stable under different conditions including food storage, processing, cooking, or simulated digestion. Moreover, they seem to survive after long incubation in serum, or are detected in the gastric content of mice, suggesting that plant miRNAs are more resistant to degradation than synthetic or animal miRNAs."

ncsRNA uptake from the gut

Contrary to assumptions made by EFSA, research by Davalos et al. (2019) shows that the uptake of ncsRNA from plants and microorganisms via the gut into the cells of humans and animals is an established fact.

It is known that there are many barriers between the intestine, the blood stream, the cells and the cell nuclei, which lower the likelihood of such RNAi effects occurring. However, according to Davalos et al. (2019), there are mechanisms that can allow the molecules to pass through these barriers: plant ncsRNA is protected against degradation by methylation, it can be excreted and taken up in vesicles (such as exosomes); nano-particles are also produced by plants which can serve as transport elements.

The ncsRNA molecules originating from plants can reportedly be found in many bodily fluids of humans and animals, including blood and milk. Similar findings have been reported by Nawaz et al., (2019): “Strong evidence suggested that plant-food-miRNAs can survive digestion, enter the body and affect gene expression patterns.”

In this context, Davalos et al. (2019) see the need for further research to explore the uptake and biological effects of ncsRNA: “Exogenous plant-derived ncRNAs have been found in exosomes or macrovesicles. How they reach these types of structures in biological fluids is unknown. In summary, supporting and contradicting evidence concerning the existence of systemic effects of dietary plant-derived exogenous ncRNAs is heavily debated. Important aspects such as the precise mechanism/s of transport of plant ncRNAs from food into the systemic circulation, the

amount of exogenous ncRNAs reaching tissues or the molecular mechanisms of cellular uptake need to be determined."

Interactions on the level of the microbiome

There is strong evidence that ncsRNAs originating in the host (e.g. produced by intestinal epithelial cells) are taken up by the gut microbiota and can manipulate its gene regulation. The same evidence is available for ncsRNA produced in the gut microbiome: it can be taken up by the host and enact RNAi in its cells, demonstrating the existence of bidirectional ncRNAs based host-microbial interactions (for details see Davalos et al., 2019).

In this context, Davalos et al. (2019) show that plant-derived ncsRNA does not necessarily have to be taken up from the intestine to exert its effects. Instead, interaction with the intestinal microbiome can emerge which, in a next step, may impact the health of the animal or human host.

This is of specific interest in the case of DvSnf7: the Snf7 gene which is targeted by the dsRNA produced in maize MON 87441, is involved in important biological processes in insects as well as in yeast. The essential role of the Snf7 as part of the ESCRT pathway is well described (see www.yeastgenome.org/locus/S000004015).

Thus, there is a plausible hypothesis on how the additional dsRNA might affect the gut microbiome community after ingestion, and further research is needed to understand the impact of exogenous dsRNA in mammalian host microbiota composition and identify microbial targets along with their effect on physiological conditions.

There is broad consensus on the role of the gut microbiome in human and animal health. For example, in 2019, in the study commissioned by EFSA, Parenti et al. (2019) states that "one of the most important drivers of immune response is the gut microbiota and other microbial constituent of the human body which are able to regulate host-pathogen balance and to produce systemic pro-inflammatory stimuli. The lifelong antigenic load represented by foods and bacteria/bacterial products leads to a profound remodeling of the gut microbiota and these changes are emerging as a driving force of the functional homeostasis of the immune system. As a matter of fact, a perturbation of the gut microbiota homeostasis due to irregular lifestyles, stress and age may lead to gut microbiota dysbiosis. This condition may predispose the host to metabolic disorders and inflammation."

Therefore, the interaction between the ncsRNAs produced by GE plants and the microbiome of humans or animals has to be considered in food and feed safety assessment. In this context, the barrier for ncsRNA to pass from plants to gut microorganisms seems to be much lower compared to those identified in the human or animal body.

Conclusions on toxicity assessment of ncsRNA DvSnf7

In summary, it is clear that interference with gene regulation following the absorption and processing of dsRNAs to siRNA within humans and animals after ingestion of RNAi-based GM

crops is both feasible and plausible. As Nawaz et al. (2019) conclude: “Based on the currently available evidence, off-target effects from the ingestion of novel siRNA present in foods derived from either GM crops or foliar insecticidal or anti-viral spray application, cannot be ignored and thus should form an integral part of the risk assessment of these products.”

As shown by Davalos et al., (2019) and Nawaz et al. (2019), the uptake of ncsRNA from plants via ingestion in sufficient amounts to exert effects on gene regulation in mammalian cells must be seen as a certainty. Further, the impact on the host via its microbiome is another way in which human or animal health could be affected.

Therefore, further risk assessment has to be performed

• to trace the fate of the artificial ncsRNA after ingestion

• to identify the potential target site in the microbial community in the gut and mammalian cells

• to assess the magnitude of potential effects if identified.

Additional questions have arisen from risk assessment in respect to the mixed toxicity of the stacked Maize. These questions are highly relevant for demonstrating the safety of the plants because other newly expressed proteins, residues from spraying or plant constituents, can influence the impact on the microbiome in the gut or the uptake from the gut.

Allergenicity

EFSA assessment of allergenic risks (EFSA, 2022d) is not based on a sufficiently realistic exposure to newly introduced proteins and their interactions. Different routes of exposure, the timing of exposure, microbial exposure, oral and gut microbiota composition, epithelial barrier integrity and/or non-allergenic components of the food matrix, such as immune-modulating components (adjuvants) of allergenic sources that facilitate immune responses, all have to be considered. In particular, the high number of proteins additionally expressed in the plants make it essential for appropriate data to be made available.

