TOXICITY OF PESTICIDES ON HEALTH AND ENVIRONMENT

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TOXICITY OF PESTICIDES ON HEALTH AND ENVIRONMENT

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Public policy is regularly shaken by health crises or unexpected discoveries; future directions in toxicology assessment are therefore urgently needed.

Convergent evidences suggest endocrine or nervous disrupting effects of pesticides, as well as effects on wildlife and the environment. These effects are amplified by the use of surfactants and/or combinations of different active principles.

The usual concepts of regulatory toxicology are challenged by endocrine, nervous or immune disruption, or epigenetic effects. Indeed, most pollutants alter cell-cell communication systems to promote chronic diseases. They may accumulate in the food chain. Mixtures effects with other pollutants may change their bioavailability and their toxicity. The lack of scientific knowledge in these matters has large costs for public health.

This Research Topic focuses on the toxic effects of pesticides associated with large scale cultivation of genetically modified (GM) plants.

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EDITORIAL
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Editorial: Toxicity of Pesticides on Health and Environment

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Editorial on the Research Topic

Toxicity of Pesticides on Health and Environment

The aim of this research topic was to explore different aspects of the effects of pesticides on human health and the environment from a multidisciplinary point of view. The sustainability of agricultural cropping systems is a fundamental question on which the future of humanity is relying. Several indicators tend to suggest that the current system of agricultural production is reaching its limits and become unsustainable (Nicolopoulou-Stamati et al.). One hallmark of modern intensive agriculture, as well as a cause of farming system decline, is the intensive use of pesticides. They are used to kill insects, fungi or undesirable plants, reducing the biodiversity of agricultural landscapes to only one edible crop. This type of crop management has long-term detrimental effects on farming systems as the lack of biodiversity directly affects soil resilience.

Public policy is regularly shaken by health crises due to unexpected toxic effects of commonly used chemicals. This is the case for pesticides and their metabolites which can directly affect human and animal health (Nicolopoulou-Stamati et al.). Authors contributing to this research topic focused on pesticides associated to large scale cultivation of crops, for which the toxicity is debated, such as glyphosate-based herbicides (Cuhra et al.; Székács and Darvas) and neonicotinoids-based insecticides (Mullin et al.). It should also be borne in mind that the introduction of genetically modified (GM) crops at the end of the 1990s has considerably modified agricultural practices, including the use of pesticides. Almost all GM crops cultivated nowadays have been modified to tolerate an herbicide (mostly glyphosate-based herbicides) or/and produce their own modified insecticide. The toxicological properties of these insecticides is thoroughly addressed by Hilbeck and Otto in a review article, with a focus on combinatorial effects of Cry toxins.

The different studies published in our research topic shared a common conclusion. All revealed that the toxicity of pesticides is generally underestimated. For instance, pesticides are always commercialized as mixtures of different ingredients but only one declared of these ingredients is regulated and tested for human health effects. Ingredients such as surfactants, also named "inerts" or "formulants," are poorly tested although they can be the most toxic ingredients in a pesticide formulation (1). This is clearly illustrated in the work by and colleagues, showing that organosilicone surfactants are potent standalone pesticides, and that they are toxic to honey bees (Mullin et al.). This work also shows for the first time that surfactant use could be linked with declining health of honey bee populations. Another important study investigated the inflammatory effects of a plant protection product, composed of crushed fenugreek seeds, on human peripheral blood mononuclear cells (Teyssier et al.). This work reminds us that although bio-based pesticides are of natural origin, direct toxicity of these products to human can be observed. They thus must be studied carefully to avoid non-target health effects as it is done for synthetic pesticides.

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However, the problem goes beyond considerations on the toxicity of pesticides. It has social, political, ethical, and legal implications that could only be embraced through multidisciplinary research. Research on human health effects of environmental chemicals is highly specialized and few studies address the question from a multidisciplinary point of view. The debate on glyphosate is a topic for which multidisciplinary research bring meaningful insights. This idea is well supported by the analysis of the glyphosate case by Cuhra and colleagues arguing that specific aspects of the history, chemistry and safety of glyphosate and glyphosate-based herbicides should be thoroughly considered in present and future re-evaluations (Cuhra et al.). It is impossible to ignore structural changes in glyphosate uses. The use of glyphosate-based herbicides increased exponentially since their introduction on the market in the 1970s. It was amplified in the last decades by the introduction of agricultural genetically modified organisms (GMOs) designed to tolerate Roundup.

Perspectives from political economy are equally important. Glyphosate market is currently highly concentrated, and around 50% of global revenues are shared by only 4 companies. It has been estimated that Monsanto company made $4.76 billion in sales and $1.9 billion in gross profits from herbicide products, mostly consisting in Roundup (US securities and exchange commission, document 10-K, 1 mon-20150831x10k). It has been amplified now by the fusion with Bayer (2018). This may have critical consequences on political decisions related to the commercialisation of pesticides and GM crops designed to tolerate their residues. A similar line of thought is found in the perspective article published by Benbrook, describing 10 reforms and initiatives to create a more robust, science-driven regulatory infrastructure in the U.S.

Feeding 9 billion people or more with a healthy food through sustainable farming systems is one of the main challenges humanity has to face in the future. Agronomic and socioeconomic factors such as food availability, disparity in wealth, waste management, as well as dietary choices, are equally important to ensure global food security. A democratization of science is crucial in the current context of agricultural innovation that is increasingly driven by industrial interests (Vélot). Strategies to restore links between science, policy makers, and civil society are presented by (Vélot). This is well illustrated by the example of a participatory research project, in which the research work is shared between non-profit organizations from civil society or groups of citizens and academic researchers (from universities or major research organizations) like it was performed in CRIIGEN since 1999 (Vélot). In this line of though, the Cornell Alliance for Science launched an initiative in which "citizen scientists" are called upon to evaluate studies on health risks of GM crops and foods. The meaningfulness and limits of this project is examined by Antoniou and Robinson.

Our research topic confirms that new directions in agriculture are urgently needed to evaluate pesticide effects on health and environment. New agricultural policies should target sustainable development and protection of the consumers' health.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Chemical Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture

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The industrialization of the agricultural sector has increased the chemical burden on natural ecosystems. Pesticides are agrochemicals used in agricultural lands, public health programs, and urban green areas in order to protect plants and humans from various diseases. However, due to their known ability to cause a large number of negative health and environmental effects, their side effects can be an important environmental health risk factor. The urgent need for a more sustainable and ecological approach has produced many innovative ideas, among them agriculture reforms and food production implementing sustainable practice evolving to food sovereignty. It is more obvious than ever that the society needs the implementation of a new agricultural concept regarding food production, which is safer for man and the environment, and to this end, steps such as the declaration of Nyéléni have been taken.

Keywords: pesticides, agrochemicals, environmental health, endocrine disruptors, food sovereignty

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INTRODUCTION

Pesticides are substances or mixtures of substances that are mainly used in agriculture or in public health protection programs in order to protect plants from pests, weeds or diseases, and humans from vector-borne diseases, such as malaria, dengue fever, and schistosomiasis. Insecticides, fungicides, herbicides, rodenticides, and plant growth regulators are typical examples (1–3). These products are also used for other purposes, such as the improvement and maintenance of non-agricultural areas like public urban green areas and sport fields (4, 5). Furthermore, there are other less known applications of these chemical substances, such as in pet shampoos (4), building materials, and boat bottoms in order to eliminate or prevent the presence of unwanted species (6).

Many of the pesticides have been associated with health and environmental issues (1, 2, 7–12), and the agricultural use of certain pesticides has been abandoned (2). Exposure to pesticides can be through contact with the skin, ingestion, or inhalation. The type of pesticide, the duration and route of exposure, and the individual health status (e.g., nutritional deficiencies and healthy/damaged skin) are determining factors in the possible health outcome. Within a human or animal body, pesticides may be metabolized, excreted, stored, or bioaccumulated in body fat (1, 2, 13). The numerous negative health effects that have been associated with chemical pesticides include, among other effects, dermatological, gastrointestinal, neurological, carcinogenic, respiratory, reproductive, and endocrine effects (1, 2, 8, 10, 14–30). Furthermore, high occupational, accidental, or intentional exposure to pesticides can result in hospitalization and death (1, 31).

Residues of pesticides can be found in a great variety of everyday foods and beverages, including for instance cooked meals, water, wine, fruit juices, refreshments, and animal feeds (32–39). Furthermore, it should be noted that washing and peeling cannot completely remove the residues

(40). In the majority of cases, the concentrations do not exceed the legislatively determined safe levels (36, 39, 41, 42). However, these "safe limits" may underestimate the real health risk as in the case of simultaneous exposure to two or more chemical substances, which occurs in real-life conditions and may have synergistic effects (1, 43). Pesticides residues have also been detected in human breast milk samples, and there are concerns about prenatal exposure and health effects in children (13, 44–46).

This current review aims at highlighting the urgent need for a new concept in agriculture involving a drastic reduction in the use of chemical pesticides. Given the fact that the health effects have been extensively discussed in the current literature, this paper focuses on the major chronic health effects and recent findings regarding health effects that have been associated with exposure to common classes of chemical pesticides, i.e., organochlorines, organophosphates, carbamates, pyrethroids, triazines, and neonicotinoids. More emphasis is given to the widely used herbicide "glyphosate," which is an organophosphate pesticide very closely related to current agriculture (47). The important health effects, as discussed below, reveal the urgent need for implementing alternative solutions.

ORGANOCHLORINE PESTICIDES

The most widely known organochlorine pesticide is dichlorodiphenyltrichloroethane, i.e., the insecticide DDT, the uncontrolled use of which raised many environmental and human health issues (2, 48, 49). Dieldrin, endosulfan, heptachlor, dicofol, and methoxychlor are some other organochlorines used as pesticides.

There are a few countries that still use DDT or plan to reintroduce it for public health purposes (13, 48, 49). Furthermore, DDT is also used as a solution in certain solvents (2). It is a ubiquitous chemical substance, and it is believed that every living organism on Earth has a DDT body burden, mainly stored in the fat (48, 50). There is also evidence that DDT and its metabolite p,p-dichlorodiphenyldichloroethylene (DDE) may have endocrine-disrupting potential and carcinogenic action (48). *In utero* exposure to both DDT and DDE has been associated with neurodevelopmental effects in children (51). Moreover, a recent study related DDE to hepatic lipid dysfunction in rats (50).

The general class of organochlorine pesticides has been associated with health effects, such as endocrine disorders (10, 52), effects on embryonic development (53), lipid metabolism (54), and hematological and hepatic alterations (55). Their carcinogenic potential is questioned, but concerns about possible carcinogenic action should not be underestimated (38, 39, 56, 57).

ORGANOPHOSPHORUS PESTICIDES

Organophosphates, which were promoted as a more ecological alternative to organochlorines (58), include a great variety of pesticides, the most common of which is glyphosate. This class also includes other known pesticides, such as malathion, parathion, and dimethoate; some are known for their endocrine-disrupting potential (10, 59, 60). This class of pesticides has been associated with effects on the function of cholinesterase enzymes (58), decrease in insulin secretion, disruption of normal cellular metabolism of proteins, carbohydrates and fats (54), and also with genotoxic effects (61) and effects on mitochondrial function, causing cellular oxidative stress and problems to the nervous and endocrine systems (54).

Population-based studies have revealed possible relations between the exposure to organophosphorus pesticides and serious health effects including cardiovascular diseases (62), negative effects on the male reproductive system (63) and on the nervous system (58, 64–66), dementia (67), and also a possible increased risk for non-Hodgkin's lymphoma (68). Furthermore, prenatal exposure to organophosphates has been correlated with decreased gestational duration (69) and neurological problems occurring in children (70).

Regarding glyphosate, the safety of which is the subject of an ongoing scientific controversy (60, 71–76), it is the most widely used herbicide in current agriculture (47, 75), especially since the introduction of glyphosate-tolerant genetically modified crops, such as certain types of soybean and maize (60, 77–80). Its extensive use in genetically modified soybean cultivation has raised concerns about possible synergistic estrogenic effects due to the simultaneous exposure to glyphosate and to the phytoestrogen "genistein," which is a common isoflavone present in soybeans and soybean products (80, 81).

Glyphosate can display endocrine-disrupting activity (80, 82), affect human erythrocytes *in vitro* (83), and promote carcinogenicity in mouse skin (84). Furthermore, it is considered to cause extreme disruption in shikimate pathway, which is a pathway found in plants and bacteria as well as in human gut bacteria. This disruption may affect the supply of human organism with essential amino acids (85). Commercial glyphosate formulations are considered to be more toxic than the active substance alone (80, 83, 86, 87). Glyphosate-based herbicides, such as the well-known "Roundup," can cause DNA damages and act as endocrine disruptors in human cell lines (60) and in rat testicular cells (88), cause damages to cultured human cutaneous cells (89), and promote cell death in the testicular cells of experimental animals (88, 90). There is evidence also for their possible ability to affect cytoskeleton and intracellular transport (91).

A recent study examined the possible relation between glyphosate, genetically modified crops, and health deterioration in the USA. Correlation analyses raised concerns about possible connections between glyphosate use and various health effects and diseases, such as hypertension, diabetes, strokes, autism, kidney failure, Parkinson's and Alzheimer's diseases, and cancer (82). Furthermore, there are concerns about the possible ability of glyphosate to cause gluten intolerance, a health problem associated with deficiencies in essential trace metals, reproductive issues, and increased risk to develop non-Hodgkin's lymphoma (92).

CARBAMATE PESTICIDES

Carbamate pesticides, such as aldicarb, carbofuran, and ziram, are another class of chemical pesticides that have been associated with endocrine-disrupting activity (10, 93), possible reproductive disorders (63, 93), and effects on cellular metabolic mechanisms and mitochondrial function (54). Moreover, *in vitro* studies have

revealed the ability of carbamate pesticides to cause cytotoxic and genotoxic effects in hamster ovarian cells (94) and to induce apoptosis and necrosis in human immune cells (95), natural killer cells (96, 97), and also apoptosis in T lymphocytes (98).

Furthermore, it has been confirmed that carbaryl, which belongs to the category of carbamate pesticides, can act as a ligand for the hepatic aryl hydrocarbon receptor, a transcription factor involved in the mechanism of dioxin toxicity (99). There is also evidence for the ability of carbamate pesticides to cause neurobehavioral effects (65, 100), increased risk for dementia (67), and non-Hodgkin's lymphoma (101).

OTHER CLASSES OF CHEMICAL PESTICIDES

Triazines, such as atrazine, simazine, and ametryn, are another class of chemical pesticides that have been related to endocrine-disrupting effects and reproductive toxicity (10, 102, 103). Moreover, it was found that there is a possible statistical relationship between triazine herbicides and breast cancer incidence (104). Atrazine is the most known of the triazines, and it is a very widely used herbicide that has been associated with oxidative stress (103), cytotoxicity (105, 106), and dopaminergic effects (107, 108). Furthermore, the exposure of experimental animals to atrazine has been associated with reproductive toxicity (109) and delays in sexual maturation (110).

Synthetic pyrethroids, such as fenvalerate, permethrin, and sumithrin, are considered to be among the safer insecticides currently available for agricultural and public health purposes (111, 112). However, there is evidence for their ability to display endocrine-disrupting activity (10, 113–115), and to affect reproductive parameters in experimental animals including reproductive behavior (114, 116). Furthermore, a recent study related more than one pyrethroid metabolite to DNA damages in human sperm, raising concerns about possible negative effects on human reproductive health (117). It should also be mentioned that there are also concerns about their possible ability to display developmental neurotoxicity (25, 118, 119).

