

# Maize MON 87427 x MON 87460 x MON 89304 x 1507 x MON 87411 x 59122

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

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## **a. Assessment:**

### **4. Conclusions and recommendations**

Toxic, toxic, toxic. For years, the European GMO-free Citizens have resolutely maintained that GM crops are toxic. Undeterred, you have stuck to your story that this crop is the same as the “normal” one. That was never the case. When will you finally live up to your responsibility and ban these rotten, poisonous crops from the European market? We are also writing on behalf of Stichting Ekopark, Lelystad, which shares our objections. This genetically modified maize must be removed from the EU market.

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## **5. Others**

Even then

GMWatch via Twitter. Brazil's National Cancer Institute names GM crops as cause of massive pesticide use <https://www.gmwatch.org/en/news/latest-news/16067> gmwatch.org  
Brazil's National Cancer Institute names GM crops as cause of massive pesticide use Details  
Published 09 April 2015 After the WHO's classification of glyphosate as a “probable carcinogen” Brazil's cancer institute condemns GM crops for placing the country in the top ranking globally for pesticide consumption.

<http://www.biodiversidadla.org/Recomendamos/La-relacion-entre-el-glifosato-y-el-cancer-el-informe-argentino-del-Instituto-Nacional-del-Cancer> Claire Robinson reports

The links between glyphosate and cancer - report from Argentina's National Cancer Institute. Biodiversity in Latin America (biodiversidadla.org). The links between glyphosate and cancer - report from Argentina's National Cancer Institute. By Marcos Filardi and Claudio Lowy. Fragment ‘CANCER IN HUMANS. There is limited evidence of the carcinogenicity of glyphosate in humans. A positive association has been observed for the risk of non-Hodgkin's lymphoma and exposure to glyphosate. CANCER IN ANIMAL TESTING. There is sufficient evidence of the carcinogenicity of glyphosate in laboratory animals.’

Country: Argentina 28 January 2021

GMWatch

Argentinas National Cancer Institute confirms glyphosate is a probable carcinogen  
<http://biodiversidadla.org/Recomendamos/La-relacion-entre-el-glifosato-y-el-cancer-el-informe-argentino-del-Instituto-Nacional-del-Cancer...> Important because since GMO crops came along Argentina has become the largest consumer of glyphosate per population on the planet

Moms Across America According to a new study we have not even begun to see the true impact of glyphosate. This new study by Kubsad et al published in Scientific Reports shows that glyphosate alone not the surfactants impacts the germline of the fetus in female rats. The germline means the sperm and eggs inside the fetus which will ultimately become the fetuses' offspring. The sperm and eggs are harmed by glyphosate affecting the health and viability of future generations.  
<https://www.nature.com/articles/s41598-019-42860-0> Assessment of Glyphosate Induced Epigenetic Transgenerational Inheritance of Pathologies and Sperm Epimutations Generational Toxicology Scientific Reports nature.com • Article • Open Access • Published 23 April 2019 Assessment of Glyphosate Induced Epigenetic Transgenerational Inheritance of Pathologies and Sperm Epimutations Generational Toxicology • Deepika Kubsad • Eric E. Nilsson • Stephanie E. King • Ingrid Sadler-Riggelman • Daniel Beck • Michael K. Skinner GMO Free USA 1u • “Independent Egyptian scientists have found that Bayer-Monsantos GMO Bt toxic corn is NOT substantially equivalent to its Non-GMO parent. While Monsanto claims there is no evidence of toxicity in their voluntary safety assessments these independent scientists will tell you otherwise. By the 91st day of their studies they found evidence of kidney liver intestinal toxicity as well as male infertility. And an investigation by Testbiotech found that Monsanto's own data from 30 years ago revealed that Bt proteins expressed in genetically modified plants are significantly more toxic than natural Bt toxins.”

Morphological and Biochemical Changes in Male Rats Fed on Genetically Modified Corn  
Ajeeb YG Journal of American Science 2012

Adel shatta

Ahmed Rayan Histopathological Changes in Some Organs of Male Rats Fed on Genetically Modified Corn  
Ajeeb YG Marsland Press 2012

Ahmed Rayan

READPDF Morphological and Biochemical Changes in Male Rats Fed on Genetically Modified Corn  
Ajeeb YG Adel shatta - Academia.edu  
[https://www.academia.edu/3138607/Morphological\\_and\\_Biochemical\\_Changes\\_in\\_Male\\_Rats\\_Fed\\_on\\_Genetically\\_Modified\\_Corn\\_Ajeeb\\_YG\\_fbc](https://www.academia.edu/3138607/Morphological_and_Biochemical_Changes_in_Male_Rats_Fed_on_Genetically_Modified_Corn_Ajeeb_YG_fbc) lidIwAR1AgiMWdRhS0p9JRSyRp9NUQXmGm7jlpz360pXbcV-yEWeJ45ZiUva4LJI

READ PDF Histopathological Changes in Some Organs of Male Rats Fed on Genetically Modified Corn  
Ajeeb YG Ahmed Rayan - Academia.edu  
[https://www.academia.edu/3405345/Histopathological\\_Changes\\_in\\_Some\\_Organs\\_of\\_Male\\_Rats\\_Fed\\_on\\_Genetically\\_Modified\\_Corn\\_Ajeeb\\_YG\\_fbc](https://www.academia.edu/3405345/Histopathological_Changes_in_Some_Organs_of_Male_Rats_Fed_on_Genetically_Modified_Corn_Ajeeb_YG_fbc) lidIwAR3H251MrJKb7EmWax\_15UyTjxhUkBJRIDGqWfCmVcMVILJfBUTGbXYHNa4

READ Are GM plants with Bt toxins 20 times more toxic than previously known  
gmwatch.org <https://gmwatch.org/en/news/archive/2020-articles/19632-are-gm-plants-with-bt->

toxins-20-times-more-toxic-than-previously-knownfbclidIwAR0o11oHEy6BDptpH0IsM3ET0e iwQk5PEQjEueGuQSI\_T2gZi56ORp2MtNQ

Glyphosate and Roundup disturb gut microbiome and blood biochemistry at doses that regulators claim to be safe gmwatch.org New study reveals evidence for potential cancer-causing damage. Report Claire Robinson Glyphosate and the glyphosate-based herbicide Roundup disrupt the gut microbiome by the same mechanism by which the chemical acts as a weedkiller and these effects happen even at low doses that regulators claim to be safe a newly published study has found.1 <https://www.gmwatch.org/en/news/latest-news/19677-glyphosate-and-roundup-disturb-gut-microbiome-and-blood-biochemistry-at-doses-that-regulators-claim-to-be-safe>

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 nih.gov

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 Robin Mesnage<sup>1</sup> Maxime Teixeira<sup>2</sup> Daniele Mandrioli<sup>3</sup> Laura Falcioni<sup>3</sup> Quinten Raymond Ducarmon<sup>4</sup> Romy Daniëlle Zwittink<sup>4</sup> Francesca Mazzacuva<sup>5</sup> Anna Caldwell<sup>5</sup> John Halket<sup>5</sup> Caroline Amiel<sup>2</sup> Jean-Michel Panoff<sup>2</sup> Fiorella Belpoggi<sup>3</sup> and Michael Nicolas Antoniou<sup>1</sup> <https://ehp.niehs.nih.gov/doi/pdf/10.1289/EHP6990>

GMWatch Are GM plants with Bt toxins 20 times more toxic than previously known Details Published 21 December 2020 EFSA has for decades ignored crucial data from Monsanto

Data from Monsanto revealed that Bt proteins expressed in genetically modified GM plants are significantly more toxic than natural Bt toxins reports Testbiotech. It is more than 30 years ago since in 1990 Monsanto data first showed that if mixed with plant material from e.g. soybeans cotton and maize toxicity could be up to 20 times higher. This is due to enzymes naturally present in the tissues of many crop plants.

