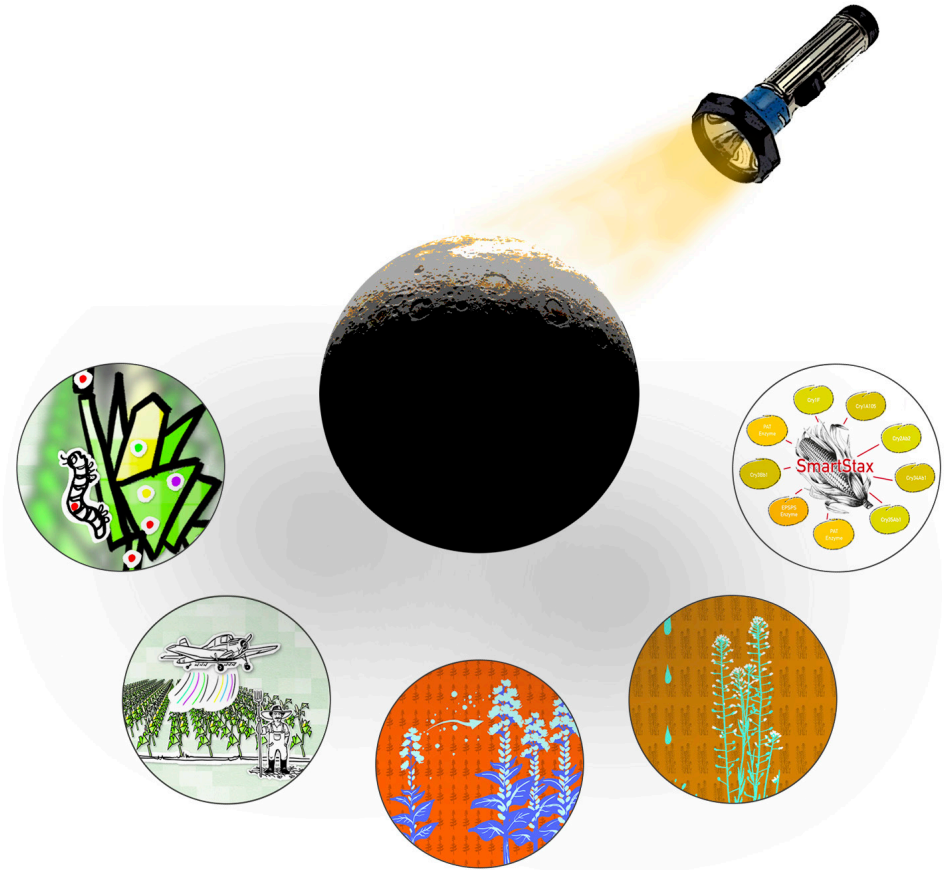

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Institute for Independent Impact Assessment in Biotechnology

Risk assessment of GE plants in the EU: Taking a look at the 'dark side of the moon'

Christoph Then & Andreas Bauer-Panskus

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Testbiotech e. V.
Institute for Independent Impact Assessment in Biotechnology
Frohschammerstr. 14
D-80807 Munich
Tel.: +49 (0) 89 358 992 76

info@testbiotech.org
www.testbiotech.org

Executive Director: Dr. Christoph Then

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Summary

The RAGES project (Risk Assessment of genetically engineered organisms in the EU and Switzerland) started in 2016 and ended in 2020. Its objective was to carry out an in-depth analysis of European Food Safety Authority practices (and its counterpart in Switzerland) in regard to the risk assessment (RA) of genetically engineered (GE) plants in Europe. The project was led by ENSSER (European Network of Scientists for Social and Environmental Responsibility) and its Swiss branch CSS (Critical Scientists Switzerland) as well as GeneWatch UK and Testbiotech. RAGES was funded by the Mercator Foundation Switzerland.[1]

The European Food Safety Authority (EFSA) and the EU Commission had the opportunity to discuss the findings during the course of the project. The outcomes were published on the Testbiotech website in January 2020, and subsequently, after peer-review, in several scientific journals.[2]

EFSA published its response to the RAGES findings in June 2020 (EFSA, 2020a). As expected, EFSA defended its scientific work and rejected most of the RAGES findings.[3] EFSA states: *"Overall, EFSA concludes that the final RAGES reports do not contain elements that would lead the GMO Panel to reconsider the outcome of its previous scientific opinions on GMPs4. Therefore, EFSA considers that the previous GMO Panel risk assessment conclusions remain valid."* EFSA concluded that the results of its GMO risk assessments remained sufficient, but also referred to some planned or ongoing improvements.

In this report, Testbiotech analyses the EFSA response and discusses the most recent scientific findings. During the first 20 years of its existence EFSA published more than 100 opinions on the risk assessment of GE crops, but was nevertheless unable to present robust criteria and test designs to allow empirical testing and reliable risk assessment of GE crop plants.

Testbiotech is aware that some aspects will need further discussion and analysis, e.g. RAGES has introduced the concept of the holobiont, which places organisms in the context of their microbiome. Even so, RAGES has brought questions to light in regard to changes in GE plants that affect biologically active molecules, such as non-coding small RNAs (ncRNA), and their interactions with the closer and wider environment. As far as these issues are concerned, the discussions on risk assessment are still in their infancy.

However, some evidence has been found of basic failures in EFSA's risk assessment of GE plants; these failures have been perpetuated over the years and can no longer be denied. This report aims to shed light on the dark areas of risk assessment, some of which appear to have been intentionally created, constructed and upheld. If divided into two categories, the most important findings are:

Failure to request sufficiently reliable data

- The field trials with herbicide-resistant (or tolerant) GE plants - carried out by the companies for the approval process - are not representative of the agricultural conditions in which the plants are grown. The herbicide application rates in these trials are generally much lower compared to the data from cultivation. Therefore, the data presented by industry are insufficient to conclude on the impact of herbicide applications in regard to the relevant biological characteristics of the plants, such as gene expression, plant composition or food and feed safety.

1 https://www.testbiotech.org/en/content/overview-rages-project
2 https://www.testbiotech.org/en/content/research-project-rages
3 https://www.testbiotech.org/en/news/efsa-defends-risk-assessment-ge-plants
4 GMP: genetically modified plants

- The field trials carried out by industry and accepted by EFSA are not representative of the bio-climatic regions where the plants are grown. Nevertheless, EFSA routinely accepts data from field trials carried out for only one year and in only one country (the US). This means that the much broader range of environmental factors in other growing areas to which the plants are exposed and which influence gene expression, e.g. the effects of climate change, regional conditions, plant diseases or other stress factors, are not taken into consideration. Therefore, the data presented by industry are insufficient to conclude on the impact of environmental factors on gene expression, plant composition, other biological plant traits or the safety of food and feed for consumption.
- EFSA accepts data from tests with isolated Bt proteins to assess their toxicity. However, the toxicity (and immunogenicity) of Bt proteins in plants, such as maize, cotton and soybeans, has to be assumed to be much higher than the isolated Bt toxins. All these plants produce protease inhibitors (PI) which delay the degradation of the toxins. Even small amounts of PI can enhance exposure to the Bt toxins, potentially causing toxicity to be up to 20 times higher. Even so, EFSA has failed to assess any combinatorial effects despite this being relevant for all Bt plants approved for import and cultivation.
- Most of the approved GE plants carry a combination of (several) Bt toxins and (several) herbicide resistances. Consequently, all food and feed derived thereof will contain mixtures of the Bt toxins and herbicide residues from spraying. Nonetheless, EFSA has never asked for empirical data on combined toxicity (and immunogenicity).
- EFSA assessment of risks affecting protected European butterfly species and other non-target organisms due to Bt cultivation, continues to suffer substantially from an absence of empirical data.

Failure to develop adequate methodology

- EFSA rejects 90-day feeding studies with the plant harvest. At the same time, EFSA has failed to develop adequate alternative methodology to empirically test risks linked to the consumption of such products. For example, criteria for suitable in-vitro methods were never made available.
- Despite discussing plausible hypotheses on higher toxicity and lower selectivity of Bt toxins for many years, EFSA has never managed to develop and apply a suitable methodology to gather reliable data to assess risks linked to consumption of the plants.
- Despite discussing plausible hypotheses on the immunogenicity of Bt toxins for many years, EFSA has never managed to develop and apply a suitable methodology to gather reliable data to assess their impact on the immune system after consumption.
- GE plants which have been deliberately changed in their nutritional composition pose specific challenges in risk assessment. However, EFSA has never developed adequate methodology to assess the intended and unintended effects.
- Unintended next generation effects are known to occur in the offspring of genetically engineered plants; these are caused by the heterogeneous genetic backgrounds of other varieties or wild relatives that are absent in the original GE events. EFSA has no methodology to investigate or assess these effects.
- Although it is known that the insertion of additional genes can unintentionally give rise to various biologically active molecules, such as non-coding small RNAs (ncRNA), EFSA does not take these into consideration.

› Although it is known that the microbiome plays a decisive role in safety for human health and the environment, (see EFSA, 2020d, Parenti et al., 2019), EFSA has never developed methodology to integrate this into the risk assessment of GE plants.

Safety never demonstrated

Current cultivation of GE plants for food production means that huge numbers of organisms enter agro-ecosystems and food chains without having gone through evolutionary adaptation. There can be no doubt that over longer periods of time, there will be unintended changes and environmental reactions. These unintended effects might not always be a cause for concern. However, it is worrying that current risk assessment is insufficient to identify the magnitude of the changes or to determine their consequences for human health and the environment.

EFSA has never taken all the relevant risks into account, but instead focused on the risks that can be most easily assessed. One could say that significant risks associated with genetically engineered plants were intentionally placed on 'the dark side of the moon'. There is evidence for the manifest, intended and systematic ignorance of EFSA in regard to the most critical aspects of risk assessment.

It has to be assumed, for example, that Bt proteins contained in crops, such as maize, cotton and soybeans, are inherently much more toxic than isolated Bt toxins. This is because protease inhibitors (PI) are present in the plant tissue. The mechanism by which PIs, even in very small quantities, potentiate the toxicity of the Bt proteins might be a delay in Bt toxin degradation. It is 30 years since Monsanto first showed enhanced toxicity - up to 20 times higher - from mixing Bt toxins with seeds from plants, such as soybeans, cotton and maize (MacIntosh et al., 1990). Since then these findings have been repeatedly confirmed in scientific studies. However, these effects have never been taken into account in EFSA risk assessment, even though they are relevant for all Bt plants approved for import or cultivation in the EU. These findings are also relevant for mixtures of the plants with material from other plants in diets.

A further example of repeated and intended ignorance on the part of the EFSA: several publications show that the insertion of epsps gene constructs not only confers resistance to glyphosate, it can also confer generally higher plant fitness (see, for example, Fang et al., 2018). This significantly increases the risk of uncontrolled spread of the genetically engineered plants in the environment, thus indirectly promoting the emergence of weeds with higher fitness. However, EFSA has never considered the multi-functional effects of EPSPS enzymes produced at increased levels in nearly all herbicide-resistant transgenic plants over the past twenty years.

Another example: Long term exposure to glyphosate residues due to these plants may lead to disruption in the gut microbiome. It has to be considered a plausible hypothesis that a combination of Bt toxins and residues from spraying with glyphosate, can trigger effects on the immune system or other adverse health effects via the microbiome (see for example Parenti et al. 2019). However, EFSA does not request empirical examination of mixed toxicity of food and feed which is derived from GE plants with a combination of traits.

Further uncertainties and unknowns in risk assessment have emerged from recent research on microbiomes and small interfering RNA (ncRNA). The RAGES project has clearly highlighted new fundamental challenges in the risk assessment of GE organisms that EFSA failed to address in its response.

In conclusion, evidence has been provided to show that the genetic engineering of food plants has layers of complexity that go far beyond what can be assessed by current standards of risk assessment. The safety of the plants is claimed on basis of approval processes that only consider risks that are easiest to assess.

Conclusions and necessary consequences

Current standards of risk assessment are insufficient to fulfill legal requirements to determine the safety of genetically engineered organisms by applying the *"highest possible standard"* to *"any risks which they present"* as requested by EU Regulation 1829/2003. In addition, EFSA, in a self- assigned mission, developed its own new guidance (EFSA, 2015) and therefore escaped the strict and specific requirements of EU Implementing Regulation 503/2013 for several years.

However, it is far from simply the failure of EFSA that needs to be highlighted, there are also failures on the part of the EU Commission. Sufficiently detailed regulation and standards of risk assessment are mostly lacking. Therefore, the EU Commission, which is responsible for the political dimension of risk assessment, needs to become actively engaged. The EU Commission is also mostly responsible for developing and implementing a sufficiently reliable system of post-market monitoring (PMM) after approval. However, PMM is still just a label without substantial scientific content and does not provide sufficiently reliable data.

To avoid further damage to the credibility of the EU Commission and EFSA, both EU institutions should openly discuss these problems and no longer hide in the shadows of an intentionally created ignorance. Whatever the case, the safety of approved GE organisms should no longer be claimed if none of the necessary data being available.

1. Introduction

The RAGES project (Risk Assessment of genetically engineered organisms in the EU and Switzerland) started in 2016 and ended in January 2020. Its objective was to carry out an in-depth analysis of European Food Safety Authority practices (and its counterpart in Switzerland) in regard to the risk assessment (RA) of genetically engineered (GE) plants in Europe. The project was led by ENSSER (European Network of Scientists for Social and Environmental Responsibility) and its Swiss branch CSS (Critical Scientists Switzerland) as well as GeneWatch UK and Testbiotech. RAGES was funded by the Mercator Foundation was funded by the Mercator Foundation Switzerland.[5]

What did RAGES examine?

The analyses carried out in the RAGES project are based on case studies of published EFSA opinions, peer reviewed scientific publications and other scientific data/expertise. RAGES compiled six reports on specific topics that were identified as particularly important in this context. These topics are:

- Health risks associated with the consumption of products derived from herbicide tolerant GE plants (RAGES, 2020b);
- The assessment of environmental risks associated with the cultivation of insecticidal Bt crops (RAGES, 2020c);
- Health risks associated with the consumption of products derived from GE plants with altered nutritional composition (RAGES, 2020d);
- Health risks associated with the consumption of products derived from GE plants with a combination of traits ('stacked events') (RAGES, 2020e);

5 www.testbiotech.org/en/content/overview-rages-project

- Environmental risks due to persistence, self-propagation and uncontrolled spread of GE plants (RAGES, 2020f) and
- Risk assessment of GE organisms derived from new genetic engineering technologies (RAGES, 2020g).

Cross-cutting issues and other findings are summarised in a full report (RAGES, 2020a) and accompanied by a tabled overview.

The results of the RAGES project were presented at workshops in Brussels and Neuchatel (Switzerland) in 2018 and 2019. The EU Commission, EFSA and Swiss authorities all participated; this was very much appreciated even though there was no consensus on many of the findings. The RAGES findings were updated to correspond with the results from the workshops. Some of the outcomes were submitted for further review in scientific journals.