Neonicotinoid pesticides, such as imidacloprid, thiacloprid, and guadipyr, are relatively new and also the most extensively used insecticides (120) that were promoted for their low risk for non-target organisms (121). However, there is plenty of evidence to the contrary (115, 122–125); their effect on bees is a common example (124, 125). There is also evidence for possible effects on the endocrine and reproductive systems of animals (115, 126, 127). Moreover, a recent study demonstrated that neonicotinoids are able to increase the expression of the enzyme aromatase, which is engaged in breast cancer and also plays an important role during developmental periods (128).

URGENT NEED TOWARD CLEANER AND SAFER AGRICULTURAL PRACTICES

Current agricultural practices include the wide production and extensive use of chemicals known for their ability to cause negative health effects in humans and wildlife and to degrade the natural environment. Therefore, an urgent strategic approach is needed for a reduction in the use of agrochemicals and for the implementation of sustainable practices. Furthermore, current agriculture has to implement environmentally friendlier practices that pose fewer public health risks. Reforming agricultural practices aligned to fulfill these criteria is a step toward the sustainability of the agricultural sector in contrast to precision agriculture (129–134).

However, the reduction in the use of agrochemicals by applying them only when and where they are necessary, the spatiotemporal variability of all the soil and crop factors of a given field must be taken into consideration. This variability includes yield, field, soil, and crop variability but also factors, such as wind damage or flooding. Technological systems, such as geographical information systems, global positioning systems, and various sensors, can be useful (130–132, 135). These technological systems are developed by precision agriculture which of course we do not endorse, but we consider that selected technological tools can be used to decrease risks for environmental pollution and water pollution and to enhance economic benefits stemming from the reduction in the use of chemical products (130, 132).

It should be clear that the reform into an aggregate of machine-centered procedures and losing a human-centered character are not the desired. In contrast, the reduction in the use of pesticides assisted by innovative technological methods we strongly believe that may reduce the use of chemical substances or maybe it can lead to a total abandonment in many cases, such as in the case of urban green areas. The decision of the Italian village of Mals near the Austrian and Swiss borders to ban the use of pesticides and produce pesticide-free foods can be considered as a pioneer example across Europe. In 2014, more than 70% of the inhabitants of Mals who participated in a referendum voted against the use of pesticides (136). This historical decision apart that is consistent with the food sovereignty concept, which is discussed in the following section, also declares the need for disseminating information for raising awareness of the public in order to develop informed consents.

An innovative idea developed by the international movement "Via Campesina," was the democratic concept of food sovereignty that has accompanied the progress toward sustainability for more than 20 years. It acquired a strong basis in 2007 in the African village Nyéléni in Mali, where representatives from more than eighty countries adopted the "Declaration of Nyéléni." According to its principles, all the people of the world have the right to choose their own national and local policies to eliminate poverty, malnutrition, and hunger, to protect their traditions and also the natural environment (137–141).

The industrialization of agriculture has brought a series of problems including economic, social, and environmental impacts that local populations cannot manage. Furthermore, the overproduction of food, export-oriented monocultures, the demand for cheap labor, and the other characteristics of industrialization have clearly failed to solve the problems of hunger and malnutrition. On the contrary, inequitable food distribution, overexploitation of land and water sources, the overuse of agrochemicals, and the degradation of the natural environment are some of the results of the dominant agricultural model (138, 142–144). Food sovereignty

promotes social, economic, and environmental sustainability, for instance, through the protection of the indigenous population and the production of food for distribution in local markets, and there is an ongoing effort for its recognition as a basic human right (138–140, 142, 145).

The dominant agricultural model has increased the chemical burden on natural environment (140, 142). Moreover, international agrochemical companies absorb traditional agricultural companies, leading to an industrialized agriculture model and leaving the local farmers and small producers to face the consequences (138, 143). In many cases, these people are obliged to adopt environmentally unfriendly techniques to increase their production in order to survive in the market, causing more environmental degradation (138). However, due to the fact that food sovereignty does not necessarily mean pesticide-free, organic food production, and because it does not determine pesticide use levels, for this reason, international eco-friendly standards should be implemented. People must be free to decide the method of production of their own food, and an important component of this decision concerns agrochemical products. The decision of the people of Mals to reject pesticides can be considered a step in this direction.

DISCUSSION

The need for protection against pests is a given and has its roots in antiquity, when both organic and chemical substances were applied as pesticides (146). Since then, numerous chemical pesticides have been produced, and now multinational agrochemical companies, which mostly control global food production, apply new chemical substances with pesticide properties and implement biotechnological advances, thus diverging from traditional agricultural methods. Furthermore, current agricultural practices are based on the wide use of chemical pesticides that have been associated with negative impacts on human health, wildlife, and natural environment (9, 11, 120, 147, 148).

Current agriculture has to deal with important factors, such as population growth, food security, health risks from chemical pesticides, pesticide resistance, degradation of the natural environment, and climate change (149–155). In recent years, some new concepts regarding agriculture and food production have appeared. A concept as such is climate-smart agriculture that seeks solutions in the new context of climate change (152, 153). Another major ongoing controversy exists between the advocates and the opponents of genetically engineered pesticide-resistant plants, regarding not only their safety (29, 156, 157) but also their impact on pesticide use (158–160).

Furthermore, the real-life chronic exposure to mixture of pesticides with possible additive or synergistic effects requires an in depth research. The underlying scientific uncertainty, the exposure of vulnerable groups and the fact that there are numerous possible mixtures reveal the real complex character of the problem (161–163). The combination of substances with probably carcinogenic or endocrine-disrupting effects may produce unknown adverse health effects. Therefore, the determination of "safe" levels of exposure to single pesticides may underestimate the real health effects, ignoring also the chronic exposure to multiple chemical substances.

Taking into consideration the health and environmental effects of chemical pesticides, it is clear that the need for a new concept in agriculture is urgent. This new concept must be based on a drastic reduction in the application of chemical pesticides, and can result in health, environmental, and economic benefits (164) as it is also envisaged in European Common Agricultural Policy (CAP) (165).

We believe in developing pesticide-free zones by implementing a total ban at local level and in urban green spaces is easily achievable. Furthermore, alternative procedures to the current model of food production should be implemented in new agricultural policies targeting sustainable development and protection of the consumers' health. Despite the difficulties of establishing an innovative concept, the transition to a new cleaner and safer agricultural model is necessary.

AUTHOR CONTRIBUTIONS

Professor PN-S and Professor LH are the principal authors. Mr. SM contributed with proof reading, literature review, and editing. Mrs. CK and Mr. PS contributed with literature review and editing.

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Glyphosate: Too Much of a Good Thing?

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Although previously accepted as the less toxic alternative, with low impact on animals, farmers as well as consumers who are exposed to residues in food, glyphosate chemicals are now increasingly controversial as new evidence from research is emerging. We argue that specific aspects of the history, chemistry and safety of glyphosate and glyphosate-based herbicides should be thoroughly considered in present and future re-evaluations of these dominant agrochemicals:

- Glyphosate is not a single chemical, it is a family of compounds with different chemical, physical, and toxicological properties.
- Glyphosate is increasingly recognized as having more profound toxicological effects than assumed from previous assessments.
- Global use of glyphosate is continuously increasing and residues are detected in food, feed, and drinking water. Thus, consumers are increasingly exposed to higher levels of glyphosate residues, and from an increasing number of sources.
- Glyphosate regulation is predominantly still based on primary safety-assessment testing in various indicator organisms. However, archive studies indicate fraud and misbehavior committed by the commercial laboratories providing such research.

We see emerging evidences from studies in test-animals, ecosystems indicators and studies in human health, which justify stricter regulatory measures. This implies revising glyphosate residue definitions and lowering Maximum Residue Limits (MRLs) permissible in biological material intended for food and feed, as well as strengthening environmental criteria such as accepted residue concentrations in surface waters. It seems that although recent research indicates that glyphosates are less harmless than previously assumed and have complex toxicological potential, still regulatory authorities accept industry demands for approving higher levels of these residues in food and feed.

Keywords: glyphosate safety-assessment, history of glyphosate-herbicides, chemical diversity of glyphosates, glyphosate tolerant transgenic crops, Roundup

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INTRODUCTION

In *As You Like It* by Shakespeare, Rosalind asks Orlando: "*Can one desire too much of a good thing?*" ...

The phytotoxic properties of glyphosate were recognized around 1970 and the new compound was enthusiastically embraced as a *good thing*; it was perceived as a *practically non-toxic alternative*, a *safe chemical* and a *benefit to society*. And, best of all, it proved to be an efficient herbicide. After introduction of first commercial formulations around 1975, glyphosate-based herbicides (GBHs)

have become globally dominant for eradication of unwanted weed species and lately also have found other use, e.g., as desiccants on agriculture crops. At the moment of writing, glyphosate is the globally dominating herbicide, measured in tonnage, and revenue.

Archive film from a commercial biotechnology laboratory in 1987 shows George H. W. Bush (at that time vice-president of the USA) as he asks the assembled researchers; "this gene of yours, what does it do in the plant?" Before any of the superiors have a chance to answer, a junior scientist excitedly proclaims; "we have this fabulous herbicide..." (Robin, 2008). The fabulous herbicide was glyphosate and the gene in question was the commercially promising EPSPS gene isolated from *Agrobacterium*, which made it possible to modify agriculture crops into *glyphosate-tolerant varieties*. Leading agronomists later described the fabulous herbicide in a widely acknowledged publication bearing the title; "Glyphosate: a once-in-a century herbicide" (Duke and Powles, 2008). The headline for this present writing also refers to the 2012–2015 detailed evaluation of glyphosate recently completed by the European Food Safety Authority: "Glyphosate: EFSA updates toxicological profile" (EFSA, 2015c), in which EFSA concludes that glyphosate is probably not a human carcinogen, but on the other hand also acknowledges the need for tighter regulation, specifically by adjusting consumer exposure.

GBHs were primarily intended for pre-emergence application in conventional industrial agriculture. However, genetically modified cultivars (GM) allow post-emergence application in herbicide-tolerant genetically modified soybean, corn, cotton, and canola. These crops are engineered to withstand the effects of glyphosate and seen as a main incitement for increasing production of and application of these chemicals (Charles, 2001; Benbrook, 2012, 2016; Bonny, 2014; Cuhra, 2015a). Annual global production figures for glyphosate have recently been estimated at 825,800,000 kg (Benbrook, 2016), while less investigative sources estimate even higher production volumes, surpassing 1 million ton annually (Székács and Darvas, 2012; Bøhn et al., 2016) and there are few indications of reduced use, other than challenges from resistant weed and emerging evidence.

A string of previous studies investigated aspects such as toxicity of glyphosate and Roundup toward aquatic invertebrates (Cuhra et al., 2013), accumulation of glyphosate in glyphosate-tolerant soybean (Bøhn et al., 2014) and potential effects of such residues in test animal feed (Cuhra et al., 2014, 2015). Furthermore, we have reviewed reports from industry studies investigating these issues. No studies other than our own were found to specifically assess effects of glyphosate residues (Cuhra, 2015b). We have also published preliminary results from studies of documentation in archives from US FDA and US EPA, obtained via freedom of information act requests (Cuhra, 2015c).

Importantly, glyphosate is not one single clearly defined compound, but rather a family of chemicals that can be synthesized through different chemical processes, which in turn will cause various qualitative differences e.g., impurities and byproducts. Glyphosates exist in several chemical mixtures and/or forms, primarily as either glyphosate technical acid or as various salts of glyphosate.

REVIEW OF RECENT FINDINGS

Compositional analysis of soybean samples from major production areas in USA and Argentina determined that such transgenic glyphosate-tolerant crops accumulate glyphosate, causing surprisingly high levels of glyphosate residues (Bøhn et al., 2014), even far above the spacious maximum residue limits which exists for soybean at present (Then, 2013; Cuhra, 2015b). Such high levels of glyphosate residues are endemic for the glyphosate-tolerant GM-varieties. In samples of organic soy and conventionally grown soy from industrial agriculture, no such residues were detected ($n = 21$, LOQ = 0.1 mg/kg) (Bøhn et al., 2014). Subsequent research indicated that (a) soy from organic agriculture gave better growth, survival and reproduction in the indicator organism *Daphnia magna*, compared to conventional GM and non-GM varieties of soy (Cuhra et al., 2014). Furthermore, (b) subsequent testing demonstrated that when *D. magna* was fed diets made with soybean meal from Roundup-ready soybean, the biological parameters growth and reproduction were negatively correlated with the magnitude of glyphosate residues (in a 42-day experiment involving 300 animals allocated to eight separate diets with known glyphosate-residue concentrations, all below existing legal limits) (Cuhra et al., 2015). Furthermore, we found that glyphosate isopropyl amine salt (glyphosate IPA) in water, had 100-300-fold higher acute toxicity toward test-organism *D. magna*, as compared with industry studies using same species of test-animal and similar methodology of testing. And, in long-term studies we found that low concentrations (0.05–0.45 ppm) of either glyphosate (glyphosate IPA) or GBH Roundup formulation (contains glyphosate IPA as active ingredient) had adverse effects on growth and reproduction (Cuhra et al., 2013). Several problematic issues relating to existing assumptions on ecotoxicity of glyphosate were identified, amongst these the fact that glyphosates are a family of chemicals with distinctly differing physical properties and biological effects, notably levels of contaminants from different manufacturing processes, and basic properties such as solubility in water (Cuhra, 2015c). Also, numerous studies on toxicity and ecotoxicity of glyphosate and glyphosate-based herbicides were performed by commercial laboratories at a time when such research did not adhere to later quality requirements. Thus, it has been concluded that amongst the industry-funded studies providing data for the regulatory basis by documenting glyphosate safety, there are studies which should be reviewed and discarded as evidence of safety (Cuhra, 2015b,c).

The lack of relevant risk-assessment data may come from lack of valid studies, since research commissioned and funded by industry is found to ignore the question of herbicide use as well as residue levels in the plant material, and possible effects from these (Millstone et al., 1999; Viljoen, 2013; Cuhra, 2015b). Millstone et al. documented serious flaws in initial assessments presented by industry as evidence of safety: Most safety assessments had been conducted using herbicide tolerant plant material which was not sprayed with its belonging herbicide, and thus could not have the levels of glyphosate residues which would be expected under normal agriculture practice. Later, Viljoen confirmed this

to still be the case in most feeding studies performed to test the quality of herbicide tolerant GM plants. Our recent review of the issue highlights that not only are the test materials for research cultivated in artificial environments, but the question of glyphosate residues continues to be an ignored issue. We argue that such unfortunate gap in knowledge originates in a societal acceptance of industry autonomy and that the responsibility for providing data for safety-assessment studies is delegated to the producer of the product (Cuhra, 2015b). Only one of 30 reviewed studies was found to address the question of glyphosate residues, and that was a compositional study performed by us (Bøhn et al., 2014). Such methodological flaws in industry studies not only discredit and undermine the claimed substantial equivalence of GM cultivars, they also point to an insufficient regulatory oversight over knowledge gaps related to important safety-issues (Cuhra, 2015b).

Glyphosates and formulated GBH products such as Roundup have been subjected to a large number of studies: Researchers have investigated glyphosates and their role in industrialized farming practices, from various scientific disciplines and from a wealth of perspectives; agronomy (Duke and Powles, 2008; Benbrook, 2012, 2016; Bonny, 2011, 2014), socio-economy (Binimelis et al., 2009; Bonny, 2014), Ecology (Giesy et al., 2000; Samsel and Seneff, 2015b) and health (Williams et al., 2000; Samsel and Seneff, 2013, 2015a,b; Mesnage et al., 2015a). Few independent scientists (researchers not employed by industry) have voiced such univocal praise as the agronomists who published the initially mentioned commentary in which glyphosate is stated to be "a once-in-a-century herbicide," "a precious herbicidal resource" and a "unique ideal herbicide" (Duke and Powles, 2008). Some of these claims seemed justified at the time of writing, especially since these evaluations arose before the more recent; (1) findings of high levels of accumulation in food and feed, (2) findings of destructive outbreaks of glyphosate-resistant weeds, and (3) indications of complex toxic effects.