However, the necessary methodology is neither provided nor requested by EFSA. Therefore, the outcome of assessing allergenicity cannot be regarded as being sufficient.

5. Environmental risk assessment

The appearance of teosinte in Spain and France (see Testbiotech, 2016; Trtikova et al., 2017) has to be considered in more detail. Maize volunteers can be found in the EU on a regular basis as has been reported from Palaudelmàs et al. (2009) in Spain or from Pascher (2016) in Austria. Further, in awareness of the biological characteristics of the GE maize and the findings of Fang et al. (2018), the stacked maize needs to be examined in detail regarding next generation effects, volunteer potential (persistence) and gene flow. Under these circumstances, even a rare single outcrossing that goes unnoticed can have a huge long-term impact on the agro-ecosystems.

Furthermore, the EFSA (2022a) opinion is also wrong for several reasons:

• Without more data on the teosinte species growing in the EU, the likelihood of gene flow from the maize to teosinte cannot be assessed (Trtikova et al., 2017). The same is true for gene flow from teosinte to genetically engineered plants.

• Furthermore, the characteristics of potential hybrids and next generations have to be investigated and cannot be predicted simply from the data of the original event. It is well known that there can be next generation effects and interference from genetic background that cannot be predicted from the assessment of the original event (Bauer-Panskus et al., 2020). This issue is relevant for gene flow from maize to as well from teosinte to maize.

EFSA should have requested data from the applicant to show that no adverse effects can occur through gene flow from the maize to teosinte and / or from teosinte to the maize volunteers. In the absence of such data, the risk assessment and the authorisation have to be regarded as not valid.

Without detailed consideration of the hazards associated with the potential gene flow from maize to teosinte and from teosinte to maize, no conclusion can be drawn on the environmental risks of spillage from the stacked maize.

Consequently, environmental risk assessment carried out by EFSA is not acceptable.

Testbiotech is aware of a recent EFSA statement (2022c) regarding the teosinte situation in France and Spain. Here, EFSA comes to the conclusion:

“The new evidence retrieved confirms that where maize and EU teosinte plants co-occur and flower synchronously, maize alleles (transgenic or not), can move into teosinte populations at rates that depend on different factors. Hence, the possible introgression of transgenes from maize MON810, Bt11, 1507 and GA21 into EU teosinte may only provide a selective advantage to GM teosinte hybrid progeny under high infestation of target pests and/or when glufosinate-ammonium- and/or glyphosate-based herbicides are applied. However, this fitness advantage will not allow GM teosinte hybrid progeny to overcome other biological and abiotic factors limiting their persistence and invasiveness. Therefore, EFSA considers that the growth habits of EU teosinte plants and teosinte hybrid progeny are such that the acquisition of insect resistance and/or herbicide tolerance is unlikely to change their relative persistence and invasive characteristics under EU conditions.”

However, in the updated risk assessment, EFSA still does not consider that epsps genes as such may induce fitness advantages (as noted, for example, by Fang et al., 2018; Wang et al., 2014; Yang et al., 2017). The updated teosinte risk assessment is therefore too narrow to conclude on possible environmental effects and provides no answers to relevant risk related questions.

6. Others

For monitoring and methods to identify the specific event, Implementing Regulation 503/2013 requests:

The method(s) shall be specific to the transformation event (hereafter referred to as 'event-specific') and thus shall only be functional with the genetically modified organism or genetically modified based product considered and shall not be functional if applied to other transformation events already authorised; otherwise the method cannot be applied for unequivocal detection/identification/quantification. This shall be demonstrated with a selection of non-target transgenic authorised transformation events and conventional counterparts. This testing shall include closely related transformation events.

However, no such method for identification was made available. Based on the information available, it will not be possible to distinguish the stacked event from a mixture of single parental events or stacked events that overlap with the actual stack.

If approval for import is given, the applicant has to ensure that post-market monitoring (PMM) is developed to collect reliable information on the detection of indications showing whether any (adverse) effects on health may be related to GM food or feed consumption. Thus, the monitoring report should at very least contain detailed information on: i) actual volumes of the GE products imported into the EU, ii) the ports and silos where shipments of the GE products were unloaded, iii) the processing plants where the GE products was transferred to, iv) the amount of the GE products used on farms for feed, and v) transport routes of the GE products. Environmental monitoring should be run in regions where viable material of the GE products such as kernels are transported, stored, packaged, processed or used for food/feed. In case of losses and spread of viable material (such as kernels) all receiving environments need to be monitored. Furthermore, environmental exposure through organic waste material, by-products, sewage or faeces containing GE products during or after the production process, and during or after human or animal consumption should be part of the monitoring procedure (see also comments from Member States experts, EFSA, 2022b).

In addition, the example of the stacked maize highlights some general problems. These are:

(1) Due to current EFSA practices it is not possible to access the original data from the companies within the period of consultation. Therefore, the opinion has to provide all the necessary data to allow other experts to conclude whether the provisions of GMO regulation (esp. 503/2013) are fulfilled. We are making this comment after our recent experiences in requesting access to documents, which in many instances took months to achieve. The Commission should advise EFSA to improve transparency.

(2) A Testbiotech report published in 2021 (Testbiotech, 2021), shows how the European Food Safety Authority (EFSA), which is responsible for risk assessment of GE plants, intentionally puts crucial issues aside. This careless approach exemplifies the overall decrease in general food safety standards that has been ongoing since the introduction of GE plants. The number of events authorised for import has, at the same time, steadily increased. In light of these findings, the Commission should try to avoid 'rubber stamping' all applications for import of GE plants, and

thus reduce the overall number of products entering the market, while ensuring that these products undergo much more thorough risk assessment.

