RAGES

RISK ASSESSMENT OF GENETICALLY ENGINEERED ORGANISMS IN THE EU AND SWITZERLAND

Assessment of health risks associated with the consumption of products derived from genetically engineered plants with a combination of traits

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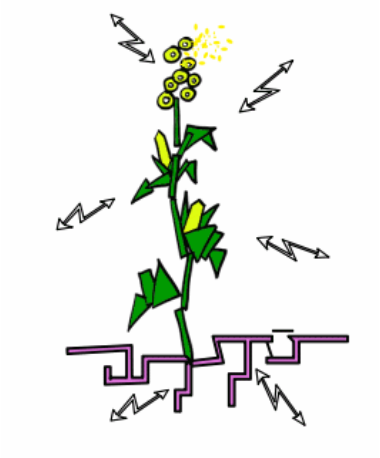

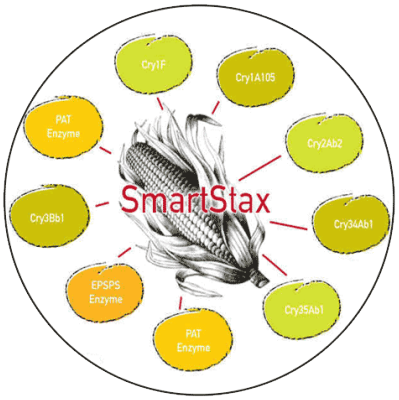

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Content

Summary

Most GE plants (events) allowed for import, processing and usage for food and feed into the EU, show a combination of several traits. These combinations can be derived from the stacking of plants (crossing of parental GE plants) as well as by co-transformation of single events. Most GE plants with stacked traits combine herbicide tolerance (HT) (also called herbicide resistance) and production of insecticidal toxins (IT) (also called insect resistance). The GE plants on the market with trait combinations, especially those produced through stacking, are increasing and this trend is expected to continue in the future.

These combinations should always be addressed by the risk assessors. The harvests of HT plants regularly contain residues from spraying with the complementary herbicides such as glyphosate and others. In addition, one or several Bt toxins can be present. 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 as stressors with potential additive, antagonistic or synergistic effects and interactions.

EU legal provisions such as Regulation 1829/2003 (Recital 9) state that *"…any risks which they present for human and animal health and, as the case may be, for the environment…"* have to be avoided. Therefore, potential adverse effects that result from combinatorial exposures of various stressors need specification and their assessment needs priority. However, so far, the EU does not have a systematic and coherent approach to how health effects or environmental impacts stemming from such combinations of stressors should be assessed. In this report, the current gaps in risk assessments as performed by European Food Safety Authority (EFSA) are exemplified by two case studies.

We have analysed the concepts and methodologies for combined, cumulative and aggregated exposure to mixtures of stressors in GE plants with trait combinations. In addition, we discuss how other biologically active substances present in plants (e.g. oestrogens, allergens and anti-nutritional compounds in soybean) may interact with the trait-related characteristics and resulting stressors.

We have concluded that the health risk assessments as currently performed by EFSA for stacked GE plants are unacceptable. EFSA's approach does not take account of adverse health effects arising from GE plants, which could simultaneously introduce multiple potential stressors into our food chains. 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*" (EFSA 2019). For regulatory purposes, the plants should be considered as being 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).

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.

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.

There are ethical concerns that need to be taken into account by the risk manager: a cost-benefit assessment in addition to the risk assessment might become necessary. However, it is important to clearly separate the risk assessment from the cost-benefit analysis. Safety always has to be shown in accordance with scientific standards. Thus, if the cost-benefit assessment does result in a negative outcome, the approval process might be stopped, to avoid unnecessary animal feeding studies. But if the approval process goes ahead, risk assessment cannot be replaced by lower scientific standards.

As a next step, EFSA risk assessments and monitoring of mixtures of GE plants in diets that will lead to co-exposures of multiple potential stressors will need to fully assess the risks of combinatorial, aggregated and/or cumulative effects.

1. Definitions and the scope of this report

The largest category of genetically engineered plants (events) being allowed for import, processing and usage for food and feed into the EU, shows a combination of several traits. Most combinations concern herbicide tolerance (HT) and production of insecticidal toxins (IT). It is expected that the overall number of these plants with a trait combination will rise in future.

The harvest of these plants can be expected to regularly contain residues from spraying with the complementary herbicides such as glyphosate and others. In addition, one or several Bt toxins can be present. These residues and insecticidal toxins, also in combination with specific plant constituents (such as those showing hormonal or immunogenic properties) can be considered as potential stressors with potential additive, antagonistic or synergistic interaction.

Several terms are used to address the impacts and potential adverse effects of exposing organisms to multiple stressors. Since the usage of the specific terms is not always self-explanatory, it is helpful to provide some definitions.

For the purpose of this report, we think the definitions as provided by EFSA 2019 are helpful, although EFSA (2019) does not mention risk assessment of genetically engineered organisms (or GMOs). Some of the terms and definitions which we consider of specific relevance in this context are summarized in Table 1

Table 1: Some terms and definition as provided by EFSA (2019)

Term	Explanation
Component-based approach	An approach in which the risk of combined exposure to multiple chemicals is assessed based on exposure and effect data of the individual components
Concentration addition	A component-based model in which the components are treated as if having a similar action. The components may vary in toxic potency. Components contribute to the combined effect relative to the ratio between their concentration and toxic potency. Concentration is often the exposure metric used as a proxy for dose in in vitro studies and also commonly in ecological risk assessment.
Interaction	In risk assessment practice, the term interaction is used to refer to combined effects that differ from an explicit null model, i.e. dose and/or response addition. Interactions are categorised as less than additive (antagonism, inhibition, masking) or greater than additive (synergism, potentiation).
Mixture	Any combination of two or more chemicals that may contribute to effects regardless of source and spatial or temporal proximity.
Mixture of concern	A group of chemicals or whole mixture that is the subject of a risk assessment because there are indications that the chemicals in the group or whole mixture may contribute to the risk.
Mode of action (MoA)	Biologically plausible sequence of key events in an organism leading to an observed effect, commonly supported by robust experimental observations and mechanistic data. It refers to the major steps leading to an adverse health effect following interaction of the chemical with biological targets. It does not imply full understanding of mechanism of action at the molecular level.
Whole mixture approach	A risk assessment approach in which the mixture is treated as a single entity, similar to single chemicals, and so requires dose–response information for the mixture of concern or a (sufficiently) similar mixture.

EFSA (2019) gives the following further explanations which we consider especially useful in this context (emphasis added):

“The whole mixture approach is particularly relevant for mixtures whose composition is only partially known or difficult to characterise. The mixture is treated as a single entity, similar to single chemicals, and so requires dose-response information for the mixture of concern. In some instances, dose-response data might not be available for the mixture of concern itself, but may be obtained by read-across from similar mixtures (sometimes referred to as sufficiently similar mixtures). (….)” (page 14)

“If the components of the mixture and their exposure levels are chemically defined, the component-based approach can be applied using exposure and hazard data of the individual components. (…)” (page 14)

There are additional terms and definitions used in EFSA (2019) such as reference point, reference value and response addition which are derived from risk assessment of chemicals and would require some transition and consideration to be applied in the context of genetically engineered organisms, and not deem to be absolutely necessary for the purpose of this report.

In the following we try to develop a rationale regarding how combinatorial, aggregated and cumulative effects associated with genetically engineered plants can be assessed. To reduce the complexity of the underlying problem, we mostly deal with the question of how combinatorial health effects should be assessed that are triggered by single genetically engineered plants (events) that exhibit more than one trait. This approach is especially relevant for risk assessment of genetically engineered plants (so-called events) with a fixed combination of traits such as herbicide tolerance or production of insecticidal toxins. Therefore, we are mostly dealing with what is called by EFSA (2019) “intentional mixtures” (described by EFSA as “manufactured formulated products that are marketed as such, for example a formulated plant protection product or a flavouring agent used in food or feed.” page 11)

Such plants with a combination of traits can be derived from co-transformation of a single event and/ or by further crossing (stacking) of several genetically engineered parental plants. Most of the plants being currently authorised for import in the EU inherit a combination of traits (see table 2). Each of these events has to undergo risk assessment by European Food Safety Authority (EFSA).