Hence, although glyphosate was initially found to be environmentally benign, to have low toxicity to farm workers and other non-target organisms, and to be biodegradable, several of these assumptions of the "unique ideal herbicide" have recently been scrutinized and questioned.

In addition, GBHs include a large diversity of herbicidal products, i.e., more than 750 formulated products are found on the market (Guyton et al., 2015), with unknown additive ingredients, making evaluation and testing even more difficult.

At present, the global database at www.weedscience.org has registered 32 different species of weeds tolerant to glyphosate (Heap, 2015). Arguably, the reaction to these recent challenges has partly contributed to increase the ecological challenges: we see that a main strategy applied by agroindustry has been to further develop technical and biological modifications of agriculture crops, in order to facilitate even higher application dosage of glyphosates as active ingredients in products (Cuhra, 2015b). This is increasingly affecting local biota and farming systems as application rates on individual fields increase, in an unsustainable spiraling development which should be evaluated carefully (Binimelis et al., 2009). Another approach is to combine tolerances to several herbicides in the same transgenic plant (Green, 2009).

Chemistry and History of Glyphosates

The common name "glyphosate" is used indiscriminately in published literature, denominating various chemical compounds that differ substantially from the glyphosate-IPA salt (chemical identity CAS# 38641-94-0), e.g., the technical grade glyphosate (CAS# 1071-83-6). Toxicological data for technical grade glyphosate are not relevant when assessing ecological effects of glyphosate herbicides, which contain water-soluble forms of glyphosate, e.g., the IPA-salt, as the active ingredient.

In this context we again find it relevant to highlight the types of glyphosate which are used in agriculture as active chemical ingredient in glyphosate-based herbicides (GBH). These are primarily glyphosate isopropyl amine, glyphosate ammonium, glyphosate sesquisodium, and glyphosate trimesium salts. It is these glyphosate-salts that are the primary glyphosate chemicals released into the environment and which are sources of residues or metabolites subsequently found in various feed- and foodstuff.

Different glyphosate compounds have slightly or profoundly different properties. An overview can be found at the PubChem online database (hosted by the US National Institutes of Health at https://pubchem.ncbi.nlm.nih.gov) presenting a synthesis of information on physical, chemical, and toxicological properties of chemicals. Glyphosates are pooled in Compound identity CID #3496. This entry includes glyphosate technical acid, but also various other glyphosate chemicals such as the isopropylamonium salts (IPA-salts), which are commonly used in commercial herbicides. The PubChem database also provides common synonyms and lists major producers of glyphosate, including a range of different glyphosate chemicals which these producers offer onto the commercial market. Links to hundreds of records on related compounds in the database present confusing information, especially as the commercially and environmentally important glyphosate salts obscurely are also listed in other subdivisions of the database.

Also, we notice that there are several independent systems for nomenclature of chemicals including glyphosates. The PubChem database employs CID-codes for chemical compounds. These are different from the universally recognized CAS-codes. Also, although US EPA documentation on glyphosates refers to CAS-codes, additional codes (e.g., internal codes and "Shaughnessy" codes) are used. Authorities such as the US Department of Labor use an altogether different nomenclature for glyphosate (OSHA–IMIS codes, in which glyphosate-IPA is given the identity "R107"). This diversity of codes results in confusing nomenclature which subsequently complicates scientific assessments and regulatory approvals.

The following examples illustrate the challenges for identifying correct type of glyphosate for testing: For many years (and to some degree still) the US EPA Reregistration Eligibility Decision (RED) on glyphosate (US EPA, 1993) has been the main document on glyphosate in the US administration and an important reference for assessment of potential effects on health and environment. However, the supporting technical dossier (Shaughnessy Case No. 0178) confuses the physical

properties of two different main glyphosate chemicals: The IPA-salt specification gives data on melting point, density, and water-solubility. Again, amongst these properties, the water-solubility is most important in a chemical intended to be diluted with water. However, the documentation presents the very low solubility of the glyphosate technical acid (at 10 g/l this is relatively insoluble and not relevant as an active ingredient in commercial formulations, in comparison the IPA-salt has solubility exceeding 1000 g/l). The RED is largely based on data provided by the industry manufacturer of the glyphosate chemicals (partly confidential information protected by national and international patents) and evidently has been compiled without the necessary differentiation between glyphosate forms.

Numerous published experiments on ecotoxicological effects of GBH in various species and environments have tested the glyphosate technical acid (the parent compound). However, studies on effects of glyphosate technical acid are not relevant for assessing the potential effects of the glyphosate active ingredient in herbicides. We argue that this is a possible explanation for the contradictory published results in specific species of test-animals and specific test-systems, presenting EC50 values which span several orders of magnitude (Cuhra et al., 2013).

Furthermore, analysis of glyphosate residues in environmental samples, food and feed, have quantified only "glyphosate" (as N-phosphonomethyl glycine) and the defined main metabolite "AMPA" (aminomethylphosphonic acid). The newest revision of central EFSA documents on glyphosate (EFSA, 2015a) begins to take these questions into account. The document specifies that the IPA-salt of N-phosphonomethyl glycine (glyphosate-IPA) is the relevant compound for assessment and also presents some details on other metabolites (N-acetylglyphosate (NAG), N-acetyl-AMPA), and impurities. As presented in the EFSA document, N-acetylglyphosate, and N-acetyl-AMPA are newly proposed to be part of the residue definition for monitoring and for dietary risk assessment. They occur in certain genetically modified plants such as soybeans or maize following application of glyphosate and were evaluated by EFSA with regard to setting of import tolerances. It was noted that formaldehyde may occur as an impurity and a content of 1 g/kg or higher in the active ingredient would result in a classification as a 1B carcinogen (EFSA, 2015a).

We find that the chemical and biological processes of glyphosate degradation are insufficiently documented and we expect that other potential metabolites and additional residues could also be of importance. Also, the break-down rates of glyphosates are relevant. Glyphosate and AMPA residues in samples of Roundup-ready soybean were analyzed two years after harvest. We found high concentrations of both chemicals (mean 3.3 mg/kg of glyphosate and 5.7 mg/kg of AMPA) (Bøhn et al., 2014), i.e., somewhat more AMPA (63% of the total) than glyphosate. This indicates that in stored seeds, glyphosate degrades slowly.

A classic and somewhat morbid joke states that five out of six scientists conclude, that Russian roulette is safe. The evidence on glyphosate safety is of this nature, as a majority of previous studies (before 2010) find that glyphosate is safe, contrasted by only a minority of studies which find that glyphosate causes harm. Returning to the metaphor of the revolver in the undoubtedly dangerous game of Russian roulette, an inspection would reveal that only one chamber is loaded with a functional cartridge, the others are blanks. Based on our review of published glyphosate safety assessments we conclude that the mentioned metaphor is highly relevant. We see that an important cluster of publications, which can be said to be at the core of evidence demonstrating safety of glyphosate herbicides, was not performed using the relevant type of glyphosate chemical. Thus, those safety assessments investigated "blanks," whereas a few supplementary studies have tested the actual glyphosate herbicide or the active ingredients correctly representative of the actual chemicals dispersed onto farmlands and into the environment.

We recommend focusing further on the studies which investigate representative glyphosate, instead of concluding from studies that have investigated the parental compounds of glyphosate. Regulatory authorities must be capable to separate real bullets from blanks when assessing evidence for risk-assessment. Only the effects of real bullets are relevant.

Toxicity and Ecotoxicity of Glyphosates and GBHs

Roundup and similar formulated glyphosate herbicides contain various adjuvants and inert ingredients. We have described some of the confusion that enshrouds ecotoxicological and toxicological assessments of these compounds, which are seen as significantly contributing to toxicological properties of formulated herbicides (Cuhra et al., 2013; Cuhra, 2015c). Recognizing the inherent complexity of assessing compounds which are protected commercial products and which have properties known to producers, but partly unavailable to scientists and regulators, we suggest that all ingredients in herbicide formulations should be regulated and subject to mandatory declaration. Present regulation allows producers of formulations to simply declare various additives and adjuvants as "inert ingredients," although such GBH-compounds were initially recognized to have biological and toxicological effects in non-target organisms (Folmar et al., 1979).

The best-known GBH products are Roundup formulations that contain additional surfactants, chemical adjuvants. Recent papers have reviewed published literature on GBH-formulation toxicity (Mesnage et al., 2014, 2015a). Typically, Roundup contains glyphosate as IPA-salt, polyethoxylated tallow amine (POEA) and additional substances. These adjuvants may in some cases be more toxic than the glyphosate active ingredient itself (Howe et al., 2004; Peixoto, 2005). The phenomenon of potentially higher toxicity in formulated herbicides, as compared to the active ingredient only, is documented for glyphosate-based herbicides as well as for a number of other herbicide active ingredients (Mesnage et al., 2014). Recent evidence indicates that glyphosate has complex toxic effects (Samsel and Seneff, 2015b) and supports the hypothesis that co-formulants to glyphosate in Roundup are endocrine disruptors in human cells (Defarge et al., 2016). Relative to this, our ecotoxicological comparative testing of glyphosate (IPA-salt) and Roundup "Weed & Grass Killer Concentrate Plus" in *D. magna*, has shown that the

active ingredient and the formulated product have approximately the same acute toxicity (short-term), although the formulated product did produce more severe effects in long-term exposure (life-long) (Cuhra et al., 2013).

GBH (Roundup) has been shown to disturb male reproductive systems through Ca^{2+}-mediated toxicity, oxidative stress and disruption of signaling mechanisms in rats (Cavalli et al., 2013). This also happened at concentrations below what farm workers typically are exposed to Cavalli et al. (2013). Further, both acute and chronic exposure to Roundup may cause oxidative stress and neurotoxicity in rats (Cavalli et al., 2013; Cattani et al., 2014), justifying claims of being a neurotoxic hazard also for humans (Malhotra et al., 2010; Grandjean and Landrigan, 2014). Some evidence of arrhythmic and cardiac electrophysiological changes mediated by GBH also indicate cardiovascular risk to animals and humans (Gress et al., 2015).

A recent study investigated gene expression changes in rats after long-term exposure to Roundup at very low concentrations (0.1 μg/kg) in the drinking water. The results showed that 263 genes from kidney and liver had a fold-change >2, indicating liver and kidney damage and potential health implications also in other animals including humans (Mesnage et al., 2015b). Roundup, but not "pure glyphosate" (not clarified what type), was shown to cause endocrine disruption in Leydig cells (Walsh et al., 2000), indicating significant activity in other components of formulations. An additional recent review by Mesnage et al. summarizes further evidence that Roundup at or below regulatory limits may be toxic or cause teratogenic, tumorigenic, and hepatorenal effects (Mesnage et al., 2015a). Such effects can be linked to endocrine disruption and oxidative stress (Gasnier et al., 2009).

Glyphosate Mode-of-Action

The herbicidal properties of glyphosate (N-phosphonomethyl-glycine) inhibit biosynthesis of chorismate from shikimate (Amrhein et al., 1980), thereby lethally disrupting photosynthesis and plant cell metabolism. It has been claimed that since only plants (and some lichens and microorganisms) have the 5-enolpyruvylshikimic acid-3-phosphate synthase metabolic pathway (EPSPS pathway) defined as glyphosate target-site, only such organisms can be expected to be targeted by toxic effects of this chemical (Duke et al., 2012). Arguably, such general deduction of safety toward non-target organisms is scientifically unfounded. It is not justified to assume that specific chemicals have only one mechanism or mode-of-action in ecosystems, biota and species. Toxins can interact with numerous biochemical processes in cells, tissues, and organs of various organisms.

Published Evidence on Glyphosate and Safety

A brief database search on term "glyphosate," alternatively the term "glyphosate" combined with term "safety" or term "risk" determined by Boolean operator "AND" and "OR" via the Google Scholar search engine yields data presented in **Figure 1**.

The total number of peer reviewed scientific articles and related posts such as technical reports and patent documents on "glyphosate" published 1965–2014 (search date 24/09/2015) is found to be 62.200. Using at least one of the terms "safety" or "risk" in addition to "glyphosate" returns 20.900 scientific articles and related posts. These total figures on glyphosate are found to be comparable to the available evidence on herbicide atrazine and insecticides malathion and dieldrin determined by similar searches using same search-terms and conducted in the same period (**Table 1**).

The annual total publications on "glyphosate" are visualized in **Figure 1**. We extracted data for each year from 1970 to 2014, thus covering glyphosate research over 45 years. The quantity of publications on glyphosate rise exponentially (gray line) to the present level of 9.435 registrations in 2014. Although there are some fluctuations in the rates (percentages) of safety-related studies (dotted curve), the general tendency over time is that there is an increasing proportion of glyphosate-related publications which satisfy the related search terms "safety" and/or "risk." This brings us to conclude that safety and risk are relevant terms in present and recent research on glyphosate, as reflected by the indexed publications.

We have highlighted some of the studies which have been performed by chemical industry (A), the period of patent applications and first safety studies by independent researchers (B) and the time of introduction of GBH- tolerant transgenic crops (C), as this development has been identified as a most important single factor accelerating demand for GBH. Also, two important reviews (Giesy et al., 2000; Williams et al., 2000) were published around the time when several important national and international patents on glyphosates expired (D). The reviews are syntheses of evidence available at the time, notably including data and conclusions from numerous studies performed by industry. These industry reports had been reviewed by US authorities (EPA and FDA) but were recently found to lack the standards of peer reviewed studies (Samsel and Seneff, 2015b; Cuhra, 2015c). In addition, the actual reports from the laboratory work, specification of methodology, chemistry etc, have previously been inaccessible for independent verification, due to commercial interest. Several important studies were subsequently published by independent scientists (not affiliated with industry) presenting findings on higher toxicity in test animals and environment, and thus challenging the previously accepted view of negligible toxicity toward non-target organisms. Series of new findings also focused on effects in aquatic environments, finding evidence of higher toxicity toward amphibians (Relyea, 2005) and

TABLE 1 | Search results as number of publications 1965–2015 on four pesticide active ingredients (a.i): herbicides glyphosate and atrazine, insecticides dieldrin, and malathion.

a.i	Scholar	Scholar ++	PubMed	PubMed ++	Science Direct
Glyphosate	62.200	20.900	2021	19	7.061
Atrazine	55.500	21.900	3595	19	12.172
Dieldrin	27.800	20.100	3337	14	10.161
Malathion	32.900	19.200	3235	18	8.534

Databases: Google Scholar (http://www.scholar/google.no), PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and Science Direct (http://www.sciencedirect.com). Additional search limited by term "safety" or "risk" (++).