References

Bauer-Panskus, A., Miyazaki, J., Kawall, K., Then, C. (2020) Risk assessment of genetically engineered plants that can persist and propagate in the environment. Environmental Sciences Europe, 32(1): 1-15. https://doi.org/10.1186/s12302-020-00301-0

Benevenuto, R.F., Venter, H.J., Zanatta, C.B., Nodari, R.O., Agapito-Tenfen, S.Z. (2022) Alterations in genetically modified crops assessed by omics studies: Systematic review and meta-analysis. Trends in Food Science & Technology, 120: 325-337. https://doi.org/10.1016/j.tifs.2022.01.002

Beres, Z.T. (2019) Ecological and evolutionary implications of glyphosate resistance in Conyza canadensis and Arabidopsis thaliana. Dissertation presented in partial fulfillment of the requirements for the degree Doctor of Philosophy in the graduate school of the Ohio State University. http://rave.ohiolink.edu/etdc/view?acc_num=osu1555600547328876

Beres, Z.T., Yang, X., Jin, L., Zhao, W., Mackey, D.M., Snow, A.A. (2018) Overexpression of a native gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) may enhance fecundity in Arabidopsis thaliana in the absence of glyphosate. Int J Plant Sci, 179(5): 390-401.

https://doi.org/10.1086/696701

Boccioni, A.P.P.C., García-Effron, G., Peltzer, P.M., Lajmanovich, R.C. (2021) The effect of glyphosate and ciprofloxacin exposure on the gut bacterial microbiota diversity of Rhinella Arenarum (Anura: Bufonidae) tadpoles. [Preprint]. https://www.researchsquare.com/article/rs-492368/v1

Bøhn, T. (2018) Criticism of EFSA's scientific opinion on combinatorial effects of ‘stacked’GM plants. Food Chem. Toxicol, 111: 268-274. https://doi.org/10.1016/j.fct.2017.11.023

Bøhn, T., Rover, C.M., Semenchuk, P.R. (2016) Daphnia magna negatively affected by chronic exposure to purified Cry-toxins. Food Chem. Toxicol. 91: 130-140. https://doi.org/10.1016/j.fct.2016.03.009

Bondzio, A., Lodemann, U., Weise, C., Einspanier, R. (2013) Cry1Ab treatment has no effects on viability of cultured porcine intestinal cells, but triggers hsp70 expression. PloS One, 8: e67079. https://doi.org/10.1371/journal.pone.0067079

Broderick, N.A., Robinson, C.J., McMahon, M.D., Holt, J., Handelsman, J., Raffa, K.F. (2009) Contributions of gut bacteria to Bacillus thuringiensis-induced mortality vary across a range of Lepidoptera. BMC Biol, 7: 11. https://doi.org/10.1186/1741-7007-7-11

Carman, J. A., Vlieger, H. R., Ver Steeg, L. J., Sneller, V. E., Robinson, G. W., Clinch-Jones, C. A., ... & Edwards, J. W. (2013). A long-term toxicology study on pigs fed a combined genetically modified (GM) soy and GM maize diet. J Org Syst, 8(1), 38-54. http://www.organic-systems.org/journal/81/8106.pdf

Cesco, S., Lucini, L., Miras-Moreno, B., Borruso, L., Mimmo, T., Pii, Y., ... & Trevisan, M. (2021) The hidden effects of agrochemicals on plant metabolism and root-associated microorganisms. Plant Science, 311: 111012. https://doi.org/10.1016/j.plantsci.2021.111012

Chávez-Ortiz, P., Tapia-Torres, Y., Larsen, J., & García-Oliva, F. (2022) Glyphosate-based herbicides alter soil carbon and phosphorus dynamics and microbial activity. Applied Soil Ecology, 169: 104256. https://doi.org/10.1016/j.apsoil.2021.104256

Chowdhury, E.H., Kuribara, H., Hino, A., Sultana, P., Mikami, O., Shimada, N., Guruge, K.S., Saito, S., 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. J Anim Sci, 81(10): 2546-2551. https://doi.org/10.2527/2003.81102546x

Commission Committee for the Common Organisation of Agricultural Markets (2021) EU Cereals Trade 2021/22, Marketing Year July – September, 26 November 2021). https://circabc.europa.eu/sd/a/a1135630-e8e9-4531-a522-23670f75e2c5/cereals-trade-2017-18-marketing-year-july-december.pdf

Dávalos, A., Henriques, R., Latasa, M.J., Laparra, M., Coca, M. (2019) Literature review of baseline information on non-coding RNA (ncRNA) to support the risk assessment of ncRNA-based genetically modified plants for food and feed. EFSA Supporting Publication, 16(8): EN-1688. https://doi.org/10.2903/sp.efsa.2019.EN-1688

de Souza Freire, I., Miranda-Vilela, A.L., Barbosa, L.C.P., Martins, E.S., Monnerat, R.G., Grisolia, C.K. (2014) Evaluation of cytotoxicity, genotoxicity and hematotoxicity of the recombinant spore- crystal complexes Cry1Ia, Cry10Aa and Cry1Ba6 from Bacillus thuringiensis in Swiss mice. Toxins, 6: 2872-2885. https://doi.org/10.3390/toxins6102872

Dong, T., Guan, Q., Hu, W., Zhang, M., Zhang, Y., Chen, M., ... & Xia, Y. (2020). Prenatal exposure to glufosinate ammonium disturbs gut microbiome and induces behavioral abnormalities in mice. Journal of Hazardous Materials, 389: 122152. https://doi.org/10.1016/j.jhazmat.2020.122152