In June 2020, EFSA published its final response to RAGES in a report titled *"Assessment of the outcomes of the project 'Risk Assessment of Genetically Engineered Organisms in the EU and Switzerland' (RAGES)"* (EFSA 2020a). In this response, EFSA defends its work and rejects most of the RAGES findings.[6] It also made some statements in regard to planned or ongoing improvements in GMO risk assessment. EFSA commented in its response to the RAGES reports, but did not take the peer-reviewed publications into account.

Even though the RAGES project finished at the beginning of 2020, Testbiotech, as one of the RAGES partners, is still following the debate closely. In this backgrounder, we analyse the EFSA response and discuss this against the backdrop of the most recent findings. However, we have left the issue of 'New Genetic Engineering' aside as it is discussed elsewhere (Kawall, 2020; Testbiotech, 2020a).

2. RA of herbicide-tolerant GE plants and food & feed safety

Conclusions

The herbicide-tolerant (or -resistant) GE plants tested in field trials by the companies are not grown in representative agricultural conditions. The herbicide application rates in the trials are much lower compared to the data from cultivation. Therefore, the data presented by industry are insufficient to conclude on the impact of the herbicide applications on gene expression, plant composition and the safety of food and feed derived thereof as requested by EU Regulation 503/2013.

Instead EFSA, has been engaged in a self-assigned task, i.e. drawing up its own guidance (EFSA, 2015), including a transition period which effectively postpones the enforcement of Implementing Regulation 503/2013 requirements for about four years. It is highly problematic that EFSA can prevent an EU Regulation from coming into force on the date foreseen by EU legislation in this way.

In conclusion, current EFSA practice violates the provisions of Implementing Regulation 503/2013 related to assessing whether the expected agricultural practices influence outcome of the studied endpoints.

6 www.testbiotech.org/en/news/efsa-defends-risk-assessment-ge-plants

General overview

RAGES (2020b) and a peer-reviewed publication (Miyazaki et al., 2019) show that the introduction of herbicide-tolerant (HT) genetically engineered (GE) soybeans creates new challenges in the European risk assessment of imported food and feed. Food and feed products derived from these plants may show specific patterns of chemical residues and altered nutritional composition. At the same time, the emergence of resistant weeds has led to a substantial increase in the use of herbicides in soybean production. This is linked in particular to glyphosate-based herbicides and also other herbicides.

In this context, RAGES (2020b) and Miyazaki et al. (2019) give an overview of available data on glyphosate application to HT GE soybeans in North and South America. Furthermore, they compare these data with herbicide applications in experimental field trials conducted by industry. They conclude that the field trials carried out for risk assessment purposes do not generally represent the real agronomic conditions in commercial HT GE plant cultivation. In most cases, neither the applied dose nor the number of applications match real conditions.

This finding is highly relevant for the risk assessment of HT GE plants: Miyazaki et al. (2019) also show that the amount and timing of spraying glyphosate as a complementary herbicide on HT GE plants can impact their composition; these changes in plant composition can arise from, or be influenced by, or even impact the expression of the additionally inserted genes. In this context, particular attention should be paid to stacked events and the gene expression of all additionally inserted genetic elements. Therefore, the compositional analysis and assessment of phenotypical characteristics of HT GE plants have to take dose, the number of sprayings and the timing of herbicide application into account. It is evident that these factors can influence plant and product safety. However, these criteria have still not been integrated into EFSA risk assessment.

Surprisingly, EFSA in its response to RAGES, states that in regard to the field trials *"the timing and rate of the applied intended herbicides are in line with the recommendations of the manufacturers. This information is routinely verified by the GMO Panel and specifically discussed in the section of the scientific opinion on management practices."* This is in stark contrast to the findings presented by RAGES and Miyazaki et al. (2019). Moreover, as Miyazaki et al. (2019) show, the real rate of herbicide applications is in many cases much higher than officially recommended by the companies.

The claim as cited also contradicts further statements made by EFSA: instead of requesting details of the specific agricultural conditions in which the GE HT plants will be grown, EFSA states that the application rates between the sites with conventional varieties (grown for comparison) and GE plants (!) *"should not differ too strongly "* (EFSA, 2020b; Testbiotech, 2020b). Consequently, the standards imposed by EFSA undermine risk assessment as required by Regulation 503/2013, which requests an assessment of whether the expected agricultural practices influence the outcome of the studied endpoints. As a result, risk assessment as required under EU regulation is eroded by unrealistic EFSA measures.

There is more disturbing information in the EFSA response: according to Implementing Regulation 503/2013, all applications filed after 8 December 2013 have to fulfill the standards defined in the regulation. However, in 2015, EFSA in a self-assigned mission (!), published its own new guidance on the agronomic and phenotypic characterisation of GE plants that is intended to define the criteria required by Regulation 503/2013 (EFSA, 2015). As explained in the response to RAGES, this guidance is meant *"to further clarify and standardise the type of information the applicants should report with regards to the application of herbicides (e.g. timing, dose, volumes, coadjuvants)."* Together with this guidance, EFSA introduced a further 2-year transitional period to fulfill the

requirements. As a consequence, the specific requirements of Implementing Regulation 503/2013 only became binding for applications filed after the end of 2013, i.e. they only became binding for those applications filed more than four years later. Legally, it is highly problematic that the EFSA can self-assign guidance preventing an EU Regulation from coming into force on the date foreseen in EU legislation. Consequently, even up until 2020, there appear to be hardly any examples of risk assessment in which the relevant criteria were applied. In addition, the EFSA guidance (EFSA, 2015) still does not request that field trials must reflect agricultural practices in the countries where the plants are cultivated, i.e. the real conditions in which the crop is likely to be grown.

Thus, current EFSA practice does not fulfil the provisions of Implementing Regulation 503/2013 to assess whether the expected agricultural practices influence outcomes of the studied endpoints. According to the Regulation, this is especially relevant for herbicide-resistant plants. It is evident that this requirement is not fulfilled by EFSA.

Consequently, the GE plants tested in field trials do not represent the imported GE plants. The data requested by EFSA are insufficient to conclude on the impact of the herbicide applications on gene expression, plant composition and the biological characteristics of the plants as requested by the EU Regulation. Consequently, current EFSA practice violates EU GMO legislation.

Tabled overview

RAGES (2020b) findings and EFSA response (2020a)* regarding RA of HT GE plants and health impacts at the stage of consumption

*** In the first and second column, quotes are taken from RAGES and from the EFSA response insofar as possible, however some edits were introduced to improve readability**

Selected RAGES findings	Selected statements taken from the EFSA response (2020a)	Testbiotech conclusions
RAGES and the published peer-reviewed paper (Miyazaki et al., 2019) show that the introduction of herbicide-tolerant (HT) genetically engineered (GE) soybeans has created new challenges in the European risk assessment of imported food and feed. Food and feed products derived from these plants may show specific patterns of chemical residues and altered nutritional composition. There has also been a substantial increase in the use of herbicides in soybean production due to the emergence of resistant weeds. This is related to particular glyphosate-based herbicides and also other herbicides.	EFSA suggests that the timing and rate of the applied herbicides are in line with the recommendations of the manufacturers. This information is routinely verified by the GMO Panel and specifically discussed in the section of the scientific opinion on management practices.	The EFSA statement contradicts the findings presented by RAGES and Miyazaki et al. (2019). They both show that herbicide applications in the field trials are neither in accordance with the manufacturer's recommendations nor with current practice in North and South America. Thus, the claim made by EFSA is not substantiated by any evidence. Moreover, as Miyazaki et al. (2019) show, the real rate of herbicide application is in many cases much higher than officially recommended by the companies.

Selected RAGES findings	Selected statements taken from the EFSA response (2020a)	Testbiotech conclusions
RAGES and Miyazaki et al. (2019) give an overview of available data on glyphosate applications on HT GE soybeans in North and South America. They compared the data from countries where the crops are cultivated with herbicide applications in experimental field trials conducted by industry. They conclude that field trials carried out for risk assessment purposes do not generally represent the real agronomic conditions in commercial HT GE plant cultivation. In most cases, neither the applied dose nor the number of applications match real conditions.	EFSA also states that in the field trials for comparative analysis of HT GMPs, the complementary herbicides should be kept at a similar application rate across sites to ensure comparability between locations.	This EFSA statement contradicts the one above and shows that current practice violates Regulation 503/2013, which requests an assessment of whether the expected agricultural practices influence outcomes of the studied endpoints. According to the Regulation, this is especially relevant for herbicide-resistant plants. It is evident this requirement is not fulfilled by EFSA.
Miyazaki et al. (2019) show that the amount and timing of spraying the complementary herbicide glyphosate onto HT GE plants can impact their composition; these changes in plant composition can be caused, or influenced by, or impact expression of the additionally inserted genes. In this context, particular attention should be paid to stacked events and gene expression of all additionally inserted genes.	In 2015, EFSA, in a self-assigned mission, published a new guidance on the agronomic and phenotypic characterisation of GE plants, which is meant to define the criteria required by Regulation 503/2013 (EFSA, 2015). As explained in the answer to RAGES, this guidance is meant to further clarify and standardise the type of information the applicants should report with regard to the application of herbicides (e.g. timing, dose, volumes, co-adjuvants).	The guidance (EFSA, 2015) does not request that the rate and number of applications has to be representative of the agricultural practices in the countries where the plants are cultivated, i.e. the real conditions in which the crop is grown. Therefore, this guidance is not sufficient to close the gaps in risk assessment exposed by RAGES and Miyazaki et al. (2019). Secondly, EFSA permitted an additional 2-year transitional period to fulfill the requirements of the guidance. Consequently, the specific requirements of Implementing Regulation 503/2013 were not binding for applications filed after the end of 2013 (as foreseen by EU legislation), but only for applications filed after 2017.
Closely related issues were identified which overlapped with EU GMO and pesticide regulation, but these are not currently considered. Such issues are related to indirect, cumulative and combinatorial effects as well as the assessment of mixed toxicity.		In its response, EFSA does not address the issue of gaps between the pesticide regulation and GMO regulation.
Long-term effects arising from the consumption of the products and their impact on the immune system, the endocrine system and the gut microbiome completely escape the risk assessment by EFSA.		EFSA, in its response, does not address the issue of feeding studies and long-term aspects, including effects on the microbiome.

Further findings and Testbiotech comments

When EFSA was first established, it did not initially request the testing of whole food derived from GE plants after exposure to the complementary herbicides. This gap was closed some years ago. Currently, EFSA still does not request that the plants are submitted to conditions representing agronomic practice in the most relevant countries. This means that the material tested in the approval process is not representative of the material that is actually imported. Therefore, the assessment is not in accordance with Implementing Regulation 503/2013 which requests that:

- *1.2.2.3: "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).*
- 1.3.1: "*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.*"

In regard to applications of the complementary herbicide, the response from EFSA is surprising: The authority suggests that *"the timing and rate of the applied intended herbicides are in line with the recommendations of the manufacturers. This information is routinely verified by the GMO Panel and specifically discussed in the section of the scientific opinion on management practices."* This is in stark contrast to the findings presented by RAGES and Miyazaki et al. (2019).

The EFSA statement cited above also contradicts other EFSA statements asserting that the complementary herbicides are not applied in realistically high dosages: *"In the field trials for comparative analysis of HT GMPs, the intended herbicides* [explanation: complementary herbicides] *are kept at a similar application rate across sites, to ensure comparability between locations, while the combinations of conventional herbicides applied at the selected sites reflect different weed management practices, chosen to maintain the weed pressure under control."* This is in line with another EFSA statement made to the Experts of the Members States in 2020: *"for the experimental treatments to be comparable between different locations, the application rate should not differ too strongly between them." (EFSA*, 2020b; see also Testbiotech, 2020b)

In summary, it is evident that the data from field trials as requested by EFSA are not representative of current agronomic practices. Thus, current EFSA assessment violates the provisions of Implementing Regulation 503/2013.

In the context of assessing the EFSA response, Testbiotech became aware that the effects of glyphosate applications on plant metabolism in herbicide-resistant plants had also been confirmed by de Campos et al. (2020) and Zanatta et al. (2020). This underlines the need to test the GE plants under the agronomic conditions specific to GE HT plants, and not to keep to similar application rates across all the field trial sites.

EFSA asserts that current gaps in risk assessment would have been closed by its 2015 guidance (EFSA, 2015), which must be applied to all applications filed after 2017. However, taking a closer look at the guidance (EFSA, 2015), it does not request that the rate and number of applications has to be representative of the agricultural practices in the countries where the crops are cultivated, i.e. the real conditions under which the crop is grown. This guidance is therefore not suitable for closing the gaps in risk assessment that were exposed by RAGES and Miyazaki et al. (2019); and is not suitable to fulfill the requirements of Regulation 503/2013.

It is also disturbing that EFSA introduced a 2-year transition period alongside its 2015 guidance to fulfill the requirements. Consequently, the specific requirements of Implementing Regulation 503/2013 were not binding for applications filed after the end of 2013; they are only binding for applications filed after 2017. It appears that EFSA has intentionally undermined EU legislation through its self-assigned actions.

Legally, it is highly problematic that the EFSA guidance de facto prevents an EU Regulation from coming into force as foreseen in EU legislation. However, this is exactly how EFSA has used its guidance. As seen, for example, in a response to experts from Member States 2020 regarding an application from 2017 (EFSA, 2020b): *"The field trials were conducted in typical maize growing areas of North America, representing regions of diverse agronomic practices and environmental conditions, which is supported by the geographic map indicating the locations, the information provided on the variety of agronomic practice, soils and meteorological factors. In order to improve the representativeness of the selected field trials, EFSA published a guidance document on the agronomic and phenotypic characterisation of genetically modified plants (EFSA GMO Panel, 2015). Application EFSA-GMO-DE-2017-142 was submitted during the transitional period of the GMO Panel guidance. Therefore, the requirements of the guidance document were not fully applicable for this application."*

Overall, the conclusions presented in the RAGES report are confirmed in the EFSA response. Risk assessments of HT GE crops do not take the application of high dosages and repeated spraying of the complementary herbicides into account, which is standard practice in commercial cultivation.

Therefore, the GE plants tested in field trials do not represent the imported GE plants. These 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. Consequently, current EFSA practice violates the EU GMO legislation.

Further, it should no longer be ignored that the most obvious question in regard to GE HT plants, i.e. the toxicity of the residues from spraying with glyphosate in the imported material was never fully assessed. This is acknowledged by the EFSA Pesticide Panel (EFSA, 2018a). In this context, it also has to be considered that in the case of glyphosate resistant plants, there is a specific situation in regard to chronic exposure via the route of food consumption, since glyphosate is known to show antibiotic activity. Glyphosate has indeed been shown to have negative effects on the composition of the intestinal flora of cattle (Reuter et al., 2007), poultry (Shehata et al., 2013; Ruuskanen et al., 2020) and rodents (Mao et al., 2018; Mesnage et al., 2020 (preprint); 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 is not unlikely to trigger significant changes in intestinal bacteria, but these effects are escaping risk assessment completely (see also Parenti et al., 2019).