Further information

The Monsanto publication <https://pubs.acs.org/doi/10.1021/jf00094a051>

Recent information on the pending approval processes <https://www.testbiotech.org/en/news/eu-close-approving-new-ge-plants>

Source Testbiotech <https://www.testbiotech.org/en/press-release/are-ge-plants-bt-toxins-20-times-more-toxic-previously-known>

Resistance Through resistance the herbicide accumulates in plants in the form of an acetylated product from which the herbicide is subsequently released into the gastrointestinal tract of warmblooded animals. This has been demonstrated in the case of rats chickens and goats used as test animals by M.N. Huang et al Metabolism of <sup>14</sup>C Glufosinate and in <sup>14</sup>C—N-Acetyl Glufosinate in lactating goats and laying hens – Agrevo Frankfurt whereby the herbicide also enters the human food chain – with all the consequences that that entails. Source Eijsten and Van der Meulen also see page 15 20 21 26 30 and 31 in this book

<https://www.gentechvrij.nl/2020/11/27/book-eijsten/> . This must be assessed with certainty in your EFSA comment. For the EFSA to write that it is not its job to assess this is fallacious we are dealing here with processes taking place within plants. Glyphosate residue is also present in this glyphosate-resistant genetically modified Soybean DAS-81419-2 x DAS-44406-6 as a protein adduct. Here too the herbicide is released in the intestinal tract. We are not talking in general about herbicide residues but highly specifically about the residues of herbicides to which crops have been made resistant and highly specifically about the properties of the particular residues and highly specifically about the mechanisms that enable these herbicide residues to enter the food chain. And hence we find the proposal inconceivable.

<https://sustainablepulse.com/2019/12/30/togos-agriculture-minister-announces-total-ban-on-glyphosate-herbicides/> Togos Agriculture Minister Announces Total Ban on Glyphosate Herbicides - Sustainable Pulse

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

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**a. Assessment:**

**b. Food Safety Assessment:**  
**Toxicology**

08-02-21 Supplementary 1 of GMO-free Citizens.

<https://www.nature.com/articles/s41598-021-82552-2>

Low-dose exposure of glyphosate-based herbicides disrupt the urine metabolome and its interaction with gut microbiota Jianzhong Hu Corina Lesseur Yu Miao Fabiana Manservisi Simona Panzacchi Daniele Mandrioli Fiorella Belpoggi Jia Chen Lauren Petrick Scientific Reports volume 11 Article number 3265 2021 Cite this article

Abstract Fragment Glyphosate-based herbicides GBHs can disrupt the host microbiota and influence human health. In this study we explored the potential effects of GBHs on urinary metabolites and their interactions with gut microbiome using a rodent model.

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## 5. Others

Also on behalf of Stichting Ekopark Lelystad NL.

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**Organisation: Testbiotech e.V. - Institute for Independent Impact Assessment of Biotechnology**

**Country: Germany**

**Type: Non Profit Organisation**

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**a. Assessment:**

**Molecular characterisation**

Annex II of Implementing Regulation 503/2013 requests that

“The risk assessment of genetically modified food and feed containing stacked transformation events shall also include an assessment of the following aspects a stability of the transformation events b expression of the transformation events c potential synergistic or antagonistic effects resulting from the combination of the transformation events shall be subject to an assessment in accordance with Sections 1.4 Toxicology 1.5 Allergenicity and 1.6 Nutritional assessment.” Introduction

“Protein expression data including the raw data obtained from field trials and related to the conditions in which the crop is grown in regard to the newly expressed proteins.” Scientific requirements 1.2.2.3

“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.” Scientific requirements 1.3.1

“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.” Scientific requirements 1.3.2.1

Assessment of open reading frames The process of genetic engineering involved several deletions and insertions in the parental GE maize plants. 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 concerns therefore no detailed investigations were carried out in this regard.

Furthermore other biologically active gene products such as unintended non coding small RNAs ncsRNAs from additional open reading frames were not assessed. Thus uncertainties remain in regard to other biologically active substances arising from the method of genetic engineering and the newly introduced gene constructs.

Consequently EFSA neither took into account all the relevant data requested by EU regulation nor potential synergistic or antagonistic effects resulting from the combination of the transformation events. This would however be needed to draw reliable conclusions on health safety including the assessment of toxicity and impact on the immune system.

Impact of environmental factors agricultural practice and genetic backgrounds There are several reasons why the data presented do not meet the requirements of Implementing Regulation 503/2013 1 the field trials were not conducted in all relevant regions where the Maize is to be cultivated and no extreme weather conditions were taken into account 2 the field trials did not take current agricultural management practices into account 3 only one transgenic variety was included in the field trials.

Data on environmental factors stress conditions and their impact on gene expression Environmental stress can cause unexpected patterns in expression of the newly introduced DNA see for example Trtikova et al. 2015 Lohn et al. 2020. There is plenty of evidence showing that drought or heat can significantly impact the content of Bt in the plant tissue Adamczyk Meredith 2004 Adamczyk et al. 2009 Chen et al. 2005 Dong Li 2006 Luo et al. 2008 Then Lorch 2008 Trtikova et al. 2015 Jiang et al. 2018 Girón-Calva et al. 2020. Therefore to assess gene expression the plants should have been grown under conditions of severe drought with and without irrigation with and without application of the complementary herbicide and in comparison to more moderately severe climate conditions. However no such data were requested or used for detailed comparison to assess the genome x environment interactions.

More specifically Fang et al. 2018 and Yang et al. 2017 show that stress responses can lead to unintended changes in plant metabolism inheriting additional EPSPS enzymes. In this context there are strong indications that the EPSPS enzyme which confers glyphosate tolerance also interferes with the auxin metabolism in the plants Fang et al. 2018. This plant hormone plays a key role in growth fecundity and adaptation to environmental stressors. Thus changes in the auxin content can also result in changes in plant composition that can raise safety concerns.

Several publications support these findings showing unintended effects in plants inheriting additional EPSPS genes Beres 2019 Beres et al. 2018 Wang et al. 2014. In addition other authors show the need for further investigations Vila-Aiub et al. 2009 and Vila-Aiub et al. 2019.

The EPSPS enzymes occur in the stacked Maize in higher concentration compared to the parental plants. Therefore the Maize should have been subjected to a broad range of defined environmental conditions and stressors to gather reliable data on gene expression and functional genetic stability. This is especially relevant for the stacked Maize since these plants are the first in which expression of the epsps gene constructs are intended to be exposed to more extreme climate conditions.

Therefore the Maize should have been tested in all major maize producing countries such as North and South America. Furthermore the Maize should have been subjected to defined drought conditions since it must be assumed it will be grown in accordance with its intended purpose. However this was not the case Only five sites suited in the US were used in this case. They are all relatively close and therefore only represent a very limited region of the maize-producing areas in the US no other maize-producing country was included.

No extreme weather conditions were reported during the cultivation. In summary the available publications strongly indicate that plants inheriting a combination of EPSPS and CSPB are likely to show gene expression responses when grown under stress conditions such as drought. In addition Bt toxin expression in GE plants is known to be influenced by environmental conditions such as drought.

Thus in the stacked Maize there is a combination of a trait which is meant to expose the plant to extreme climate conditions and gene constructs such as for the EPSPS enzyme and the production of Bt toxins which are likely to show or to cause major changes in gene expression if exposed to environmental stressors such as drought. There are also no data on gene expression and the dsRNA DvSnf7 under stress conditions. Furthermore data on the concentration of the biologically active molecules in the plants are missing.