EFSA (2019) Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. EFSA Journal, 17(3): 5634. https://doi.org/10.2903/j.efsa.2019.5634

EFSA (2020) Editorial: Exploring the need to include microbiomes into EFSA's scientific assessments. EFSA J, 18(6): e18061. https://doi.org/10.2903/j.efsa.2020.e18061

EFSA (2022a) Scientific Opinion on the assessment of genetically modified maize DP4114 × MON 89034 × MON 87411 × DAS-40278-9 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA GMO-NL-2020-171). EFSA J, 20(11): 7619. https://doi.org/10.2903/j.efsa.2022.7619

EFSA (2022b) Comments and opinions submitted by Member States during the three-month consultation period. OpenEFSA portal. https://open.efsa.europa.eu/

EFSA (2022c) Statement on the update of environmental risk assessment conclusions and risk management recommendations of EFSA (2016) on EU teosinte. EFSA J, 20(4): 7228. https://doi.org/10.2903/j.efsa.2022.7228

EFSA (2022d) Scientific Opinion on development needs for the allergenicity and protein safety assessment of food and feed products derived from biotechnology. EFSA J, 20(1): 7044. https://doi.org/10.2903/j.efsa.2022.7044

Fang, J., Nan, P., Gu, Z., Ge, X., Feng, Y.-Q., Lu, B.-R. (2018) Overexpressing exogenous 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) genes increases fecundity and auxin content of transgenic Arabidopsis plants. Front Plant Sci, 9: 233. https://doi.org/10.3389/fpls.2018.00233

González-González, E., García-Hernández ,A.L., Flores-Mejía, R., López-Santiago, R., Moreno-Fierros, L. (2015) The protoxin Cry1Ac of Bacillus thuringiensis improves the protection conferred by intranasal immunization with Brucella abortus RB51 in a mouse model. Vet Microbiol, 175(2-4): 382-388. https://doi.org/10.1016/j.vetmic.2014.11.021

Grisolia, C. K., Oliveira, R., Domingues, I., Oliveira-Filho, E. C., Monerat, R. G., & Soares, A. M. (2009) Genotoxic evaluation of different δ-endotoxins from Bacillus thuringiensis on zebrafish adults and development in early life stages. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 672(2), 119-123. https://doi.org/10.1016/j.mrgentox.2008.10.017

Guerrero, G.G., Dean, D.H., Moreno-Fierros, L. (2004) Structural implication of the induced immune response by Bacillus thuringiensis cry proteins: role of the N-terminal region. Mol Immunol, 41(12): 1177-1183. https://doi.org/10.1016/j.molimm.2004.06.026

Guerrero, G.G. & Moreno-Fierros, L. (2007) Carrier potential properties of Bacillus thuringiensis Cry1A toxins for a diphtheria toxin epitope. Scand J Immunol, 66(6): 610-618. https://doi.org/10.1111/j.1365-3083.2007.01992.x

Gujar, T., Kalia, V., Kumari, A., Prasad, T.V. (2004) Potentiation of insecticidal activity of Bacillus thuringiensis subsp. kurstaki HD-1 by proteinase inhibitors in the American bollworm, Helicoverpa armigera (Hübner). Indian J Exp Biol, 42: 157-163. http://hdl.handle.net/123456789/23352

Hilbeck, A. & Otto, M. (2015) Specificity and combinatorial effects of Bacillus thuringiensis Cry toxins in the context of GMO risk assessment. Front Environ Sci, 3: 71. https://doi.org/10.3389/fenvs.2015.00071

Hu, J., Lesseur, C., Miao, Y., Manservisi, F., Panzacchi, S., Mandrioli, D., ... & Petrick, L. (2021) Low-dose exposure of glyphosate-based herbicides disrupt the urine metabolome and its interaction with gut microbiota. Scientific Reports, 11(1): 1-10. https://doi.org/10.1038/s41598-021-82552-2

Huffmann, D.L., Abrami, L., Sasik, R., Corbeil, J., van der Goot, G., Aroian, R.V. (2004) Mitogenactivated protein kinase pathways defend against bacterial pore-forming toxins. PNAS USA, 101: 10995-11000. https://doi.org/10.1073/pnas.0404073101

Ibarra-Moreno, S., García-Hernández, A.L., Moreno-Fierros L. (2014) Coadministration of protoxin Cry1Ac from Bacillus thuringiensis with metacestode extract confers protective immunity to murine cysticercosis. Parasite Immunol, 36(6): 266-270. https://doi.org/10.1111/pim.12103

Ibrahim, M.A., & Okasha, E.F. (2016). Effect of genetically modified corn on the jejunal mucosa of adult male albino rat. Exp Tox Pathol, 68(10): 579-588. https://doi.org/10.1016/j.etp.2016.10.001

Ito, A., Sasaguri, Y., Kitada, S., Kusaka, Y., Kuwano, K., Masutomi, K., Mizuki, E., Akao, T., Ohba, M. (2004) Bacillus thuringiensis crystal protein with selective cytocidal action on human cells. J Biol Chem, 279: 21282-21286. https://doi.org/10.1074/jbc.M401881200

Jarillo-Luna, A., Moreno-Fierros L., Campos-Rodríguez R., Rodríguez-Monroy, M.A., Lara-Padilla,E., Rojas-Hernández, S. (2008) Intranasal immunization with Naegleria fowleri lysates and Cry1Ac induces metaplasia in the olfactory epithelium and increases IgA secretion. Parasite Immunol, 30(1): 31-38. https://doi.org/10.1111/j.1365-3024.2007.00999.x