3. RA of insecticidal GE Bt plants for cultivation

Conclusions

EFSA accepts data from isolated Bt proteins to assess toxicity. However, the toxicity (and immunogenicity) of Bt proteins produced in plants, such maize, cotton and soybeans, is always higher than the isolated Bt toxins. All these plants produce protease inhibitors, which delay the degradation of the toxins and increase exposure to the toxins, and can thus cause to up to 20-fold higher toxicity. Even though these effects are relevant for all Bt plants approved for import and cultivation, such combinatorial effects were never mentioned or assessed by EFSA.

Furthermore, even though several plausible hypotheses on higher toxicity and lower selectivity of Bt toxins have been discussed for many years, EFSA has never managed to develop and apply suitable methodology to gather reliable data on non-target organisms or the food and feed chain. In fact, EFSA never fully explored even the more recent findings on mode of action, selectivity and co-factors.

Although EFSA has been in existence for almost 20 years, its assessment of the risks from cultivation of Bt plants to protected European butterfly species and other non-target organisms still suffers from a significant lack of empirical data.

Finally, EFSA considerably underestimates the risks of gene flow from transgenic maize to teosinte and any associated risks.

General overview

RAGES (2020c) showed that EFSA primarily arrives at its conclusions using data from isolated Bt toxins produced by an artificial bacterial surrogate system. There are only a limited number of tests on non-target organisms using the GE Bt plants, e.g. some tests with pollen or leaves. Thus, EFSA largely assesses the environmental risks of GE plants on the basis of an isolated chemical.

Furthermore, EFSA assumes that Bt toxins have a single-target specific mode of action with high selectivity and, consequently, assumes safety for non-target organisms. However, RAGES showed that a much broader spectrum of non-target organisms can be affected by Bt toxins, and there is a much broader diversity of modes of action than considered by EFSA. The high selectivity paradigm is largely outdated; instead, a much broader variety of models is proposed and accepted today than three decades ago when Bt crops were first introduced

RAGES also showed that a large number of non-target organisms are exposed to Bt toxins, in particular from the uptake of Bt plant material. Furthermore, the Bt toxins are continuously present above and below ground throughout the growing season and beyond, including in aquatic ecosystems such as headwater streams running through the agricultural landscapes where Bt crops are grown.

RAGES concludes a body of evidence has accumulated showing that the assumptions on which EFSA risk assessments are based need to be corrected.

In regard to the appearance of teosinte in Spain and France, RAGES showed that there is no scientific basis for any conclusion that the damage caused by potential crossings with GE maize would be moderate. The data needed for conclusive risk assessment are simply missing. Apparently, the teosinte found in Spain deviates in its genotype from that found in Latin America. One of the identified reasons for this are additional crossings with maize varieties. This increases the risk of gene flow between GE maize and the European teosinte.

In its response, EFSA (EFSA, 2020a) disagrees with most of the findings in the RAGES reports and emphasises that the newly produced proteins are not just assessed in isolation. EFSA also defends its approach to the risk assessment of non-target organisms. However, EFSA does acknowledge that their current concept of measures to protect non-target *Lepidoptera* suffers extensively from a lack of empirical data and needs to be revised.

As a further literature research by Testbiotech shows, recent data highlight the need for the revision of the current EFSA model. We identified other publications that strongly support the need to revise the toxicity assessment of Bt proteins produced in plants, i.e. in regard to toxicity and selectivity.

Finally, there is new evidence showing that EFSA greatly underestimates the risk of hybrids between teosinte and GE maize. As recent research shows, teosinte in Europe has changed its biological characteristics in ways that will facilitate further genetic exchange with maize plants. Therefore, the likelihood of hybridisation with the GE maize has strongly increased.

Tabled overview

RAGES (2020c) findings and EFSA response (2020a)* regarding RA of insecticidal GE Bt plants for cultivation

*** In the first and second columns, quotes were taken from RAGES and from the EFSA response insofar as possible, however some edits were introduced to improve readability**

Selected RAGES findings	Selected statements taken from the EFSA response (2020a)	Testbiotech conclusions
RAGES (2020b) showed that EFSA arrives at its conclusions primarily by using data from isolated Bt toxins produced by an artificial bacterial surrogate system. There are only a limited number of tests on non-target organisms using the GE Bt plants, e.g. some tests with pollen or leaves. Thus, to a great extent, EFSA assesses the environmental risks of GE plants as an isolated chemical.	It is incorrect to claim that EFSA's risk assessments for Non-target organisms (NTO) only address the newly expressed proteins (NEPs). In line with internationally agreed risk assessment practices, potential harmful effects on NTOs associated with the newly expressed proteins are evaluated within different tiers that progress from laboratory studies representing highly controlled, worst-case expected environmental concentrations using microbial-derived (purified) proteins (Tier 1a), to laboratory bioassays with more realistic exposure to the newly expressed protein (Tier 1b), semi-field (Tier 2) and field (Tier 3) studies carried out under less controlled conditions.	Further literature research shows that Bt proteins produced in plants, such as maize, cotton and soybeans, can be expected to be much more toxic than isolated Bt toxins. The reason for this is protease inhibitors (PI) present in the plant tissue. PIs substantially delay the degradation of Bt toxins and enhance their toxicity, e.g. up to 20-fold. These effects were shown by Monsanto 30 years ago (MacIntosh et al., 1990). Since then these findings have been confirmed in several scientific publications. They have, however, never been taken into account in risk assessment, even though they are relevant for all Bt plants. Therefore, starting risk assessment (Tier 1) by feeding isolated proteins to represent worst-case scenarios does not make any sense. The data gathered cannot be used to conclude upon the safety, or the design, or the need for further tests in the following stages of risk assessment.
Furthermore, EFSA assumes Bt toxins have a single-target specific mode of action with high selectivity and assumes safety for non-target organisms. However, as RAGES showed, a much broader spectrum of non-target organisms can be affected by the Bt toxins; there is also a much broader diversity in modes of action than considered by EFSA. The high selectivity paradigm is largely outdated; instead, a much broader variety of models is proposed and accepted today than was the case decades ago when Bt crops were first introduced.	It is incorrect to claim that EFSA does not consider the spectrum of activity of insecticidal Bt proteins when assessing their environmental risk on NTOs. For all GE plant applications for cultivation, earlier-tier studies have been requested from applicants on a range of NTOs belonging to different taxonomical groups, going beyond that of the target pests.	Since the toxicity of Bt proteins produced in plants is much higher than for isolated Bt proteins, the selectivity of the GE plant proteins is likely to be lower (Then, 2010), and a much broader range of non-target organisms can be affected than considered by EFSA, which mostly bases its assessment on tests with isolated proteins. In addition, combinatorial effects with other stressors, such as residues from spraying with the complementary herbicides, also have to be considered as additional factors that enhance toxicity and lower selectivity. This is especially relevant in stacked events, but the mixed toxicity of residues from spraying and Bt toxins was never assessed by EFSA in detail. Since these factors are still not taken into account, risk assessment in regard to non-target organisms is substantially flawed.

Selected RAGES findings	Selected statements taken from the EFSA response (2020a)	Testbiotech conclusions
RAGES also showed that a large number of non-target organisms are exposed to Bt toxins, particularly from uptake of Bt plant material. Furthermore, these Bt toxins are continuously present above and below ground throughout the growing season and beyond, including in aquatic ecosystems such as headwater streams running through the agricultural landscapes where Bt crops are grown.	EFSA's risk assessments of GE plants for cultivation cover all relevant direct and indirect exposure pathways to which terrestrial, soil and aquatic NTOs can be exposed to different matrixes (plant, soil, aquatic environments), as well as the level of exposure to such proteins. EFSA has quantified the risk to non-target Lepidoptera associated with the ingestion of maize MON 810, Bt11 and 1507 (referred to hereafter as Bt -maize) pollen deposited on their host plants through estimates of larval mortality based on mathematical models developed by Perry et al. (2010, 2011, 2012, 2013). Since 2009, EFSA and its GMO Panel have published seven scientific outputs on this topic, either on their own initiative or on request of the European Commission, applying and further refining the model in a stepwise approach, whilst taking into account new relevant scientific publications, like those cited by RAGES. In these scientific outputs, EFSA and its GMO Panel acknowledge that: (1) uncertainties pertaining to the structure of the Perry et al. models, mostly caused by the lack of data from bioassays estimating the sensitivity of a wider range of 'real' NT *Lepidoptera* for most of the assessed Bt -maize events; and (2) uncertainties contributing to the variability in exposure of NT *Lepidoptera* to Bt -maize pollen.	Testbiotech welcomes the announcement made by EFSA that they will improve their methodology for exposure assessment.
RAGES concludes a body of evidence has accumulated, showing that the assumptions on which EFSA risk assessments are based need to be corrected.	When assessing the relevance of NTO studies for the risk assessment of GE plants for cultivation, it is important that results seen under worst case exposure conditions in laboratory settings are brought in the context of expected environmental exposure levels. To characterise the risk of GE plants to NTOs, EFSA assembles, weighs and integrates all available evidence. Overall, these studies have collectively concluded that non-target effects of Bt plants are minimal or negligible, especially in comparison to the negative effects of the use of insecticides for controlling of target organisms.	In regard to NTOs, Testbiotech is aware of only very few studies which try to empirically assess combinatorial effects which are known to enhance toxicity and to lower selectivity of Bt toxins. Since these effects ALWAYS have to be expected when Bt toxins are produced in maize, cotton and soybeans, we do not agree with the EFSA findings. The toxicity of isolated Bt toxins cannot be used to conclude on the safety of Bt proteins present in plants, which also produce protease inhibitors. We also do not agree with the approach to compare chemical insecticides (with a strong effect over shorter period of time) with Bt toxins (potentially more subtle effects, but permanent exposure). These strategies for controlling plant pests have to be assessed separately.

Selected RAGES findings	Selected statements taken from the EFSA response (2020a)	Testbiotech conclusions
In regard to the appearance of teosinte in Spain and France, RAGES showed that there is no scientific basis for any conclusion that the damages caused by potential crossings with GE maize would be moderate. The data necessary for conclusive risk assessment are simply missing. Apparently the teosinte found in Spain deviates in its genotype from that found in Latin America. One reason identified are additional crossings with maize varieties. This increases the risk of gene flow between GE maize and the European teosinte.	Following a mandate from the European Commission in 2016, EFSA assessed whether the scientifically relevant information on teosinte contained new evidence that would change or invalidate its previous ERA conclusions and risk management recommendations on the cultivation of maize MON 810, Bt11, 1507 and GA21 (EFSA, 2016). In its assessment, EFSA explored whether plausible pathways to harm from the cultivation of GE maize could be hypothesised for situations where GE maize and teosinte would grow sympatrically, focusing on four specific areas of risk that are typically considered in ERAs of GE plants: (1) altered persistence and invasiveness of GM maize × teosinte hybrids; (2) cross-pollination of maize by GM maize × teosinte hybrids; (3) interactions of GM maize × teosinte hybrids with other organisms; and (4) interactions of GM maize × teosinte hybrids with abiotic environment and biogeochemical processes. For each of these pathways, EFSA considered unlikely that environmental harm would be realised. EFSA is not aware of new evidence that would invalidate the conclusions of EFSA (2016).	In 2016, EFSA published a first opinion on the risks of teosinte spreading in Spain and France. At that time, EFSA was not aware of publications showing that the genome of teosinte which emerged in the EU is very different to teosinte in Latin America. Apparently, crossings between European maize varieties and teosinte have occurred which will probably impact gene flow and invasiveness of the European teosinte. Therefore, EFSA should correct its opinion. Furthermore, any assessment of the risks of crossings between teosinte and maize have to take into account as next generation effects. These effects cannot be predicted solely on the basis of data from the parental plants. However, EFSA never requested any data on biological characteristics of hybrid generations resulting from crossings with Bt maize.

Further findings and comments by Testbiotech

(1)Testbiotech has become aware of further relevant findings in regard to selectivity and efficacy since RAGES was finalised: in general, selectivity and efficacy of Bt toxins can be influenced by many co-factors (see, for example, Then, 2010; Hilbeck & Otto, 2015). One crucial impact factor are protease inhibitors (PI), which delay the degradation of Bt proteins and so enhance their toxicity. In many of its comments on EFSA opinions, Testbiotech has highlighted this flaw by referring, for example, to Pardo-López et al. (2009). However, EFSA has never provided a detailed response.

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

Therefore, any risk assessment which does not take a combination of plant material with the Bt toxin into account is not reliable and systematically underestimates the risks. However, as can be concluded from the EFSA response, most of the data on the toxicity of Bt proteins are based on tests using isolated Bt toxin.

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. EFSA has however never assessed this crucial aspect in any of its opinions, despite all its concerns about Bt plant applications. The only explanation for this situation is that EFSA has intentionally set aside assessment of these specific combinatorial effects as they would otherwise impact ALL applications for cultivation, import and cultivation.

It is known from scientific publications that co-factors which enhance the toxicity of the Bt proteins can also impact their selectivity (for overview see 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. In addition, there has never been any systematic research into these combinatorial effects. There are just a few publications available which indicate effects on non-target insects from protease inhibitors combined with Bt toxins (Babendreier et al., 2005; Liu et al., 2005a; Liu et al., 2005b; Han et al., 2010). Again, Testbiotech has no explanation about why EFSA constantly ignores these facts and findings which are relevant for non-target organisms as well as for food and feed safety.

(2) EFSA has, as far as we are aware, for the first time clearly admitted weaknesses in the Perry et al. (2010, 2012) models in regard to the exposure of non-target organisms to Bt plants. EFSA points to *"the lack of data from bioassays estimating the sensitivity of a wider range of 'real' non-target Lepidoptera for most of the assessed Bt -maize events"*. This is a significant finding since all EFSA opinions on the risk assessment of MON810, Bt11 and Maize 1507 were based on this model and led EFSA to conclude that a 20-meter distance to fields with Bt maize would be sufficient to safeguard protected species of *Lepidoptera* (butterfly species).

Testbiotech criticised the Perry et al. models as far back as 2016 because of a lack of data on the susceptibility of European butterfly species (Lepidoptera) in regard to the Bt toxins produced in the Bt maize: *"Because this modelling system is by no means based on empirical data (…) it cannot be used to reliably assess the real environmental risks."* (Testbiotech, 2016)

Using the example of *Aglais* io in Catalonia (Baudrot et al., 2020), it has recently been shown that the model used by EFSA, by assuming an average susceptibility, underestimates the risks in particular for susceptible subpopulations of *Lepidoptera. "When looking at the average lethal effect, Bt-pollen seems to have negligible impact on Non-target Lepidopteras, but when looking at the most Bt-sensitive individuals (i.e. combining highest exposure and lowest survival), we observed a dramatic change in their survival probability."*

The authors also state that *"the high complexity of the physiological mechanisms of ingestion, solubilisation, activation, binding, storage, depuration, bio-transformation of Cry protein are not enough described and quantified"* to use a standard mechanistic modelling. Therefore, they "*cannot estimate a quantitatively accurate lethal damage effect from a given exposure profile."*

Further, the researchers explain *"that also other factors such as sub-lethal effects combined with other stressors (e.g., parasitism, chemical compounds, resource depletion) should be taken into account."*

As a result, the publication, which is co-authored by former EFSA experts, confirms the uncertainties and gaps in current EFSA risk assessment.