Further additional proteins are expressed which are absent in conventional maize NPTII which confers antibiotic resistance and should have been avoided as requested by EU Directive 2001/18 and PMI which was used for selecting process during plant production. These traits and genetic elements may synergise and interact with each other. The resulting effects are not unlikely to impact plant composition and biological characteristics crucial for the assessment of food and feed safety. However no specific data were requested or used for detailed comparison to assess genome x environment interactions.

At the very least the unintended effects of the EPSPS enzymes plausibly interfere with the activity of the other gene constructs for example via the auxin hormone see above. This is especially relevant if the plants are exposed to stress conditions. Even if no such effects were observed in the parental plants which to our knowledge were not tested in field trials under stressful conditions these enzymes are produced in the stacked Maize at higher concentrations. Therefore the likelihood of interaction between the gene constructs and gene expression is higher in the stacked compared to the parental plants.

It has to be expected that the stacked maize will be grown under drought conditions to an extent that most of the parental GE plants were not tested for in their previous risk assessment. Moreover it is the first time that the combination of artificial gene constructs will be exposed to more extreme drought conditions. It is obvious that in the absence of adequate data there can be no assessment of whether interactions will occur under stress conditions. Therefore there is no scientifically sound way of arguing why much more specific data is not necessary. Consequently the stacked plants should have been grown under conditions of severe drought with and without irrigation with and without application of the complementary herbicide and in comparison to more moderately severe climate conditions.

In summary while the plants are expected to show improved performance under more extreme drought conditions no such data have been reported from the field trials. No data are made available as requested by 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. Furthermore no such data are available from the parental plants or any subcombinations.

These gaps in risks assessment are further emphasised in other meteorological data

- US weather data from 2014 show that for example there was more precipitation than normal at the trial sites in Illinois. This means that a relevant stressor that might influence gene

expression composition or phenotype of the Maize may not be covered by the field trial data.

- Trial sites such as in Illinois neither represent the climatic conditions in other maize growing regions of the US nor the conditions in other relevant maize producing countries such as Brazil. USDA data show relevant maize production in North Dakota Minnesota Mississippi Louisiana and other states.
- According to climate data precipitation in maize production regions such as Illinois is significantly different to that of other maize producing regions like South Dakota .1

[https://ipad.fas.usda.gov/cropexplorer/cropview/comm\\_chartview.aspxcropid0440000regionidusnationalGraphFalsecntryidUSAse1\\_year2021startRow1fctypeid23fcattributeid1](https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspxcropid0440000regionidusnationalGraphFalsecntryidUSAse1_year2021startRow1fctypeid23fcattributeid1) The same is true for lower average temperatures in northern maize growing states e.g. Minnesota in comparison to other maize growing regions in the US.2

[https://ipad.fas.usda.gov/cropexplorer/cropview/comm\\_chartview.aspxcropid0440000regionidusnationalGraphFalsecntryidUSAse1\\_year2021startRow1fctypeid24fcattributeid1](https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspxcropid0440000regionidusnationalGraphFalsecntryidUSAse1_year2021startRow1fctypeid24fcattributeid1) • Even more striking is the difference between the climatic conditions in the major maize growing regions of the US and the conditions in maize growing regions in Brazil another major producer of GE maize. Data show much more precipitation in maize producing states like Paraná or Mato Grosso compared to the US.3

[https://ipad.fas.usda.gov/cropexplorer/cropview/comm\\_chartview.aspxcropid0440000regionidbrnationalGraphFalsecntryidBRAse1\\_year2021startRow1fctypeid23fcattributeid1](https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspxcropid0440000regionidbrnationalGraphFalsecntryidBRAse1_year2021startRow1fctypeid23fcattributeid1) Furthermore the average temperature in most Brazilian maize production regions is much higher compared to US regions e.g. Iowa Illinois or Ohio.4

[https://ipad.fas.usda.gov/cropexplorer/cropview/comm\\_chartview.aspxcropid0440000regionidusnationalGraphFalsecntryidUSAse1\\_year2021startRow1fctypeid24fcattributeid1](https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspxcropid0440000regionidusnationalGraphFalsecntryidUSAse1_year2021startRow1fctypeid24fcattributeid1)

Consequently the Maize plants tested in field trials do not sufficiently represent the imported Maize. The data presented by the applicant are insufficient to conclude on the impact of environmental factors and stress conditions on gene expression plant composition and the biological characteristics of the plant as requested by the EU Regulation 503/2013.

Data on herbicide application rates and their impact on gene expression Due to increased weed pressure it must be assumed that these plants will be exposed to high and also repeated dosages of glyphosate. Higher rates of herbicide application will not only lead to a higher burden of residues in the harvest but can also influence the expression of the transgenes or other genome activities in the plants. These observations are evidenced by a large amount of data showing substantial changes in the composition of GE herbicide-resistant soybeans Miyazaki et al. 2019 while more specific data on GE maize seem to be lacking.

It is likely that glyphosate was applied at a rate of 0.87 kg a.e./ha only. At the same time industry recommendations suggest dosages of up to approx. 35 kg a.i./ha glyphosate post-emergence 7 kg per season and even higher rates can be sprayed on herbicide-resistant maize.5 [www.greenbook.net/monsanto-company/roundup-weathermax](http://www.greenbook.net/monsanto-company/roundup-weathermax)  
[www.greenbook.net/monsanto-company/roundup-ultra](http://www.greenbook.net/monsanto-company/roundup-ultra)

Fang et al. 2018 and Yang et al. 2017 show that stress responses can lead to unexpected changes in plant metabolism inheriting additional EPSPS enzymes. These findings showing unintended effects in plants inheriting additional EPSPS genes are supported in several other publications Beres 2019 Beres et al. 2018 Wang et al. 2014. In addition other authors show the need for further investigations Vila-Aiub et al. 2009 and Vila-Aiub et al. 2019.



In this context there are strong indications that the EPSPS enzyme also interferes with the auxin metabolism in the plants Fang et al. 2018. Auxin has a role in multiple metabolic pathways in plants. Thus changes in the auxin content can also result in changes in plant composition that can raise safety concerns.

The EPSPS enzymes occur in the stacked Maize in much higher concentrations compared to the parental plants. Therefore it should be taken into account that unintended effects caused by high concentrations of EPSPS enzymes in combination with higher rates of spraying may also affect gene expression and plant composition. When the Maize is exposed to higher rates of glyphosate application the plants may experience stress conditions impacting gene expression and plant composition as well as the biological characteristics of the Maize.

Consequently the Maize plants tested in field trials do not sufficiently represent the Maize as imported. 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 by the EU Regulation 503/2013.

Data on genetic backgrounds and their impact on gene expression It is very well known that the genomic background of the variety can influence the expression of the inserted genes and / or the concentration of the additional proteins being present in the GE plants see Trtikova et al. 2015. For example Lohn et al 2020 in a multigenerational study with Bt maize show that the Bt content was generally lower in offspring if the maize was crossed with Brazilian varieties but much higher than expected in offspring from South African plants.

Therefore EFSA should have requested additional data from several varieties including those cultivated in South America. However no such data were provided or requested. Furthermore no such data are available for the parental plants or any subcombinations.

In conclusion the Maize plants tested in field trials do not represent the Maize as imported. The data presented by the applicant are insufficient to conclude on the impact of genetic background on gene expression plant composition and biological characteristics. However reliable data are needed to demonstrate safety as requested by EU Regulation.