Jneid, R., Loudhaief, R., Zucchini-Pascal, N., Nawrot-Esposito, M. P., Fichant, A., Rousset, R., ... & Gallet, A. (2022). Bacillus thuringiensis Cry1A toxins divert progenitor cell fate toward enteroendocrine lineage by diminishing cell adhesion with intestinal stem cells. BioRxiv. https://doi.org/10.1101/2022.04.13.488147

Khalique, F. & Ahmed, K. (2005) Compatibility of bio-insecticide with chemical insecticide for management of Helicoverpa armigera Huebner. Pak J Biol Sci, 8: 475-478. https://dx.doi.org/10.3923/pjbs.2005.475.478

Kramarz, P., de Vaufleury, A., Gimbert, F., Cortet, J., Tabone, E., Andersen, M.N., Krogh, P.H. (2009) Effects of Bt-maize material on the life cycle of the land snail Cantareus aspersus. Appl Soil Ecol, 42: 236-242. https://doi.org/10.1016/j.apsoil.2009.04.007

Kramarz, P.E., de Vaufleury, A., Zygmunt, P.M.S., Verdun, C. (2007) Increased response to cadmium and Bacillus thuringiensis maize toxicity in the snail Helix aspersa infected by the

nematode Phasmarhabditis hermaphrodita. Environ Toxicol Chem, 26: 73-79. https://doi.org/10.1897/06-095R.1

Legorreta-Herrera, M., Oviedo Meza, R., Moreno-Fierros L. (2010) Pretreatment with Cry1Ac protoxin modulates the immune response, and increases the survival of plasmodium -infected CBA/Ca mice. BioMed Research International: 198921. https://doi.org/10.1155/2010/198921

Liu, J. B., Chen, K., Li, Z. F., Wang, Z. Y., Wang, L. (2022) Glyphosate-induced gut microbiota dysbiosis facilitates male reproductive toxicity in rats. Science of The Total Environment, 805: 150368. https://doi.org/10.1016/j.scitotenv.2021.150368

Lohn, A.F., Trtikova, M., Chapela, I., Van den Berg, J., du Plessis, H., Hilbeck, A. (2020) Transgene behavior in Zea mays L. crosses across different genetic backgrounds: Segregation patterns, cry1Ab transgene expression, insecticidal protein concentration and bioactivity against insect pests. PLoS ONE, 15(9): e0238523. https://doi.org/10.1371/journal.pone.0238523

MacIntosh, S.C., Kishore, G.M., Perlak, F.J., Marrone, P.G., Stone, T.B., Sims, S.R., Fuchs, R.L. (1990) Potentiation of Bacillus thuringiensis insecticidal activity by serine protease inhibitors. J Agric Food Chem, 38: 1145-1152. https://doi.org/10.1021/jf00094a051

Ma, Y., Zhang, Y., Chen, R.-R., Ren, X.-L., Wan, P.-J., Mu, L.-L., Li, G.-Q. (2013) Combined effects of three crystalline toxins from Bacillus thuringiensis with seven proteinase inhibitors on beet armyworm, Spodoptera exigua Hübner (Lepidoptera: Noctuidae). Pestic Biochem Physiol, 105: 169-176. https://doi.org/10.1016/j.pestbp.2013.01.007

Mao, Q., Manservisi, F., Panzacchi, S., Mandrioli, D., Menghetti, I., Vornoli, A., Bua, L., Falcioni, L., Lesseur, C., Chen, J., Belpoggi, F., Hu, J. (2018) The Ramazzini Institute 13-week pilot study on glyphosate and Roundup administered at human-equivalent dose to Sprague Dawley rats: effects on the microbiome. Environmental Health, 17: 50. https://doi.org/10.1186/s12940-018-0394-x

Mason, K.L., Stepien, T.A., Blum, J.E., Holt, J.F., Labbe, N.H., Rush, J.S., Raffa, K.F., Handelsman, J. (2011) From commensal to pathogen: translocation of Enterococcus faecalis from the midgut to the hemocoel of Manduca sexta. mBio 2: e00065-00011. https://doi.org/10.1128/mBio.00065-11

Mesén-Porras, E., Dahdouh-Cabia, S., Jimenez-Quiros, C., Mora-Castro, R., Rodríguez, C., Pinto-Tomás, A. (2020) Soybean protease inhibitors increase Bacillus thuringiensis subs. israelensis toxicity against Hypothenemus hampei. Agronomía Mesoamericana, 31: 461-478. https://doi.org/10.15517/am.v31i2.36573

Mesnage, R., Clair, E., Gress, S., Then, C., Székács, A., & Séralini, G. E. (2013). Cytotoxicity on human cells of Cry1Ab and Cry1Ac Bt insecticidal toxins alone or with a glyphosate-based herbicide. Journal of Applied Toxicology, 33(7), 695-699. https://doi.org/10.1002/jat.2712

Mesnage, R., Agapito-Tenfen, S.Z., Vilperte, V., Renney, G., Ward, M., Séralini, G.E, Nodari, R.O., Antoniou, M.N. (2016) An integrated multi-omics analysis of the NK603 Roundup-tolerant GM maize reveals metabolism disturbances caused by the transformation process. Sci Rep, 6: 37855. https://doi.org/10.1038/srep37855

Mesnage, R., Calatayud, M., Duysburgh, C., Marzorati, M., Antoniou, M. (2021a) Alterations in human gut microbiome composition and metabolism after exposure to glyphosate and Roundup and/or a spore-based formulation using the SHIME® technology. BioRxiv [Preprint]. https://www.biorxiv.org/content/10.1101/2021.12.16.472928v1