Therefore, this investigation mostly deals with combinatorial effects (or potential mixed toxicity) emerging from simultaneous exposure to a fixed combination of potential stressors emerging from genetically engineered plants at the stage of consumption. The relevant effects might be caused by similar or dissimilar modes of action affecting the same or different toxic endpoints (or organs). Besides toxicity in regard to specific organs, we also try to consider immune reactions and hormonal activities (endocrine disruption) which are not considered by EFSA (2019) but are relevant for the overall health safety assessment of whole food & feed as requested by GMO Regulation (1829/2003).

While the approach as elaborated here is especially useful for risk assessment of single genetically engineered plants, it should be taken into account that, in real-life situations, it is the overall combination of all stressors we are exposed to which may harm us. The same is true for the environment. Thus, realistic assessment of real-life exposure would need additional concepts and methods. This is not discussed within this publication.

2. Some legal background

Since we are focusing on combinatorial health effects in this report, caused by genetically engineered plants (with more than one trait) at the stage of consumption, the most relevant legal background is EU food law and EU GMO regulation. In the following we give a condensed overview of the key elements.

2.1 Regulation 178/2002

Regulation 178/2002 lays down the general principles and requirements of food law, establishing the European Food Safety Authority (EFSA) and defining procedures in matters of food safety upon which European measures, such as the GMO Regulation, should be based. These include the

"General Objective" of "a high level of protection of human life and health and the protection of consumers' interests[1] and the principle of "Risk Analysis".

According to Article 6 of the Food Safety Regulation:

> "(1) In order to achieve the general objective of a high level of protection of human health and life, food law shall be based on risk analysis except where this is not appropriate to the circumstances or the nature of the measure.
> (2) Risk assessment shall be based on the available scientific evidence and undertaken in an independent, objective and transparent manner."

Regulation 178/2002 explicitly refers to the "Precautionary Principle": According to Article 7(1) of the Food Safety Regulation:

> "In specific circumstances where, following an assessment of available information, the possibility of harmful effects on health is identified but scientific uncertainty persists, provisional risk management measures necessary to ensure the high level of health protection chosen in the [Union] may be adopted, pending further scientific information for a more comprehensive risk assessment."

For human health, Article 14 (4) of Regulation 178/2002 states:

> "In determining whether any food is injurious to health, regard shall be had. . .to the probable cumulative toxic effects".

2.2 GMO regulation

"Genetically modified organism" is defined in Article 2(2) of Directive 2001/18[2] as "an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination", where an "organism" is defined in Article 2(1) as "any biological entity capable of replication or of transferring genetic material". Food and/or feed that consists of, contains, or is produced from, genetically modified organisms must not:

- "have adverse effects on human health, animal health or the environment" (Articles 4(1)(a) and 16(1)(a) of the GM Regulation); or
- "differ from the food which it is intended to replace to such an extent that its normal consumption would be nutritionally disadvantageous for the consumer" and/or "differ from feed which it is intended to replace to such an extent that its normal consumption would be nutritionally disadvantageous for animals or humans" (Articles 4(1)(c) and 16(1)(d) of the GM Regulation) respectively;
- be placed on the market "unless it is covered by an authorisation granted in accordance with" the GM Regulation.[3]

Recitals (2), (3) and (9) of Regulation 1829/2003 make clear that: (a) "A high level of protection of human life and health should be ensured in the pursuit of [Union] policies"; (b) In order to protect human and animal health, food and feed consisting of, containing or produced from genetically modified organisms...should undergo a safety assessment through a [Union] procedure before being placed on the market within the [Union]"; and (c) "…genetically modified food and feed should only be authorised for placing on the Community market after a scientific evaluation of the highest

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1 Article 5 of the Food Safety Regulation (also reflected in Recital (3)).

2 Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC.

3 Articles 4(2) and 16(2) of the GMO Regulation.

possible standard, to be undertaken under the responsibility of [EFSA], of any risks which they present for human and animal health and, as the case may be, for the environment...”

There are some specific requirements provided on the assessment of cumulative effects within the Directive 2001/18. In Annex II of the Directive it is stated:

> “A general principle for environmental risk assessment is also that an analysis of the cumulative long-term effects relevant to the release and the placing on the market is to be carried out. Cumulative long-term effects refers to the accumulated effects of consents on human health and the environment, including inter alia flora and fauna, soil fertility, soil degradation of organic material, the feed/ food chain, biological diversity, animal health and resistance problems in relation to antibiotics.”

Furthermore, Recital 20 requests that

> “Monitoring of potential cumulative long-term effects should be considered as a compulsory part of the monitoring plan.”

There are further EU regulations for assessment of mixture toxicity that are relevant in this context which will be discussed below.

3. Overview: Genetically engineered plants with a combination of traits

As table 2 shows, most events allowed for import into the EU, show a combination of traits. These combinations can be derived from stacking of plants (crossing of parental genetically engineered plants) but also by methods like co-transformation. According to the data we have collected (until April 2018) 41 of the overall 64 events (64 %) authorised in the EU for import can be categorized in this group. Most of them (29) are stacked events. Most combinations involve herbicide tolerance (HT) and production of insecticidal toxins (IT). It can be expected that the overall number of these plants with a trait combination will rise in future, due to increasing pressure from herbicide resistant weeds (Schütte et al., 2017) and ongoing adaptation of pest insects (Gressel et al., 2017; Siegfried and Jurat-Fuentes, 2016).

We are aware of only a few empirical studies being conducted to assess the combined toxicity of complementary herbicides. However, some of them come up with relevant observations: for example, evidence for synergistic effects is published for the impact of combined application of 2,4-D and dicamba in a tropical fish species (Ruiz de Arcaute et al., 2018) and for glyphosate and dicamba formulations in introducing primary DNA breaks on circulating blood cells of South American common toad *Rhinella arenarum* tadpoles (Soloneski et al., 2016).

Looking to the EU legal provisions such as that of Regulation 1829 / 2003 that states that “*any risks which they present for human and animal health and, as the case may be, for the environment*” have to be avoided, the assessment of potential adverse effects that result from combinatorial exposure of various potential stressors is a challenge that deserves high priority.

Table 2 Overview of genetically engineered events being authorised for import in the EU, exhibiting more than one trait till December 2019 (HT: Herbicide tolerance; IT: production of insecticidal toxins[4]).

Event	Species	Trait	Applicant
1507 x 59122 x MON810 x NK603	Maize	HT, IT (two herbicides, four insecticides)	Dow AgroSciences, Pioneer/DuPont
281-24-236 × 3006-210-23 × MON88913	Cotton	HT, IT (two herbicides, two insecticides)	Dow AgroSciences
281-24-236 x 3006-210-23	Cotton	HT, IT (one herbicide, two insecticides)	Dow AgroSciences
4114	Maize	HT, IT (one herbicide, two insecticides)	Pioneer/DuPont
Bt11	Maize	HT, IT	Syngenta
Bt11 × 59122 × MIR604 × 1507 × GA21	Maize	HT, IT (two herbicides, five insecticides)	Syngenta
Bt11 x GA21	Maize	HT, IT (two herbicides, one insecticide)	Syngenta
Bt11 x MIR162 x 1507 x GA21	Maize	HT, IT (two herbicides, three insecticides)	Syngenta
Bt11 x MIR162 x MIR604 x 1507 x 5307 x GA21	Maize	HT, IT (two herbicides, five insecticides)	Syngenta
Bt11 x MIR162 x MIR604 x GA21	Maize	HT, IT (two herbicides, three insecticides)	Syngenta
DAS-81419-2	Soybean	IT (two Bt toxins)	Dow AgroSciences
DAS1507	Maize	HT, IT	Dow AgroSciences, DuPont
DAS1507 x DAS59122	Maize	HT, IT (one herbicide, three insecticides)	Dow AgroSciences, DuPont
DAS1507 x NK603	Maize	HT, IT (two herbicides, one insecticide)	Dow AgroSciences, DuPont
DAS44406-6	Soybean	HT (three herbicides)	Dow AgroSciences, DuPont
DAS59122	Maize	HT, IT	Dow AgroSciences, DuPont
DAS59122 x DAS1507 x NK603	Maize	HT, IT (two herbicides, three insecticides)	Pioneer/DuPont
DAS59122 x NK603	Maize	HT, IT (two herbicides, two insecticides)	Pioneer/DuPont
DAS68416-4	Soybean	HT (two herbicides)	Dow AgroSciences, Pioneer/DuPont
DP305423	Soybean	Changed oil composition, HT	Pioneer/DuPont
DP305423 × 40-3-2	Soybean	Changed oil composition, HT	Pioneer/DuPont
FG72	Soybean	HT (two herbicides)	Bayer CropScience
FG72 x A5547-127	Soybean	HT (three herbicides)	Bayer CropScience
GHB119	Cotton	HT, IT	Bayer CropScience
GHB614 x LLCotton25	Cotton	HT (two herbicides)	Bayer CropScience
GHB614 x LLCotton25 x MON15985	Cotton	HT, IT (two herbicides, two insecticides)	Bayer CropScience