Taken together, there are strong indications that the current EFSA approach to risk assessment of non-target *Lepidoptera* has to be comprehensively revised.

(3) Some RAGES experts are also actively researching teosinte, which has emerged as a weed in Spain and France. Up until 2016, hazards arising from the gene flow of the insecticidal Bt trait in GE maize to wild and weedy relatives were dismissed as there are no wild or weedy relatives of currently cultivated GMOs, i.e. Bt maize MON810, in Europe. This situation changed dramatically when, in 2009, Spanish farmers discovered a new, fast-spreading and highly destructive weed in their maize fields (Pardo et al., 2014, Trtikova et al., 2017). It proved to be the case that the new weed is the ancestor of maize, teosinte, however, the exact species remains unclear, as does the route of introduction.

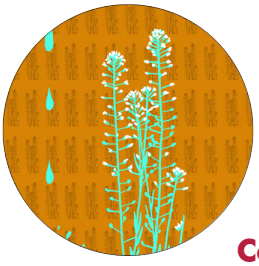
In 2016, EFSA in 2016 concluded that *"...it is unlikely that environmental harm will be realised"*. This conclusion was repeated in another publication in 2018 by a group of authors led by an EFSA staff member and an industry representative (Devos et al., 2018). EFSA tried to establish a consensus that, given the ecology and biology, the risk of seeing teosinte emerge as a problematic weed in a temperate climate was remote. This problem is not even mentioned in the monitoring report assessment of Bt maize cultivation in Spain (EFSA, 2020c).

However, RAGES showed there is no scientific basis for assuming a low likelihood of severe damage from the emergence of teosinte because the data necessary for reliable risk assessment are missing. In the context of assessing the EFSA response, Testbiotech has become aware of further publications confirming that the risks from crossings of GE maize and teosinte cannot be predicted from the data assessed by EFSA (2020a): as already shown by Trtikova et al. (2017), another publication, Le Corre et al. (2020), confirms that European teosinte plants from Spain and France have, 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. 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. The scientists have therefore explicitly warned that the risk of the plants becoming invasive should not be underestimated.

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.

Le Corre et al. (2020) emphasise that their results show that risks of crop-wild introgression should not be underestimated when forecasting invasiveness risks. They also show that crop-wild introgression can be a two-way street, enhancing the gene flow to both partners, maize and teosinte. These findings strongly underline the risk of the European teosinte acquiring Bt gene constructs and, potentially, also further herbicide resistance genes (such as in Bt11 and Maize 1507) from the GE maize cultivated in Spain.



4. RA of nutritionally-altered GE crops and food & feed safety

Conclusions

Plants which are deliberately changed in their nutritional composition for intended health benefits create specific challenges in risk assessment. However, EFSA has not delivered an adequate methodology to assess the intended and unintended effects. Post-market monitoring is also inadequate for identifying adverse health effects.

Gene-environment interactions will affect nutrient expression, but the field trials that have been conducted are inadequate for the characterization of the resulting variability in nutrient levels. The field trials that were conducted and accepted by EFSA do not represent the bioclimatic regions in which the plants are grown. EFSA simply routinely accepts field trials from only one bioclimatic region for one year (such as the US), not taking into account the much broader range of environmental impacts (due to climate change, regional conditions, infestation with plants pests, other stressors) to which the plants will be exposed during cultivation in various regions and which will impact gene expression.

Despite it being known that the insertion of additional genes can unintentionally give rise to various biologically active molecules (see, for example, Ben Ali et al., 2020), EFSA only looks at unintended proteins in its molecular risk assessment.

Testbiotech sees the need to further develop specific guidance and methodologies for the risk assessment of plants with complex changes in their genotype and / or phenotype. This guidance should also request data which allow EFSA, from a technical perspective, to assess whether the intended nutritional characteristics are actually achieved by the GE plants, and if there is even a reasonable expectation that these can be achieved.

General overview

As RAGES (2020d) states, nutritional changes are complex and not limited to a single nutrient; their impacts may vary with dose and also depend on the receiving population, which will include vulnerable individuals. This complexity makes both risk assessment and labelling (in terms of the exact wording) challenging. Nutritionally-altered GM crops have been approved for use as food and feed within the EU without specific guidance for their risk assessment. This means that many important issues have not been considered adequately.

The issues include:

- The GM traits all affect multiple nutrients and the overall effect on health is poorly understood: health claims (of benefits) are not substantiated;
- Unlike previous GM crops, nutritionally-altered GM crops are engineered to produce molecules that are biologically active in humans; there is an increase in the risk of adverse medical effects either from over exposure to the intended product or from unintended by-products whose health hazards are unknown;
- Gene-environment interactions will affect nutrient expression and the field trials conducted are inadequate to characterise the resulting variability in nutrient levels;
- There has been no full nutritional/food safety analysis (instead the focus is on comparing the main altered nutrient with standard dietary recommendations);
- Potentially vulnerable subgroups need to be considered;
- Impacts of food processing and storage need to be considered for all food types;

- Use of the GM crop as feed can alter nutrient content of (unlabelled) meat and dairy products;
- Food labelling proposals are inadequate to provide sufficient information for consumers;
- Post-market monitoring is inadequate to identify adverse health effects.
- Other nutritionally altered crops may in future contain altered levels of vitamins and minerals, which will create additional challenges for risk assessment.

No applications for the commercial cultivation of nutritionally-altered GM crops have yet been filed in the EU. However, there are many examples of unintended effects described in the published literature. These include:

- Direct adverse effects on wildlife from consumption of altered nutrients;
- Complex ecological effects associated with introducing new or enhanced levels of nutrients into ecosystems;
- Increased attractiveness to pests and/or susceptibility to pathogens, associated with altering biochemical pathways in the plant;
- Adverse impacts on yield and agronomic properties.

EFSA, in its response to the RAGES reports (EFSA, 2020a), states that its guidance documents already provide principles, strategy and data requirements in the risk assessment of GE plants for food and feed uses, including food and feed derived with nutritional traits. Accordingly, EFSA claims that it carries out a comprehensive nutritional assessment of all GE plants, in particular of those with a modified nutrient profile, using a stepwise approach that also takes into account the use of different dietary intake scenarios. EFSA also claims that current post monitoring plans are adequate monitor potential health effects.

Testbiotech is aware that so far only three GM crops with altered oil content have been approved for import to the EU and use in food and feed. However, in future, other nutritionally-altered GM crops – for example, with altered vitamin or mineral content – might be proposed for import or for cultivation. Therefore, Testbiotech sees the need to further develop specific guidance and methodologies for the risk assessment of plants with complex changes in their genotype and / or phenotype. This guidance should also request data which allow EFSA, from a technical perspective, to assess whether the intended characteristics are actually achieved by the GE plants, and whether there is a reasonable expectation of the health claim being achieved.

Tabled overview

RAGES (2020d) findings and EFSA (2020a)* response to RA of nutritionally-altered GE crops and health impacts at the stage of consumption

*** In the first and second columns, quotes were taken from the RAGES reports and from the EFSA response insofar as possible, however some edits were introduced to improve readability.**

Selected RAGES findings	Selected statements from the EFSA assessment (2020a)	Testbiotech conclusions
Nutritionally-altered crops create challenges in risk assessment. Nutritional changes are complex and not limited to a single nutrient, their impacts may vary with the amount of the altered or newly produced substances and will also depend on the receiving population, which will include vulnerable people. Nutritionally-altered GM crops have been approved for use as food and feed within the EU with no specific guidance for their risk assessment. This means that many important issues have not been adequately considered.	EFSA does not support the claim that the risk assessment for nutritionally altered GE plant needs the development of specific guidance, and considers the current GMO Panel guidance documents adequate to conduct the risk assessment of these GE plants.	Looking at the range of GE plants being developed by genome editing to have a nutritionally-altered composition, there is no doubt that EFSA will in the near future need specific guidance for the assessment of plants which do not have conventional comparators due to complex changes in their genotype and / or phenotype.
The GM traits all affect multiple nutrients and the overall effect on health is poorly understood: health claims (of benefits) are not substantiated.	EFSA agrees with RAGES on the importance of a regular reviewing of the scientific literature regarding the effect of the nutrients on human health (positive and/or negative), considering the vast number of studies that continuously becomes available, often with contradictory outcomes. At the same time, EFSA wants to point out that the EU risk assessment of GMOs focuses on their safety and not on potential human and animal health benefits (irrelevant for the authorisation).	We agree that potential benefits should not be assessed by EFSA and are irrelevant for risk assessment. However, from a technical perspective, EFSA should assess whether the intended characteristics are actually achieved by the event and whether there is a reasonable expectation they are achievable.
Unlike previous GM crops, nutritionally-altered GM crops are engineered to produce molecules that are biologically active in humans, therefore there is an increase in the risk of adverse medical effects either from over exposure to the intended product, or from unintended by-products whose hazard to health is unknown.	For new compounds not present in the conventional plant (e.g. specific fatty acids), and even in the absence of any type of reference value, scientific evidence must be provided to support their safety (e.g. information on levels in commonly consumed foods, scientific literature on the absence of safety concerns, etc.).	Due to the complexity of the underlying issues, EFSA should develop specific guidance on how to assess the safety of all biologically active compounds produced in GE plants meant to impact animal or human health. For example, despite it being known that the insertion of additional genes can unintentionally give rise to various biologically active molecules (see, for example, Ben Ali et al., 2020), only unintended proteins are considered by EFSA in the molecular risk assessment.

Selected RAGES findings	Selected statements from the EFSA assessment (2020a)	Testbiotech conclusions
Gene-environment interactions will affect nutrient expression and the field trials conducted are inadequate to characterise the resulting variability in nutrient levels.	RAGES claims lack of specific requirements for the compositional data of nutritionally altered plants to take into account the importance of genome-environment (G × E) interactions. EFSA points out that the applicable GMO Panel guidance documents cover the analysis of possible G × E interactions for all GE plants, i.e. also for nutritionally altered GE plants.	We do not think the guidance EFSA has spoken of is sufficient from a legal or scientific perspective: (1) The most pertinent guidance for the EFSA (EFSA, 2015) is the introduction of a 2-year transition period to fulfil EU Regulation criteria 503/2013. This means that EFSA is undermining the regulation, which is legally binding for all filed GE plant applications for import since December 2013. (2) From a scientific perspective, the guidance does not request that the plants are exposed to a sufficiently wide range of defined environmental stressors to observe all relevant genome x environment interactions.
Potentially vulnerable subgroups need to be considered.	EFSA acknowledges that, in certain cases, consumption data on particular vulnerable populations are needed for the assessment of nutritionally altered plants as well as representative data across European countries.	Due to the complexity of the underlying issues, EFSA should develop specific guidance to assess the safety of biologically active compounds in GE plants meant to impact animal or human health, especially taking the most vulnerable groups into account.
Impacts of food processing and storage need to be considered for all food types.	EFSA does not support the RAGES claim that the effect of processing and storage on the nutritionally altered GE plants is not considered in the nutritional assessment. Composition data of both raw agricultural commodities (i.e. seeds) and processed products (i.e. oil) are compared and considered during the risk assessment of GE plants with modified fatty acid profile.	We suggest that EFSA, from a technical perspective, should develop guidance to assess whether the intended characteristics are actually achieved by the event and how the harvest should be stored and processed to deliver what it sets out to achieve.
Use of the GM crop as feed can alter nutrient content of (unlabelled) meat and dairy products.	The assessment of the safety of food products obtained from animals fed with genetically modified feed is not in the scope of the GMO Regulation.	If there is a gap in regulation, EFSA should actively to point this out to the political decision- makers. Further, EFSA should pick up on this issue in its opinions even if not requested. There appears to be no legal barrier to stop EFSA from considering these effects. Specific guidance is needed to make sure all relevant risks are assessed.

Selected RAGES findings	Selected statements from the EFSA assessment (2020a)	Testbiotech conclusions
Food labelling proposals are inadequate to provide sufficient information for consumers.	The appropriate labelling of food products containing, consisting of or produced from the GE plants as required in the authorizations, allows incorporating or excluding these products from the diet depending on individual health needs.	The gaps in labelling referred to by RAGES are not about the GE information, but about the specific intended or unintended effects the products may have.
Post-market monitoring is inadequate to identify adverse health effects.	EFSA disagrees with RAGES regarding the inadequacy of post-market monitoring (PMM) plans and labelling proposals for nutritionally altered GE plants. Once the safety of the GE plant is demonstrated, current PMM is appropriate to confirm the predicted consumption, the application of conditions of uses, or identified effects.	It is obvious that current standards of PMM do not allow the gathering of sufficiently reliable information to detect indications of any (adverse) effects on health that might be related to GE food or feed consumption. It is the task of the Commission in its role as a risk manager, to develop sufficiently robust standards for tracking health impacts of plants intentionally changed in their nutritional composition.

Further findings and comments by Testbiotech

When plants are intentionally changed in their nutritional composition, specific guidance is needed not only to assess complex health risks but also the intended biological characteristics.

From a technical perspective, EFSA should assess whether the intended characteristics are actually achieved in the event as stated in the application, and whether there is a reasonable expectation that it can achieve its intended purpose while also taking storage and processing into account.

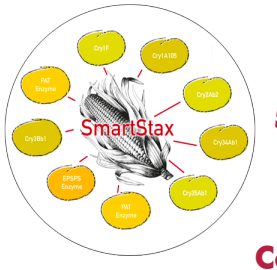
A case in point is Golden Rice (Testbiotech, 2018a): applications were filed to several authorities by the IRRI for import of Golden Rice, e.g. at the Food Standards Australia New Zealand (FSANZ). The data provided to FSANZ were mostly from a specific line of the GR2E event (see Paine et al., 2005) showing a low level of carotenoids, especially of beta-carotene. If additional losses in carotenoids during storage and heating are taken into account, this event is very unlikely to deliver on its intended purpose. Only minor levels of beta carotenoids can be expected at the stage of consumption.

There are several possible explanations why just these data were used for the applications. One is that the applicants wanted to establish product safety by using data from an event with low transgene activity. Higher transgene activity might affect overall plant composition to a degree that raises specific questions on safety. Therefore, lower transgene activity can ease to claim safety.

There is a serious dilemma in this case: if the purpose (to combat Vitamin A deficiency) cannot be achieved, there will be no interest in growing the plants. However, if lines of Golden Rice that produce higher amounts of carotenoids are cultivated for human consumption, the data provided by the applicant will not allow conclusions to be drawn on safety.

Thus, risk assessment has to ensure that the data in the application are in line with the purpose of the GE plants. If not, they should be rejected and suitable data, e.g. from different events or from different trials, should be requested. To solve this problem, specific guidance is necessary, which also requests the assessment of technical and biological characteristics in regard to the intended purposes.

This specific guidance should also consider that although the role of the microbiome is known to be crucial for environmental and health risks (see EFSA, 2020d), EFSA has never developed any methodology on how to integrate it into the risk assessment of GE plants.