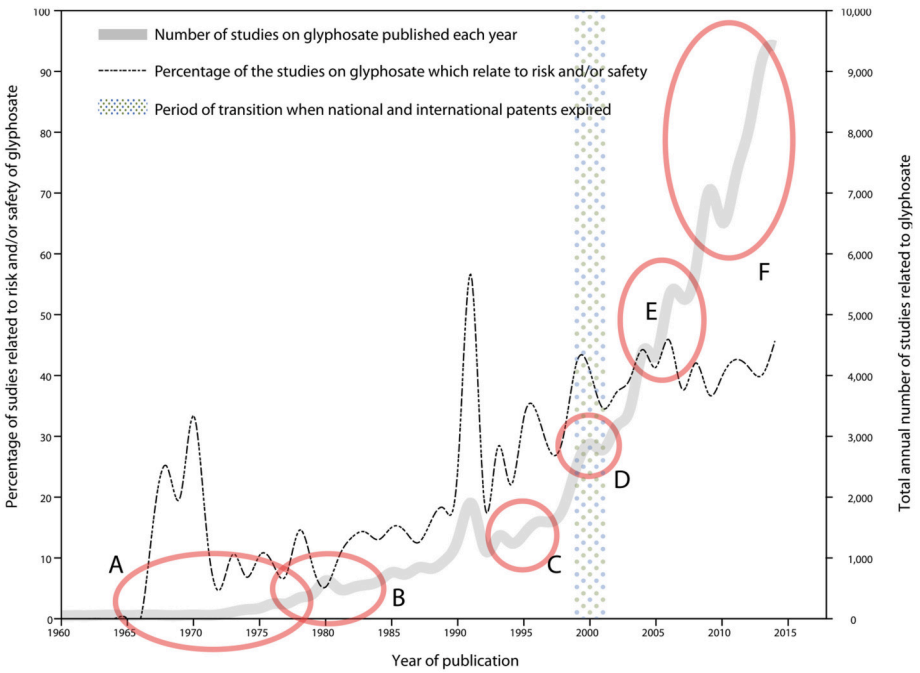

FIGURE 1 | Glyphosate research and development. A: Period of development, approval and patenting. B: Initial safety assessments. C: Advent of glyphosate-tolerant-crops. D: Several important reviews on health and ecotoxicology documenting low toxicity. E: Emerging evidence on crop damage, residue accumulation and impact on non-target organisms. F: Evidence on complex effect.

invertebrates. We have previously presented a small review of this evidence (Cuhra et al., 2013).

Resistant weeds and the lowered costs of GBH in recent years (after the patents expired) have led to crops being subjected to increasing application rates of glyphosates. Studies on glyphosate have investigated effects on soil microbiomes (Kremer and Means, 2009) and as an important parallel, on gastrointestinal communities in consumers (Samsel and Seneff, 2013). Also, a review of rodent studies and analysis of common commercial types of feed formulations for laboratory animals, have disclosed that such feed to a surprising extent is contaminated by various toxins and pesticide residues, including glyphosate (Mesnage et al., 2015c). These contaminations can affect the controls in the experiments and induce *false positives* as well as mask relevant effects. Thus, such feed-quality issues are a serious setback for the entire analytic community which depends on trustworthy data from rodent studies. The findings indicate fundamental systemic defects in rodent studies in general.

Historic Data on Glyphosate Non-Toxicity

For regulatory assessment of glyphosate health effects by US Food and Drug Administration (US-FDA) and parallel regulatory assessment of glyphosate ecotoxicity by US Environmental Protection Agency (US-EPA), the manufacturer carried out a wide range of laboratory testing in 1975–1985, in the first decade following its introduction. The tests were primarily performed by private subcontracting analytical laboratories according to established protocols at that time and submitted as evidence for regulatory assessment. Archive reports of numerous of these tests have been accessed but will not be discussed in detail here, other than noting that this archive base is not of peer-reviewed standard but rather has three levels of quality control; (a) at the performing laboratory, (b) at the commissioning industry, and (c) at the regulatory authority.

Through FOIA requests (FOIA, 2009, 2011) we have re-evaluated specific documentation extracted from the archives of the US Environmental Protection Agency (US EPA) and demonstrated faults in historical data assessing glyphosate toxicity in aquatic invertebrates. For example conclusions had been changed, regulatory importance exaggerated and wrong type of glyphosate had been tested (Cuhra, 2015c). Further investigations into industry safety-studies on glyphosate disclose notable early indications of carcinogenicity in rodents, albeit in high doses. A 2-year industry feeding study of glyphosate technical acid in rat showed significantly heightened incidences of tumors in high dose groups (US EPA, 1983). The industry applicant reported this to the US EPA including raw data and documentation. In a string of memos and letters these results were discussed internally in the EPA. Following (a) grouping of adenoma and carcinoma detections in treatment groups and in controls, and (b) re-evaluations of original histological slides from organs and tumor tissue, it was concluded that the incidence of tumors in treatment groups was not higher than controls (US EPA, 1983). Aspects of this case have recently been reviewed (Samsel and Seneff, 2015a) although the authors do not exhaustively discuss the role of the regulatory interpretation. A parallel case was highlighted in a 1985 internal EPA memorandum *on false positives* which points out methodological faults and mistakes in a 24-month study of glyphosate in mouse (unspecified type of glyphosate) submitted

as evidence of safety to the EPA (US EPA, 1985). The main EPA argument against the interpretation presented by the applicant relates to the cancer incidence in the control group, which was claimed to be comparable to the treatment groups. The internal EPA memorandum explicitly states that the industry applicant interpretation should be overruled. The EPA conclusion from the statistical review is that the data demonstrates that glyphosate at 5000 and 30,000 ppm levels in feed, induce renal tubular adenomas.

Thus, we see buried historical evidence from two long-term studies of glyphosate in rodents, indicating carcinogenicity. The main reason these studies failed to achieve regulatory importance, seems to be the approval process conducted internally in the US EPA, which re-interpreted data and modified conclusions. It must be noted however, that both of these studies used high dosage of glyphosate and thus are not indications of tumorigenicity at lower dosage, such as found in a controversial recent two-year rat study with a glyphosate commercial formulation given in drinking water (Séralini et al., 2014).

GBH Aimed at New Targets

Agrochemicals such as GBHs will affect both the quality of agricultural products as well as the surrounding environment, notably as chemical residue levels in agricultural commodities and as impact on non-target organisms respectively.

Glyphosate was originally developed and patented as a broad-spectrum herbicide, with disrupting activity and lethal effects toward a broad spectrum of plants with active photosynthesis. Therefore, the use of glyphosate was restricted to pre-plant clearance of agriculture fields or forestry. In those early days of glyphosate use, the GBHs were not applied onto growing crops and thus the question of residues accumulating in plant material intended for consumption was less relevant. Furthermore, it was assumed that plants do not take up glyphosate from soil and thus even if a soil reservoir of un-metabolized glyphosate had been allowed to build up in agriculture soils, it was not perceived to be a problem. However, two developments in modern industrial agriculture have brought new challenges: (1) transgenic bypassing of the vital plant EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) metabolic pathway, which is specifically targeted by glyphosate, allows for herbicide application onto growing crops, and (2) increasing use of GBHs as desiccants to kill and force-ripen semi-mature crops. Both of these developments have resulted in substantial amounts of GBHs being applied onto the crops intended for feed and food use. Thus, (1) the target organisms must be re-defined, and (2) the chemicals intended to eradicate weeds, have increasingly found a way into the food and feed supply of consumers.

In 2012 EFSA, the European Food Safety Authority, proposed a dramatic relaxation (increase) of maximum residue levels (MRLs) of herbicide glyphosate in lentils. The MRL for glyphosate in lentils was 0.1 mg/kg in the EU. The proposal aimed at raising the MRL for glyphosate in lentils to 15.0 mg/kg or alternatively 10.0 mg/kg, effectively by a factor of 150 (or 100) from the existing level. The proposal was submitted by the rapporteur member state Germany to the European Commission for approval on behalf of the applicant, Monsanto Europe (EFSA, 2012). The background for the proposal was findings of high residue levels of glyphosate in lentils grown in Canada. Residue levels ranging from 0.5 to 4.17 mg/kg were reported by the applicant, with one extreme high value of 8.8 mg/kg driving the proposed MRL target value of 15.0 mg/kg. At the same time when the application was submitted, a notification of food withdrawal from market was given by EU member state the Czech Republic, based on our detections of 10.5 mg/kg of glyphosate in lentils originating from Canada, which were confirmed by analysis of lentil samples taken from the Czech market (RASFF, 2012). This indicated that glyphosate residues in Canadian lentils occur at even more extreme values than envisaged in the requested relaxation of MRLs.

A communication from the Agriculture and Rural Development Department, Government of Alberta, Canada, describes the common practice of pre-harvest application of glyphosate to lentils in Canada as desiccant and recommends that the practice be terminated in harvest batches intended for export to the European Union (Agri-News, 2011). The newsletter discloses that glyphosate application immediately before harvest is widely used by farmers to force-ripen the lentil seed and though this practice is not estimated to conflict with the relatively relaxed MRLs in Canada, it will produce residues higher than the former MRLs for glyphosate in lentils in the European Union.

In the EU there seems to be lack of focus on the evidence of glyphosate use as desiccant and ripener in agriculture. Anecdotal evidence from rural areas in Denmark indicates that GBHs (Roundup) is routinely being used for ripening of wheat, and the practice is well-known from Germany; " (...) in der EU seit einigen Jahren vermehrt Herbizide zur Sikkation von Erntebeständen, insbesondere von Getreide, Kartoffeln, Raps und Hülsenfrüchten, eingesetzt werden. Bei dieser Methode werden Herbizide kurz vor der Ernte direkt auf die zu erntenden Kulturpflanzen gespritzt. Das Totspritzen, wie die Sikkation treffender bezeichnet werden sollte, erleichtert durch gleichmäßig abgestorbene Pflanzen die Ernte (...)" (Brändli and Reinacher, 2012).

Further German studies (Haalck and Reinken, 2010) provide details on the practice of "Totspritzen" and document that a wide variety of herbicides in addition to glyphosates, such as glufosinate-ammonium, diquat, carfentzarone, cyanamid, cinidon-ethyl, and pyraflufen are used for this killing and forced ripening of crops.

The European Union maximum residue levels for glyphosate in barley grain are 20 ppm. For barley straw, the MRL is 200 ppm. These high MRLs are set to accommodate the use of glyphosate as desiccant in farming of barley. The main issue here may have implications far beyond the practicalities concerning the European Union maximum residue levels for glyphosate in lentils or barley. We find it disturbing that dominant agricultures are developing in such a way that toxins are used rather indiscriminately in order to ease harvesting. This use of herbicides is non-essential and from the perspective of both health of environment, hazardous. Again, here we see a development which contributes to the increasing total load of

pesticides, and glyphosate in particular, into biota, fields and consumer organisms (**Box 1**).

Residues in Plants and Food/Feed Products

Recognizing the fact that consumers are ingesting more glyphosate residues via our food supply, it is also relevant to review this exposure. Bio-active herbicides interact with biomass and ultimately get into soil and water systems through processes such as drifting, leaching, and surface runoff (Mensah et al., 2012). Glyphosate is present in ground water, human and animal urine, human breast milk, and farmed-animal flesh (Borggaard and Gimsing, 2008; Krüger et al., 2013, 2014; Honeycutt and Rowlands, 2014; Niemann et al., 2015). Thus, potential interaction with other stressors in biological systems or in the environment need to be studied in more realistic settings (Then, 2009; Nørgaard and Cedergreen, 2010; Bjergager et al., 2011). Glyphosate or GBH should not be evaluated or discussed in isolation. In organisms and biota exposure to glyphosate will co-occur with exposure to other pesticides. Monitoring programmes generally detect more than 7-8 different pesticides in single samples from the environment, and cocktails of multiple pesticides are routinely present in foods and feedstuffs (EFSA, 2014). In spite of that, current testing regimes for relevant agrochemicals are predominately based on acute exposure (short term) and specific testing of isolated single chemicals (Martin et al., 2003; Nørgaard and Cedergreen, 2010). Studies of combinatorial effects of multiple toxins are however increasingly acknowledged as missing (van Haver et al., 2008; Al-Gubory, 2014).

In the late 1997–1999, levels of 1.9–4.4 mg/kg glyphosate was found in Roundup Ready soy plant parts other than the grains, and 0.1–1.8 mg/kg was found in the grains (Arregui et al., 2004). A study from the US noted that repeated herbicide applications increased the residue levels of both glyphosate and AMPA in the soybeans. At three applications the highest residue level found was at 3.08 mg/kg for glyphosate and 25 mg/kg for AMPA (Duke et al., 2003). Thus, applications closer to time of harvest induce relatively high residue levels in the soybeans, leading to high residues in commodities.

The scarcity of published data on glyphosate residues in glyphosate-tolerant crops such as Roundup-ready soybean is unfortunate. In this situation estimates must be based on the few existing data: an earlier publication from Duke et al. (2003), our recent data from USA (Bøhn et al., 2014) and Argentina (Then, 2013). Data presented in Cuhra (2015b, Figure 1) are recalculated from Bøhn et al. (2014) and shows AMPA as glyphosate equivalents conforming to the FAO standards of the data presented by Then (2013). Average glyphosate-equivalent residue concentrations are 11.87 mg/kg in tests of soybeans from USA and 39.87 mg/kg in tests of soybeans from Argentina. These *average* concentrations are in compliance with the maximum residue levels defined by the US FDA (40 mg/kg) and the results from USA are also in compliance with the EU MRL of 20 mg/kg. However, individual samples from Argentina exceed current MRLs.

BOX 1 | GLYPHOSATE FACTS

Facts 1: Global Omnipresence

- Glyphosate herbicides (GBHs) such as Roundup have been on the market since 1975 and their use is still increasing, making GBHs the primary category of pesticides world-wide. By volume and revenue, GBHs are globally dominant.
- Glyphosate is detected in water, air, animal feed, animal urine, and animal flesh. Glyphosate is also found in human food, human milk, and human urine.

Facts 2: Higher dosage and increased ingestion

- Regulation of glyphosate has gradually been relaxed, allowing for increasing maximum residue limits in important food and feed commodities.
- GBHs are used for late-season application and pre-harvest desiccation. Such practices cause high residue levels.
- Animal- and human consumer ingestion is increasing due to higher residue levels in food and increasing number of glyphosate sources.

Facts 3: Safety-assessements are flawed

- Reviews of older safety assessment studies of glyphosate have uncovered flaws and misinterpretations in the regulatory base.
- Numerous safety assessments have been performed with glyphosate technical acid instead of the glyphosate salts actually used in GBH herbicide formulations.
- Lack of labeling and low traceability of food/feed, combined with unknown levels of glyphosate in such biomass, is prohibitive for research on effects in consumers.

Facts 4: Recent developments

- Recently, regulators such as the EFSA have reduced the annual frequency of analysis for glyphosate residues in food and feed, giving glyphosate lower priority
- New research indicates that glyphosate should be recognized as having potentially more complex and severe effects on health and environment than previously assumed.
- Other research upholds that since humans and animal consumers do not have the EPSPS photosynthesis pathway, they will not be affected by glyphosate.

In comparison to the level of glyphosate in crops, other pesticides are typically found in much lower concentrations. For example, in US soybeans we found Fluazifop-P (0.078 mg/kg, one sample "Roundup-ready"), malathion (0.02 mg/kg, one sample "conventional"), and dieldrin (0.002 mg/kg, one sample, "organic"). In pooled samples alpha-endosulfane, trans-nonachlor, and trans-chlordane was found at levels close the detection limit of 0.05 μg/kg (Bøhn et al., 2014).

Thus, there are striking concentration differences between glyphosate and other pesticides in food and feed crops. Contrary to other pesticides that are measured in low ppm or ppb levels, glyphosate is detected at ppm-levels, orders of magnitude higher.