[4] herbicide resistance (often named herbicide tolerant) or production of insecticidal toxins (often named as insect resistant).

Event	Species	Trait	Applicant
MON 87427 × MON 89034 × 1507 × MON 88017 × 59122	Maize	HT, IT (two herbicides, six insecticides)	Monsanto
MON 87427 x MON 89034 x NK603	Maize	HT, IT (one herbicide, two insecticides)	Monsanto
MON 89034 x 1507 x MON 88017 x 59122 x DAS-40278-9	Maize	HT, IT (three herbicides, six insecticides)	Dow AgroSciences
MON 89034 x 1507 x NK603 x DAS-40278-9	Maize	HT, IT (three herbicides, three insecticides)	Dow AgroSciences
MON531 x MON1445	Cotton	HT, IT	Monsanto
MON87411	Maize	IT, IT (Bt and RNAi)	Monsanto
MON87701 x MON89788	Soybean	HT, IT	Monsanto
MON87705	Soybean	Changed oil composition, HT	Monsanto
MON87705 × MON89788	Soybean	Changed oil composition, HT	Monsanto
MON87708 × MON89788	Soybean	HT (two herbicides)	Monsanto
MON87751	Soybean	IT (two insecticides)	Monsanto
MON88017	Maize	HT, IT	Monsanto
MON88017 x MON810	Maize	HT, IT (one herbicide, two insecticides)	Monsanto
MON88302 x MS8 x RF3	Oilseed rape	HT (two herbicides)	Bayer CropScience, Monsanto
MON89034	Maize	IT (two insecticides)	Monsanto
MON89034 × MON88017	Maize	HT, IT (one herbicide, two insecticides)	Monsanto
MON89034 x 1507 x MON88017 x DAS59122	Maize	HT, IT (two herbicides, six insecticides)	Dow AgroSciences, Monsanto, DuPont
MON89034 x 1507 x NK603	Maize	HT, IT (two herbicides, three insecticides)	Dow AgroSciences, Monsanto
MON89034 x NK603	Maize	HT, IT (one herbicide, two insecticides)	Monsanto
MS8, RF3, MS8 x RF3	Oilseed rape	HT, Male sterility	Bayer CropScience
MZHG0JG	Maize	HT (two herbicides)	Syngenta
NK603 x MON810	Maize	HT, IT	Monsanto
NK603 x T25	Maize	HT (two herbicides)	Monsanto
T304-40	Cotton	HT, IT	Bayer CropScience

4. Two case studies

To exemplify current risk assessment as performed by European Food Safety Authority (EFSA) we provide two case studies: (1) Genetically engineered maize, produced by Monsanto, expressing several insecticidal toxins and being tolerant to two herbicides; and (2) genetically engineered soybean, produced by Bayer, which can be sprayed with two complementary herbicides in parallel.

4.1. Case study MON 89034 × 1507 × MON88017 × 59122

4.1.1 The event

In November 2013, the EU Commission authorised products from genetically engineered maize MON 89034 × 1507 × MON88017 × 59122 grown under the brand name “SmartStax” to be used in food and feed in the EU (EFSA, 2010). SmartStax, which combines four genetically engineered plants by stacking, was submitted for import into the EU by Monsanto and Dow AgroSciences and produces six insecticidal proteins (Cry1A105, Cry1F, Cry2Ab2, Cry34Ab1, Cry35Ab1, Cry3Bb1). Further, it is tolerant to two herbicides (glyphosate and glufosinate). The combination of traits was achieved by crossing (stacking) four parental genetically engineered plants (MON 89034, DP1507, MON88017, DP59122, see also Figure 1).

The maize is grown in the US on a large-scale (Edgerton et al., 2012). The combination of toxins and herbicides are meant to overcome increasing problems with herbicide resistant weeds and pressure from pest insects that occur due to the extreme monoculture and industrialisation of US agriculture (Schütte et al., 2017; Siegfried and Jurat-Fuentes, 2016).

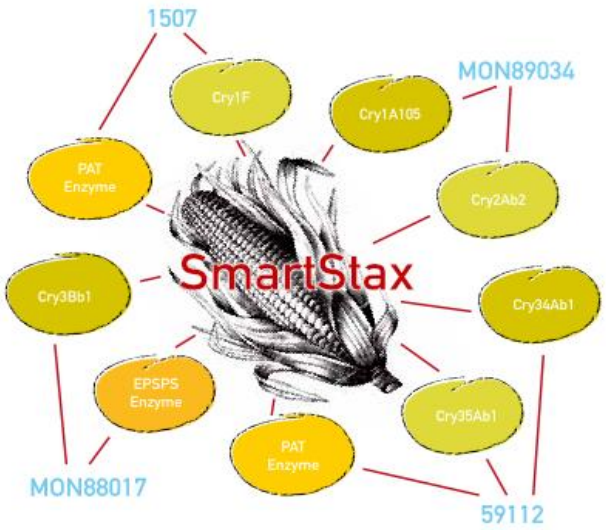

Figure 1: SmartStax, produced by Monsanto and Dow AgroSciences. This maize is a combination of four genetically engineered events (MON88017, MON89034, 59122, 1507), produces six insecticide-producing toxins (Cry toxin is derived from several strains of Bacillus thuringiensis, one of which, Cry1A105, is synthetically manufactured) and is tolerant to two herbicides (glufosinate through the PAT enzyme and glyphosate through the EPSPS enzyme) (source: Testbiotech).

4.1.2 Some aspects relevant for the risk assessment of cumulative effects in this case

Combinatorial effects enhancing toxicity of Bt toxins

There are several publications that show that Bt proteins are able to trigger synergies and interactions with other stressors/plant enzymes (for an overview see Table 3, derived from Then and Bauer-Panskus, 2017.

Table 3: Documented combinatorial effects between Bt toxins and other factors

Factors that modify the toxicity of Bt toxins	
Availability of several co-factors such as specific gut bacteria	Broderick et al., 2009
Parallel or successive exposure to biotic and/ or abiotic stressors	Kramarz et al., 2007; Kramarz et al., 2009; Khalique and Ahmed, 2005; Singh et al., 2007; Zhu et al., 2005; Mason et al., 2011; Reardon et al., 2004
Delaying degradation of Bt toxins by plant enzymes (protease inhibitors)	Zhang et al., 2000; Zhu et al., 2007; Pardo-López et al., 2009
Presence of other Bt toxins	Sharma et al., 2004; Tabashnik et al., 2013; Bøhn et al., 2016;
Presence of glyphosate-based herbicide (Roundup)	Bøhn et al., 2016

While some of these effects might be relevant mostly for non-target organisms, as considered under environmental risk assessment, there are also risks and uncertainties regarding potential health effects for several reasons:

(1) There are several and partially diverging theories about the exact mode of action of the Bt toxins at the molecular level (see Then, 2010; Hilbeck & Otto, 2015), thus it cannot be concluded *a priori* that the toxins are inert in regard to human and animal health as considered under risk assessment for food and feed.
(2) There are further uncertainties regarding the specificity of Bt toxins (Venter and Bøhn, 2016). Changes in specificity may emerge from structural modifications performed to render higher efficacy. For example, the proteins are truncated to become activated (see Hilbeck and Schmidt, 2006) or fused like Cry1A105 produced in SmartStax. As non-target organisms that were not expected to be harmed by Bt toxins show negative effects, like higher mortality, reduced reproduction and other stress-responses (e.g. Bøhn et al., 2008; Bøhn et al., 2010; Holderbaum et al., 2015) the claim that a limited range of organisms is affected by Bt toxins is not trustworthy.
(3) In addition, there are findings in mammalian species showing that Bt toxicity is a relevant topic for detailed health risk assessment: some Cry toxins are known to bind to epithelial cells in the intestines of mice (Vázquez-Padrón et al., 1999; Vázquez-Padrón et al., 2000).
(4) As far as potential effects on health are concerned, several publications (Thomas and Ellar, 1983; Shimada et al., 2003; Mesnage et al., 2012; Huffman et al., 2004; Bondzio et al., 2013) show that Cry proteins indeed may have an impact on the health of mammals. For example, de Souza Freire et al., (2014) confirm the haematoxicity of several Cry toxins. Some of these effects seem to occur where there are high concentrations and tend to become stronger over longer period of time.

It should be noted that just recently, Shu et al., (2018) performed studies on potential synergistic effects of the Bt toxins as produced in SmartStax. To our knowledge this was the first publication with empirical data investigating a combination of these toxins. The authors could not identify interactions between these toxins. Interestingly, the authors not only mention that the study was the first to test this combination in aphids and spiders, but also emphasize the difficulties of measuring

the correct concentration of Bt toxins produced by these plants (see also Székács et al., 2012). Without validated test methods to measure the concentration of the Bt toxins, risk assessment for food and the environment suffers from substantial methodological gaps. Consequently, this study should trigger further research.

Assessement of immune system responses to Bt toxins

Besides toxic effects, Bt toxins such as Cry1Ac (which has high similarity to Cry1A.105) are also known to invoke and boost immune reactions. Rubio-Infante and Moreno-Fierro, (2015) concluded by classifying Cry1Ac as a potent mucosal and systemic immunogen and adjuvant (Moreno-Fierros et al., 2003; Vázquez-Padrón et al., 1999). They mention the high immunogenicity of the Cry1Ac protoxin: demonstrated by its capacity to induce significant specific antibody responses in serum and mucosal-secretions recovered from the small and large intestine, bronchoalveolar and vaginal lavages of mice after immunisation by every tested route, such as intraperitoneal, intragastric, intranasal, rectal (Moreno-Fierros et al., 2000; Vázquez-Padrón et al., 1999) and vaginal (Moreno-Fierros et al., 2002). 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 (Román Calderón et al., 2007).

The adjuvant effects of Cry1Ac protoxin were evaluated regarding the specific antibody responses attained at both mucosal and systemic levels to co-administered antigens of different nature. In a further publication (Torres-Martínez et al., 2016), it is shown in more detail how Cry1Ac induces macrophage activation. Rubio-Infante and Moreno-Fierros (2015) conclude that further risk research is necessary:

> "(...) *the immunogenicity of these proteins and their possible risks in humans after short- and long-term exposure must be determined. Evaluation of the risks of Cry proteins in other systems such as the respiratory and nervous systems is also needed. The toxicity definition must include the adverse effects caused by these toxins not only in the short term; therefore, subchronic and chronic studies in humans should be performed, and the immunotoxicological features of these toxins should be determined.*"

Chowdhury et al. (2003) as well as Walsh et al. (2011) have found that Cry1A proteins can frequently and successfully be found in the colon of pigs, at the end of digestion after being fed with Bt maize. Thus, Bt toxins are not degraded quickly in the gut and can persist in larger amounts till the end of digestion. This means that further interactions between Bt toxins and the complex gut ecosystem, including various food compounds, are possible.

Potential immune reactions are likely to be dose dependent and might be additive or combinatorial. Moreover, in outbred organisms, where the genetic diversity is high (e.g. humans), immune responses can be highly individual and affect groups that are particularly vulnerable (young, old, allergic, immunocompromised, etc). SmartStax (and other events with multiple Bt toxins expressed) has a higher concentration of some individual Bt toxins compared to events with a single Bt toxin expressed (Stillwell and Silvanovich, 2008) and a much higher total concentration of Bt toxins (Venter and Bøhn 2016). This needs further investigation.

Interactions and residues from spraying

In the context of the risk assessment of the stacked event SmartStax, the residues from spraying with the complementary herbicides have also to be considered as potent co-stressors. The impact on cells and organisms exposed to several stressors in parallel can be of importance for the efficacy and effects of Bt toxins. As, for example, Kramarz et al. (2007, 2009) show, parallel exposure to

chemical toxins can render effects of Bt toxins on organisms that are normally not susceptible. In addition, Bøhn et al. (2016) show that Roundup exposure strongly modifies the effect of purified Cry toxins when these stressors are co-exposed to *Daphnia magna*.

Furthermore, the interactions between the chemical residues from spraying with multiple herbicides, as intended with genetically engineered plants that have multiple herbicide tolerances (i.e. various combinations of glyphosate, glufosinate, 2,4-D, dicamba and others) also need to be considered. These aspects will be discussed in more detail in the second case study.

4.1.3 Risk assessment as performed by EFSA on combinatorial effects

EFSA (2010), as well as the applicants, seem to acknowledge that the assessment of combinatorial effects is relevant for food safety assessment. Thus the combinatorial effects of some of the Bt toxins in pest insects were tested (MacRae and Stork, 2008). Furthermore, EFSA (2010) considered combinatorial effects between the two enzymes produced in the plants that provide herbicide tolerance.

Importantly however, no investigations were performed to assess combined toxicity of all stressors in vitro or in vivo. Also, potential adjuvant effects emerging from the combinatorial exposure to multiple Bt toxins were not discussed. Further, other relevant compounds that can trigger combinatorial or synergistic effects such as components from food or feed (such as proteinase inhibitors, see above), the bacterial community or pharmaceutical products (such as antibiotics) were completely left aside (see Table 2, above).

From EFSA, no sub-chronic or chronic feeding study has been requested with the whole food or feed. Instead of considering these relevant but complex issues, the responsible authority on food safety has taken the position that data derived from the feeding studies with the parental plants with single traits is sufficient to exclude health risks (EFSA 2010).

4.1.4 The position of the Commission

The EU Commission accepted the assessment as provided by EFSA. The only additional issue raised by the EU Commission concerned the risk assessment of all sub-combinations of the parental events. In reaction and without requesting any further data from the applicant, EFSA considered it unlikely that any combination of the single maize events MON89034, 1507, MON88017 and 59122 would have an adverse effect on human and animal health or the environment.

> *"Based on the known functional characteristics and modes of action of the newly expressed proteins and the outcomes of the comparative analysis of compositional, phenotypic and agronomic characteristics of maize MON 89034 x 1507 x MON 88017 x 59122, the EFSA GMO Panel considers it unlikely that interactions between the single maize events in all possible sub-combinations will occur that may impact on the food and feed safety and the nutritional properties of the whole food and feed."*(EFSA, 2011)

In 2013, the EU Commission authorised the import of products derived from the stacked event for use in food and feed. The Commission did not request case specific monitoring. From a technical point of view, it is difficult to distinguish the stacked event from a sample which contains a mixture of single events. Therefore, post market monitoring of potential health effects from stacked events is hardly possible.

4.1.5 Conclusion:

In general, potential health impacts of single and multiple insecticidal proteins as produced in the plants and relevant combinatorial effects have not been sufficiently assessed in the European risk

assessment system. Specifically, i) potential impacts on the immune system (such as adjuvant effects) have not been investigated at all, ii) residues from spraying with the complementary herbicides have not been analysed or taken into account, nor were any combinatorial effects with the insecticidal proteins tested or evaluated, iii) no *in vitro* or *in vivo* tests (feeding studies) have been performed with stacked GE events to investigate the effects emerging from exposure to combination of multiple potential stressors, iv) no investigations have been carried out to examine the impact of long-term exposures, and v) no effective system for post market monitoring of health effects has been established.