Mesnage, R., Panzacchi, S., Bourne, E., Mein, C.A., Perry, M.J., Hu, J., ... & Antoniou, M.N. (2021b) Glyphosate and its formulations Roundup Bioflow and RangerPro alter bacterial and fungal community composition in the rat caecum microbiome. BioRxiv [Preprint]. https://www.biorxiv.org/content/10.1101/2021.11.19.468976v1

Mesnage, R., Teixeira, M., Mandrioli, D., Falcioni, L., Ducarmon, Q.R., Zwittink, R.D., ... & Antoniou, M.N. (2021c) Use of shotgun metagenomics and metabolomics to evaluate the impact of glyphosate or Roundup MON 52276 on the gut microbiota and serum metabolome of Sprague-Dawley rats. Environmental Health Perspectives, 129(1): 017005. https://doi.org/10.1289/EHP6990

Mezzomo, B.P. (2013) Hematotoxicity of Bacillus thuringiensis as spore-crystal strains Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa in Swiss albino mice. J Hematol Thromb Dis, 1(1): 1-9. http://repositorio.unb.br/handle/10482/18532

Miyazaki, J., Bauer-Panskus, A., Bøhn, T., Reichenbecher, W., Then, C. (2019) Insufficient risk assessment of herbicide-tolerant genetically engineered soybeans intended for import into the EU. Environmental Sciences Europe, 31(1): 1-21. https://doi.org/10.1186/s12302-019-0274-1

Moreno-Fierros, L., García, N., Gutiérrez, R., López-Revilla, R., Vázquez-Padrón, R.I. (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(8): 885-890. https://doi.org/10.1016/S1286-4579(00)00398-1

Moreno-Fierros, L., García-Hernández, A.L., Ilhuicatzi-Alvarado, D., Rivera-Santiago, L., Torres-Martínez, M., Rubio-Infante, N., Legorreta-Herrera, M. (2013) Cry1Ac protoxin from Bacillus thuringiensis promotes macrophage activation by upregulating CD80 and CD86 and by inducing IL-6, MCP-1 and TNF-α cytokines. International Immunopharmacology, 17(4): 1051-1066. https://doi.org/10.1016/j.intimp.2013.10.005

Motta, E.V., Mak, M., De Jong, T.K., Powell, J.E., O'Donnell, A., Suhr, K.J., Riddington, I.M., Moran, N.A. (2020) Oral and topical exposure to glyphosate in herbicide formulation impact the gut microbiota and survival rates of honey bees. Appl Environ Microbiol, 6: e01150-20. https://doi.org/10.1128/AEM.01150-20

Nawaz, M.A, Mesnage, R., Tsatsakis, A.M., Golokhvast, K.S., Yang, S.H., Antoniou, M.N., Chung, G. (2019) Addressing concerns over the fate of DNA derived from genetically modified food in the human body: a review. Food Chem Toxicol, 124: 423-430. https://doi.org/10.1016/j.fct.2018.12.030

Nawrot-Esposito, M. P., Babin, A., Pasco, M., Poirié, M., Gatti, J. L., & Gallet, A. (2020). Bacillus thuringiensis bioinsecticides induce developmental defects in non-target Drosophila melanogaster larvae. Insects, 11(10), 697. https://doi.org/10.3390/insects11100697

Owagboriaye, F., Mesnage, R., Dedeke, G., Adegboyega, T., Aladesida, A., Adeleke, M., ... & Antoniou, M.N. (2021) Impacts of a glyphosate-based herbicide on the gut microbiome of three earthworm species (Alma millsoni, Eudrilus eugeniae and Libyodrilus violaceus): A pilot study. Toxicology Reports, 8: 753-758. https://doi.org/10.1016/j.toxrep.2021.03.021

Pardo-López, L., Muñoz-Garay, C., Porta, H., Rodríguez-Almazán, C., Soberón, M., Bravo, A. (2009) Strategies to improve the insecticidal activity of Cry toxins from Bacillus thuringiensis. Peptides, 30(3): 589-595. https://doi.org/10.1016/j.peptides.2008.07.027

Parenti, M.D., Santoro, A., Del Rio, A., Franceschi, C. (2019) Literature review in support of adjuvanticity/immunogenicity assessment of proteins. EFSA Supporting Publications, 16(1): 1551E.

https://doi.org/10.2903/sp.efsa.2019.EN-1551

Pascher, K. (2016) Spread of volunteer and feral maize plants in Central Europe: recent data from Austria. Environmental Sciences Europe, 28(1):28-30. https://doi.org/10.1186/s12302-016-0098-1

Palaudelmàs, M., Peñas, G., Melé, E., Serra, J., Salvia, J., Pla, M., Nadal, A., Messeguer, J. (2009) Effect of volunteers on maize gene flow. Transgenic Research, 18(4): 583-594. https://doi.org/10.1007/s11248-009-9250-7

Reardon, B.J., Hellmich, R.L., Sumerford, D.V., Lewis, L.C. (2004) Growth, Development, and Survival of Nosema pyrausta -Infected European Corn Borers (Lepidoptera: Crambidae) Reared on Meridic Diet and Cry1Ab. J Econ Entomol, 97: 1198-1201. https://doi.org/10.1093/jee/97.4.1198

Reuter, T., Alexander, T.W., Martínez, T.F., McAllister, T.A. (2007) The effect of glyphosate on digestion and horizontal gene transfer during in vitro ruminal fermentation of genetically modified canola. J Sci Food Agric, 87(15): 2837-2843. https://doi.org/10.1002/jsfa.3038