5. RA of GE plants with a combination of traits and food & feed safety

Conclusions

There are a number of plausible and relevant hypotheses that were never investigated and assessed in regard to combinatorial effects and risk assessments of stacked events: (i) the higher toxicity of Bt toxins if combined with residues from spraying; (ii) the higher toxicity and immunogenicity of Bt toxins if combined with protease inhibitors; (iii) the higher toxicity and/or immunogenicity of a combination of traits (several Bt toxins or several herbicide resistances and combinations thereof) if combined in stacked events or mixed in a diet; (iv) changes in the intestinal flora after long-term consumption of food and feed derived from herbicide-resistant or Bt producing plants, or combinations thereof.

EFSA rejected a 90-day feeding study with whole food and feed and never requested feeding studies on stacked events - without ever having developed methodology to empirically test the health effects of whole food and feed at the stage of consumption.

In addition, even though the role of the microbiome is known to be crucial for environmental and health risks (see EFSA, 2020d; Parenti et al., 2019), EFSA has never developed any methodology on integrating this into the risk assessment of GE plants.

Although EFSA has already assessed around 60 stacked events for import,[7] the authority has not developed robust criteria and test designs to allow empirical testing and reliable assessment of the risks of products derived from the plants or those mixed into diets.

General overview

As RAGES (2020e) showed, most GE plants (events) allowed for import, processing and use in food and feed into the EU, carry a combination of several traits. These combinations can arise from the stacking of plants (crossing of parental GE plants) as well as co-transformation of single events. Most GE plants with stacked traits combine herbicide-tolerance (HT) (also known as insect-resistance) and production of insecticidal toxins (IT) (also known as insect resistance). The number of GE plants on the market with trait combinations, especially those produced through stacking, is increasing and this trend is expected to continue in the future.

7 https://www.testbiotech.org/en/database

As RAGES explains, the combined presence of herbicide residues and insecticidal toxins (also in combination with specific plant constituents, e.g. with hormonal or immunogenic properties) have to be considered as stressors with potentially additive, antagonistic or synergistic effects and interactions.

However, RAGES shows that EFSA is following a logic of assessing and testing single components to conclude on the overall risks of GE plants. This causes its opinions to be incomplete, inconclusive and also potentially wrong in their findings. Tests on isolated proteins, single parental plants and an assessment of changes in plant components that just looks at each component individually and not at their overall composition, are not sufficient to assess the potential health impacts of food derived from GE organisms. In addition, active herbicide ingredients are only assessed in isolation (by the EFSA Pesticide Panel) but not as the actual mixtures present in GE plants.

Bt proteins are a case in point for the problems in the RA of EFSA: it has been known since the 1990s that the protease inhibitors (PI) produced in food plants, such as maize, cotton and soybeans, enhance the toxicity of Bt toxins by delaying their degradation. This can lead to a 20-fold plus increase in toxicity. Tests with isolated Bt proteins, e.g. 28-day animal feeding studies, cannot capture their toxicity and immunogenicity under real conditions if consumed in combination with plant material. However, the EFSA fails to mention these synergistic effects in their opinions. Despite a large body of evidence accumulating over the past 30 years, this complete denial of the most relevant facts cannot simply be explained by accidental failures.

Furthermore, co-stressors, such as toxic chemicals, are known to potentially enhance toxicity and lower selectivity of Bt toxins. These risks are also relevant for food and feed safety, especially in stacked events which combine a number Bt proteins with resistance to several herbicides. However, the most obvious question in regard to these stacked plants, i.e. the combined toxicity of the residues from spraying with the Bt toxins, was never investigated.

In conclusion, EFSA never asked for experimental data on the overall toxicity of stacked events and / or mixtures of several traits in one diet. Also no feeding studies were ever requested with material derived from stacked events and/ or from plants mixed into a diets. However, without such data, the real health impact at the stage of consumption cannot be assessed.

In its response, EFSA states that animal feeding studies should only be performed to test a defined hypothesis. However, in regard to combinatorial effects and the risk assessment of stacked events, there are a number of plausible and relevant hypotheses that were never investigated or assessed: (i) the higher toxicity of Bt toxins if combined with residues from spraying; (ii) the higher toxicity and immunogenicity of Bt toxins if combined with protease inhibitors; (iii) the higher toxicity and/or immunogenicity of a combination of traits (several Bt toxins or several herbicide resistances and combinations thereof) if combined in stacked events or mixed into a diet; (iv) changes in intestinal flora after chronic consumption of food and feed derived from herbicide-resistant or Bt-producing plants or their combinations.

In this context, it also has to be considered a plausible hypothesis that a combination of Bt toxins and residues from spraying, can trigger effects on the immune system or other adverse health effects via the microbiome (see Parenti et al., 2019). This hypothesis needs to be tested, including taking into account synergistic effects due to protease inhibitors which may increase exposure to Bt toxins in the gut, before any conclusion can be drawn on the safety of food and feed derived thereof.

As RAGES explains, as yet animal feeding studies are the only accepted method of generating experimental data on whole food and feed. However, this does not mean there is no other adequate method. There are

several possibilities to test specific issues such as combined toxicity by in vitro methods. However, EFSA has so far completely failed to establish a list of criteria for suitable methods and designs for experimental testing of health impacts of whole food and feed.

Tabled overview

RAGES (2020e) findings and EFSA (2020a)* response to GE plants with a combination of traits and health impacts at the stage of consumption

*** In the first and second columns, quotes were taken from the RAGES reports and from the EFSA response insofar as possible, however some edits were introduced to improve readability**

Selected RAGES findings	Selected statements from EFSA assessment (2020a)	Testbiotech conclusions
In regard to food safety, the combined presence of herbicide residues and insecticidal toxins (also in combination with specific plant constituents, e.g. with hormonal or immunogenic properties) have to be considered stressors with potential additive, antagonistic or synergistic effects and interactions. There are many biologically active substances, such as estrogens, allergens and anti-nutritional compounds, present in plants such as soybeans, which may interact with trait-related characteristics and act as stressors.	EFSA does not support the statement that the current EU assessment of stacked events is not sufficiently addressing the identification of risks for animals and consumers.	EFSA does not address the specific issues of the combination of residues from spraying, Bt toxins or plant components.
Bt toxins are an example of possible interactions: they can result in effects on the immune system, e.g. because Bt toxins act as an adjuvant for other plant components. These effects can be enhanced by higher concentrations of Bt toxins in the plants (for example due to stacking) as well as by enzymes produced in the plant, such as trypsin inhibitors, that can delay the degradation of the toxins in food composition. Finally, the effect on the immune system will also depend on the concentration of the allergens produced by the plants.	In relation to allergenicity and the immune system in general, EFSA performs its risk assessment according to relevant guidelines, the principles of which are aligned with international documents. Furthermore, because none of the newly expressed proteins in the assessed GE plants showed potential for allergenicity, considering current knowledge, no reasons for concerns regarding the simultaneous presence of these newly expressed proteins in GE plants are expected.	EFSA does not address the issue of enhanced effects from plants components such as protease inhibitors (PI). EFSA does not discuss dosage- dependent effects of adjuvant components. EFSA does not mention that Cry1Ac is under discussion to be allergenic (Santos-Vigil et al., 2018).[8]

8 See also: www.testbiotech.org/en/press-release/can-bt-toxins-cause-allergies

Selected RAGES findings	Selected statements from EFSA assessment (2020a)	Testbiotech conclusions
It is known that changes in the efficacy and selectivity of Bt toxins have to be considered if they occur in mixtures with potential stressors such as residues from spraying.	The assessment of potential interactions (synergistic or antagonistic effects) resulting from the combination of events in stacks and of relevance for humans, animals and the environment is based on molecular characterisation data, on the outcome of the comparative analysis studies and on the safety assessment of interactions among the newly produced proteins.	EFSA does not address the issue of lowered selectivity and higher toxicity due to the presence of co-factors.
Besides toxic effects, Bt toxins such as Cry1Ac, are also known to invoke and boost immune system reactions. The ability of Cry1Ac toxin to cause adjuvant effects in mice has been used to suggest that Cry toxins can be used as adjuvants for the administration of heterologous antigens. The adjuvant effects of Cry1Ac protoxin were evaluated in regard to the specific antibody responses attained at both mucosal and systemic levels to co-administered antigens of a different nature. A further publication shows in more detail how Cry1Ac induces macrophage activation. Cry1A proteins can frequently and successfully be found in the colon of pigs at the end of digestion after being fed with Bt maize. This shows that Bt toxins are not degraded quickly in the gut and can persist in larger amounts until the end of the digestion process. This means that further interactions between Bt toxins and the complex gut ecosystem, including various food compounds, are possible.	According to RAGES, the potential impacts on the immune system (such as adjuvant effects) have not been appropriately investigated. In particular, RAGES claims that the potential immune adverse effects of Bt proteins were not addressed because their additive or combinatorial effects were not sufficiently assessed and because they are not degraded in the gut upon oral consumption. EFSA previously published comprehensive scientific reports addressing similar questions on the EFSA assessment of GE plants and the potential effects of Bt proteins on the immune system.	In the published reports and also in references made by EFSA (2020a), there is a general lack of empirical data. Consequently, EFSA can only conclude on an absence of evidence, but not on evidence of safety for the immune system. None of the reports mention, discuss or assess the potential enhancement of toxic or immunogenic effects caused by interaction with plant components such as PI.

Selected RAGES findings	Selected statements from EFSA assessment (2020a)	Testbiotech conclusions
Our report shows that combinatorial effects (or potential mixed toxicity) emerging from simultaneous exposure to a fixed combination of potential stressors, emerging from GE plants at the stage of consumption, need to be assessed in far more detail. We recommend that these plants should be tested following the whole mixture approach, considering them as "insufficiently chemically defined to apply a component-based approach". For regulatory purposes, the plants should be considered to be equivalent to UVCB substances (substances of unknown or variable composition, complex reaction products or biological materials) as defined by the provisions of Regulation (EC) No 1907/2006 (REACH).	EFSA is actively working at developing new methodologies for the assessment of mixed toxicity that can be horizontally relevant for food and feed risk assessment.	Testbiotech acknowledges that EFSA is doing this. However, this cannot be used as excuse for a lack of data on long outstanding safety issues. EFSA was founded 20 years ago but has been unable in all that time to establish robust criteria and test designs, which would allow empirical testing and assessment of health impacts from whole GE food and feed actually meant for consumption.
Currently, the most appropriate method to test these substances is life-time feeding studies with whole plant materials. This material should be relevant to the product consumed as food or feed, including the residues from spraying with complementary herbicides (with dosages that are in accordance with the conditions of commercial agricultural practices). To generate reliable data for products that are used daily in the food chain, the feeding studies will need to be long-term, including several generations.	Use of animal feeding studies with appropriate stack GE material to investigate toxicological, reproductive, hormonal, immunological effects on consumers EFSA confirms its opinion that the use of animal studies to investigate possible effects of GE plants whole food and feed on consumers should be conducted only when suited to investigate specific hypotheses.	There are several hypotheses that need to be tested, such as (i) the higher toxicity of Bt toxins if combined with residues from spraying; (ii) the higher toxicity and immunogenicity of Bt toxins if combined with protease inhibitors; (iii) the higher toxicity and/or immunogenicity of a combination of traits (several Bt toxins or several herbicide resistances and combinations thereof) if combined in stacked events or mixed in a diet; (iv) changes in the intestinal flora after chronic consumption of food and feed derived from herbicide-resistant or Bt producing plants or combinations thereof.

Selected RAGES findings	Selected statements from EFSA assessment (2020a)	Testbiotech conclusions
In addition, in vitro testing systems and testing systems using non-vertebrates should also be required and developed further to establish risk-hypotheses and to reduce the overall number of animals needed for feeding studies. Further methodologies need to be developed for testing whole mixtures in addition to, or as reliable replacements for, animal feeding studies. More scientific studies should be initiated to better understand combinatorial, aggregated or cumulative exposure and effects from mixtures of GE plants in the diets of humans and animals.	EFSA underlines that the sensitivity of animal studies to indicate the presence of adverse effects related to the whole food and feed is in general limited due to various hurdles, such as limitations in dose level selection. In case a clear test-hypothesis is identified, a fit-for purpose design would allow to investigate specific endpoints addressing the risk assessment question. EFSA disagrees with RAGES on the use of animal studies on whole GM food and feed to resolve possible gaps in the assessment of long-term, reproductive or immunological adverse effects.	Until now, animal feeding studies are the only way to generate experimental data on whole food and feed. However, this does not mean that they always have to be conducted in the first tier. There are several other possibilities to test specific issues such as combined toxicity. However, EFSA has so far completely failed to establish a list of suitable methods and designs for testing. In addition, although it is known that the role of the microbiome is crucial for environmental and health risks (see EFSA, 2020d), EFSA has never developed any methodology to integrate this into the risk assessment of GE plants.
As a next step, EFSA risk assessments and monitoring of mixtures of GE plants in diets leading to co-exposures of multiple potential stressors will need to fully assess the risks of combinatorial, aggregated and/or cumulative effects.	EFSA also strives for continuously improving the assessment of combined exposure to multiple substances, horizontally applicable to risk assessment areas.	Testbiotech acknowledges the actions that the EFSA is taking. However, this cannot be used as an excuse for a lack of data on long outstanding safety issues. Although EFSA was founded 20 years ago it has in all that time been unable to develop robust criteria and test designs, which would allow empirical testing and monitoring of the health impacts associated with whole GE food and feed actually meant for consumption.

Further findings and comments by Testbiotech

RAGES has provided evidence that EFSA is following its own logic of assessing and testing single components to conclude on the overall risks of GE plants. However, it is known that Bt proteins tested in isolation never represent their actual toxicity and immunogenicity when consumed in combination with plant material containing protease inhibitors (PI). Testbiotech has in many comments on EFSA opinions, directed attention to this issue mostly by referring to Pardo-López et al. (2009). This is something that EFSA has never responded to in detail. In the context of assessing the EFSA response, Testbiotech is now aware of many more publications all confirming this gap in risk assessment that EFSA has until now either steadfastly ignored or denied.

In 1990, Monsanto published data on proteinase inhibitors produced in food plants, such as maize, cotton and soybeans, showing that the toxicity of the Bt toxins is enhanced by a delay in their degradation (MacIntosh et al., 1990). This mechanism is used to enhance the toxicity of Bt toxins in the fields, but is ignored in the assessment of risks to non-target organisms and the food chain from Bt toxins. A large body of evidence has accumulated over the past 30 years showing that this complete denial of the most relevant facts cannot simply be explained by accidental failures.

Basically, PIs cause the Bt toxin to degrade much more slowly than in isolation. This causes a much higher toxicity of the Bt toxins if they are taken up together with the plant tissue than just the isolated toxin (MacIntosh et al., 1990; Zhao et al., 1999; Zhang et al., 2000; Gujar et al., 2004; Zhu et al., 2007; Pardo-López et al., 2009; Ma et al., 2013; Mesén-Porras et al., 2020). The effects that are described indicate, for example, a 20-fold higher toxicity (Pardo-López et al., 2009) for Bt toxins in the presence of PIs, which are known to be present in all the relevant food plants.