Given the very large quantities of soybean material produced, it is relevant to calculate or estimate the total amount of residues thus transported and mediated to consumers (mainly farm animals). The majority (82%) of global soybean production stems from glyphosate tolerant soy (James, 2014). The total global production in the 2013/14 growing season, was estimated to be 320 million ton (USDA, 2016), of this 290 million ton is estimated to be cultivated in glyphosate-tolerant varieties (Roundup ready

soy). Based on the findings of residue concentrations in US soybeans (11.87 mg/kg glyphosate-equivalents) the quantity of glyphosate residues which are accumulated, translocated, and consumed via glyphosate tolerant soy, is ∼3440 ton. Recalculating by using the data from Argentina (39.87 mg/kg glyphosate-equivalents), this figure could be as high as 11560 ton (Cuhra, 2015a). However large it may seem, this quantity is just a fraction of the total load of glyphosate herbicides applied in soybean cultivation. Exact figures are difficult to obtain, not least since the cultivation of transgenic soybean is continuously expanding and application rates of glyphosate active ingredient are increasing. Based on data from the US Soybean association, the USDA, the Penn State University online Agronomy-Guide and similar readily accessible sources it is not unreasonable to use production figures of 2.5–2.9 ton/ha for present soybean yield and estimates of 90 Million hectares for the total area currently in global cultivation with glyphosate tolerant soybean. Based on the same sources, realistic seasonal application rates for glyphosate herbicides are likely not <1.7 kg active ingredient per hectare, probably closer to 2.5 kg. A conservative estimate can be based on the USDA maximal single-pass application rate of 1.5 lb/acre, and total area in cultivation. With one seasonal pass of maximum allowed application, the total quantity of glyphosate active ingredient applied on glyphosate tolerant soybean globally would be in the magnitude of 153,000 ton. This would indicate that roughly 14% of the global production of glyphosate is used in agriculture of glyphosate-tolerant soybean. Assessing the application figures via the available production data for soybean yield, the estimates are similar, ∼140,000 ton. These figures indicate that 2–7% of the applied glyphosate active ingredient is accumulated in the soybean commodity. This represents a sizeable amount of pesticide residues directed at consumers, via the herbicide tolerant GM crops. We argue that regulators/governments need to respond and re-evaluate the potential human and animal health risks from this exposure.

Duke et al. (2003) found a low glyphosate/AMPA ratio in soybean following late application. The data on residues in Roundup ready soybean from Iowa show a similar trend (Bøhn et al., 2014). However, the glyphosate/AMPA ratios in analyzed samples were found to be inconsistent. Glyphosate is known to interact with biochemical processes in metabolism of transgenic glyphosate tolerant plants (Zobiole et al., 2011). The scarcity of published data on glyphosate residues accentuates the relevance of further investigating the dynamics of glyphosate degradation and transformation in plants. EFSA noted in the annual monitoring report (EFSA, 2015b) that for certain pesticides covered by the 2013 European coordinated monitoring programme (EUCP), including glyphosate, the number of determinations reported was significantly below the number needed to derive statistically sound conclusions. In comparison to e.g., some pyrethroids or organophosphates, the number of analyzed samples for glyphosate was ∼25 times lower (chlorpyriphos–70943 samples, glyphosate–2866 samples). The reason is that glyphosate is impossible to include in multi-residual methods as it requires the application of a single and specific method, which is expensive, demanding, and time consuming. Only a limited number of laboratories are able to perform it. For the same reason, not only the number of samples, but also the number of commodities involved in monitoring programmes (and thus also in risk assessment) are limited. Analyses were performed on a limited set of commodities (e.g., apples; oats; rye; wine, grapes, wheat) in the EU monitoring programme in 2013. In spite of this, a total of 7.9% of samples were glyphosate positive (i.e., above LOQ). In some commodities, high ratio of glyphosate positive samples were found—e.g., for oats, 44% of samples were found as positive. According to the EFSA, reporting countries should extend the scope of the analytical methods used for enforcement of MRLs to make sure that the detection rate and the MRL exceedance rate is not biased by the low number of determinations or lack of data from certain countries. It is clear, that at present there is lack of reliable and representative results for most of food commodities in the food basket. In addition, the main metabolite AMPA is not included in coordinated EU monitoring programme.

Accepted Levels of Glyphosate Residues

Regulatory threshold of accepted levels of glyphosate residues are continuously being raised. At present the maximal residue levels (MRLs) of glyphosate in soybean in the USA has been increased from 20 up to 40 mg/kg in the fall of 2013. Again, we accentuate that such ppm-levels are high when compared to other pesticide active ingredients such high MRLs should only be accepted for compounds with very low toxicity. Review of regulatory documents such as the US EPA (1993) RED on glyphosate shows that such MRLs are defined pragmatically; to accommodate existing residue levels and existing agriculture practice (US EPA, 1993). Furthermore, we find that even the recently raised acceptance levels will not be enough for the concentrations of residues found in the transgenic soybean material tested in Argentina.

The global annual soy production equals ∼43 kg per capita. Of this quantum, ∼39 kg is from glyphosate tolerant varieties. Direct human consumption of soy is minimal as the majority of the global production at present is utilized in production of feed for farmed animals. Many species of farmed animals (cattle, poultry, pigs, fish, prawns etc) are fed diets with a considerable proportion of soy. Such feeding is daily and throughout the whole life span. This fact alone accentuates the relevance of adequate testing for chronic exposure to, and potential effects from, glyphosate residues. A recent report of glyphosate residues in aborted and malformed piglets from sows in intensive animal farming is remarkable albeit inconclusive (Krüger et al., 2014). Although this important indication necessitates further research, we note that due to faults in methodology, lack of a proper control group, and missing information on feed composition, the reported abortion rates and malformations cannot be irrefutably linked to lifelong feeding with GMO ingredients containing normal levels of glyphosate residues.

Environmental Impact Quotient (EIQ) of Glyphosates

Herbicides and other chemical substances intended for dispersal into the environment are evaluated for unintended and

undesirable effects in indicator organisms representing non-target species. The results of testing is extrapolated to other taxonomic groups and extended to ecosystem levels, thus providing information for regulatory decisions. Furthermore, indicators from e.g., oral ingestion in representative test species, dermal exposure, inhalation, and cell culture studies are amongst the indicators important for assessment of effects on human biology. Kovach et al. (1992) established the Environmental Impact Quotient for pesticides (EIQ) as a measure to condense into one indicative denominator the relative toxicity of specific chemical compounds, by collecting fragmented evidence on effects in a variety of indicators. Main components of the EIQ are three categories of effects defined as; Farmworker component, Consumer component, and Ecological component. Such relative indicators can be used as general comparators and the process involved in determining the EIQ of a novel pesticide compound can in itself be a useful exercise for regulators and stakeholders. However, the validity of such a relative indicator is dependent on regular revisions of the basis, the scientific evidence, which supplies numerical values to the individual factors in the equation from which the quotient is calculated.

The Dynamics of Pesticide Regulation

In a time with considerable confusion regarding possible toxic effects of glyphosate herbicide toward health and environment, with contradictory findings on potential impacts and strong voices arguing on one side for precaution, on the other side incentives for continued high volume use of a chemical, we find it useful to mention the key elements which constitute the basis for regulation. In a commercially driven market economy, the dominating societal model in the world, industry interests seek to market and employ products. Some products may have unwanted consequences. In general, the regulation of potentially toxic chemicals, e.g., pesticides, is largely based on scientific information produced by the industry which often has strong financial incitement for unrestricted use. Thus, in society there is an antagonistic tension between commercial vs. public interests concerning the regulation of global and local application of e.g., glyphosate herbicides. This leads to a dynamic interplay driven by two main vectors, of which one represents commercial forces (in this case primarily manufacturing chemical industry and farmers), and the other represents societal interest (health, environmental protection, qualitative requirements) (Cuhra, 2015c).

The arguments supporting and enhancing the opposing vectors, are furnished by scientists and other professionals working within private sector research firms, in publicly funded university laboratories, in regulatory authorities, as consultants or in non-governmental organizations representing defined interests. All of these, we commonly call "experts." Resulting policy should be a careful balance of these expert opinions, based on factual findings from e.g., laboratory testing (**Figure 2**). Thus, when scientific evidence shows that a compound or groups of compounds has low toxicity for consumers and environment, restrictions on use are relaxed (society accepts more). However, in the opposite case, if science demonstrates that compounds are more toxic than previously assumed, their penetration into environment and food chains should be reduced through regulatory measures (society accepts less,—such as in the cases of DDT and PCBs).

In a previous commentary, we have reflected upon the quality of evidence supporting the notion of glyphosate non-toxicity, finding that serious flaws confuse the current regulatory basis (Cuhra, 2015c).

Socioeconomic Aspects

Important societal challenges related to production of glyphosate-tolerant crops such as Roundup-ready soybean include ecological damage through deforestation and degradation of natural habitats (Pengue, 2005) and glyphosate pollution of the environment. The large-scale cultivation of glyphosate-tolerant crops, such as Roundup-ready soy (RR-soy), RR-maize, and RR-canola has also been identified as a main cause for emergence and widespread occurrence of numerous glyphosate-resistant agricultural weeds (Duke and Powles, 2008; Benbrook, 2012). The weed-challenges will be met with alternative and more potent mixes of herbicides (Green, 2009), whereby older and arguably more toxic herbicides, such as atrazine, may be reintroduced (Binimelis et al., 2009). This development has been linked to increased occurrence of severe medical problems in farmers and farm village populations in Argentina, in areas where Roundup-ready soybean is produced (Vazquez and Nota, 2011).

Here, the evolution of glyphosate use and risk-assessment has been defined as five distinct periods (each a decade) following the discovery and commercialization of glyphosate

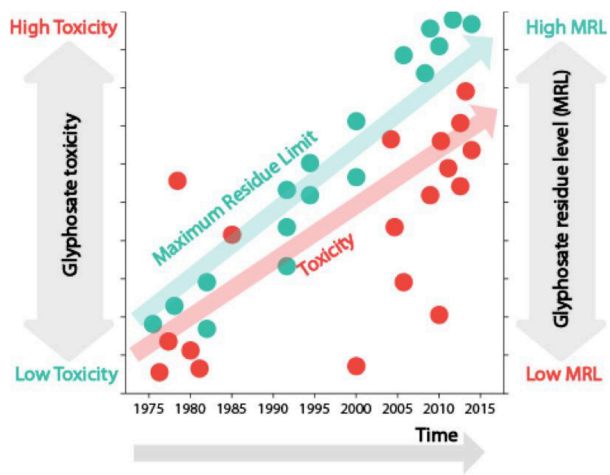

FIGURE 2 | Conceptual model of glyphosate toxicity and maximum residue level (MRL) over time. The paradox of glyphosate regulation. Red dots symbolize evidence of toxic effects, measured as a relative denominator defined in the left y-axis "Glyphosate toxicity." The red arrow shows the trend over time, as more evidence demonstrates higher toxicity. Green dots symbolize acceptance (MRLs) on a relative scale (the right side y-axis). Green arrow shows trend over time, as MRLs are increased. In science-based policy evidence of higher toxicity should lead to lowering of acceptance levels (MRLs). In the case of glyphosate, the development is the opposite: increasing acceptance is positively correlated with toxicity.

in 1975 (**Figure 3**). The first decade (1975–1985) represents "glyphosate optimism." Glyphosate was discovered as a very efficient herbicide, with a systemic action on a broad spectrum of agriculture weeds. At that time, glyphosate was perceived to have very low toxicity toward users, non-target organisms and consumers of agriculture produce. The following two decades (1986–2005) saw global implementation of glyphosate based herbicides such as Roundup and a dramatic increase in glyphosate use. The introduction and successful commercialization of several glyphosate-tolerant genetically modified crops in 1995 was a development later identified as the most important factor accelerating the use of glyphosate herbicides (Charles, 2001; Duke and Powles, 2008; Benbrook, 2012, 2016). However, although the use of glyphosate has accelerated even further in the following decade (2006–2015) this has also been a decade of increasing and sobering challenges, notably caused by the advent of tolerant and resistant weed species, globally disrupting the efficacy of this agrochemical system. We define this latest decade "the decade of glyphosate skepticism" in our model. Numerous research programs, reviews and laboratory findings have documented that the safety assumptions of glyphosate are mature for revision. The decade culminated with a string of published evidence in 2015 detailing the challenging issues (Mesnage et al., 2015a; Samsel and Seneff, 2015b) even concluding that glyphosate should be categorized as a probable carcinogen (Guyton et al., 2015), in contrast to previously accepted conclusions concerning these chemicals. EFSA recently reviewed the evidence of glyphosate carcinogenicity and concluded that glyphosate is not a carcinogen (EFSA, 2015c). Other research in 2015 indicated that previous assumptions of safety, have in part been based on flawed evidence or misinterpretations (Cuhra, 2015c; Samsel and Seneff, 2015a).

Future Developments

Agricultural industry in general depends on more-or-less toxic pesticides. This is a generally accepted normality for conventional agriculture, which has developed gradually since the latest great war (Alston et al., 2010) and now constitutes an "agroecological-prison-situation," in which pesticides and other chemicals are now unavoidable in order to make industrial farming cost-effective. Thus, farmers are trapped and dependent on a combination of selected seeds and selected poisons.

Despite the challenges associated with both the continued use of glyphosate as the principal herbicide and the continued cultivation of glyphosate tolerant crops, there are few attractive chemical-biotechnological alternatives at present. Several crop varieties tolerant to herbicidal chemicals glufosinate-ammonium, dicamba, and 2,4-D are currently either in development, awaiting approval or already on the market. But, it is still an unresolved issue whether these crop varieties and agrochemical systems (which are relying on "old" herbicide technology) are as efficient, cost-effective or "better or worse" for the receiving environment, as the existing glyphosate-tolerant varieties currently available.

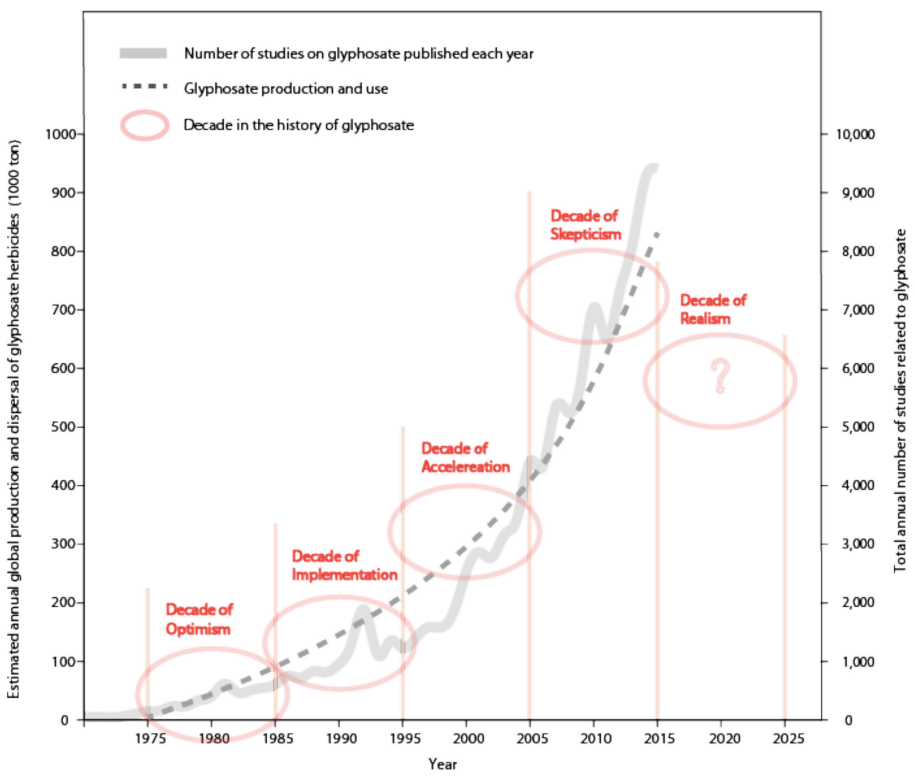

FIGURE 3 | Concept model to visualize how societal perception of glyphosate has evolved through five decades (1975–2025), related to trends in glyphosate use (Benbrook, 2016) and increasing annual rate of publications on glyphosate.