4.2 Case study FG72

4.2.1 The FG72 soybean event

In November 2013, the EU Commission authorised products from genetically engineered soybean FG72 grown under the brand name “Balance GT” to be used in food and feed in the EU. FG72 is developed by Bayer CropScience and is made tolerant to applications of two complementary herbicides, glyphosate and isoxaflutole, by co-transformation (the genetic constructs were inserted as one cassette).

4.2.2 Some relevant aspects for the risk assessment of cumulative effects from the FG72 soybean

Due to the specific agricultural practices that go along with cultivation of these soybeans, it is evident that the products imported for food and feed are very likely to contain residues from spraying with the complementary herbicides (see for example Duke et al., 2003; Bøhn et al., 2014).

In this context, isoxaflutole is classified as a “suspected human carcinogen” (EFSA, 2016). Further, glyphosate has been characterized as probably carcinogenic to humans (IARC, 2015), although this is disputed by EFSA (EFSA, 2015a). Thus, in regard to carcinogenicity, one could assume increased risk by being exposed to these two chemicals simultaneously. According to Reuter (2015), glyphosate, isoxaflutole and their metabolites share the following common effects, endpoints or modes of action:

- Effects on the liver
- Tumours in liver and thyroid
- Teratogenic effects (reduced ossification of embryos)

For the overall risk analysis there are two major problems in the assessment of the herbicide residues present in the FG72 soybean plant:

(1) In 2016, the EFSA Panel on Plant Protection Products and their Residues (the PPR Panel) presented its peer review of the pesticide risk assessment of the active substance isoxaflutole. This clearly shows major data deficiencies in regard to the requirements of Pesticide Regulation 396/2005 (EFSA, 2016). The results in short are:

- Carcinogenicity and developmental toxicity were confirmed for the active substance.
- In soybean seed three different metabolites of isoxaflutole were found, most of them at higher levels than compared to other uses.
- Risk assessment of these residues in food and feed derived from genetically engineered soybeans FG72 could not be concluded and no maximum residue level (MRL) could be determined due to lack of data.
- Further data gaps concern the method for the determination of residues in food and feed of plant origin.

(2) In 2015, the PPR Panel of EFSA presented the result on risk assessment of glyphosate. In its opinion, EFSA stated that not enough data were available on the application of glyphosate to genetically engineered plants tolerant to the herbicide:

> "*In the framework of the renewal, representative uses were proposed for conventional crops only and residue trials on glyphosate tolerant GM crops were not provided*." (EFSA, 2015a)

This is the reason why EFSA's risk assessment on effects on health from glyphosate is limited to conventional crops:

> "*Based on the representative uses, that were limited to conventional crops only, chronic or acute risks for the consumers have not been identified*." (EFSA, 2015a)

Further, EFSA (2015a) states that more investigations are needed, for example, in regard to the carcinogenicity of the formulations that are applied commercially:

> "*In particular, it was considered that the genotoxic potential of formulations should be addressed; furthermore EFSA noted that other endpoints should be clarified, such as long-term toxicity and carcinogenicity, reproductive/developmental toxicity and endocrine disrupting potential of formulations*."

In addition, EFSA provided an assessment of POE-tallowamine additives, which are used in several formulations with glyphosate, and concluded that these are more toxic than glyphosate:

> "*Compared to glyphosate, a higher toxicity of the POE-tallowamine was observed on all endpoints investigated*." (EFSA, 2015b)

However, no data were made available on the actual load of residues from spraying the crop plants with these formulations:

> "*The genotoxicity, long-term toxicity and carcinogenicity, reproductive/developmental toxicity and endocrine disrupting potential of POE-tallowamine should be further clarified. There is no information regarding the residues in plants and livestock. Therefore, the available data are insufficient to perform a risk assessment in the area of human and animal health for the co-formulant POE-tallowamine.*" (EFSA, 2015b)

In conclusion, EFSA was unable to deliver a conclusive risk assessment on the actual risks of residues from spraying with glyphosate, various glyphosate formulations, and isoxaflutole.

The consequence is that the product safety of the genetically engineered FG72 soybean event, sprayed with isoxaflutole and glyphosate, was not demonstrated.

4.2.3 The risk assessment by EFSA in regard to combinatorial effects

The GMO panel of EFSA has stated that assessment of residues from spraying is outside its remit and should be performed by the Panel on Plant Protection Products and their Residues (PPR) of EFSA. However, as shown in 4.2.2, the pesticide experts of EFSA did not come to any conclusion on the actual toxicity of the residues from spraying with glyphosate and isoxaflutole and were not able to define a Maximum Residue Level (MRL) for the metabolites of isoxaflutole.

A 90-day feeding study with FG72 was provided by the company, however, the data was rejected by EFSA due to methodological flaws. Still, no further study was requested by EFSA. It should be mentioned that in 2018, a feeding study with FG72 was published by the company Bayer, which did not show health effects in rats being fed for 90 days (Xie et al., 2018). However, no information is given in this study regarding whether the crop was sprayed at all.

4.2.4 The position of the EU Commission

The EU Commission authorised the import of FG72 in 2016. No case-specific monitoring was requested. However, several written statements of the EU Commission are available showing that the EU Commission is aware of several problems with the risk assessment of the FG72 soybean:

In regard to risk assessment of combinatorial effects of residues from spraying, in August 2016, the EU Commission sent a letter to several civil society organisations stating (European Commission, 2016a):

> "*It is true that the legislation requires cumulative and synergistic effects of pesticide residues to be considered in the MRL setting, but only when the methods will be available. This is not yet the case and the legislation recognises that further work in this respect is needed. The Commission is working with the Member States, EFSA and other scientists to develop such a methodology for cumulative risk assessment*."

This statement fails to mention that in the case of isoxaflutole being sprayed on soybeans as a complementary herbicide, no MRL could be set at all.

In regard to combinatorial effects emerging from the commercial herbicide formulations being applied on the plants, the EU Commission recommends the prohibition of spraying of formulations containing POE-tallowamine in the EU (Michalopoulos, 2016). However, this initiative does not solve the problem with residues from spraying of POE-tallowamine residues in imported soybeans. The EU Commission seems to be aware of this problem because in February 2016 a letter was sent to EFSA stating (European Commission, 2016b):

> "*A significant amount of food and feed is imported into the EU from third countries. This includes food and feed produced from glyphosate-tolerant crops. Uses of glyphosate-based plant protection products in third countries are evaluated by the competent authorities in those countries against the locally prevailing regulatory framework, but not against the criteria of Regulation (EC) No 1107/2009. (…) EFSA is hence requested under Article 31 of Regulation (EC) No 178/2002 to assess the available information on glyphosate residues in feed, including particular feed imported from outside the EU/ third countries e.g. glyphosate-tolerant GM crops, and conclude on the possible impact of those residues on animal health*."

4.2.5 Conclusion:

Even though no appropriate investigation of the impact of spraying the genetically engineered soybeans has been undertaken, marketing authorisation under the GMO regulation has been granted. This creates a clear gap in the safety assessment of the FG72 soybeans, which has not been filled by assessment performed under the pesticide regulation.

This is a clear-cut breach of the EU regulation 1829/2003, which requires that "…*genetically modified food and feed should only be authorised for placing on the Community market after a scientific evaluation of the highest possible standard, to be undertaken under the responsibility of [EFSA], of any risks which they present for human and animal health and, as the case may be, for the environment...*".

The impact of spraying residues should have been fully assessed before FG72 soybeans could be declared safe under the GMO regulation. Consequently, the FG72 soybean cannot be declared as a fully risk assessed and safe product.

5. Current approaches in the EU for the assessment of mixtures and cumulative toxicity

There are two approaches within the EU to assessing effects emerging from a combination of potential stressors at the stage of consumption. One is applied in the context of the REACH regulation which provides an approach for the assessment of substances that are mixtures themselves. The other is currently developed by EFSA and is based on Monte Carlo Risk Assessment (MCRA) that tries to calculate cumulative toxicity of pesticide residues in food, based on the defined maximum residue levels (MRLs). In 2019, EFSA presented the "Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals". None of these approaches addresses the risk assessment of genetically engineered plants. In the following we explore which of these approaches could be suitable to address the specific issues and questions raised in this report.