Therefore, any risk assessment which does not take a combination of plant material with the Bt toxin into account is not reliable and underestimates the risks. However, EFSA does not mention this crucial aspect in any of its opinions.

The synergistic effects described by MacIntosh et al. (1990), Zhao et al. (1999), Zhang et al. (2000) Gujar et al. (2004), Zhu et al. (2007), Pardo-López et al. (2009), Ma et al. (2013), Mesén-Porras et al. (2020) causing higher toxicity of the Bt toxins are also relevant to risk assessment in regard to the immune system: 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 chronic inflammation and allergies.

In this context, it is relevant that Bt toxins produced by plants can indeed survive digestion to a much higher degree than has been assumed by EFSA. 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. In addition, a study testing corn with a combination of Bt toxins (Cry1Ab and Cry34Ab1) indicates health impacts in rats (Zdziarski et al., 2018). Currently, around 40 events that produce Bt toxins are already authorised for import, many of them producing several Bt toxins in combination.

Moreover, it is known from scientific publications that co-factors which enhance the toxicity of the Bt proteins can also lower their selectivity (for overview see 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. These effects are also relevant for the assessment of food and feed health impacts, but were never assessed by EFSA in any detail. Again, Testbiotech can find no excuse as to why EFSA constantly ignores these facts and findings.

In summary, there is evidence of synergisms emerging in food and feed derived from Bt plants which enhance toxicity and immunogenicity as well as lower selectivity of the toxins. EFSA must request data which allow robust conclusions on dosages and effects to be drawn. Without such data, the safety of the GE plants and products derived thereof cannot be demonstrated.

The same problem – a systematic denial of the most relevant question for risk assessment – can be observed in the case of stacked herbicide-resistant plants: the most obvious question, i.e. the mixed toxicity of the residues from spraying, including the additives, was never investigated. The lack of relevant data was explicitly confirmed by the EFSA Pesticide Panel (EFSA, 2018a).

Furthermore, it should be taken into account that health effects might be transmitted or enhanced by interactions with the gut microbiome; these have still not been considered by EFSA but were recently mentioned (EFSA, 2020d). It is an obviously relevant question for risk assessment if the intestinal flora can be affected by constant exposure to Bt proteins and residues from spraying with the complementary herbicides.

It has to be considered that in the case of glyphosate resistant plants, there is a specific situation in regard to chronic exposure via the route of food consumption, since glyphosate is known to show antibiotic activity. Glyphosate has indeed been shown to have negative effects on the composition of the intestinal flora of cattle (Reuter et al., 2007), poultry (Shehata et al., 2013; Ruuskanen et al, 2020) and rodents (Mao et al., 2018; Mesnage et al., 2020 (preprint); 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 is not unlikely to trigger significant changes in intestinal bacteria, but these effects are escaping risk assessment completely.

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

These findings are highly relevant for the risk assessment of all GE plants inheriting additional epsps genes that confer enhanced resistance to glyphosate. As explained, long term exposure to glyphosate residues due to these plants may lead to disruption in the gut microbiome. Furthermore, stacking of the GE plants very often results in a combination of EPSPS enzymes and Bt toxins that is known to trigger possible immune reactions. It has to be considered a plausible hypothesis that a combination of Bt toxins and residues from spraying, can trigger effects on the immune system or other adverse health effects either directly or via the microbiome. This hypothesis needs to be tested, including taking into account synergistic effects due to protease inhibitors which may increase exposure to Bt toxins in the gut, before any conclusion can be drawn on the safety of food and feed derived thereof.

6. RA of GE crops that can persist and spontaneously propagate in the environment

Conclusions

Effects arising from heterogeneous genetic backgrounds of varieties, wild relatives and hybrid offspring (such as next generation effects) are known to occur. These effects can, for example, affect the persistence and propagation of GE plants in the environment, but EFSA has no adequate methodology to investigate and assess them.

This is exemplified by two cases: (i) EFSA largely underestimates the possible gene flow between teosinte and GE maize in Spain as well as an uncontrolled spread of transgenes; (ii) EFSA ignores that the insertion of epsps gene constructs may not only confer resistance to glyphosate, but also increase the plants' general fitness, which in case of GE oilseed rape is highly relevant for risk assessment.

General overview

As RAGES (2020f) and a peer-reviewed paper (Bauer-Panskus et al., 2020) show, applications for the approval of GE plants for import as well as cultivation in the EU market, raise specific challenges in risk assessment if these plants are able to persist and propagate in the environment.

In general, the risk assessment of GE organisms which can persist and spontaneously propagate in the environment (within or beyond their production systems) has to consider the spatio-temporal dimension, which is far more complex in comparison to GE plants only grown for one season.

More specifically, next generation effects can be impacted by interactions with heterogeneous genetic backgrounds. Unexpected effects can be triggered in interaction with environmental conditions. This observation is especially relevant for the assessment of long-term impacts under changing environmental conditions such as those caused by climate change.

Therefore, risk assessment under these conditions cannot be reduced to the traits and characteristics that are known when the application is filed, it also has to consider effects that can emerge after a number of generations, in other genetic backgrounds or under stress conditions.

However, in regard to environmental risk assessment (ERA) as currently performed by EFSA, the safety of the next generation resulting from spontaneous propagation is hardly considered. The only potential hazards which EFSA considers in more detail are those which exacerbate weed problems and displacement or extinction of native plant species.

However, these potential hazards are not the only risks that can emerge from the persistence and self-propagation of GE crops. Potential hazards include plant interactions and biological signalling pathways within the food web, with soil organisms or insects such as pollinators and other organisms. These pathways and networks can be disturbed or disrupted, for example, by changes in the composition of volatile compounds or biochemical pathways and changes in nutritional quality.

Depending on the specific spatio-temporal dimensions, environmental risk assessment creates problems for both risk management arise due to a high level of uncertainty. To deal with these problems, Testbiotech recommends establishing 'cut off criteria' in risk assessment that take into account the factual limits of knowledge. It proposes the introduction of 'cut-off criteria' based on a specific step of 'spatio-temporal controllability' within risk assessment (Bauer-Panskus et al., 2020).

It is suggested that, in cases where it is known that GE organisms can escape 'spatio-temporal controllability', the authorisation process should be stopped and the release of the GE organisms not permitted. The reason for stopping the approval process under these conditions would be a lack of conclusiveness in risk assessment.

The suggested criteria should not only be relevant to applications for commercial cultivation but also to imports that are likely to cause spillage of viable kernels of events / species.

In its response to RAGES, EFSA appears to intentionally come to incorrect assumptions, misunderstandings and conclusions which are all beside the point. Furthermore, EFSA neglects existing evidence showing that GE plants can overcome biological and abiotic factors limiting the persistence and invasiveness of their conventional counterparts.

Tabled overview

RAGES (2020f) findings and EFSA assessment EFSA (2020a)*of RA of GE crops that can persist and spontaneously propagate in the environment

*** In the first and second columns, quotes were taken from the RAGES reports and from the EFSA response insofar as possible, however some edits were introduced to improve readability.**

Selected RAGES findings	Selected statements from the assessment of EFSA (2020a)	Testbiotech conclusions
New challenges arise for risk assessment if genetically engineered (GE) organisms can persist and propagate in the environment and also produce viable offspring. This review shows that next generation effects can be substantially influenced by interactions with heterogeneous genetic backgrounds.	RAGES and EFSA have different perspectives on protection goals, and thus on the framing of the ERA. There is no consensus between RAGES and EFSA on what constitutes environmental harm arising from the persistence/ invasiveness and vertical gene flow of GE plants. For example, RAGES considers the potential for escape from "spatio-temporal controllability" as a "cut off criterion" based on which GE plant applications for authorisation should be rejected. In contrast, for EFSA the fact that GE plants can persist and propagate in the environment and produce viable offspring is not harmful per se – as this will depend on the associated environmental/agronomic impacts, which must be assessed on a case-by-case basis.	EFSA assumptions are incorrect as far as RAGES findings are concerned: In regard to the protection goals, RAGES does not claim that GE organisms that escape into the environment and produce viable offspring are harmful per se. Instead, RAGES has produced evidence that these organisms pose specific challenges that are likely to render risk assessment inconclusive. Under such circumstances, no releases can be allowed.
It has been suggested that in cases where it is known that GE organisms can escape 'spatio-temporal controllability' due to propagation within natural populations, and where there is no effective control of spread or persistence, then the authorisation process cannot proceed and the release of the GE organism cannot be permitted. The reason for including such criteria in the approval process under these conditions is a lack of conclusiveness in risk assessment.	EFSA notes that Directive 2001/18/ EC and Commission Directive (EU) 2018/305 on the deliberate release into the environment of GE organisms do not mention/refer to "spatio-temporal controllability", so it is not considered a protection goal by law at present.	EFSA assumptions are incorrect as far as the RAGES findings are concerned: spatio-temporal controllability as presented in the report is not a protection goal. Rather, spatio-temporal controllability is introduced as part of the cut-off criteria in risk assessment, allowing decisions to be made in the face of greater uncertainties.

Selected RAGES findings	Selected statements from the assessment of EFSA (2020a)	Testbiotech conclusions
However, these potential hazards are not the only risks that can arise from the persistence and self -propagation of GE crops. There are potential hazards related to plant interactions and biological signalling pathways within the food web, with soil organisms, or insects such as pollinators and other organisms. These pathways and networks can be disturbed or disrupted, for example, by changes in the composition of volatile compounds or biochemical pathways and changes in nutritional quality.	Possible alteration of the plant's interactions and biological signalling pathways within the food web, with soil organisms or insects such as pollinators and other organisms is addressed in the area of risk dedicated to the assessment of potential adverse effects of GE plants to NTOs.	EFSA does not address the problem raised by RAGES. Risk assessment as described by EFSA, is only based on the intended characteristics of the original event, without considering effects in following generations that are known to be likely to occur. RAGES focusses on these next generation effects. EFSA appears to be intentionally ignoring the fact that the RAGES findings concentrate on biological characteristics that emerge unexpectedly.
The suggested criteria should not only be applied to applications for commercial cultivation, but also to imports that are likely to cause spillage of viable kernels of relevant events / species. In general, the release of genetically engineered plants should not be allowed if their persistence in the environment cannot be controlled in the spatio-temporal dimension.	EFSA notes that some of the RAGES demands pertaining to the assessment of persistence, invasiveness and vertical gene flow are disproportionate, and not in tune with the nature of the former and current GE plants for market release in the EU, their intended uses, and the expected level of environmental exposure. At present, the bulk of applications for authorisation of GE plants covers the import/processing for food/feed uses of highly domesticated plants with a low potential to survive until subsequent seasons, or to establish occasional feral plants under European environmental conditions in case of accidental release into the environment of viable grains/seeds. Owing to the nature of most of the former/current novel traits, it is unlikely that such traits will enable GE plants to overcome other biological and abiotic factors limiting their persistence and invasiveness.	EFSA neglects evidence, e.g. from Japan, showing the establishment of self-sustaining GE oilseed rape originating from spillage of imports. These populations exhibit a higher fitness than expected. One probable reason is the presence of the inserted epsps gene construct. It is known to confer higher fitness even if glyphosate is not applied. There are strong indications that these plants, although not designed for this purpose, overcome natural biological barriers and become persistent as self-sustaining populations.

Selected RAGES findings	Selected statements from the assessment of EFSA (2020a)	Testbiotech conclusions
New challenges arise in risk assessment if genetically engineered (GE) organisms can persist and propagate in the environment and produce viable offspring. This review shows that effects in following generations can be substantially influenced by interactions with heterogeneous genetic backgrounds. Furthermore, unexpected effects can be triggered in interaction with environmental conditions. This observation is especially relevant for the assessment of long-term impacts under changing environmental conditions, such as those caused by climate change. Therefore, the risk assessment of genetically engineered plants that can persist and propagate in the environment cannot be reduced to the specific traits and characteristics known when the application is filed; it also has to take into account effects that can emerge after a number of generations, in other genetic backgrounds or under stress conditions.	Consequently, through the problem formulation process, case-specific information requirements must be defined on a case-by-case basis for each GE plant for deliberate release into the environment. ERA will vary dependent on the biology of the GE plant under consideration, the introduced traits, the intended uses of the GE plant, the scale and frequency of the deliberate release, the receiving environments, and the interactions amongst these variables. Case-specific information from applicants is typically requested by the EFSA GMO Panel, as appropriate. In this respect, it should be noted that no application for authorisation of the cultivation of GE plants with enhanced potential for persistence/invasiveness and vertical gene flow has been submitted and thus considered at EU level at the time of writing.	EFSA does not address the problem raised by RAGES. EFSA reiterates that their risk assessment is only based on the intended characteristics of the original event. The assessment as described does not consider next generation effects from heterogeneous genetic backgrounds or the reaction of the GE organisms to changes in the environment. EFSA appears to intentionally ignore the fact that the RAGES findings concentrate on the biological potential which can emerge unexpectedly.

Further findings and comments by Testbiotech

As shown above, EFSA appears to be intentionally misinterpreting the RAGES findings. In regard to the protection goals, RAGES does not claim that GE organisms which escape into the environment and produce viable offspring are harmful per se. RAGES has however produced evidence that these organisms create specific challenges in risk assessment that are likely to make RA inconclusive. Therefore, EFSA should develop cut-off criteria which allow decisions to be made when faced with substantial uncertainties.

EFSA rejects the concept of cut-off criteria without sufficient scientific reasoning and by making incorrect assumptions in regard to the RAGES findings.

Indeed, EFSA statements confirm that their risk assessment is based solely on the intended characteristics of the original event; neither does it consider any effects in following generations which are likely to occur. EFSA intentionally ignores the RAGES findings on biological potentials emerging unexpectedly after further crossings with heterogeneous genomic backgrounds and under changing environmental conditions. Instead, EFSA bases its assumptions on the unrealistic expectations that GE organisms will not change their biological characteristics after crossing with wild relatives or non-GE varieties.

In the context of assessing the EFSA response, Testbiotech has become aware of three further publications confirming that next generation effects in GE plants cannot be predicted from the original events and need to be assessed carefully:

(1) In a multigenerational study with Bt maize, it was shown 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. Surprisingly, no correlations were observed between the amount of mRNA for Cry1Ab and the corresponding Cry1Ab protein concentrations (Lohn et al., 2020).

(2) In GE soybean, proteomics and metabolomics were used to evaluate different generations of transgenic (cp4-epsps gene) and non-transgenic soybean plants. In this case, differences also occurred between the offspring of GE plants and conventional plants, e.g. in storage proteins and flavonoids, which were not predictable from the characteristics of the parental plants (de Campos et al., 2020).

(3) New research shows that the risks emerging from crossings of GE maize and teosinte cannot be predicted from the data used by EFSA (2016): Le Corre et al. (2020) show that European teosinte plants, by integrating larger parts from European maize varieties, have changed their biological characteristics in ways that will facilitate further genetic exchange with maize plants. Similarly, Diaz et al. (2019) show theses new weeds seem to have a complex origin. Therefore, the likelihood of hybridisation with the GE maize has strongly increased.