Despite the aforementioned challenges posed by glyphosate-tolerant GMOs, several large biotech firms are now releasing "second-generation" glyphosate-tolerant cultivars touted as being even more efficient. Developing a new herbicide and getting it approved for use is very costly. According to some estimates, the financial investments of industry can amount to US $180 million and the regulatory approval can take a decade (Smith et al., 2008; McDougall, 2010). Furthermore, it is challenging for industry to meet societal demands in such developments; new compounds are expected to have high target specificity and low general toxicity (for the environment, the users and the consumers of agricultural commodities). The biotech-agrochemical industry therefore adheres to two general strategies: it develops and registers new transgenic cultivars and chemical compounds for the market (ISAAA, 2014); and it uses existing chemical compounds in new ways, notably through introduction of transgenic varieties that tolerate higher doses of approved agrochemicals such as glyphosate (e. g., Cao et al., 2012, 2013). The role of glyphosate herbicides can therefore be expected to remain predominant in global industrial agriculture, especially in cultivation of glyphosate-tolerant varieties. As such, it is relevant to consider the possible benefits vs. challenges associated with continued or increased glyphosate use.

Returning to the history of glyphosate as depicted in **Figure 3**, we suggest that the decade which we are entering at the time of this writing, should be later seen as the period of "glyphosate realism." Hopefully a time when glyphosate will be recognized as a chemical which has to be stewarded carefully and restricted. This would allow that glyphosate can be used sensibly, in moderation, and play a reduced role in global agriculture *as the lesser evil*, until an alternative is found.

Returning to Shakespeare, let us join the young prince of Denmark as he exclaims to his friends: *"Why, then,'tis none to you, for there is nothing either good or bad, but thinking makes it so. To me it is a prison"* (Hamlet, Act 2, scene 2).

CONCLUSION

The recognized higher toxicity and the stronger potential for negative effects on health and environment should be important arguments for restrictions in use of glyphposate and GBHs. Despite this evidence, regulatory authorities have gradually allowed more sources of glyphosate into the food-supply and higher residue levels, in an ongoing development contrary to toxicological principles and common sense.

AUTHOR CONTRIBUTIONS

All listed authors have contributed to the work. The corresponding author initiated and structured the manuscript, drew the figures, coordinated various input and elaborated the wording.

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frontiers in Public Health

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Toxicological Risks of Agrochemical Spray Adjuvants: Organosilicone Surfactants May Not Be Safe

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Agrochemical risk assessment that takes into account only pesticide active ingredients without the spray adjuvants commonly used in their application will miss important toxicity outcomes detrimental to non-target species, including humans. Lack of disclosure of adjuvant and formulation ingredients coupled with a lack of adequate analytical methods constrains the assessment of total chemical load on beneficial organisms and the environment. Adjuvants generally enhance the pesticidal efficacy and inadvertently the non-target effects of the active ingredient. Spray adjuvants are largely assumed to be biologically inert and are not registered by the USA EPA, leaving their regulation and monitoring to individual states. Organosilicone surfactants are the most potent adjuvants and super-penetrants available to growers. Based on the data for agrochemical applications to almonds from California Department of Pesticide Regulation, there has been increasing use of adjuvants, particularly organosilicone surfactants, during bloom when two-thirds of USA honey bee colonies are present. Increased tank mixing of these with ergosterol biosynthesis inhibitors and other fungicides and with insect growth regulator insecticides may be associated with recent USA honey bee declines. This database archives every application of a spray tank adjuvant with detail that is unprecedented globally. Organosilicone surfactants are good stand alone pesticides, toxic to bees, and are also present in drug and personal care products, particularly shampoos, and thus represent an important component of the chemical landscape to which pollinators and humans are exposed. This mini review is the first to possibly link spray adjuvant use with declining health of honey bee populations.

Keywords: adjuvant, agrochemical formulation, organosilicone surfactant, non-target effects, spray tank mix

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INTRODUCTION

Applications of modern pesticide formulations, particularly in combinations, are often accomplished using proprietary spray adjuvants to achieve high efficacy for targeted pests and diseases (1). An adjuvant is an additive or supplement used to enhance the performance or aid in the stability of formulations of active ingredients (2). Adjuvant products are formulated combinations of surfactants,

Abbreviations: AI, active ingredient; CCD, colony collapse disorder; EBI, ergosterol biosynthesis inhibitor; EC, emulsifiable concentrate; IGR, insect growth regulator; NMP, *N*-methyl-2-pyrrolidone; OSSA, organosilicone surfactant adjuvant; ppm, part per million.

penetrant enhancers, activators, spreaders, stickers, cosolvents, wetting agents, pH modifiers, defoaming agents, drift retardants, nutrients, etc., depending on their proposed utility. Usually, adjuvants are much less expensive than formulated active ingredients and can reduce the active ingredient dose needed by an order or more of magnitude (3, 4). Similarly, contemporary drug delivery to humans and animals transdermally (5) and orally (6) is often mediated *via* adjuvant technologies that enhance penetration. Newer agrochemical technologies include co-formulants such as polyethoxylated tallow amines, cosolvents such as *N*-methyl-2-pyrrolidone (NMP), and spray adjuvants such as organosilicone polyethoxylates (7).

Numerous studies have found that pesticide active ingredients elicit very different physiological effects on non-target organisms when combined with their co-formulants and tank adjuvants (7–9). Despite the widespread assumption that formulation ingredients and spray adjuvants are biologically inert, substantial evidence suggests that this is often not the case. Indeed, glyphosate has weak ecotoxicity and systemic movement without tallow amines and other adjuvants (10–12), including its toxicity to mammals (13) and human cells (14). Noteworthy is the fact that spray tank adjuvants by themselves harm non-target organisms from all taxa studied. Adjuvant-dependent toxicities, often more than the associated formulations of herbicides and fungicides, have been reported for bacteria (15), cyanobacteria (16), algae (17), and snails (18). The non-ionic spray adjuvant R-11 synergized the acute toxicity of the insecticides spinosad (19) and imidacloprid (20) on aquatic crustaceans and, in the absence of an insecticide, reduced the growth rate of *Daphnia pulex* at relevant field concentrations found after application near aquatic systems (21). Aquatic organisms are particularly vulnerable to the general ecotoxicity of adjuvant surfactants ranging from invertebrates (22, 23) to fish (19, 24, 25) and amphibians (26). Terrestrial insects, in turn, have long been shown susceptible to insecticide synergisms associated with spray adjuvants (27, 28). Many of the classical cases of ecotoxicities found with spray adjuvants and used with pesticides other than glyphosate are due to older surfactant classes, such as nonylphenol polyethoxylates, which environmentally degrade to the endocrine disrupting nonylphenols (29). It is clear that agrochemical risk assessment that takes into account only pesticide active ingredients and their formulations in absence of the spray adjuvants commonly used in their application (30, 31) will miss important toxicity outcomes that may prove detrimental, even to humans. Here, we attempt to characterize the scope of spray adjuvant use, especially organosilicone surfactants, and explore a possible link between their increasing presence in California almonds and the declining health of honey bee populations.

SPRAY ADJUVANTS CONTRIBUTE TO THE TOXIC LOAD

Supplemental adjuvants used in tank mixes generally enhance the pesticidal efficacy as well as inadvertently the non-target effects of the active ingredient after application (7, 14). Dramatic impacts of agrochemical formulants on the bee toxicity of pesticide active ingredients have been documented (32). Formulations are generally more toxic than active ingredients, particularly fungicides, by up to 26,000-fold based on published literature. The highest oral toxicity of three insecticide formulations tested was for Vertimec® 18 EC that was 8,970 times more toxic to the stingless bee *Melipona quadrifasciata* and 709 times more toxic to the honey bee than the topically applied active ingredient abamectin in acetone (33). However, the largest documented formulation compared to active ingredient differences in bee toxicity have been with the least toxic pesticides, particularly fungicides. Among the 300 pesticide formulations tested for oral toxicity to adult honey bee in China, a 25% EC formulation of the fungicide tebuconazole was equally toxic to the most bee-toxic insecticide known, emamectin benzoate (LD_{50} = 0.0035 μg/bee), whereas a 5% suspension concentrate of tebuconazole was > 25,000 times less toxic (34). This product-dependent range in toxicity is presumably determined by the undisclosed fungicide co-formulants. While technical glyphosate has virtually no toxicity for honey bees, common formulations such as WeatherMAX® do (35). Commercial formulations of fumagillin acid used to control *Nosema* and other microsporidian fungal diseases in honey bees and mammals, respectively, are actually salts of the base dicyclohexylamine. This co-formulant is five times more toxic and persistent than the active ingredient to rodents and other organisms, serving as a sensitive bioindicator of fumagillin pollution (36). Most studies documenting pesticide effects on honey bees are performed without the formulation or other relevant spray adjuvant components used when applying the active ingredient, most often due to lack of such required tests for product registration (7).

Less potent bee toxicities are usually found when spray adjuvants are tested alone or relative to the pesticide formulations used in tank combinations. About one-third of non-ionic, organosilicone and other surfactant spray adjuvants at up to a 0.2% aqueous solution have been shown to deter or kill honey bees (37–39). Exposure to the nonylphenol polyethoxylate adjuvant N-90 by itself at field rates impaired nest recognition behavior of two managed solitary bees, *Osmia lignaria* and *Megachile rotundata* (40). While the organosilicone adjuvant Break-Thru® fed to nurse bees at 200 ppm with or without 400 ppm of the fungicide Pristine® did not impact honey bee queen development or survival (41), toxic interaction of the co-occurring insect growth regulator (IGR) dimilin with this adjuvant is likely [cf., Ref. (42)]. Higher toxicities were found when honey bees are fed related commercial organosilicone surfactants in 50% sucrose with oral LC_{50}s around 10 ppm (7). A discontinued agrochemical surfactant perfluorooctylsulfonic acid is highly and orally toxic to *Bombus terrestris* (43). The penetration enhancing solvent NMP commonly present in agrochemical formulations is a dietary toxicant for honey bee larvae at 100 ppm (44).

Organosilicone surfactants are particularly potent as super-penetrants, super-spreaders, and probable ecotoxicants (7). They are used worldwide at up to 1% (10,000 ppm) of the spray tank mix, while other adjuvant classes require higher amounts up to 5% of the spray tank mix (3, 32). All organosilicone surfactant adjuvants (OSSA) tested (Dyne-Amic®, Syl-Tac®, Sylgard 309®, and Silwet L-77®) impaired honey bee olfactory learning much more than other non-ionic adjuvants (Activator 90®, R-11®, and

Induce®), while the crop oil concentrates (Penetrator®, Agri-Dex®, and Crop Oil Concentrate®) were inactive at 20 μg per bee (45). The greater surfactancy of organosilicones over other non-ionic adjuvants and crop oil concentrates can drive the stomatal uptake of large bacterial-sized mineral particles (46) and *Agrobacterium* transformation of grape plantlets (47), and thus may aid movement of pathogens into bee tissues.

SPRAY ADJUVANT USE DURING POLLINATION OF CALIFORNIA ALMONDS

Pollination of California almonds during February and March is the single largest pollination event in the world. Over 60% (1.5 million) of USA honey bee colonies are transported to California each year to pollinate the crop. A workshop convened to address reduced overwinter survivorship of commercial honey bee colonies used in almond pollination since the 2006 onset of colony collapse disorder (CCD) judged neonicotinoids unlikely to be a sole factor and *Varroa* mites plus viruses to be a probable cause (48). However, fungicides, herbicides, and spray adjuvants were not evaluated. Recent surveys of migratory beekeepers who pollinate almonds do not self-report overwintering losses greater than the majority of non-migratory beekeepers, although their summer colony losses tend to be higher (49). Better management practices employed by migratory beekeepers who pollinate almonds may explain their lower winter losses in comparison with sideline or backyard beekeepers (50). Nevertheless, it has been surmised by beekeepers and documented by researchers that decreasing honey bee health issues are initiated in almonds, a winter/early spring pollinated crop, and then progressed over the course of the year as colonies are employed to pollinate other crops including apples, blueberries, alfalfa, cotton, pumpkin, cantaloupe, etc. Although the rates of foraging honey bees were not reduced over time during almond pollination in contrast to those pollinating cotton and alfalfa, there was no corresponding increase in foraging population though a significant increase in colony size occurred (51). Some of the highest pesticide residues, especially fungicides, were found on almonds, which represents a notable pesticide exposure risk and ranked fifth in hazard among the eight crops assessed (51). Ironically, increasing fungicide load in pollen has been associated with increased probability of fungal *Nosema* infection in exposed bees (52).

California law defines adjuvants packaged and sold separately as pesticide products that require registration (53). Every application of a spray tank adjuvant is reported with detail that is unprecedented globally. California almond exposes most USA honey bees to highly documented pesticide and adjuvant applications and is an unique crop to assess all other agrochemical inputs in the absence of neonicotinoids, presently considered to be the primary pesticide factor associated with pollinator decline (54). There are no substantial applications of neonicotinoids to this monoculture (55), particularly when honey bees are present, and almond pollen and nectar tend to be the sole food source unless supplemental sugar feeding is employed (52). Pesticide usage information for California has been archived since 1990 in the pesticide use reporting (PUR) database maintained by the California Department of Pesticide Regulations (55). The great utility of this data for assessing environmental risks of spatial and temporal pesticide use in California almonds to aquatic organisms and earthworms has been demonstrated (56). However, our study is the first to include spray adjuvants as potentially toxic agrochemical inputs in risk evaluation.

We analyzed annual trends in applications of tank adjuvants and associated formulated products of active ingredients during almond pollination (February and March). January applications were also included since their foliar residues may pose toxicity risks for newly arriving bee colonies. Over 3.3 million records for almond applications were downloaded from PUR (55) and sorted for January to March of 2001–2013 using Microsoft Excel (Mac 2011). Only synthetic pesticides were analyzed for trends, thereby excluding bulky applications of older natural products and biologicals, such as sulfur, petroleum and mineral oils, copper salts, and microbials, since CCD was first noted in 2006, decades after major regular inputs of these natural pesticides were initiated. While overall statewide synthetic fungicide and insecticide use on almonds has not increased over this evaluation period, applications of herbicides and spray adjuvants, the latter including nutrient and buffer supplements, have doubled (**Figure 1**). Yearly application rates were normalized to total almond bearing acres, which increased from 530,000 in 2001 to 850,000 acres in 2013 (57), indicating that the total synthetic pesticide load has increased on almonds since the onset of CCD (**Figure 1**). Because herbicide applications are generally made to the understory and not to the flowering canopy where pollinator exposure is likely, we focused on actual tank adjuvant and pesticide mixes that may provide direct exposure risks for bees. Among adjuvant classes, the organosilicone surfactants pose the greatest toxicity risks for honey bees (7).