It also should have been taken into account that previous research indicates the 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. Several publications point out the unintended changes in the genome the transcriptome the proteome or the metabolome of transgenic maize. see Agapito-Tenzen et al. 2013 Agapito-Tenzen et al. 2014 Benevenuto et al. 2017 Mesnage et al. 2016 Ben Ali et al. 2020.

Several of these transgenes are also present in the stacked Maize. In addition four other proteins are expressed which are absent in conventional maize NPTII which confers antibiotic resistance which should have been avoided as requested by EU Directive 2001/18 PMI which was used for the selecting process during plant production dsRNA DvSnf7 insecticidal toxicity and CSPB for drought tolerance. These traits and genetic elements can synergise and interact with each other. These effects are also likely to impact plant composition and biological characteristics crucial for the assessment of food and feed safety. However no specific data for a detailed comparison to assess the genome x environment interactions were presented by the applicant.

Robust data should have been requested to assess whether metabolic changes with relevance to food and feed safety occur in the stacked maize and EFSA should have requested a much more detailed investigation into potential biologically active gene products and changes in metabolic pathways under more extreme climate conditions.

However EFSA risk assessment has not yet taken these issues into account. Nor does EFSA use more sensitive methods such as transcriptomics, proteomics and metabolomics to explore and assess unintended changes in the stacked Maize.

In summary EFSA did not take into account all relevant data as requested by EU regulation to come to reliable conclusions regarding health safety including the assessment of toxicity and impact on the immune system.

Consequently the Maize plants tested in the field trials do not sufficiently represent the Maize as imported. The data presented by the applicant are insufficient to conclude on the impact of environmental factors, stress conditions, herbicide application rates, genetic backgrounds and stacking on gene expression, plant composition or the biological characteristics of the plant as requested by the EU Regulation 503/2013.

**Conclusions on molecular characterisation and gene expression** We conclude that the available data strongly indicate gene expression of several of the additional genes is likely to depend on or be influenced by stacking, varietal background, herbicide spraying or environmental factors such as stress conditions.

Therefore the plants should have been exposed to a much broader range of defined environmental conditions and stressors to gather reliable data on gene expression and functional genetic stability. This is especially relevant in this case since the stacked Maize is meant to be cultivated under more extreme drought conditions such as those caused by ongoing climate change. Whatever the case it should have been tested in the maize producing countries in South America. Furthermore EFSA should have requested the applicant to submit data from field trials which represent current agricultural practices with much higher rates of complementary herbicide applications on the plants including repeated spraying. In addition EFSA should have requested data for several varieties including those cultivated in South America.

Plant material should have also been assessed by using -omics techniques to investigate changes in the gene activity of the transgene and the plant genome as well as to investigate changes in metabolic pathways and the emergence of unintended biologically active gene products.

However only 4 samples each for grain and forage from 5 closely located field trials were used to generate the data on gene expression. Furthermore only one variety of the stacked Maize was used in the trials. The impact of environmental factors and agricultural practices was assessed without taking more extreme climate conditions into account although cultivation in drought conditions is one of the intended traits in the stacked Maize. For example according to weather data in 2014 there was more precipitation in most field trial sites than normal. Herbicide applications in the field trials did not represent current agricultural practices the rates were much lower. Relevant data on comparison of sprayed and unsprayed GE Maize plants are completely missing.

In summary the Maize plants tested in field trials do not sufficiently represent the imported Maize. Consequently the data presented by the applicant and accepted by EFSA are insufficient to conclude on the impact of the combination of traits and gene constructs stacking or the impact of environmental factors herbicide applications and of genetic background on gene expression and plant metabolism. However reliable data are needed to demonstrate safety as requested by EU Regulation.

Based on the available data no final conclusions can be drawn on the safety of the plants. Consequently the data do not fulfill the requirements of Implementing Regulation 503/2013 and Regulation 1829/2003.

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### **Comparative analysis (for compositional analysis and agronomic traits and GM phenotype)**

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.”

However the data provided by Monsanto do not represent expected agricultural practices or the different meteorological and agronomic conditions under which the crop is to be grown. There are three reasons 2.2.1 the field trials were not conducted in all relevant regions where the Maize will be cultivated and no extreme weather conditions were taken into account 2.2.2 the field trials did not take the current agricultural management practices into account 2.2.3 only one transgenic stacked variety was included in the field trials.

Data on environmental factors and stress conditions and their impact on plant composition and agronomic and phenotypic characteristics Field trials for the assessment of plant composition and agronomic and phenotypic characteristics of the stacked Maize were conducted in the US for one year only but not in other relevant maize production areas such as Brazil and Argentina. As shown in the EFSA opinion 2021a “no exceptional weather conditions were reported at any of the selected field trial sites”.

Taking into account the purpose of the genetic engineering in this case it is not acceptable that EFSA failed to require further studies • No field trials were conducted that lasted more than one season. Thus based on current data it is hardly possible to assess site-specific effects. However as our analysis on gene expression shows specific site by site and year by year effects have to be expected. • No data were generated representing more extreme environmental conditions such as those caused by climate change resulting in more extreme

droughts. • No data were generated that represent the growing conditions in other relevant maize growing regions outside the US.

These gaps in risks assessment are further emphasised in other meteorological data

- US weather data from 2014 show that for example there was more precipitation than normal at the trial sites in Illinois. This means that a relevant stressor that might influence gene expression composition or phenotype of the Maize may not be covered by the field trial data.
- Trial sites such as in Illinois neither represent the climatic conditions in other maize growing regions of the US nor the conditions in other relevant maize producing countries such as Brazil. USDA data show relevant maize production in North Dakota Minnesota Mississippi Louisiana and other states.
- According to climate data precipitation in maize production regions such as Illinois is significantly different to that of other maize producing regions like South Dakota .1

[https://ipad.fas.usda.gov/cropexplorer/cropview/comm\\_chartview.aspxcropid0440000regionidusnationalGraphFalsecntryidUSAse1\\_year2021startRow1fctypeid23fcattributeid1](https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspxcropid0440000regionidusnationalGraphFalsecntryidUSAse1_year2021startRow1fctypeid23fcattributeid1) The same is true for lower average temperatures in northern maize growing states e.g. Minnesota in comparison to other maize growing regions in the US.2

[https://ipad.fas.usda.gov/cropexplorer/cropview/comm\\_chartview.aspxcropid0440000regionidusnationalGraphFalsecntryidUSAse1\\_year2021startRow1fctypeid24fcattributeid1](https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspxcropid0440000regionidusnationalGraphFalsecntryidUSAse1_year2021startRow1fctypeid24fcattributeid1) • Even more striking is the difference between the climatic conditions in the major maize growing regions of the US and the conditions in maize growing regions in Brazil another major producer of GE maize. Data show much more precipitation in maize producing states like Paraná or Mato Grosso compared to the US.3

[https://ipad.fas.usda.gov/cropexplorer/cropview/comm\\_chartview.aspxcropid0440000regionidbrnationalGraphFalsecntryidBRAse1\\_year2021startRow1fctypeid23fcattributeid1](https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspxcropid0440000regionidbrnationalGraphFalsecntryidBRAse1_year2021startRow1fctypeid23fcattributeid1) Furthermore the average temperature in most Brazilian maize production regions is much higher compared to US regions e.g. Iowa Illinois or Ohio.4

[https://ipad.fas.usda.gov/cropexplorer/cropview/comm\\_chartview.aspxcropid0440000regionidusnationalGraphFalsecntryidUSAse1\\_year2021startRow1fctypeid24fcattributeid1](https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspxcropid0440000regionidusnationalGraphFalsecntryidUSAse1_year2021startRow1fctypeid24fcattributeid1)

Fang et al. 2018 as well as Yang et al. 2017 show that stress responses can lead to unintended changes in plant metabolism inheriting additional EPSPS enzymes. In this context there are strong indications that the EPSPS enzyme which confers glyphosate tolerance also interferes with the auxin metabolism in the plants Fang et al. 2018. This plant hormone plays a key role in growth fecundity and adaptation to environmental stressors. Thus changes in the auxin content can also result in changes in plant composition that can raise safety concerns.