As the new publication shows, gene flow to conventionally bred maize in Europe has already been established. As a consequence, there is a much a higher likelihood of teosinte acquiring MON810 transgene constructs and becoming insecticidal. For example, teosinte has an altered flowering time increasing the potential for hybridisation. Furthermore, teosinte has already acquired herbicide-resistance from conventional European maize varieties.

The authors state that the risk of teosinte emerging as a problematic weed in a temperate climate was remote. Nevertheless, 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 the gene flow to both partners, maize and teosinte. The findings underline the high capacity of the European teosinte to acquire Bt gene constructs and, potentially, further herbicide resistance genes, such as those present in Bt11 and Maize 1507, for which applications for cultivation have been filed in the EU.

Unlike maize, teosinte can overwinter in the fields and pass the 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.

7. Other RAGES findings

Other findings were presented in the overall report on cross-cutting issues (RAGES, 2020a), accompanied by a tabled overview. These were, however, not mentioned in the EFSA response.

RAGES emphasises that GE organisms always have to be seen in the context of their environment. The project introduces the concept of the 'holobiont' to show that the biological characteristics of organisms, such as plants, insects or mammals, cannot be considered separately from their associated microbiomes.

The microbiome can be seen as the common the network of life, circumventing and closely interacting with plants, animals and humans. These networks are thought to co-evolve with their hosts and develop a mutualistic relationship that benefits both the host and microorganisms. It acts at the interphase between the organisms and their environment and is considered to be key for human, animal and plant health.

Therefore, risk assessment of GE plants not only has to consider their interactions with their macroscopic, wider environment (such as pollinators and the food web), but also interactions with their microscopic, closer environment, such as soil organisms. Furthermore, changes in the composition of the microbiome of humans and animals can be caused by GE plants at the stage of consumption. However, these issues are still mostly hidden away when it comes to the risk assessment of GE plants.

In this context, it has to be acknowledged that plants communicate and interact with their environments via multiple bio-chemical pathways. Various compounds are involved, such as volatile substances, secondary metabolites and biologically active compounds, including small non-coding RNA (sncRNA).

The role of the microbiome in risk assessment

Just recently, a document published by EFSA (EFSA, 2020d), called attention for the first time to the role of the microbiome in environmental risk assessment and food and feed safety.

As EFSA states, the soil microbial community represents the greatest reservoir of biological diversity in the world. The collective genome of the rhizosphere microbiome is referred to as 'the plant second genome' which has a crucial function for the plant, ranging from the recruitment of essential nutrients to boosting its defensive capacity against pathogens. It is mentioned that plants may also secrete biologically active molecules interfering with gene expression in the soil community. The balance within soil microorganisms is considered to be directly related to plant health and soil fertility.

Therefore, the soil microbiome is presented as vital for the conservation of soil health, particularly in changing environmental and/or management conditions. Therefore, the preservation of its integrity is important in environmental risk assessment. However, according to EFSA, clarification is still needed on how current environmental risk assessments and possible indirect effects of plant and soil microbiomes on soil fertility and plant health could be captured.

According to EFSA (2020d), there are still no standardised approaches to characterise healthy soil from a microbiome perspective. Furthermore, the fungal, viral and archaeal diversity of the plant microbiome still cannot be evaluated in its entirety.

In regard to food and feed safety, EFSA (2020d) 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 this context, it also has to be considered that in the case of glyphosate resistant plants, there is a specific situation in regard to chronic exposure via the route of food consumption, since glyphosate is known to show antibiotic activity. Glyphosate has indeed been shown to have negative effects on the composition of the intestinal flora of cattle (Reuter et al., 2007), poultry (Shehata et al., 2013; Ruuskanen et al, 2020) and rodents (Mao et al., 2018; Mesnage et al., 2020 (preprint); 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 is not unlikely to trigger significant changes in intestinal bacteria, but these effects are escaping risk assessment completely.

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

These findings are highly relevant for the risk assessment of all GE plants inheriting additional epsps genes that confer enhanced resistance to glyphosate. As explained, long term exposure to glyphosate residues due to these plants may lead to disruption in the gut microbiome. Furthermore, stacking of the GE plants very often results in a combination of EPSPS enzymes and Bt toxins that is known to trigger possible immune reactions. It has to be considered a plausible hypothesis that a combination of Bt toxins and residues from spraying, can trigger effects on the immune system or other adverse health effects either directly or via the microbiome. This hypothesis needs to be tested, including taking into account synergistic effects due to protease inhibitors which may increase exposure to Bt toxins in the gut, before any conclusion can be drawn on the safety of food and feed derived thereof.

However, no attempts have been made to integrate combinatorial effects and the microbiome into the current risk assessment of food and feed derived from GE plants. This is in contradiction to Regulation 1829/2003 which requests "*genetically modified food and feed should only be authorized 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).

As stated by RAGES (2020a), the issue of the microbiome is especially relevant for risk assessment of traits in food and feed, such as herbicide resistance, insecticidal toxicity or changes in nutritional composition. However, EFSA did not address this problem in its response.

Unintended changes in the genome, the transcriptome, the proteome or the metabolome of GE plants are also highly relevant in this context (see for example Rang et al., 2005; Barbosa et al., 2012; Agapito-Tenfen et al., 2013; Agapito-Tenfen et al., 2014; Benevenuto et al., 2017; Mesnage et al., 2016; Ben Ali et al., 2020; Zanatta et al., 2020).

However, these findings have as yet not been taken into account in EFSA risk assessment. Nor do they use more sensitive methods such transcriptomics, proteomics and metabolomics to explore and assesses unintended changes in the GE plants.

The role of non-coding RNAs (ncRNAs) in risk assessment

It is known that new open reading frames can occur due to the deletion or insertion of genes that can give rise to unanticipated new gene products (such as ncRNAs), which may be biologically active through unintended and unanticipated RNAi (RNA interference) processes.

Very generally, RNAi processes are based on the silencing of gene functions. These effects can be enacted by uptake of ncRNAs across species' borders, the plant and animal kingdoms and the domains of life (bacteria, archaea and eukaryotes). For example, plants naturally use ncRNA to interact with their microbiome, such as the soil organisms. Based on these signalling molecules, there is a two-way communication between the associated microbial fauna and the plants that is directly related to plant health and soil fertility.

For example, plants can induce gene silencing in some eukaryotic pathogens, pests, parasites or symbiotic microorganisms as a defence strategy. However, pathogens also developed similar mechanisms, proving the existence of a two-way ncRNAs traffic between pathogens and their plant hosts. Similar mechanisms and pathways are also known to occur in plant-insect interaction. Therefore, genetically engineered plants were developed to produce artificial ncRNA which, after uptake by pest insects such as the corn rootworm, will downregulate gene activity in the insect and thus kill it.

At the stage of consumption, the biologically active molecules produced in plants may also actively interfere with gene regulation in humans (animals) or their intestinal microbiome.

In this context, a report commissioned and published by EFSA in 2019 (Davalos et al., 2019) considers the role of ncRNA in the risk assessment of GE plants. Davalos et al. summarise current findings on ncRNAs 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.

As Davalos et al. show, there are many matches between the ncRNA 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 cells of humans and animals, RNAi effects, such as gene silencing, can occur and, for example, genes in animals can be downregulated by plant nscRNA.

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, as also summarised by Davalos et al. (2019), there are mechanisms that can allow the molecules to pass through these barriers: plant ncRNA is protected against degradation by methylation, it can be excreted and taken up in vesicles (such as exosomes) and there are nano-particles produced by plants which can serve as transporting elements.

As research summarised by Davalos et al. (2019) shows, the uptake of ncRNA from plants and microorganisms via the gut into the cells of humans and animals is an established of fact. The ncRNA molecules stemming from plants are reported to be found in many bodily fluids of humans and animals, including blood and milk. Therapeutic effects from the uptake of ncRNA from the gut has been evidenced in several publications. Some of the research shows that biological effects can be achieved with very low dosages.

It appears that some findings depend on the specific type of ncRNA. For example, naked synthetic ncRNA used by some researchers, is degraded very quickly compared to ncRNA produced by plants. Davalos et al. (2019) see the need for further research to explore the uptake and biological effects of ncRNA: *"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."

In addition, Davalos et al. (2019) also show that plant-derived ncRNA 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.

There is well-established evidence that ncRNAs stemming from the host (e.g. produced by the intestinal epithelial cells) are taken up by the gut microbiota and can manipulate its gene regulation. The same evidence is available for ncRNA 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.

Therefore, the interaction between the ncRNAs 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 ncRNA to pass from plants to gut microorganisms seems to be much lower compared to those identified in the human or animal body.

These findings are relevant for the risk assessment of transgenic plants intended to produce additional ncRNA that are toxic to insects. However, EFSA's food and feed risk assessment of maize MON87411, which produces an insecticidal ncRNA (EFSA, 2018b), did not consider effects on the microbiome and no empirical data were made available on the uptake of the molecules from the gut (Testbiotech, 2018b).

The findings as summarised are relevant for GE plants in general since the process of genetic engineering causes new open reading frames to emerge that may not only give rise to intended proteins, but also to ncRNA. Depending on the type and dosage of the unintentionally produced ncRNA molecules, they may enact RNAi in soil organisms or pollinators as well as in microorganisms in the gut of humans and animals after consumption. From this point of view, the bioinformatic analyses currently carried out for the approval of GE plants are not adequate or sufficient and should be extended.

Further research is needed to precisely identify the potential magnitude of changes and to determine their consequences for health and the environment. Whatever the case, risk assessment of GE plants can no longer ignore the issue of unintended RNAi effects on the level of the microbiome in respect to plants, animals and humans.

8. Further discussion and conclusions

Current cultivation of GE plants for food production means that a huge number of organisms enter agro-ecosystems and food chains without having gone through evolutionary adaptation. There is no doubt that long-term exposure to these plants leads to unintended changes and reactions in the receiving environment.

For example, the extensive cultivation of glyphosate-resistant GE plants has resulted in severe adverse effects for agro-ecosystems and has caused weedy species to exert epigenetic reactions: species such as *Amaranthus palmeri* show gene duplication of their native epsps gene sequences (Gaines et al., 2019). Since the epsps gene not only confers resistance to glyphosate but also higher fitness in plants (Fang et al., 2018), the herbicide-resistant weedy plants may spread much faster and show higher vigour than expected, making it extremely difficult for the farmers to keep it out of the fields. Just recently, glyphosate-resistant *Amaranthus palmeri* that has evolved in fields in the US, was found growing in areas around ports in Japan (Shimono et al., 2020).

Furthermore, after transgenic glyphosate-resistant oilseed rape managed to escape into the environment, it became self-sustaining in populations in Canada, Japan, Australia and the US (Bauer-Panskus et al., 2013). EFSA risk assessment EFSA (2014) expected those populations to show higher fitness only in cases where glyphosate was applied and therefore considered the risk of self-sustaining populations of the GE plants in absence of the herbicide to be minor. It has to be assumed that, due to the dual effect of the additionally inserted epsps genes, the GE oilseed rape became self-sustaining and persistent even in the absence of glyphosate. These effects were neither expected nor predicted or assessed before the glyphosate-resistant GE plants were massively introduced into the environment. EFSA still does not take these risks into account in current applications for approval of such plants.

Unintended effects and changes caused by cultivation or consumption of GE plants might not always be a matter of concern. However, it is worrying that current risk assessment is insufficient to identify the magnitude of changes or determine their consequences for health and the environment.

It is even more worrying that these gaps in risk assessment do not seem to be simply accidental or arbitrary. RAGES shows that risk assessment as currently performed by EFSA, has actively or passively established systemic 'darkness' or ambiguity as well as areas of uncertainty that are crucial for the risk assessment of GE plants.

For example, it has to be assumed that the Bt proteins produced in plants, such as maize, cotton and soybean, are much more toxic than isolated Bt toxins. The reason for this are protease inhibitors (PI) present in the plant tissue. PIs substantially delay the degradation of Bt toxins and enhance their toxicity, e.g. up to 20-fold. Monsanto pointed out these effects 30 years ago (MacIntosh et al., 1990). Since then these findings have been confirmed in several scientific publications, but have never been taken into account in EFSA risk assessment, even though they are relevant for all Bt plants approved for import or cultivation in the EU.

In addition, new uncertainties and unknowns have emerged from recent findings on microbiomes and ncRNA. In this context, RAGES introduces the concept of the holobiont, which puts GE organisms into their factual biological context, including their microbiome. RAGES also raises questions about changes in GE organisms which affect biologically active molecules, such as non-coding small RNAs (ncRNA), and their interactions with the near and wider environment.

In conclusion, there is evidence that the genetic engineering of food plants interferes with layers of complexity which go far beyond what can be assessed by current standards of risk assessment. Safety is claimed on basis of approval processes which focuses solely on those risks that can most easily be assessed. One could say that crucial risks of genetically engineered plants were intentionally placed on 'the dark side of the moon'. One could also say that there is evidence for manifest, intended and systemic ignorance of EFSA in regard to many crucial aspects of risk assessment.

However, it is not only the failure of EFSA which has to be put under the spotlight, but also the EU Commission. In several cases, there is a lack of sufficiently detailed risk assessment regulation and standards. Therefore, the EU Commission which is responsible for risk assessment policies has to take action. It is also the Commission which is mostly responsible for developing a meaningful and sufficiently reliable system for post-market monitoring (PMM). However, PMM is still just a nice label without substantial scientific content and does not provide sufficiently reliable data.

References

Agapito-Tenfen, S.Z., Guerra, M. P., Wikmark, O.G., Nodari, R.O. (2013) Comparative proteomic analysis of genetically modified maize grown under different agroecosystems conditions in Brazil. Proteome Science 11(1): 46. https://doi.org/10.1186/1477-5956-11-46

Agapito-Tenfen, S.Z., Vilperte, V., Benevenuto, R.F., Rover, C.M., Traavik, T.I., Nodari, R.O. (2014) Effect of stacking insecticidal cry and herbicide tolerance epsps transgenes on transgenic maize proteome. BMC Plant Biology, 14: 1-19. https://doi.org/10.1186/s12870-014-0346-8.