We then conducted a detailed analysis of temporal trends in organosilicone applications for Stanislaus Co., a major almond producing county in California (57), which had the largest number of pesticide applications over our evaluation period. PUR records (55) were sorted by date, county/meridian/township/range/section (COMTRS) location, and amount of treated almond acres. Co-occurring and synonymous records were assumed to represent combined pesticide and adjuvant products within the same tank application mix. Based on this premise, most of the spray combinations comprised, in addition to one or more pesticide formulations, at least one tank adjuvant. Focused assessment was then made out of the total number and percentages of applications containing an OSSA, which included 45 products (Table S1 in Supplementary Material) dominated by Dyne-Amic®, Syl-Tac®, Sylgard 309®, RNA Si 100®, First Choice Break-Thru®, Freeway®, Kinetic®, Multi-Spred®, Widespread Max®, and Silwet L-77®. Similar combinations of products were assigned unique tank mix codes and resorted. Almost 10,000 pesticide applications on almonds in Stanislaus Co. contained an OSSA over the years evaluated, each on average to 40 acres. The greatest increase in major agrochemical inputs observed before and after onset of CCD in 2006 was the tripling of total pesticide applications containing an OSSA from 587 in January–March 2001 to 1,781 in January–March 2006 (**Figure 2A**). Greater than 80% of these applications contained fungicides, followed

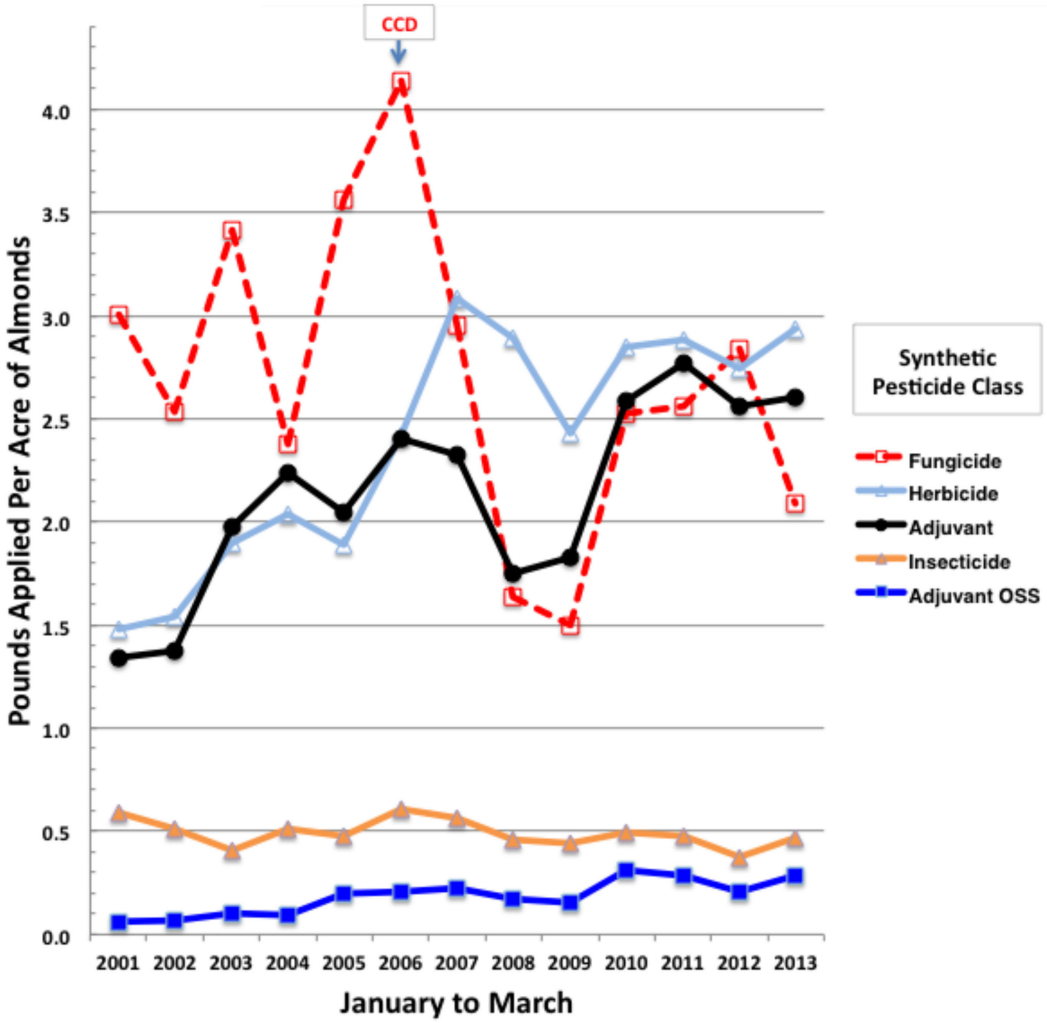

FIGURE 1 | Total pounds of synthetic pesticides by class applied per acre of California almonds during January to March of 2001 through 2013. Yearly total almond bearing acres were from the CA Department of Food and Agriculture (57).

by 10% insecticides, and 5% herbicides. Ergosterol biosynthesis inhibitor (EBI) fungicides and IGR insecticides were greatly increased, whereas herbicide and other insecticide applications were fairly static across this period (**Figures 2A,B**). Pristine® (a combination of boscalid and pyraclostrobin), chlorothalonil, and EBIs (propiconazole > myclobutanil > fenbuconazole > metconazole > difenoconazole) dominated the increasing trends in fungicide use at the onset of CCD (**Figure 2B**). The IGRs (diflubenzuron > methoxyfenozide > pyriproxyfen > tebufenozide) displayed the greatest increases among insecticides in spray tank mixes containing OSSA during the onset and continuation of CCD (**Figure 2B**). Concomitantly, greatest decreasing tendencies in almond pesticide applications were for other fungicides (cyprodinil, iprodione, and azoxystrobin) and the older EBI myclobutanil, while inputs of herbicides (primarily glyphosate, oxyfluorfen, and paraquat) with OSSA did not change markedly. Based on the CDPR data for agrochemical applications to California almonds during pollination, increasing adjuvant use, particularly the OSSAs, in tank mixes with fungicides, including EBIs, Pristine®, and chlorothalonil, and with IGR insecticides may be associated with recent USA honey bee declines.

ORGANOSILICONES: THE MOST POWERFUL SURFACTANTS

Organosilicone surfactants are the most potent adjuvants and super-penetrants available to growers (58, 59). These polyethoxylates and those containing the nonyl- and octylphenols are widely used as non-ionic surfactants in spray adjuvants or additives in agrochemical formulations applied during bloom when bees are foraging. Organosiloxane surfactants were detected in all wax samples and 60% of pollen samples, although absent from honey (60). Their general wide occurrence as residues in beehive samples is noteworthy since spray adjuvants are not presently regulated by the EPA (61). Nonylphenol more than organosiloxane and octylphenol polyethoxylates were found in

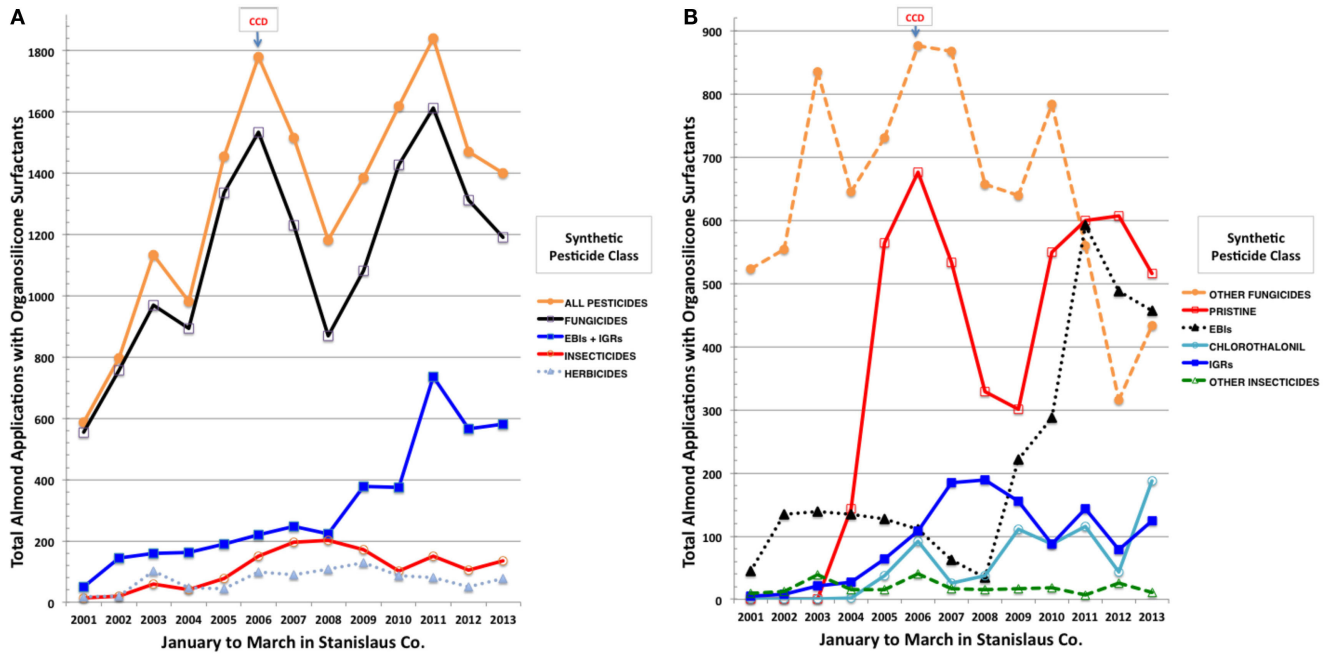

FIGURE 2 | Total applications to almonds in Stanislaus County, CA, USA of synthetic pesticides by class (A) and by more selective class and individual pesticides (B) during January to March of 2001 through 2013 using an organsilicone surfactant.

wax samples, while pollen and particularly honey residues were lower (62). Major commercial spray tank adjuvants are blends of organosilicone, nonylphenol, and octylphenol polyethoxylates, making it more difficult to associate environment residues with any specific product (63). Nevertheless, sample levels of the more abundant nonylphenol polyethoxylate residues may be used as a risk predictor for pesticide exposure because of their frequent coincidence in tank mixes of formulations and adjuvants (62). Spray tank adjuvants containing these polyethoxylates greatly influence pesticide fate (64) in pollinator or other environments, generally increasing the residue levels, of particularly fungicides (65) and herbicides (66), available to expose pollinators and other non-target species. The impact of OSSA sprays on the frequent incidence of neonicotinoid residues in bee environments (67) and their often associated roles in pollinator decline (68) may be great since the highest imidacloprid residue ever reported in pollen (7.4 ppm) was after use of Dyne-Amic® on citrus [(69), Appendix E].

Even at 10 ppm, OSSAs are good, stand-alone insecticides and miticides (7, 70), and can be more toxic to beneficial insects than the active ingredient used to control the associated pest (71). Silwet L-77® and Kinetic® are known to synergize the neonicotinoid imidacloprid used to control the psyllid vector of citrus greening disease (72). Yearly use of these potent adjuvants continues to increase, with an estimated annual global production of 1.3 billion pounds of OSSAs in 2008 among 10 billion pounds of all organosilicones (73). This is 30 times greater than the highest estimated global annual imidacloprid use of 44 million pounds (74). Silwet L-77® was the most potent endocrine disruptor among surfactants tested in a screen of 1,814 chemicals, with composite scores that placed it in the top 38 of the 465 endocrine disruptors found [(75), supplemental data], much more active than polyoxyethylene(10)nonylphenyl ether. All six neonicotinoids, including imidacloprid, were inactive in the entire battery of endocrine tests used. Organosilicone surfactants are also present in drug and personal care products, particularly shampoos (76), and thus represent an important component of the chemical landscape to which bees (32) and humans (77) are exposed. These widely used super surfactants readily move across membranes, become systemic in plants and animals, and can ultimately degrade to silica (78) causing silicosis in sensitive tissues of exposed organisms.

ARE ORGANOSILICONE SURFACTANTS CAUSING HARM AND UNDERREGULATED?

Organosilicone surfactants are the "gold" standard for effecting solution of complex mixtures of agrochemical components of wide-ranging polarites in the spray tank. Hundreds of thousands of pounds of organosilicone adjuvants are applied every year on almonds in California alone (7, 45), both during and subsequent to bloom when bee pollinators are present. The high incidence of OSSAs in USA beehives and their ability to impair adult learning and be toxic to honey bees at all stages of development points to their great potential to harm bees and other non-target species, and yet, they are typically not even considered in the risk assessment process. It is clear that relevant pesticide risk assessment for pollinators and other non-target species cannot be

addressed solely by evaluating the active ingredients without the concomitant formulation ingredients and spray tank adjuvants. Lack of risk mitigation on spray tank adjuvants presently allows major OSSA products such as Break-Thru®, Kinetic®, RNA Si 100®, Silwet Eco Spreader®, Syl-Coat®, and Widespread® to be used on any "organic" crop under a certified Organic Materials Review Institute (OMRI) label (79).

Spray adjuvants are largely assumed to be biologically inert and are not registered by EPA at the federal level in the USA (7, 55). Registration and monitoring of adjuvant use patterns are regulated at the state level in the USA, and most states do not participate in this process. To the best of our knowledge, only California, Washington, and perhaps Oregon make substantial effort to monitor use patterns or regulate these major chemical inputs into the environmental landscape. This lack of federal oversight is surprising since Department of Transportation employees of Pennsylvania and Iowa claim that herbicide applications to right-of-ways and roadways always contain a separate spray tank adjuvant (personal communications, 2015). Leaving regulation to the mandate of individual states results in a "wild west" approach that, in most cases, leaves these chemicals unaccounted for and allows for their increasing presence in our environment. Requiring regulation of spray tank adjuvants at the federal level in the USA would be a reasonable step toward addressing this problem.

While we recognize that chemical stressors alone are likely not responsible for the decline of pollinator or other non-target organisms, the true impact of chemical exposure is impossible to determine given our lack of understanding of the total chemical burden, a burden that clearly includes unknown and unevaluated materials. Coincidence of virus and pesticide exposures in declining honey bee colonies (80) is most noteworthy among other factors, which also includes malnutrition and elevated *Varroa* mites. More industry and regulatory agency disclosure of the identity of agrochemical adjuvant and formulation components would aid in evaluating risk and hazard assessment. Most adjuvants and inert ingredients are presently exempted from human safety tolerances, generally recognized as safe, and thus no environmental monitoring is required (7). A needed improvement is to include all formulation (81) and adjuvant (82) ingredients at relevant environmental input and exposure levels, and not just active ingredients, in studies to document the safety and risk for pollinators and other non-target species prior to product registration and commercialization.

AUTHOR CONTRIBUTIONS

CM and MF were the primary authors and contributed substantially to the concept, design, final drafting, and primary accountability of the content of this mini review. JF and RR were key to the acquisition, analysis, and interpretation of cited data and were involved in drafting and final approval for work cited here.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fpubh.2016.00092

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GENERAL COMMENTARY
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Commentary: "Estrogenic and anti-androgenic endocrine disrupting chemicals and their impact on the male reproductive system"

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A commentary on

Estrogenic and anti-androgenic endocrine disrupting chemicals and their impact on the male reproductive system
by De Falco M, Forte M, Laforgia V. Front Environ Sci (2015) 3:3. doi: 10.3389/fenvs.2015.00003

During the last two decades or so, endocrine-disrupting chemicals (EDCs) and their effects on human health have become one of the most researched and controversial topics in toxicology. There are a number of reviews on the health consequences of exposure to EDCs including a comprehensive report by the World Health Organization and the United Nations Environment Programme (1). Recently, De Falco et al. (2) addressed the impact of EDCs on male reproductive system, with special reference to the effects of bisphenol A (BPA), alkylphenols, and phthalates. Jeng (3) also reviewed the epidemiological data on the adverse effects of EDCs on male reproduction and experimental studies that could shed light on mechanisms (disruption of steroidogenesis, oxidative stress, and epigenetic changes) through which EDCs could impair male reproductive health. Both articles are essentially narrative reviews of the abundant and highly controversial literature on the health consequences of exposures to EDCs.