Several publications support these findings showing unintended effects in plants inheriting additional EPSPS genes Beres 2019 Beres et al. 2018 Wang et al. 2014. Other authors also show the need for further investigations Vila-Aiub et al. 2009 and Vila-Aiub et al. 2019.

The EPSPS enzymes occur in the stacked Maize in higher concentrations compared to the parental plants. Therefore the Maize should have been subjected to a broad range of defined environmental conditions and stressors to gather reliable data on gene expression and functional genetic stability. This is especially relevant to the stacked Maize since EPSPS gene constructs will for the first time be expressed in these plants in more extreme climate conditions.

The stacked Maize carries a combination of a trait making the plants more tolerant to extreme climate conditions and gene constructs such as for Bt toxins likely to show or cause major changes in gene expression if exposed to environmental stressors such as drought. In addition two other proteins are expressed which are absent in conventional maize NPTII which confers antibiotic resistance - and should have been avoided as requested by EU Directive 2001/18 - and PMI which was used in the selecting process during plant production. These traits and genetic elements can synergise and interact. The effects are not unlikely to impact plant composition and biological characteristics crucial for the assessment of food and feed safety. However no specific data necessary for detailed comparison to assess the genome x environment interactions were presented by the applicant.

Whatever the case it should have been tested in all the major maize producing countries such as North and South America. Furthermore the Maize should have been subjected to defined drought conditions as there is an expectation that it will be grown in accordance with its intended purpose.

However no experiments were requested to show to which extent specific environmental conditions will influence plant composition or agronomic and phenotypic characteristics. No data were made available as requested by 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. Furthermore no such data are available for the parental plants or any subcombinations.

It has to be assumed that the stacked maize will be grown under drought conditions to an extent that most of the parental GE plants were not tested for in their previous risk assessment. Moreover it is the first time that the artificial gene constructs in combination will be exposed to more extreme drought conditions. It is obvious that in the absence of adequate data it cannot be assessed whether interactions will occur under stress conditions. Therefore there is no scientifically sound way of arguing why much more specific data is not necessary. For this reason the stacked plants should have been grown under conditions of severe drought with and without irrigation with and without application of the complementary herbicide and in comparison to more moderately severe climate conditions.

Consequently the Maize plants tested in field trials do not sufficiently represent the imported Maize. The data presented by the applicant are insufficient to conclude on the impact of environmental factors and stress conditions on gene expression plant composition and the biological characteristics of the plant as requested by the EU Regulation 503/2013.

Data on herbicide application rates and their impact on plant composition and agronomic and phenotypic characteristics Due to high weed pressure in many maize growing regions there is an expectation that these plants will be exposed to higher amounts and repeated dosages of glyphosate. It also has to be taken into account that the herbicides can be sprayed repeatedly and at high dosages. Such agricultural practices have to be taken into account to assess whether the expected agricultural practices will influence the expression of the studied endpoints.

However this requirement was mostly ignored by EFSA and the applicant glyphosate was only sprayed at an early stage of vegetation and at comparably low dosages likely dosage 0.87 kg a.e./ha. Industry recommendations suggest dosages to be sprayed on herbicide resistant maize of up to approx. 35 kg a.i./ha glyphosate post-emergence 7 kg per season and even

higher rates.<sup>5</sup> [www.greenbook.net/monsanto-company/roundup-weathermax](http://www.greenbook.net/monsanto-company/roundup-weathermax)  
[www.greenbook.net/monsanto-company/roundup-ultra](http://www.greenbook.net/monsanto-company/roundup-ultra)

From the data that is available it has to be assumed that the specific patterns of complementary herbicide applications will not only lead to a higher burden of residues in the harvest but may also influence the composition of the plants agronomic and phenotypic characteristics for example due to stress conditions caused by high amounts of complementary herbicides and unintended stress responses as described by Fang et al. 2018 and Yang et al. 2017.

Several publications report unintended effects in plants inheriting additional EPSPS genes Beres 2019 Beres et al. 2018 Wang et al. 2014. In addition other authors show the need for further investigations Vila-Aiub et al. 2009 and Vila-Aiub et al. 2019.

In this context there are strong indications that the EPSPS enzyme which confers glyphosate tolerance also interferes with auxin metabolism in the plants Fang et al. 2018. Auxin plays a role in multiple metabolic pathways in plants. Changes in the auxin content can result in changes in plant composition which can raise concerns about safety.

The EPSPS enzymes occur in the stacked Maize in much higher concentrations compared to the parental plants. Therefore it should be taken into account that unintended effects caused by high concentrations of EPSPS enzymes in combination with higher rates of spraying may also affect gene expression and plant composition. If the Maize is exposed to higher rates of glyphosate application this may cause stress conditions which can in turn impact gene expression and plant composition as well as the biological characteristics of the Maize.

EFSA should have requested the applicant to submit data from field trials at a minimum data on complementary herbicide application rates as recommended by industry including repeated applications. However no such data were provided or requested. Furthermore no such data are available for the parental plants or any subcombinations.

Consequently the Maize plants tested in the field trials do not sufficiently represent the imported Maize. The data presented by the applicant are insufficient to conclude on the impact of the herbicide applications on gene expression plant composition and the biological characteristics of the plant as requested by the EU Regulation 503/2013.

Data on genetic backgrounds and their impact on plant composition and agronomic and phenotypic characteristics It is very well known that the genomic background of the variety can influence the expression of the inserted genes and / or the concentration of the additional proteins present in the GE plants see Trtikova et al. 2015. For example Lohn et al. 2020 in a multigenerational study with Bt maize show that the Bt content was generally lower in offspring if the maize was crossed with Brazilian varieties but much higher than expected in offspring from South African plants.

Therefore EFSA should have requested additional data from several varieties including those cultivated in South America to examine how the gene constructs interact with the genetic background of the plants. This approach is supported by the analysis of gene expression given above but was ignored in the EFSA risk assessment. However no such data were provided or requested. Furthermore no such data are available for the parental plants or any subcombinations.

In conclusion the Maize plants tested in field trials do not represent the imported Maize. The data presented by the applicant are insufficient to conclude on the impact of genetic background on gene expression plant composition and the biological characteristics. However reliable data are needed to demonstrate safety as requested by EU Regulation.

Data from compositional analysis show the need for further investigation Only data from a low number of agronomic parameters 12 were subjected to statistical analysis in accordance with EFSA guidance of these 6 no spraying and 7 spraying of the complementary herbicide were found to be statistically and significantly different.

Compositional analysis in the grain revealed many and partly major statistically significant differences Of 54 endpoints 36 were statistically significantly different in plants sprayed with the complementary herbicides 27 in plants not sprayed with glyphosate but other conventional herbicides.

Not only the number of changes but also the magnitude of differences were much greater in the Maize being sprayed with with complementary herbicides compared not the non-sprayed Maize. This is a strong indication that stacking in combination with the application of the complementary herbicides are actually impact plant composition.

Even if changes taken as isolated data might not directly raise safety concerns the overall number of significant effects in the group of the sprayed Maize has to be taken as a starting point for much more detailed investigations more than half of the parameters measured for agronomic characteristics as well as for plant composition were significantly different.