5.1 The assessment of mixture toxicity

Kortenkamp et al. (2009) provide an "EU state of the art report on mixture toxicity", contracted by the EU Commission, which is referred to in the following passages. The study gives an overview on several relevant EU regulations in this field, including those covering substances that are mixtures in themselves. The following relevant groups are distinguished:

- Substances that are mixtures themselves (Multi-Constituent Substances, MCS; materials of unknown or variable composition, complex reaction products or biological materials, UVCB).
- Products that contain more than one chemical, e.g. cosmetics or plant protection products.
- Chemicals jointly emitted from production sites, during transport processes, and consumption or recycling processes.
- Several chemicals that might occur together in environmental media (water, soil, air), food items, biota and human tissues, as a result of emission from various sources, via multiple pathways, etc.

In short, Kortenkamp et al. (2009) consider dose (concentration) addition to have proven surprisingly powerful in predicting and assessing mixture toxicities. However, according to the authors, there are some limitations and specific problems:

- Cases of synergism emerging from the mixture of toxicities are hard to predict because there are substantial knowledge gaps in current scientific understanding.
- There are studies providing strong evidence that mixture effects may arise when several chemicals are combined at doses or concentrations around, or below the NOAELs (No Observed Adverse Effect Levels). Thus, mixture effects can be expected even when each chemical in the relevant mixture is present below "zero-effect levels". This phenomenon is known as "something from nothing". This has been demonstrated repeatedly for a broad range of mixtures in toxicological and ecotoxicological studies.

According to Kortenkamp et al. (2009) there is strong evidence to predict the toxicity of mixtures consisting of defined chemical substances with reasonable accuracy and precision. Therefore, there is no need for the experimental testing of each and every conceivable mixture. On the other hand, complex environmental samples (sludge, water, soil) or biological tissue (blood or fat tissue) of unknown composition often have to be subjected to specific tests.

Kortenkamp et al. (2009) show that in regard to existing regulations, most of the 21 Directives and

Regulations examined in the report are substance- or product-oriented pieces of legislation. They control single and multi-constituent substances, preparations of chemicals and products containing chemicals that are intentionally produced and placed on the market. Typically, hazards and risks of these substances and products are assessed as if they were present in isolation.

However, some relevant regulations do take into account simultaneous or sequential exposure to multiple chemicals (Kortenkamp et al. 2009):

- Regulation (EC) No 1907/2006 (REACH) provides guidance on how substances that are in fact mixtures (isomeric mixtures, MCS and UVCB) are to be assessed for their PBT/vPvB (persistent, bioaccumulative and toxic) properties.
- Regulation 1272/2008 on the classification, labelling and packaging of substances and mixtures makes detailed prescriptions for the toxicity assessment of intentionally prepared commercial mixtures.
- Regulation (EC) No 396/2005 on maximum residue levels of pesticides in food and feed of plant and animal origin provides incentives for the development of methodologies for mixture risk assessment. The task of developing viable assessment methods has been assigned to EFSA.

5.2 The Monte Carlo Risk Assessment

In trying to accomplish its task to assess cumulative toxicity as requested by Regulation 396/2005, EFSA is currently working on a version of the Monte Carlo Risk Assessment (MCRA) software. The method of MCRA is supposed to be applied especially to the cumulative toxicity of pesticide residues in food, based on the defined maximum residue levels (MRLs). The EU-Commission, in its letter to Civil Society Organisations, referred to this ongoing process, saying:

> "*It is true that the legislation requires cumulative and synergistic effects of pesticide residues to be considered in the MRL setting, but only when the methods will be available*."[5]

For this purpose, a scalable version of a Monte Carlo Risk Assessment (MCRA) software is under development. This software is supposed to handle datasets with up to at least 100 compounds in a cumulative assessment group (CAG) and at least four million concentration records reported with the EFSA Standard Sample Description (SSD) model (van der Voet et al., 2016). Monte Carlo methods are a broad class of computational algorithms that rely on repeated random sampling to obtain numerical results. Kruisselbrink et al. (2018), on behalf of EFSA, developed a more detailed data model organising the information related to specific elements of cumulative dietary exposure assessment for pesticides, including toxicological information, the authorisation status of plant protection products, the use frequency of plant protection products and the MRLs in force. These data sets are supposed to be used in the development of Monte Carlo Risk Assessment software.

The application of the MCRA method is to the assessment of mixtures of defined chemical substances. This approach creates general problems in food which confers its biological effects as mixtures and mechanisms that are not well defined in all relevant aspects. For example, in soybeans there are many biologically active substances known, many of them having defined toxicological, hormonal, allergenic or pharmaceutical potential (Kurosu, 2011; Cabrera-Orozco et al., 2013). Each of them can be characterised in isolation regarding their biochemical composition, but not all relevant substances are known or taken into account in assessing food safety (see below). Most importantly, the effects emerging from the mixtures of these substances in the soybeans cannot be predicted on the level of the effects of the single substances.

[5] www.testbiotech.org/sites/default/files/11_letter_from%20Commission_August_2016.pdf

This problem also concerns risk assessment of genetically engineered plants: the combinatorial effects emerging from the presence of new biological active molecules within the whole food and feed derived from genetically engineered plants can hardly be predicted by assessing the single substances, which are also not defined by MRLs. Thus, in regard to the application of MCRA, we conclude that this method is not suitable when it comes to risk assessment of food and feed derived from GE plants.

Furthermore, if a fixed combination of stressors is present (as is, for example, the case in genetically engineered plants with a combination of several traits) the MCRA method is not needed anyway.

As a result, there are at least two decisive arguments why the system for assessment of UVCB, as developed within REACH, seems to be much more adequate than the method of MCRA for risk assessment of genetically engineered plants: (1) The combination of stressors is more or less fixed, being inherited in the plants. (2) The combinatorial health effects cannot be predicted from the assessment of its single compounds.

5.3 Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals

According to EFSA "*this Guidance document describes harmonised risk assessment methodologies for combined exposure to multiple chemicals for all relevant areas within EFSA's remit, i.e. human health, animal health and ecological areas.*" (page 1). However, risk assessment of genetically engineered organisms is not mentioned. Within the Guidance, and derived from the areas of defined chemical substances, "*specific considerations are given to component-based approaches, including the grouping of chemicals into assessment groups, refinement of assessment groups, the use of dose (or concentration) addition as a default assumption, the use of response addition and approaches to integrate evidence of interactions.*" (page 3). For reasons also discussed in chapter 6, we do not consider the *component based approach* to be adequate.

Nevertheless, the Guidance elements are useful for defining the different strategies in risk assessment of mixed toxicity:
"A whole mixture approach is the preferred option for mixtures that are insufficiently chemically defined to apply a component-based approach. In this context, the number of chemical substances or the complexity of the mixture is not a sufficient nor necessary reason for choosing between a whole mixture and component-based approach. A component-based approach should be adopted if sufficient exposure and effect data are available on the components governing its toxicity. However, a mixture consisting of a few components may be assessed following a whole mixture approach if interaction between the components is considered likely and toxicity data are available.
A combination of component-based and whole mixture risk assessment approaches may be considered in some circumstances, e.g. if a mixture is poorly defined but known to contain some components of concern, such as genotoxic substances (EFSA Scientific Committee, 2019). Furthermore, in subsequent tiers of a whole mixture approach, information on components in the mixture may become available, which allows for component-based approaches to be applied."(page 22).

6. Specific challenges to risk assessment of genetically engineered plants with a combination of traits

GE plants have some specific characteristics that require more detailed consideration for risk assessment. In particular, risk assessment needs to define and incorporate potential effects from stressors (such as residues from spraying and Bt-toxins) that are not well characterised or understood when they co-occur in mixtures. These mixtures may even interact with a large number of plant components.