Rubio-Infante, N. & Moreno-Fierros, L. (2016) An overview of the safety and biological effects of Bacillus thuringiensis Cry toxins in mammals. J Appl Toxicol, 36(5): 630-648.https://doi.org/10.1002/jat.3252

Rubio-Infante, N., Ilhuicatzi-Alvarado, D., Torres-Martínez, M., Reyes-Grajeda, J.P., Nava-Acosta, R., González-González, E., Moreno-Fierros, L. (2018) The macrophage activation induced by Bacillus thuringiensis Cry1Ac protoxin involves ERK1/2 and p38 pathways and the interaction with cell-Surface-HSP70. J Cell Biochem, 119(1): 580-598. https://doi.org/10.1002/jcb.26216

Ruuskanen, S., Rainio, M.J., Gomez-Gallego, C., Selenius, O., Salminen, S., Collado, M.C., Saikkonena, K., Saloniemia, I., Helander, M. (2020) Glyphosate-based herbicides influence antioxidants, reproductive hormones and gut microbiome but not reproduction: A long-term experiment in an avian model. Environ Pollut, 266(1): 115108. https://doi.org/10.1016/j.envpol.2020.115108

Sharma, H.C., Sharma, K.K., Crouch, J.H. (2004) Genetic transformation of crops for insect resistance: potential and limitations. Crit Rev Plant Sci, 23(1): 47-72. https://doi.org/10.1080/07352680490273400

Sharma, P., Nain, V., Lakhanpaul. S, Kumar, P.A. (2010) Synergistic activity between Bacillus thuringiensis Cry1Ab and Cry1Ac toxins against maize stem borer (Chilo partellus Swinhoe). Lett Appl Microbiol, 51(1):42-47. https://doi.org/10.1111/j.1472-765X.2010.02856.x

Shehata, A.A., Schrödl, W., Aldin, A.A., et al. (2013) The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. Curr Microbiol, 66(4): 350-358. https://doi.org/10.1007/s00284-012-0277-2

Shimada, N., Kim, Y.S., Miyamoto, K., Yoshioka, M., Murata, H. (2003) Effects of Bacillus thuringiensis Cry1Ab toxin on mammalian cells. J Vet Med Sci, 65(2): 187-191. https://doi.org/10.1292/jvms.65.187

Singh, G., Rup, P.J., Koul, O. (2007) Acute, sublethal and combination effects of azadirachtin and Bacillus thuringiensis toxins on Helicoverpa armigera (Lepidoptera: Noctuidae) larvae. Bull Entomol Res, 97: 351-357. https://doi.org/10.1017/S0007485307005019

Steinberg, P., van der Voet, H., Goedhart, P. W., Kleter, G., Kok, E. J., Pla, M., ... & Wilhelm, R. (2019) Lack of adverse effects in subchronic and chronic toxicity/carcinogenicity studies on the glyphosate-resistant genetically modified maize NK603 in Wistar Han RCC rats. Archives of Toxicology, 93(4): 1095-1139. https://doi.org/10.1007/s00204-019-02400-1

Steinberg, P., van der Voet, H., Goedhart, P. W., Kleter, G., Kok, E. J., Pla, M., ... & Wilhelm, R. (2020). Correction to: Lack of adverse effects in subchronic and chronic toxicity/carcinogenicity studies on the glyphosate-resistant genetically modified maize NK603 in Wistar Han RCC rats. Archives of Toxicology, 94: 1779-1781. https://doi.org/10.1007/s00204-020-02751-0

Suppa, A., Kvist, J., Li, X., Dhandapani, V., Almulla, H., Tian, A.Y., Kissane, S., Zhou, J., Perotti, A., Mangelson, H., Langford, K., Rossi, V., BrownJ.B., Orsini, L. (2020) Roundup causes embryonic development failure and alters metabolic pathways and gut microbiota

functionality in non-target species. Microbiome, 8(1): 1-15. https://doi.org/10.1186/s40168-020-00943-5

Tabashnik, B.E., Fabrick, J.A., Unnithan, G.C., Yelich, A.J., Masson, L., Zhang, J., Bravo, A., Soberón, M. (2013) Efficacy of genetically modified Bt toxins alone and in combinations against pink bollworm resistant to Cry1Ac and Cry2Ab. PLOS ONE, 8(11): e80496. https://doi.org/10.1371/journal.pone.0080496

Tang, Q., Tang, J., Ren, X., Li, C. (2020) Glyphosate exposure induces inflammatory responses in the small intestine and alters gut microbial composition in rats. Environ Poll, 261: 114129. https://doi.org/10.1016/j.envpol.2020.114129

Testbiotech (2016) Cultivation of genetically engineered maize: Risks not under control - Overview: Why the EU should not allow the cultivation of transgenic maize engineered to produce insecticidal toxins. Testbiotech Background, https://www.testbiotech.org/node/1759

Testbiotech (2021) Risk assessment of GE plants in the EU: Taking a look at the 'dark side of the moon'. https://www.testbiotech.org/content/risk-assessment-ge-plants-eu-taking-look-dark-side-moon

Then, C. (2010) Risk assessment of toxins derived from Bacillus thuringiensis: synergism, efficacy, and selectivity. Environ Sci Pollut Res Int, 17: 791-797. https://doi.org/10.1007/s11356-009-0208-3

Then, C., & Bauer-Panskus, A. (2017) Possible health impacts of Bt toxins and residues from spraying with complementary herbicides in genetically engineered soybeans and risk assessment as performed by the European Food Safety Authority EFSA. Environ Sci Eur, 29(1): 1. https://doi.org/10.1186/s12302-016-0099-0