Looking at these data EFSA should have concluded that the plants are likely to be substantially different compared to their conventional counterparts. Therefore more detailed analysis would have been necessary to investigate changes in the plant composition and phenotype caused by the stacking by spraying with the complementary herbicide including investigation into potential unintended changes in metabolic pathways and the emergence of unintended biologically active gene products.

Furthermore the data presented did not take into account cultivation of the stacked maize under more extreme drought conditions even though these are the most relevant environmental conditions in which the plants will be cultivated. Under such environmental conditions the range of differences and their significance are likely to be substantially increased.

As explained above EFSA should have requested further tests with repeated herbicide applications using higher dosages and with exposure to a much wider range of environmental conditions taking more extreme drought conditions into account. Furthermore the plant material should have been assessed by using -omics techniques to investigate changes in plant composition or agronomic and phenotypic characteristics in more detail.

However instead of assessing the overall pattern of changes in plant components their causes and possible impacts in more detail EFSA only assessed the observed changes in isolation for evidence of potential harm. This approach turns the comparative approach into a trivial concept of assessing bits and pieces and it ignores questions on the overall safety of the whole food and feed. A more in-depth investigation of unintended changes is not only necessary if

there are findings indicating adverse effects it is always needed to come to sufficiently robust conclusions to inform the next steps in risk assessment.

Previous research indicates 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. This research should have been taken into account. Several publications also report unintended changes in the genome the transcriptome the proteome or the metabolome of transgenic maize see Agapito-Tenfen et al. 2013 Agapito-Tenfen et al. 2014 Benevenuto et al. 2017 Mesnage et al. 2016 Ben Ali et al. 2020.

Several of these transgenes are also present in the stacked Maize. In addition four other proteins are expressed that are absent in conventional maize NPTII which confers antibiotic resistance - and should have been avoided as requested by EU Directive 2001/18 - PMI which was used for selecting process during plant production dsRNA DvSnf7 insecticidal toxicity and CSPB for drought tolerance. These traits and genetic elements can synergise and interact. These effects are also likely to impact plant composition and biological characteristics crucial to the assessment of food and feed safety. However no specific data needed for detailed comparison to assess the genome x environment interactions were presented by the applicant.

At a minimum the unintended effects of the EPSPS enzymes will very plausibly interfere with the activity of the other gene constructs for example via the auxin hormone see above. This is especially relevant if the plants are exposed to stress conditions. Even if no such effects were observed in the parental plants which to our knowledge were not tested in field trials under stress conditions these enzymes are now produced in the stacked Maize at higher concentrations. Therefore the likelihood of interaction between the gene constructs and gene expression plant composition as well as agronomic and phenotypic characteristics is higher in the stacked Maize compared to the parental plants.

Thus robust data should have been presented to assess whether metabolic changes with relevance to food and feed safety occur in the stacked maize. EFSA should have requested a much more detailed investigation into potential biologically active gene products and changes in metabolic pathways under more extreme climate conditions.

However these issues have not yet been taken into account in EFSA risk assessment. Neither do they use more sensitive methods such as transcriptomics proteomics and metabolomics to explore and assesses unintended changes in the stacked Maize.

Based on the available data no final conclusions can be drawn on the safety of the plants. The data do not fulfill the requirements of Implementing Regulation 503/2013.

Conclusion on comparative assessment of plant composition and phenotypic and agronomic characteristics The stacked Maize has a trait enabling cultivation of the plants under more extreme climate conditions. The trait is combined with gene constructs the EPSPS enzyme and the production of Bt toxins which are likely to cause or to show major changes in gene expression if exposed to environmental stressors such as drought. In addition three other proteins are expressed in the stacked Maize which are absent in conventional maize NPTII which confers antibiotic resistance -which should have been avoided as requested by EU Directive 2001/18 - PMI which was used in the selecting process during plant production and dsRNA DvSnf7 insecticidal toxicity. These traits and genetic elements can synergise and



interact with each other. These effects are also likely to impact plant composition phenotype especially if exposed to environmental stress conditions.

However the data provided by the applicant and accepted by EFSA are insufficient to conclude on the impact of the combination of traits and gene constructs of environmental factors of herbicide applications and the genetic background on gene expression plant metabolism plant composition and the agronomic and phenotypic characteristics. Since the field trials did not represent the conditions under which the plants are to be grown in practice the data from GE plants tested in field trials do not sufficiently represent the imported GE plants. Consequently these data do not fulfill the requirements of Implementing Regulation 503/2013. In summary the Maize plants tested in field trials do not sufficiently represent the Maize as imported. Based on the available data no final conclusions can be drawn on the safety of the plants. Consequently the data do not fulfill the requirements of Implementing Regulation 503/2013 and Regulation 1829/2003.

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## **b. Food Safety Assessment: Toxicology**

Implementing Regulation 503/2013 requests “Toxicological assessment shall be performed in order to demonstrate that the intended effects of the genetic modification has no adverse effects on human and animal health b demonstrate that unintended effects of the genetic modifications 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 if sprayed with the complementary herbicides. 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. Drought would have been especially relevant since this the Maize was developed to be grown in such conditions. 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.

It is also shown that no reliable conclusions can be drawn on the content of insecticidal proteins and the other intended new proteins from the available data. For example the overall concentration of the three insecticidal proteins is relevant for the assessment of overall toxicology as well as for the immune system. Thus without more data the true impact of intended effects on health at the stage of consumption 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 the Bt toxins** 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 Vázquez-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 Cry 1Ab 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 herbicide glyphosate and the resulting residues should be seen as potential co-stressors at the stage of consumption see also Then Bauer-Panskus 2017.

It has to be considered that the concentration of the insecticidal proteins is much higher in gluten meal produced from the maize it can reach much higher concentrations than in the kernels. Therefore the food and feed products derived from the stacked Maize need to be much more carefully risk assessed in regard to their toxicity compared to genetically engineered plants producing just one Bt toxin.

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-Porrás 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. Differences in toxicity between toxins produced in isolation compared to those produced by the plants are also described for Vip3A efficacy in transgenic plants Khan et al. 2020.

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.

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.

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 MSL0028115. 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. The lack of toxicity studies was also criticised by Member States experts EFSA 2021b. 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 Monsanto 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-Padron et al. 1999 Legorreta-Herrera et al. 2010 Jarillo-Luna et al. 2008 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 adjuvance in vaccines its risks for food consumption which can be fostered by synergistic effects need 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 or 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 response. For example a study testing corn with a combination of Bt toxins Cry1Ab and Cry34Ab1 indicates inflammatory effects in rats Zdziarski et al. 2018.

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.

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.

Effects of complementary herbicide residues specific to GE plants and mixed toxicity The residues from spraying with complementary herbicides 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 practices in the cultivation of these herbicide resistant plants there are for example specific pattern of sprayings exposure occurrence of specific metabolites and emergence of combinatorial effects that require special attention see also Kleter et al. 2011.

More detailed assessment is also in accordance with pesticide regulation that requires specific risk assessment of imported plants if the usage of pesticides is different in the exporting countries compared to the usage in the EU. In this regard it should be taken into account that EFSA 2015a 2015b and 2018a explicitly stated that no conclusion can be drawn on the safety of residues from spraying with glyphosate in genetically engineered plants resistant to this herbicide.