Coming from the analysis of Kortenkamp et al (2009) and in the light of the concept of MCRA and the Guidance of EFSA from 2019, in the following we discuss to which extent a whole mixture approach (or the concept used for assessing UVCB) alone or (in combination with) a component-based approach should be applied in risk assessment of genetically engineered plants showing a fixed combination of traits such as herbicide tolerance or production of insecticidal toxins.

6.1 A fixed combination of defined stressors

Within stacked events as listed in Table 2, there are at least two groups of defined stressors that need to be taken into account when it comes to potential health effects: residues from spraying with the complementary herbicides and the insecticidal proteins as produced in the plants. In regard to both groups, the interactions and combinatorial effects within the groups and between the groups have to be considered.

In regard to the residues from spraying, and as shown above, there is some overlap between the GMO regulation and the pesticide regulation. There are some specific issues to be assessed in the context of genetically engineered plants that inherit a combination of traits such as simultaneous tolerance to several herbicides.

The agricultural practices that go along with the cultivation of herbicide tolerant plants have specific patterns of applications, exposure and occurrence of specific metabolites that require special attention in relation to combinatorial effects:

- For example, large-scale commercial cultivation of these plants results in a strong selective pressure on weeds to develop resistance to the herbicides (Sammons and Gaines, 2014). This problem is also relevant for health risk assessment, since this practice has led to a strong increase in the overall use of glyphosate (Benbrook, 2016), subsequently leading to a higher concentration of herbicide residues in the harvest (see, for example, Cuhra, 2015).

- Furthermore, herbicide-tolerant plants are engineered to survive the application of the complementary herbicide, while most other plants will die after a short time. Thus, for example, residues of glyphosate, its metabolites and additives in the formulated product might accumulate and interact in the plants, also changing the plant's composition (Bøhn et al., 2014; Zobiole et al., 2012). The issue of residues stemming from specific metabolites and additives is also acknowledged by EFSA (2015b, 2016).

- A publication by Kleter et al., (2011) summarises some of these issues: "1. GM herbicide-resistant crops can change the way that herbicides can be used on these crops, for example: (a) post-emergent over-the-top applications (i.e. on the crop itself) instead of directed sprays,

> avoiding herbicide contact with the crop; or (b) pre-emergent and pre-harvest applications made to the conventional crop and not, or in different quantities, to the GM crop. 2. The residue profile of the applied pesticide may have been altered on the basis of the nature of the modification. 3. The overall pattern of pesticides applied to the particular crop may have been altered, leading to different exposure to pesticide residues overall."

In regard to several Bt toxins, as expressed in the plants, the basic questions are, whether or not there is a dose-response relationship for the potential adverse effects and to which extent interactions between the Bt toxins have to be considered. In assessing these questions, it has to be taken into account that, compared to its natural templates, the Bt toxins very often are truncated and changed in structure (Latham et al., 2017). Therefore, existing experience with natural occurring Cry toxins is not sufficient to assess the risks of these specific toxins and combinations as produced in the plants.

Furthermore, in stacked events such as SmartStax, a combination of many specific plant constituents (such as six Bt toxins and likely residues of two different herbicides) will be present in the products derived from genetically engineered plants. This combination of residues from spraying and of insecticidal proteins represents a fixed, unique pattern of combinatorial exposure in the feed and food chain. 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 (Then, 2010; Hilbeck & Otto 2015).

6.2 Effects emerging from a mixture of plant compounds

There are many biologically active substances, such as oestrogens, allergens and anti-nutritional compounds, present in plants such as soybeans, which may interact with trait-related characteristics and act as stressors. For example, in soybeans, there are many biologically active substances known, many of them having defined toxicological, hormonal, allergenic or pharmaceutical potential. Each of them can be characterised in isolation regarding their biochemical composition, but not all relevant substances are known or taken into account in assessing food safety.

Internationally agreed lists of nutritional compositional parameters to be analysed in soybean matrices for food use only cover a limited number of the known biological substances (OECD, 2012). But even if all biological substances would be included in compositional analysis, the effects emerging from the mixtures of these substances could not be predicted.

Thus, if new, biologically active compounds such as proteins or miRNAs are produced in genetically engineered plants, these new compounds can be described as isolated substances in regard to their biochemical characteristics. However, these substances are also likely to interact with the plant's metabolism and compounds in various ways (see below). In consequence, the combinatorial effects emerging from the presence of these new biological active molecules within the whole food and feed derived from soybeans can hardly be predicted on the level of the assessment of the single substances.

As an example for possible interactions, one can consider Bt toxins: they can result in effects on the immune system, e.g. because Bt toxins act as an adjuvant for other plant components (see Then and Bauer-Panskus, 2017). 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 by 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.

Besides the intended proteins produced in GE plants, there are other biologically relevant molecules such as micro-RNAs which can be relevant for health risk assessment. Changes in the composition, or emergence of additional micro-RNAs can be triggered by intended or unintended new open reading frames introduced by the insertion of the new gene sequences. Altered or new micro-RNAs will not necessarily give rise to new proteins, but can directly interact with gene regulation on several levels. The biological relevance of these changes will differ from case to case: miRNAs produced by plants are for example able to interfere with the immune system in humans and animals (Zhou et al., 2014; Cavalieri et al., 2016). They also might interfere with the gut microbiome (Testbiotech, 2018). So far, EFSA only takes into account potentially emerging proteins but leaves aside other gene products such as micro-RNAs.

Changes in the composition of these components cannot only be triggered by the process of genetic engineering, but also by the interactions with the complementary herbicides. As Zobiole et al. (2012) show, plant composition can be changed by the application of the complementary herbicide, following a dosage dependent pattern.

Furthermore, considerable amounts of literature indicate that glyphosate formulations can act as so-called endocrine disruptors (see, for example, Thongprakaisang et al., 2013; Çağlar and Kolankaya, 2008; de Liz Oliveira Cavalli et al., 2013; Omran and Salama, 2016). Endocrine effects were found when young rats were exposed to soy milk in combination with glyphosate (Nardi et al., 2017). There may be synergistic or additive interactions of plant components, being present in soybeans such as isoflavones (see for example de Lemos, 2001), with the residues from spraying with glyphosate formulations.

6.3. The challenge: Testing whole organisms, not single substances

Products derived from GE plants may be defined or characterised by their traits (like the production of Bt toxins), but it should not be forgotten that food and feed products from such plants are always complex mixtures with effects emerging from combinatorial effects that cannot be predicted on the level of the effects of the single substances.

Summarizing findings as discussed in the scientific literature, the following parameters seem to be most relevant for the health risk assessment of cumulative effects emerging from genetically engineered plants with a combination of traits:

- Many of the newly produced or introduced potential stressors (such as Bt toxins or residues from spraying with the complementary herbicide) will be present in a fixed combination.
- Some of these potential stressors can be well defined, others may not be and are not subjected to detailed analysis during risk assessment.
- Additive as well as synergistic or antagonistic effects are not unlikely to occur.
- Most potential stressors will have different modes of action but have a common route of exposure (e.g. through the digestive system, the intestine, etc). The stressors may have different or common toxicological endpoints.
- The exposure to the potential stressors is likely – depending on the diet – to be iterative over longer periods of time. If negative health effects occur, it will most likely not come as acute, but rather as subtle effects from sustained chronic exposure.

The EU Commission, in its Implementing Regulation (EU) No 503/2013 of 3 April 2013, that guides risk assessment by EFSA for genetically modified food and feed (for applications filed after 8 December 2013) requests that combinatorial effects are tested. For example, for stacked events,

"*potential synergistic or antagonistic effects resulting from the combination of the transformation events shall be subject to an assessment in accordance with Sections 1.4 (Toxicology), 1.5 (Allergenicity) and 1.6 (Nutritional assessment)*."

Further,

"*The applicant shall perform studies with combined administration of proteins when the genetic modification results in the expression of two or more proteins in the genetically modified plant and when, based on scientific knowledge a possibility of synergistic or antagonistic interactions of safety concerns is identified*."