Thomas, W.E. & Ellar, D.J. (1983) Bacillus thuringiensis var israelensis crystal delta-endotoxin: effects on insect and mammalian cells in vitro and in vivo. J Cell Sci, 60(1): 181-197. https://jcs.biologists.org/content/60/1/181.short

Trtikova, M., Wikmark, O.G., Zemp, N., et al. (2015) Transgene expression and Bt protein content in transgenic Bt maize (MON810) under optimal and stressful environmental conditions. PLoS ONE 10(4): e0123011. https://doi.org/10.1371/journal.pone.0123011

Trtikova, M., Lohn, A., Binimelis, R., Chapela, I., Oehen, B., Zemp, N., Widmer, A., Hilbeck, A. (2017) Teosinte in Europe – searching for the origin of a novel weed. Scientific Reports, 7:1560. https://www.nature.com/articles/s41598-017-01478-w

Tu, P., Gao, B., Chi, L., Lai, Y., Bian, X., Ru, H., & Lu, K. (2019) Subchronic low-dose 2, 4-D exposure changed plasma acylcarnitine levels and induced gut microbiome perturbations in mice. Scientific reports, 9(1), 1-11. https://www.nature.com/articles/s41598-019-40776-3

Van Bruggen, A.H.C., He, M.M., Shin, K., Mai, V., Jeong, K. C., Finckh, M.R., Morris, J.G. (2018) Environmental and health effects of the herbicide glyphosate. Science of The Total Environment, 616: 255-268. https://www.sciencedirect.com/science/article/pii/S0048969717330279

Van Bruggen, A.H.C., Finckh, M.R., He, M., Ritsema, C.J., Harkes, P., Knuth, D., Geissen, V. (2021) Indirect effects of the herbicide glyphosate on plant, animal and human health through its effects on microbial communities. Frontiers in Environmental Science, 9: 763917. https://doi.org/10.3389/fenvs.2021.763917

Vázquez-Padrón, R.I., Moreno-Fierros, L., Neri-Bazán, L., de la Riva, G.A., López-Revilla, R. (1999) Intragastric and intraperitoneal administration of Cry1Ac protoxin from Bacillus thuringiensis induces systemic and mucosal antibody responses in mice. Life Sciences, 64: 1897-1912. https://doi.org/10.1016/S0024-3205(99)00136-8

Vázquez-Padrón, R.I., Gonzáles-Cabrera, J., García-Tovar, C., Neri-Bazan, L., Lopéz-Revillac, L., Hernández, M., Moreno-Fierro, L., de la Riva, G.A. (2000) Cry1Ac protoxin from Bacillus thuringiensis sp. kurstaki HD73 binds to surface proteins in the mouse small intestine. Biochem Biophys Res Commun, 271(1): 54-58. https://doi.org/10.1006/bbrc.2000.2584

Walsh, M.C., Buzoianu, S.G., Gardiner, G.E., Rea, M.C., Gelencsér, E., Jánosi, A., Jánosi, A., Epstein, M.M., Lawlor, P.G. (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. https://doi.org/10.1371/journal.pone.0027177

Wang, W., Xia, H., Yang, X., Xu, T., Si, H.J., Cai, X.X., Wang, F., Su, J., Snow, A.A., Lu, B.-R. (2014) A novel 5-enolpyruvoylshikimate-3-phosphate (EPSP) synthase transgene for glyphosate resistance stimulates growth and fecundity in weedy rice (Oryza sativa) without herbicide. New Phytol, 202(2): 679-688. https://doi.org/10.1111/nph.12428

Yang, X., Li, L., Jiang, X., Wang, W., Cai, X., Su, J., Wang, F., Lu, B.-R. (2017) Genetically engineered rice endogenous 5-enolpyruvoylshikimate-3-phosphate synthase (epsps) transgene alters phenology and fitness of crop-wild hybrid offspring. Sci Rep, 7(1): 1-12. https://doi.org/10.1038/s41598-017-07089-9

Zdziarski, I.M., Carman, J.A., Edwards, J.W. (2018) Histopathological investigation of the stomach of rats fed a 60% genetically modified corn diet. Food Sci Nutr, 9: 763-796. https://doi.org/10.4236/fns.2018.96058

Zhang, J., Wang, C., Qin, J. (2000) The interactions between soybean trypsin inhibitor and δ-endotoxin of Bacillus thuringiensis in Helicoverpa armigera larva. J Invertebr Pathol, 74(5): 259-266. https://doi.org/10.1006/jipa.2000.4936

Zhao, J.Z., Fan, Y.L., Fan, X.L., Shi, X.P., Lu, M.G. (1999) Evaluation of transgenic tobacco expressing two insecticidal genes to delay resistance development of Helicoverpa armigera. Chin Sci Bull, 44: 1871-1874. https://doi.org/10.1007/BF02886343

Zhu, Y.C., Abel, C.A., Chen, M.S. (2007) Interaction of Cry1Ac toxin (Bacillus thuringiensis) and proteinase inhibitors on the growth, development, and midgut proteinase activities of the bollworm, Helicoverpa zea. Pestic Biochem Physiol, 87(1): 39-46. https://doi.org/10.1016/j.pestbp.2006.05.004

Zhu, Y.C., Adamczyk, J.J., West, S. (2005) Avidin, a potential biopesticide and synergist to Bacillus thuringiensis toxins against field crop insects. J Econ Entomol, 98: 1566-1571. https://doi.org/10.1093/jee/98.5.1566