Further there is a common understanding that commercially traded formulations of glyphosate such as Roundup can be more toxic than glyphosate itself. Therefore the EU has already taken measures to remove problematic additives known as POE tallowamine from the market. Problematic additives are still allowed in those countries where the genetically engineered plants are cultivated. The EU Commission has confirmed the respective gaps in risk assessment "A significant amount of food and feed is imported into the EU from third countries. This includes food and feed produced from glyphosate-tolerant crops. Uses of glyphosate-based plant protection products in third countries are evaluated by the competent

authorities in those countries against the locally prevailing regulatory framework but not against the criteria of Regulation EC No. 1107/2009. ....”<sup>1</sup> [www.testbiotech.org/content/eu-commission-request-consider-impact-glyphosate-residues-feed-animal-health-february-2016](http://www.testbiotech.org/content/eu-commission-request-consider-impact-glyphosate-residues-feed-animal-health-february-2016)

The stacked Maize combines several EPSPS enzymes conferring enhanced resistance to glyphosate therefore a higher burden of the residues from spraying might be expected compared to the parental plants. Consequently EFSA should have requested the company to submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants including repeated spraying. The material derived from those plants should have been assessed in regard to organ toxicity immune system responses and reproductive toxicity also taking combinatorial effects with other plant components into account.

Whatever the case both the EU pesticide regulation and the GMO regulation 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 to be a prerequisite for granting authorisation.

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 needs to be tested in regard to its mixed toxicity EFSA 2019b.

Glyphosate is particularly problematic in regard to chronic exposure when it is consumed in food. This is because glyphosate is known to cause shifts in the microbial composition and associated microbiomes of plants and animals glyphosate Roundup has been shown to cause shifts not only in soil organisms van Bruggen et al. 2018 but also in the composition of the intestinal flora in cattle Reuter et al. 2007 poultry Shehata et al. 2013 Ruuskanen et al 2020 and rodents Mao et al. 2018 Mesnage et al. 2021 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 the stacked Maize is not unlikely to trigger significant changes in intestinal bacteria.

Alarming similar effects are reported from the exposure to glufosinate showing that glufosinate can severely impact the microbiome Dong et al. 2020. Therefore the mixed toxicity of the stacked event needs to be assessed much more carefully compared to the parental plants.

In general the microbiome can be seen as a common network of life circumventing 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 benefit both the host and microorganisms. They act at the interphase and communicate between an organism and its wider environment while at the same time being part of the closer environment of an organism. Microbiomes are considered key for the health of higher organisms i.e. humans animal and plants.

Just recently a document published by EFSA EFSA 2020 called attention to the role of the microbiome in environmental risk assessment and food and feed safety. 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.”

In 2019 EFSA commissioned a study on the adjuvanticity / immunogenicity assessment of proteins including 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.”

This finding is highly relevant for the risk assessment of the stacked Maize. Several EPSPS enzymes are combined in the stacked Maize for enhanced resistance to glyphosate therefore a higher burden of residues from spraying can be expected compared to the parental plants. These residues may cause a perturbation of the gut microbiome. At the same time stacking in the Maize results in a combination of several Bt toxins that are likely to show immunogenicity. It is plausible that this combination of Bt toxins together with residues from spraying can trigger effects on the immune system either directly or via the microbiome. This hypothesis needs to be tested before any conclusion can be drawn on the health safety of food and feed.

However no attempts have been made to integrate the microbiome into the risk assessment of food and feed derived from the stacked Maize. This is contrary 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.

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. The findings of Davalos et al. 2019 and Nawaz et al. 2019 were not mentioned by EFSA. Therefore these publications have to be regarded as new information and also as new evidence since they go beyond the EFSA assessment.

- 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 ncsRNA. 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 EFSA 2019a 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 in the assessment of the parental plants EFSA 2018a and the stacked event EFSA 2021a 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](http://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 a 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.”

- Conclusions on toxicity assessment of ncsRNA DvSnf7 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. 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.

Overall conclusions on toxicity 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. 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.

Testbiotech is also aware that feeding studies with similar stacked maize indicated potential health impacts such as inflammatory responses in the stomach Zdziarski et al. 2018. Inflammatory responses are an alarm signal typical of many chronic diseases and therefore



require close attention. While the applicant provided some data in regard to celiac disease other diseases associated with symptoms of chronic inflammation were not considered at all.

EFSA should have requested the applicant to submit data from field trials which represent the environmental and agricultural conditions under which the Maize will be grown. Drought and high rates of complementary herbicide applications should have been taken into account. The material derived from the plants should have been assessed especially in regard to long-term organ toxicity immune responses and impact on the gut microbiome also taking combinatorial effects and mixed toxicity into account.

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## Allergenicity

Implementing Regulation 503/2013 requests “In cases when known functional aspects of the newly expressed protein or structural similarity to known strong adjuvants may indicate possible adjuvant activity the applicant shall assess the possible role of these proteins as adjuvants. As for allergens interactions with other constituents of the food matrix and/or processing may alter the structure and bioavailability of an adjuvant and thus modify its biological activity.”

“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”

Potential allergenicity EFSA does not mention that Cry1Ac is thought to be allergenic Santos-Vigil et al. 2018<sup>1</sup> see also [www.testbiotech.org/en/press-release/can-bt-toxins-cause-allergies](http://www.testbiotech.org/en/press-release/can-bt-toxins-cause-allergies) . According to Santos-Vigil et al. 2018 the Bt toxin Cry1Ac can act as an allergen if ingested. This publication is relevant the Bt toxin Cry1Ac was used as a source for the synthesis of Cry1A.105 expressed in the stacked Maize. Therefore the synthetically derived Cry1A.105 toxin produced in the Maize has structural similarity with Cry1Ac. If Cry1Ac is thought to be an allergen the source of Cry1A.105 has to be verified as allergenic and therefore investigated in detail.

The EU Commission also noted that the Santos-Vigil et al. 2018 publication was relevant for the risk assessment of genetically engineered plants producing Bt toxins and therefore requested the European Food Safety Authority EFSA for an assessment. In response EFSA EFSA 2018b came to the conclusion that the Santos-Vigil et al. 2018 publication does not provide any new information and suffers from methodological deficiencies. However this EFSA opinion is based on a rather biased interpretation of existing publications and it does not provide any evidence that the Santos-Vigil et al. 2018 findings are invalid or irrelevant for more details see Moreno-Fierros et al. 2018. Consequently the Santos-Vigil et al. 2018 publication has to be considered valid but not properly assessed by EFSA. Certainly EFSA should have requested testing of the hypothesis that the consumption of products derived from the maize can trigger allergic reactions. In conclusion the EFSA assessment of the stacked maize cannot be said to fulfil the requirements for assessing allergenicity of the source of the transgene.

Potential adjuvanticity The synergistic effects between PI and Bt toxins as described above are also relevant for risk assessment in regard to adjuvanticity the 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 relevant effects.

There are several studies indicating that immune responses such as adjuvanticity in mammals are triggered by Bt toxins and have to be considered in this context. Studies with the Cry1Ac toxin Moreno-Fierros et al. 2000 Vázquez-Padron et al. 1999 Legorreta-Herrera et al. 2010 Jarillo-Luna et al. 2008 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 need for more detailed investigations in regard to potential immunogenic effects is also pointed out in the minority opinion in another EFSA opinion Annex II of EFSA 2018c.

Given the fact that potential effects of Bt toxins on the immune system have meanwhile been discussed for many years for overview see for example Then Bauer-Panskus 2017 and already 45 GE crop events producing Bt toxins have been approved for the EU market this explanatory excuse cannot be accepted. In accordance with EU Regulation 1829/2003 safety of whole food and feed has to be demonstrated before approval for import can be issued. Since this is not the case with the stacked Maize the risk assessment is not conclusive and no market authorisation can be granted.

These issues are especially relevant for stacked events since the overall concentration of Bt toxins is higher and combinatorial effects with other stressors such as residues from spraying are more likely. This is also relevant for immune responses exerted via the microbiome see above. However neither EFSA nor the applicant considered the potential enhancement of toxic or immunogenic effects caused by interaction with plant components.