However, the Implementing Regulation (EU) No 503/2013 does not request that the whole food and feed, as derived from stacked events, needs to be tested. Sub-chronic feeding studies are requested for single events, including the residues from spraying with complementary herbicides:

"*The genetically modified food and feed analysed should be relevant to the product to be consumed. In the case of herbicide tolerant genetically modified plants, the tested material should come from the genetically modified plant exposed to the intended herbicide*".

However, no such requirement is made for stacked events. Instead, the testing of the stacked events will only be requested in specific circumstances:

"*An additional 90-day feeding study with whole food and feed in rodents with the genetically modified plant with the stacked transformation events shall be included where indications of potential adverse effects are identified during the assessment of: (i) the stability of the inserts; (ii) the expression of the inserts; and (iii) the potential synergistic or antagonistic effects resulting from the combination of the transformation events*."

This implies that if a combination of traits stems from a single transformation event (like FG72)[6], the combinatorial effects would be assessed in a different way from those combinations emerging from stacked events.

From a toxicological point of view, this lower standard for testing the stacked events compared to single events, cannot be justified. In general, the concept of Implementing Regulation (EU) No 503/2013, in the case of stacked events, seems to follow the approach of a component-based approach without giving a reasonable justification. As argued above, genetically engineered plants with a combination of traits can show synergistic, additive or antagonistic effects that can emerge from the interaction of defined substances or from interaction with various plants components. The effects of such combinatorial exposures are hard to predict. The overall mixture of relevant stressors is simply not well defined, studied or understood. These issues concern all events with a combination of traits, no matter if they are derived from a single transformation event or from the crossing of several events.

In the light of the standards developed by EFSA (2019), as well as within current EU regulation of assessing mixture toxicity, we need to distinguish between 1) defined chemicals and 2) mixtures that cannot be defined sufficiently and therefore should be regarded as substances of their own. This distinction can be used to guide the risk assessment of genetically engineered plants with a combination of traits. Given the information above, these plants mostly fulfil the criteria of UVCB substances as defined by EU Regulation 1907/2006 (REACH) and need to be assessed under a whole mixture approach (EFSA 2019).

[6] In fact, FG72 was not assessed in accordance with the Implementing Regulation (EU) No 503/2013 because the application was filed, before the Regulation came into place.

Such substances need to be tested as a mixture, not as single compounds. Indeed, Kortenkamp et al (2009) pointed this out in their advice for the European Commission: the GMO legislation requires assessment of the genetically engineered plants as whole food:

> "*GM food and feed are assessed individually on a case-by-case basis by comparing them to traditional counterparts. As foods typically are complex structured complex mixture or intact organisms, they are assessed as a whole. Potentials for interactions between GM food constituents are taken into account*."

In addition, as we have explained in detail, due to the nature of "substances of unknown or variable composition, complex reaction products or biological materials" it is not possible to make reliable predictions about whether effects will be additive, synergistic or antagonistic from a mixture of UVCB substances. If an UVCB substance is added to another UVCB substance (such for example by crossing genetically engineered plants), a new UVCB substance of higher complexity will be the result. This new substance clearly needs to be assessed on its own.

In practice, if four genetically engineered plants are crossed with each other (as presented in the first case study), the cumulative adverse effects emerging from the stacked plant cannot be determined by dose addition or a component-based approach. The whole plant, with all its potential stressors present, is what is relevant.

It should be acknowledged, that for this purpose, we have already have some established methodology. Specific fixed combinations of stressors such as Bt toxins in combination with residues from spraying might be tested *in vitro* using isolated cells. Further, some relevant combinations can be tested in non-vertebrate test systems. These investigations can help to develop hypotheses which allow targeted investigation in whole food and feed studies, including chronic or multigenerational feeding studies. Some of the relevant questions might be suitable for testing in farm animals, other will require laboratory animals.

Consequently, since legislation requires cumulative effects to be assessed and the relevant methodology is available, there is an obligation also to apply these methods to all genetically engineered plants with a combination of traits, regardless of whether these are derived from single transformation events or from stacking.

As the Guidance from EFSA (2019) shows, additional information such as the possibility of grouping and categorizing similar mixtures or applying tiered approaches with worst-case assessments at lower tiers and more probabilistic assessments at higher tiers might be considered from case to case. For example, if data are available on a specific combination, its sub-combinations might require less extensive testing. However, as a simple rule, it will always be the mixture with the highest number of combinations that will need the most detailed assessment.

Furthermore, the question of how to risk assess mixtures of genetically engineered plants needs to be answered in cases where a diet will lead to co-exposure of multiple potential stressors with the risk of combinatorial, aggregated or cumulative effects. These mixtures might be considered as "intentional mixtures" (EFSA 2019) or not, and it might be discussed as being composed of "similar mixtures", therefore a classification in accordance to EFSA (2019) is warranted.

In addition to risk assessment, mixtures of genetically engineered plants in diets should be followed up with post market monitoring, adapted to fill data and knowledge gaps (e.g. on chemical and metabolite residues) identified in the risk assessment process.

7. Regulatory needs: Conclusions and recommendations

Compared to the current practice of the EU in GMO risk assessment, combinatorial effects (or potential mixed toxicity) emerging from simultaneous exposure to a fixed combination of potential stressors emerging from genetically engineered plants at the stage of consumption have to be assessed in more detail.

We highlight that GMO Regulation 1829/2003 and Directive 2001/18 require that the whole organism and not only parts and single constituents have to be assessed. This is necessary to ensure the safety of such products before market access is granted.

Consequently, the process of risk assessment needs to be adjusted to systematically assess cumulative effects from combinatorial exposure to genetically engineered plants exhibiting more than one trait. Being aware of EFSA Guidance from 2019, we propose that these plants are tested following the whole mixture approach, considering them as "*insufficiently chemically defined to apply a component-based approach*". They should be considered as being equivalent to UVCB substances as defined under Regulation (EC) No 1907/2006 (REACH).

Compared to the area of chemicals, there are some differences, because in the case of genetically engineered plants, whole mixture means the assessment of whole food and feed. Currently, the most adequate method to test these substances are feeding studies with whole feed and food. The food and feed analysed should be relevant to the product to be consumed, including the residues from spraying with complementary herbicides (with dosages that are in accordance with the conditions of agricultural practice). To generate reliable data for products that are used daily in the food chain, the feeding studies should be long term, including several generations.

In addition, in-vitro testing systems and or testing systems using non-vertebrates might be used to develop hypotheses and to reduce the overall number of animals needed for feeding studies. The combined toxicity of the residues from spraying with complementary herbicides needs specific testing if similar toxicological endpoints are identified from different but co-occurring stressors. Further methodology should be developed for testing whole mixtures in addition to or instead of animal feeding studies. More scientific studies should be initiated to better understand combinatorial, aggregated or cumulative exposure and effects from multi-trait GE plants or from mixtures of genetically engineered plants in diets.

There are ethical concerns that need to be taken into account by the risk manager. It might be necessary to imply a cost-benefit assessment in addition to the risk assessment. This cost-benefit assessment should take into account societal and ethical considerations by assessing the potential benefits that might derive from the import and consumption of relevant food and feed products in comparison to the need to perform feeding studies.

The legislation in Norway integrates a cost-benefit approach: the Norwegian Gene Technology Act requests the notifier to provide information that will allow the Norwegian authorities to evaluate the possible contributions of the applications to sustainable development, benefit to society and other ethical considerations regarding the use of the genetically modified crop.[7] This law might serve as starting point to integrate societal and ethical considerations in the EU approval process, which is foreseen in Directive 2001/18 already.

However, it should be born in mind that it is not the risk assessment that can be weighed up against

[7] http://www.regjeringen.no/en/doc/laws/Acts/gene-technology-act.html?id=173031

potential benefits. Instead safety has always to be shown in accordance with scientific standards. Thus, if the cost-benefit assessment does result in negative outcome the approval process has to be stopped, but it cannot be replaced by lower scientific standards.

Another outstanding challenge for risk analysis in the EU is the risk assessment and monitoring of mixtures of genetically engineered plants in a diet that will lead to co-exposure of multiple potential stressors with the risk of combinatorial, aggregated or cumulative effects. The assessment of the potential mixed toxicity of these diets remains a crucial and an urgent challenge for safeguarding food safety in the EU.

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