Babendreier, D., Kalberer, N.M., Romeis, J., Fluri, P., Mulligan, E., Bigler, F. (2005) Influence of Bt-transgenic pollen, Bt-toxin and protease inhibitor (SBTI) ingestion on development of the hypopharyngeal glands in honeybees. Apidologie, 36: 585-594. https://doi.org/10.1051/apido:2005049

Barbosa, H.S., Arruda, S.C., Azevedo, R.A., Arruda, M.A. (2012) New insights on proteomics of transgenic soybean seeds: evaluation of differential expressions of enzymes and proteins. Anal Bioanal Chem, 402: 299.314. https://doi.org/10.1007/s00216-011-5409-1

Baudrot, V., Walker E., Lang A., Stefanescu, C., Rey, J.-F., Soubeyr, S., Messéan A. (2020) When the average hides the risk of Bt-corn pollen on non-target Lepidoptera: Application to Aglais io in Catalonia. Ecotoxicol Environ Saf, 207: 111215. https://doi.org/10.1016/j.ecoenv.2020.111215

Bauer-Panskus, A., Breckling, B., Hamberger, S., Then, C., (2013) Cultivation-independent establishment of genetically engineered plants in natural populations: current evidence and implications for EU regulation. Environ Sci Eur, 25: 34. https://doi.org/10.1186/2190-4715-25-34

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

Ben Ali, S.-E., Draxler, A., Poelzl, D., Agapito-Tenfen, S., Hochegger, R., Haslberger, A.-G., Brandes, C. (2020) Analysis of transcriptomic differences between NK603 maize and near-isogenic varieties using RNA sequencing and RT-qPCR. Environ Sci Eur, 32(1): 1-23. https://doi.org/10.1186/s12302-020-00412-8

Benevenuto R.F., Agapito-Tenfen S.Z., Vilperte V., Wikmark O.-G., van Rensburg P.J., Nodari R.O. (2017) Molecular responses of genetically modified maize to abiotic stresses as determined through proteomic and metabolomic analyses. PLoS ONE 12(2): e0173069. https://doi.org/10.1371/journal.pone.0173069

Chowdhury, E.H., Kuribara, H., Hino, A., Sultana, P., Mikami, O., Shimada, N., Guruge, K.S., Saito, S., Nakajima, Y. (2003) Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. J Anim Sci, 81(10): 2546-2551. https://doi.org/10.2527/2003.81102546x

de Campos, B.K., Galazzi, R.M., dos Santos, B.M., Balbuena, T.S., dos Santos, F.N., Mokochinski, J.B., Eberlin, M.N., Arruda, M.A.Z. (2020) Comparison of generational effect on proteins and metabolites in non-transgenic and transgenic soybean seeds through the insertion of the cp4-EPSPS gene assessed by omics-based platforms, Ecotoxicol Environ Saf, 202: 110918. https://doi.org/10.1016/j.ecoenv.2020.110918

Díaz, A., Taberner, A., Vilaplana, L. (2020) The emergence of a new weed in maize plantations: characterization and genetic structure using microsatellite markers, Genet Resour Crop Evol, 67: 225-239. https://doi.org/10.1007/s10722-019-00828-z

le Corre, V., Siol, M., Vigouroux, Y., Tenaillon, M.I., Délye, C. (2020) Adaptive introgression from maize has facilitated the establishment of teosinte as a noxious weed in Europe. PNAS USA, 117(41): 25618-25627. https://doi.org/10.1073/pnas.2006633117

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

Devos, Y., Ortiz-García, S., Hokanson, KE., Raybould, A. (2018) Teosinte and maize × teosinte hybrid plants in Europe - environmental risk assessment and management implications for genetically modified maize. Agric Ecosyst Environ, 259(1): 19-27. https://doi.org/10.1016/j.agee.2018.02.032

EFSA (2014) Scientific Opinion on application (EFSA-GMO-BE-2011-101) for the placing on the market of herbicide-tolerant genetically modified oilseed rape MON 88302 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Monsanto.EFSA J, 12(6): 3701. https://doi.org/10.2903/j.efsa.2014.3701

EFSA (2015) Guidance on the agronomic and phenotypic characterisation of genetically modified plants. EFSA J, 13(6): 4128. https://doi.org/10.2903/j.efsa.2015.4128

EFSA (2016) Relevance of new scientific evidence on the occurrence of teosinte in maize fields in Spain and France for previous environmental risk assessment conclusions and risk management recommendations on the cultivation of maize events MON810, Bt11, 1507 and GA21. EFSA Supporting Publications 13(9): 1094E. https://doi.org/10.2903/sp.efsa.2016.en-1094

EFSA (2018a) Review of the existing maximum residue levels for glyphosate according to Article 12 of Regulation (EC) No 396/2005. EFSA J, 16(5): e05263. https://doi.org/10.2903/j.efsa.2018.5263

EFSA (2018b) GMO Panel (EFSA Panel on Genetically Modified Organisms), Scientific Opinion on the assessment of genetically modified maize MON 87411 for food and feed uses, import and processing, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2015-124). EFSA Journal 2018;16(6):5310, 29 pp. https://doi.org/10.2903/j.efsa.2018.5310

EFSA (2020a) Assessment of the outcomes of the project "Risk Assessment of Genetically Engineered Organisms in the EU and Switzerland" (RAGES). EFSA Supporting Publication 17(7): EN-1890. https://doi.org/10.2903/sp.efsa.2020.EN-1890

EFSA (2020b) Application EFSA-GMO-DE-2017-142, Comments and opinions submitted by Member States during the three-month consultation period. Register of Questions, http://registerofquestions.efsa.europa.eu/roqFrontend/questionsListLoader?unit=GMO

EFSA (2020c) Assessment of the 2018 post-market environmental monitoring report on the cultivation of genetically modified maize MON 810 in the EU. EFSA J, 18(10): 6245. https://doi.org/10.2903/j.efsa.2020.6245

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

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

Gaines, T.A., Patterson, E.L., Neve, P. (2019) Molecular mechanisms of adaptive evolution revealed by global selection for glyphosate resistance. New Phytol, 223(4): 1770-1775. https://doi.org/10.1111/nph.15858

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

Han, P., Niu, C.-Y., Lei, C.-L., Cui, J.-J., Desneux, N. (2010) Quantification of toxins in a Cry1Ac + CpTI cotton cultivar and its potential effects on the honey bee *Apis mellifera* L.. Ecotoxicology, 19: 1452-1459. https://doi.org/10.1007/s10646-010-0530-z

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

Kawall, K., Cotter, J., Then, C. (2020) Broadening the EU GMO risk assessment in the EU for genome editing technologies in Agriculture. Environ Sci Eur, 32(1): 1-24. https://doi.org/10.1186/s12302-020-00361-2

Liu, X., Zhang, Q., Zhao, J.-Z., Li, J., Xu, B., Ma, X. (2005a) Effects of Bt transgenic cotton lines on the cotton bollworm parasitoid *Microplitis mediator* in the laboratory. Biol Control, 35: 134-141. https://doi.org/10.1016/j.biocontrol.2005.08.006

Liu, X.D., Zhai, B.P., Zhang, X.X., Zong, J.M. (2005b) Impact of transgenic cotton plants on a non-target pest, *Aphis gossypii* Glover. Ecol Entomol, 30: 307-315. https://doi.org/10.1111/j.0307-6946.2005.00690.x

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

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

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

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

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

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

Mesnage, R., Teixeira, M., Mandrioli, D., Falcioni, L., Ducarmon, Q.R., Zwittink, R.D. Amiel, C., Panoff, J.-P., Belpoggi, F., Antoniou, M.N. (2019) Shotgun metagenomics and metabolomics reveal glyphosate alters the gut microbiome of Sprague-Dawley rats by inhibiting the shikimate pathway. BioRxiv, 870105. https://doi.org/10.1101/870105

Miyazaki, J., Bauer-Panskus, A., Bøhn, T., Reichenbecher, W., Then, C. (2019) Insufficient risk assessment of herbicide-tolerant genetically engineered soybeans intended for import into the EU. Environ Sci Eur, 31(1): 92. https://doi.org/10.1186/s12302-019-0274-1

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

Paine, J., Shipton, C., Chaggar, S., Howells, R.M., Kennedy, M.J., Vernon, G., Wright, S.Y., Hinchliffe, E., Adams, J.L., Silverstone, A.L., Drake, R. (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nature Biotechnol, 23(4): 482-487. https://doi.org/10.1038/nbt1082

Parenti, M.D., Santoro, A., Del Rio, A., Franceschi, C. (2019) Literature review in support of adjuvanticity/immunogenicity assessment of proteins. EFSA Supporting Publications, 16(1): 1551E. https://doi.org/10.2903/sp.efsa.2019.EN-1551

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

Pardo, G., Cirujeda, A., Betrán, E., Fernández-Cavada, S., Fuertes, S., Rodríguez, E., Perdiguer, A., Aibar, J., Zaragoza, C. (2014) El Teosinte (*Zea mays*, spp.). https://citarea.cita-aragon.es/citarea/bitstream/10532/2734/1/2014_313.pdf

Perry, J.N., Devos, Y., Arpaia, S., Bartsch, D., Gathmann, A., Hails, R.S., Kiss, J., Lheureux, K., Manachini, B., Mestdagh, S., Neemann, G. (2010) A mathematical model of exposure of non-target Lepidoptera to Bt-maize pollen expressing Cry1Ab within Europe. Proc Royal Soc B, 277(1686): 1417-1425. https://doi.org/10.1098/rspb.2009.2091

Perry, J.N., Devos, Y., Arpaia, S., Bartsch, D., Gathmann, A., Hails, R.S., Kiss, J., Lheureux, K., Manachini, B., Mestdagh, S., Neemann, G., Ortego, F., Schiemann, J., Sweet, J.B. (2011) The usefulness of a mathematical model of exposure for environmental risk assessment. Proc Royal Soc B, 278(1708): 982-984. https://doi.org/10.1098/rspb.2010.2667

Perry, J.N., Devos, Y., Arpaia, S., Bartsch, D., Ehlert, C., Gathmann, A., Hails, R.S., Hendriksen, N.B., Kiss, J., Messéan, A., Mestdagh, S., Neemann, G., Nuti, M., Sweet, J.B., Tebbe, C.C. (2012) Estimating the effects of Cry1F Bt-maize pollen on non-target Lepidoptera using a mathematical model of exposure. J Appl Ecol, 49(1): 29-37. https://doi.org/10.1111/j.1365-2664.2011.02083.x

Perry, J.-N., Arpaia, S., Nuti, M., Sweet, J.B., Tebbe, C.C. (2013) No evidence requiring change in the risk assessment of *Inachis io* larvae. Ecol Model, 268: 103-122. https://doi.org/10.1016/j.ecolmodel.2013.08.004

RAGES (2020a) Summary report of the results from the RAGES project 2016-2019. www.testbiotech.org/en/content/summary-report-results-rages-project-2016-2019

RAGES (2020b) Subreport: Serious shortcomings in the European risk assessment of herbicide tolerant GE plants for human health. www.testbiotech.org/en/content/rages-subreport-risk-assessment-herbicide-tolerant-ge-plants

RAGES (2020c) Subreport: Insecticidal Bt crops. EFSA's risk assessment approach for GM Bt plants fails by design. www.testbiotech.org/en/content/rages-subreport-insecticidal-bt-crops

RAGES (2020d) Subreport: Health risk assessment of genetically engineered nutritionally-altered GM crops. www.testbiotech.org/en/content/rages-subreport-risk-assessment-nutritionally-altered-gm-crops

RAGES (2020e) Subreport: Assessment of health risks associated with the consumption of products derived from genetically engineered plants with a combination of traits. www.testbiotech.org/en/content/rages-subreport-assessment-combinatorial-effects

RAGES (2020f) Subreport: Environmental risk assessment of genetically engineered crops that can persist and spontaneously propagate in the environment. www.testbiotech.org/en/content/rages-subreport-risk-assessment-next-generation-effects

RAGES (2020g) Subreport: New genetic engineering technologies. www.testbiotech.org/en/content/rages-subreport-new-genetic-engineering-technologies

Rang, A., Linke, B., Jansen, B. (2005) Detection of RNA variants transcribed from the transgene in Roundup Ready soybean. Eur Food Res Technol, 220: 438-443. https://doi.org/10.1007/s00217-004-1064-5

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

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

Santos-Vigil, K.I., Ilhuicatzi-Alvarado, D., García-Hernández, A.L., Herrera-García, J.S., Moreno-Fierros, L. (2018) Study of the allergenic potential of *Bacillus thuringiensis* Cry1Ac toxin following intra-gastric administration in a murine model of food-allergy. Int Immunopharmacol, 61: 185-196. https://doi.org/10.1016/j.intimp.2018.05.029

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

Shimono, A., Kanbe, H., Nakamura, S., Ueno, S., Yamashita, J., Asai, M. (2020) Initial invasion of glyphosate resistant *Amaranthus palmeri* around grain import ports in Japan. Plants, People, Planet, 2(6): 640-648. https://doi.org/10.1002/ppp3.10156

Suppa, A., Kvist, J., Li, X., Dhandapani, V., Almulla, H., Tian, A.Y., Kissane, S., Zhou, J., Perotti, A., Mangelson, H., Langford, K., Rossi, V., BrownJ.B., Orsini, L. (2020) Roundup causes embryonic development failure and alters metabolic pathways and gut microbiota functionality in non-target species. Microbiome, 8(1): 1-15. https://doi.org/10.1186/s40168-020-00943-5

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

Testbiotech (2016) Cultivation of genetically engineeredmaize: Risks not under control Overview: Why the EU should not allow the cultivation of transgenic maize engineered to produce insecticidal toxins, Testbiotech Background 24 - 11 – 2016, www.testbiotech.org/node/1759

Testbiotech (2018a) Testbiotech on risk assessment of Golden Rice by FSANZ (February 2018). https://www.testbiotech.org/node/2149Testbiotech (2018a) Testbiotech on risk assessment of Golden Rice by FSANZ (February 2018). https://www.testbiotech.org/node/2149

Testbiotech (2018b) Testbiotech comment on Assessment of genetically modified maize MON 87411 for food and feed uses, import and processing, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2015-124) of company Monsanto. www.testbiotech.org/node/2257

Testbiotech (2020a) Why 'New GE' needs to be regulated; Frequently Asked Questions on 'New Genetic Engineering' and technical backgrounds for CRISPR & Co. www.testbiotech.org/node/2659

Testbiotech (2020b) Testbiotech comment on EFSA's assessment of genetically engineered maize MZIR098 for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-DE-2017-142) by Syngenta. www.testbiotech.org/node/2625

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

Trtikova, M., Lohn, A., Binimelis, R., Chapela, I., Oehen, B., Zemp, N., Widmer, A., Hilbeck, A. (2017) Teosinte in Europe – searching for the origin of a novel weed. Sci Rep, 7(1): 1560. https://doi.org/10.1038/s41598-017-01478-w

Walsh, M.C., Buzoianu, S.G., Gardiner, G.E., Rea, M.C., Gelencsér, E., Jánosi, A., Jánosi, A., Epstein, M.M., Lawlor, P.G. (2011) Fate of transgenic DNA from orally administered Bt MON810 maize and effects on immune response and growth in pigs. PLoS One, 6(11): e27177. https://doi.org/10.1371/journal.pone.0027177

Zanatta, C.B., Benevenuto, R.F., Nodari, R.O., Agapito-Tenfen, S.Z. (2020) Stacked genetically modified soybean harboring herbicide resistance and insecticide rCry1Ac shows strong defense and redox homeostasis disturbance after glyphosate-based herbicide application. Environ Sci Eur, 32: 104. https://doi.org/10.1186/s12302-020-00379-6

Zdziarski, I.M., Carman, J.A., Edwards, J.W. (2018) Histopathological Investigation of the Stomach of Rats Fed a 60% Genetically Modified Corn Diet. Food Sci Nutr, 9: 763-796. https://doi.org/10.4236/fns.2018.96058

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

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

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