Conclusion on allergenicity and adjuvanticity Considering these uncertainties EFSA should have requested empirical testing of allergenic or adjuvant effects. In conclusion allergenicity and adjuvanticity were not assessed to sufficiently demonstrate that the food and feed from the stacked Maize has no adverse effects on human and animal health.

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## Others

1 For monitoring and methods to identify the specific event Implementing Regulation 503/2013 requests The methods 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.

2 Finally in regard to the literature research we do not agree with the way it was carried out. The review should take into account all publications on the parental plants and provide all relevant information regarding gene expression findings from field trials and feeding studies. Further monitoring data should be provided on imports of parental plants into the EU.

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### **3. 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. In its assessment of the volunteer potential the information provided by Monsanto is largely outdated. As Pascher et al. 2016 show the volunteer potential of maize is higher than assumed by Monsanto. Further in awareness of the biological characteristics of maize MON87460 and the findings of Fang et al. 2018 the stacked maize needs to be examined in detail for next generation effects volunteer potential persistence and gene flow. In doing so the hypothesis that the maize and its offspring will show a higher fitness compared to conventional maize is evident. This might also concern adaption to colder climate due to the biological characteristics of the cold shock protein CSPB. Under these circumstances even a rare single outcrossing event that goes unnoticed can have a huge long-term impact on agro-ecosystems.

Likelihood of gene flow 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.

There are new publications confirming that the risks from crossings of GE maize and teosinte cannot be predicted from the data assessed by EFSA 2021a as already shown by Trtikova et al. 2017 another publication Le Corre et al. 2020 confirms that European teosinte plants from Spain and France in fact integrated larger genomic parts from European maize varieties.

As Le Corre et al. 2020 show teosinte has changed its biological characteristics in ways that will facilitate further genetic exchange with maize plants. Similarly Diaz et al. 2019 show that these new weeds seem to have a complex origin. Therefore the likelihood of hybridisation with the GE maize has strongly increased. For example teosinte has now altered its flowering time. Furthermore teosinte has already acquired herbicide-resistance from conventional European maize varieties. In awareness of their findings the authors emphasise that their results show that risks of crop-wild introgression should not be underestimated in forecasting the risk of invasiveness. They show that crop-wild introgression can be a two-way street enhancing gene flow to both partners maize and teosinte. The scientists have therefore explicitly warned that the risk of the plants becoming invasive should not be underestimated.

Enhanced fitness If the known characteristics of the stacked Maize were to be transferred to teosinte this would render them herbicide resistant toxic for pest insects and drought tolerant.

In addition a higher overall fitness of hybrid offspring can result from the additional EPSPS enzymes. Fang et al. 2018 showed that higher fitness does occur in GE glyphosate-resistant *Arabidopsis thaliana* in a glyphosate-free environment as an unintended effect. According to this research the expressed enzyme EPSPS 5-enolpyruvylshikimate-3-phosphate synthase not only makes the plants resistant to glyphosate it also interferes with metabolic processes associated with their growth and fecundity. As a consequence plant offspring can produce more seeds and be more resistant tolerant to environmental stressors such as drought and heat. The authors stated that the observed effects are likely to be caused by increased production of the hormone auxin in the transgenic plants. This plant hormone plays a key role in growth fecundity and adaptation to environmental stressors. The general findings regarding enhanced fitness of plants inheriting additional EPSPS genes are supported by several other publications Beres 2019 Beres et al. 2018 Wang et al. 2014 Yang et al. 2017. In addition other authors show the need for further investigations Vila-Aiub et al. 2009 and Vila-Aiub et al. 2019. Thus if gene flow occurs this effect which was completely ignored by EFSA and the applicant can strongly enhance the spread of teosinte in the fields and possibly also beyond the fields.

Next generation effects Unlike maize teosinte can overwinter in the fields and pass new genetic information to offspring - from where it has the potential to spread and become a new European super-weed. These risks are not only a concern for farmers they could also seriously damage the environment and protected species.

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 Kawata et al. 2009 Cao et al. 2009 Yang et al. 2017 Bollinedi et al. 2017 Lu and Yang 2009 Vacher et al. 2004 Adamczyk Meredith 2004 Adamczyk et al. 2009 for overview see Bauer-Panskus et al. 2020. This issue is relevant for gene flow from maize to teosinte as well from teosinte to maize.

Therefore the characteristics of potential hybrids and next generations have to be investigated and cannot be predicted simply from the data of the original event as suggested by EFSA.

Conclusion on environmental risk assessment Without detailed consideration and examination 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.

This is especially relevant for the stacked Maize which combines several traits which are likely to result in higher fitness of hybrid offspring.

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.

The risk assessment as performed does not fulfill EU regulations which require the examination of the direct and indirect as well as the immediate and delayed effects of the GMO on human health and the environment.

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#### **4. Conclusions and recommendations**

The EFSA risk assessment cannot be accepted. Given the findings there are multiple violations of EU Regulations requesting that the safety of the GE plants is demonstrated

1. The plants were not exposed to bioclimatic conditions that sufficiently represent the regions in which these plants will be cultivated. Consequently expression data and the assessment of plant composition as well as of agronomic and phenotypic characteristics are not sufficiently reliable to inform the next steps in risk assessment. This is especially relevant since i the plants are intended for cultivation in drought conditions ii the EPSPS enzymes are known to show unintended effects under stress conditions such as drought and iii the Bt content is known to be influenced by environmental factors such as drought.

2. The plants were not exposed to agricultural practices which sufficiently represent the conditions under which these plants will be cultivated. Consequently expression data and the assessment of plant composition as well as agronomic and phenotypic characteristics are not sufficiently reliable to inform the next steps in risk assessment.

3. No detailed examinations were requested despite data from other events and previous applications indicating that environmental stress factors herbicide applications rates genetic backgrounds and stacking all impact gene expression and plant metabolism.

4. Risks which indicate toxicological health impacts potentially enhanced by combinatorial effects caused by the stacking were not assessed against relevant reasoned and plausible hypotheses. For example it was not examined to which extent the toxicity of the selectivity of the Bt toxins are changed by the mixed toxicity of whole food and feed. This is especially relevant in this case since it is known that enzymes protease inhibitors produced in the plants can multiply the toxicity of the Bt toxins and prolong exposure to the toxins in the gut after ingestion.

5. Risks which indicate immunological health impacts potentially enhanced by the combinatorial effects of stacking were not assessed against relevant reasoned and plausible hypotheses. For example it was not examined to which extent changes in the microbiome caused by the consumption of the Maize will impact its immunogenic properties. This is especially relevant since it is known that enzymes protease inhibitors produced in the plants

can prolong exposure to the toxins in the gut after ingestion. Furthermore residues from spraying with glyphosate Roundup are known to impact the composition of the microbiome.

6. The interaction between the ncsRNAs produced in the GE plants and the microbiome of humans or animals was not assessed despite new findings showing that these interactions are likely to occur and adverse impacts on human and animal health are a plausible consequence. That a combination of DvSnf7 dsRNA Bt toxins and residues from spraying can trigger effects on the immune system or other adverse health effects either directly or via the microbiome even if these were absent in the parental plants has to be considered a plausible hypothesis. Therefore the stacked Maize needs to be tested for synergistic effects due to other plant constituents or additional factors which may enhance stability or uptake of DvSnf7 dsRNA from the gut.

7. The environmental risk assessment and the biological characteristics of the hybrid offspring of teosinte and the Maize were not assessed against relevant reasoned and plausible hypotheses. This is especially relevant since the offspring may show invasive characteristics due to the unintended effects caused by the EPSPS enzymes higher resistance to damage by insects and potentially higher tolerance to drought conditions.

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