A key feature that distinguishes a narrative review from a systematic review is that the former review does not include a comprehensive and meticulous search of all potentially relevant articles on specified sources, and does not use explicit and reproducible criteria to selected articles for review (4). Compared to systematic reviews, narrative reviews of the literature are more likely to error and bias in the selection of relevant studies (4, 5). Moreover, if research designs, methods, and study characteristics do not undergo a critical appraisal, summary, and conclusions of literature reviews are even more prone to bias.

De Falco et al. were unable to convey to readers an unbiased review of the empirical evidence suggesting that environmental exposures to EDCs might affect male reproduction. The authors, for instance, did not disclose the conflicting evidence on the enlargement of prostate after developmental exposure to BPA. In the mid-1990s, a set of studies by vom Saal and coworkers showed that prenatal exposure to β-estradiol (EST), diethylstilbestrol (DES), or BPA led to enlarged ventral prostate in adult mice (6, 7). The observation that enlargement of prostate resulted from prenatal exposures to low doses of estrogenic compounds (e.g., supra-physiological levels of EST), and exhibited non-monotonic dose–response relationships, fueled considerable debate over the adverse health consequences of environmental exposure to EDCs. Several studies, however, failed to reproduce these findings not only with BPA but also with EST and DES (8–10). Although reproducibility is one hallmark of experimental sciences, the foregoing discrepancy between studies by different authors has remained unexplained (11).

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Furthermore, authors' statements that "...over 50 years, the global average sperm count dropped by half..." and that "studies of the last decade strongly support that male reproductive health has been deteriorating..." were unaccompanied by any reference to the conflicting evidence on this matter (2). The widespread notion that semen quality has decreased over the past decades stands on some retrospective studies [Ref. (12, 13), and others]. Nonetheless, results from a number of other studies (not cited by the authors) are inconsistent with this hypothesis. Most studies showing downward trends in sperm counts included samples coming from different populations and places that do not necessarily allow a valid comparison over time. For instance, a re-analysis of US data used by Carlsen et al. (12) found no decline in sperm counts when data from New York were excluded from the regression analysis (14). Therefore, apparent time trend toward lower concentrations reported by Carlsen et al. (12) resulted, in fact, from geographic variations in sperm counts (14, 15). Moreover, a longitudinal study of sperm concentrations for Danish military draftees (5000 men), collected annually for 15 years (1996–2010), found no indication that semen quality has changed during the monitoring period (16). Although several studies precipitated by reports on "downward temporal trends in sperm counts" refuted its existence, the "sperm crisis" notion is still a highly controversial issue in the literature (17–20). Temporal trends to increasing birth prevalence of male reproductive tract defects such hypospadias and cryptorchidism described by some authors are far from being a consistent finding among studies (21, 22).

The "endocrine disruptor hypothesis," a landmark of which was the Wingspread Conference Statement in 1990s (23), fits like a glove to the beliefs of the public that pesticides and other manmade chemicals in the environment are undermining human health and fertility. Two seminal books by Rachel Carlson (Silent Spring, 1962) and Theo Colborn (Our Stolen Future, 1996) boosted considerably these concerns on the deleterious effects of environmental chemicals on human fertility and health. Notwithstanding the fact that ED hypothesis is instigating, the notion that "male reproductive health has been deteriorating," as asserted by De Falco et al. (2) and others, lacks an unequivocal demonstration by soundly designed epidemiology studies. It is of note that, even if a temporal trend toward a worse male reproductive health had been demonstrated consistently, it would still be missing to prove that there is a causal link between EDCs, identified as such in experimental tests, and the incidence of the adverse health outcome in the human population. A step forward to identifying relevant research gaps and to unveiling the real impact of EDCs on male fertility and reproductive health would be to conduct less and less narrative and potentially biased reviews and more and more good quality systematic reviews of the literature on the topic. Finally, we highlight that a critical appraisal of the quality of original studies is required for both a good quality narrative and a good quality systematic review. If unbiased, good quality narrative reviews can also be helpful. Systematic reviews, however, are a more reliable approach to avoid bias in the selection of studies.

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ORIGINAL RESEARCH
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Inflammatory Effects of the Plant Protection Product Stifenia (FEN560) on Vertebrates

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Plant defense stimulators (PDSs) rely on the activation of plant innate immunity in order to protect crops against various pests. These molecules are thought to be a safer alternative to classical plant protection products. Given that innate immune systems share common features in plants and vertebrates, PDS can potentially cross-react with innate immunity of non-target organisms. To test this hypothesis, we studied effects of the commercial PDS Stifenia (FEN560), which is composed of crushed fenugreek seeds. We tested various concentrations of Stifenia (0.03–1 mg mL^{-1}) on human peripheral blood mononuclear cells and checked, 20 h later, cell metabolic activity (MA) using XTT assay, cell death by flow cytometry analysis, and IL-1β inflammatory cytokine released in the culture medium using ELISA. Stifenia induced a general decrease of the cell MA, which was concomitant with a dose-dependent release of IL-1β. Our results highlight the activation of human immune cells. The inflammatory effect of Stifenia was partially inhibited by pan-caspase inhibitor. Accordingly, Stifenia induced the release of p20 caspase-1 fragment into the culture medium suggesting the involvement of the NLRP3 inflammasome. Furthermore, we observed that Stifenia can induce cell death. We also tested the effect of Stifenia on Zebrafish larvae. After 24 h of exposure, Stifenia induced a dose-dependent IL-1β and TNFα gene expression. The human-cell-based approach developed in this work revealed a high sensitivity concerning inflammatory properties of a plant protection product. These tests could be routinely used to screen the potential adverse effects of this type of compounds. Finally, our results suggest a potential danger of using extensively certain PDS for crop protection.

Keywords: peripheral blood mononuclear cells, zebrafish, IL-1β, pesticides, plant defense stimulator, fenugreek

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INTRODUCTION

In the context of pesticides reduction, alternative strategies to protect crops have emerged, including use of transgenic crops, resistant hybrids, or integrated pest management methods. Among these, stimulation of the plant immune system with various molecules is promising. Plant defense stimulators (PDSs), plant defense inducers, or elicitors define a class of compounds of diverse origins, which can induce disease resistance-related mechanisms by mimicking a pathogen attack

or a danger state, resulting in reduced levels of plant infection. They comprise a range of purified or mixture-based natural or synthetic compounds that have been shown to protect plants efficiently (1, 2). To use a new molecule for crop protection in France, an authorization is needed according to the European Union (EC No. 1107/2009) and French regulations. Various toxicological and ecotoxicological tests are required for the production of an initial draft assessment report by EU-designated rapporteur member state (RMN) (3). Stifenia (FEN560), which is exclusively composed of grounded fenugreek seeds (*Trigonella foenum-graecum*) is a PDS authorized by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) to fight powdery mildew of grape vine (*Erysiphe necator*) and powdery mildew of melon (*Podosphaera fuliginea and Golovinomyces cichoracearum*) (ANSES agreement no. 2012-1685 and 2013-0227). Fenugreek, especially its seeds and its leaves, has been used for centuries in India and North Africa as food or in traditional medicine (4).

Similar to animals, the first step in the activation of the plant immune system is the perception of pathogens or microbe-associated molecular patterns (PAMPs/MAMPs) by pattern recognition receptors (5, 6). In both organisms, their perception induces complex cell signaling events, which result in cellular re-programming. For instance, PAMP/MAMP-triggered immunity in plants is associated with the production of phytoalexins, a class of antimicrobial metabolites, and with the reinforcement of plant cell walls. In mammals, the immune response induces, for example, the production of cytokines (interleukines, TNFα) and antimicrobial peptides. One of the inflammatory cytokine that plays a major role is IL-1β. Indeed, this cytokine is produced following inflammasome activation in monocytes, macrophages, and dendritic cells upon their stimulation by PAMP or damage/danger-associated molecular patterns. IL-1β is processed from pro-IL1β by caspase1 in several inflammasome complexes (e.g., NLRP3, NLRP1, and AIM2) (7, 8). This cytokine upregulates many other inflammatory factors such as IL6 (9) or TNFα (10).

Since plant and animal immune systems share some similarities (11, 12), we hypothesized that the PDS Stifenia could cross-react with the animal immune system. In fact, both beneficial and adverse effects have been described for fenugreek in mammals (4, 13–18) and toxicity against insects has also been reported (19). We thus tested the effects of Stifenia on human peripheral blood mononuclear cells (PBMCs) from different blood donors by quantifying the amount of the inflammatory cytokine IL-1β released by exposed cells. In parallel, we evaluated the metabolic activity (MA) of the stimulated cells using a XTT assay. We also checked the intensity of cell death induced by Stifenia. Finally, we studied the effects of this compound on the larvae of the model fish zebrafish by analyzing cytokine gene induction.

MATERIALS AND METHODS

Chemicals

Stifenia (FEN560, Société Occitane de Fabrications et de Technologies, France) was extemporaneously suspended in Roswell Park Memorial Institute medium (RPMI; for cell treatment) at 17 mg mL^{-1} or in autoclaved mineral Volvic water (for zebrafish treatment) at 0.1 mg mL^{-1} and gently shaken for 30 min. Since Stifenia is not fully soluble in water, insoluble matters were isolated by centrifugation (20,000 *g*, 30 min, room temperature) and supernatant was carefully collected in a new tube. All the concentrations indicated in this work refer to the initial concentration (17 mg mL^{-1} for human experimentation and 0.1 mg mL^{-1} for zebrafish experimentation).

Z-VAD-FMK stock solution [20 mM in 100% dimethylsulfoxide (DMSO)], purchased from Promega, was first diluted in RPMI at 85 μM. This solution was used for cell treatment. The final Z-VAD-FMK concentration was 5 μM in cell culture (0.025% DMSO). LPS from *Escherichia coli* 0111:B4 stock solution (1 mg mL^{-1} in pure water), purchased from Sigma-Aldrich, was diluted in RPMI to reach a final concentration of 10 ng mL^{-1} in cell culture. TNBS (2,4,6-trinitrobenzenesulfonic acid) stock solution (1 mg mL^{-1} in pure water), purchased from Sigma-Aldrich, was diluted in autoclaved mineral water (Volvic, France) at 75 μg mL^{-1} for zebrafish treatment.

Human PBMCs

Buffy coats from healthy donors were obtained from EFS Besançon, France (Agreement No. DECO-14-0124). PBMCs were prepared using Pancoll (density 1.077 g mL^{-1}, PAN-biotech Gmbh, Germany) and Blood Sep Filter tubes (Dominique Dutscher, France). Briefly, 15 mL of Pancoll were collected into the lower part of a Blood Sep Filter tube by a short centrifugation. Then, 25 mL of buffy coat and 15 mL of Dulbecco's phosphate-buffered saline (DPBS, PAN-biotech Gmbh, Germany) were added, gently mixed, and centrifuged (400 *g*, 30 min, room temperature) without brake for the deceleration phase. The PBMC ring was collected, washed three times in DPBS without Ca^{2+} and Mg^{2+}, and centrifuged (300 *g*, 10 min, 4°C). Cells were suspended in 2–5 mL of DPBS depending on the size of the cell pellet and kept on ice. Viable PBMCs were counted using trypan blue (20), suspended in RPMI medium supplemented with 10% (bovine serum albumin, w/v) and 1% PSA (Penicilline 10,000 U mL^{-1}, Streptomycine 10 mg mL^{-1}, Amphotericin B 25 μg mL^{-1} prepared in water), and then seeded in 96-well plate with 10^5 cells per well in 150 μL of medium.

Immediately after seeding, treatments were done by adding 10 μL per well of a 17-fold concentrated solution of Stifenia made by serial dilution from the stock solution described above. For anti-inflammatory studies, 30 min after Stifenia exposure, 10 μL of LPS solution was added to reach a final concentration of 10 ng mL^{-1}. Z-VAD-FMK was added simultaneously to Stifenia treatment. The final volume of RPMI was adjusted to 170 μL for all conditions. Twenty hours after treatment, cells were centrifuged (600 *g*, 3 min). Supernatants were collected for IL-1β quantification, and cell pellets were used for measuring cell MA.

Cell MA

Cell MA, reflecting cell viability, was determined using the XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay (XTT sodium salt, Sigma-Aldrich, France), based on the reduction of a tetrazolium salt (XTT)

by dehydrogenases of viable cells into a water-soluble orange formazan product. Briefly, PBMCs were centrifuged (600 *g*, 3 min) to remove culture medium. Then, 100 µL of RPMI without phenol red and 20 µL of a mixture of 0.9 mg mL^{-1} XTT and 0.01 mM phenazine methosulfate (Sigma-Aldrich, France) were added on pelleted cells. Cells were incubated at 37°C for 4 h and absorbance at 490 nm was measured with a background subtraction at 660 nm using a microplate reader (Infinites M200 PRO, Tecan, France). The results are expressed as percentage of MA compared to control non-exposed cells.

Quantification of Inflammatory Cytokines

Production of the inflammatory cytokine IL-1β was estimated in the culture medium using ELISA assay (Human IL-1β ELISA Ready-SET-Go! eBiosciences, France) according to the supplier's instructions.

Quantification of Cell Death

Two hundred thousand cells were seeded in 24-well plates and treated with Stifenia during 20 h as described above. Cells contained in the culture medium were harvested by centrifugation (400 *g*, 5 min) and then suspended in 300 µL of PBS containing propidium iodide (PI) (10 µg mL^{-1}). PI-stained cells were detected using a LSR II flow cytometer (BD Biosciences) and acquisitions were performed during 45 s using BD FACSDiva Software 6.1.2. Flow Jaw was used for figure drawing.

p20-Caspase-1 Assay

Quantity of processed caspase-1 was evaluated by measuring the amount of the p20 fragment secreted in the culture medium using ELISA assay (Human Caspase-1/ICE Quantikine ELISA Kit, R&D Systems, France) according to the supplier's instructions.

Zebrafish Strains, Maintenance, and Treatment

According to the European Union Directive 2010/63/EU, no specific ethics approval was required for this project, as all zebrafish larvae used in this study were less than 120-h post-fertilization (hpf) old (21, 22). Wild-type fishes (WIK strain) were obtained from the ZIRC (OR, USA) and kept at 28°C with a light:dark cycle 14:10 h. They were fed twice a day with dried flake food (Gemma Mirco, Skretting, France). The fish were mated and spawning was stimulated by the onset of light. Zebrafish eggs were collected immediately after being fertilized and distributed in 24-well plates (three eggs per well) containing 1 mL of autoclaved mineral water (Volvic, France). At 4 days postfertilization, water was replaced by fresh water containing the desired concentration of Stifenia or 75 µg mL^{-1} of TNBS. Twelve to fifteen zebrafish larvae per condition were incubated during 20 h.

RNA Extraction and RT-qPCR

After 20 h of exposure (Stifenia or TNBS, see above), 12–15 zebrafish larvae (120 h postfertilization) were euthanized with tricaïne, collected, and disaggregated during 5 min at room temperature using 17-gage needles in 1 mL of Trizol reagent (Invitrogen, France) and vortexed. Total RNAs were extracted according to supplier's instructions. The RNA samples were treated with DNAse (TURBO DNA-free, Life Technologies) according to the manufacturer's instructions. Approximatively 15–20 µg of RNA diluted in water were washed with butanol and diethyl ether according to Krebs et al. (23). Diethyl ether was evaporated under a fume hood. The resultant water phase containing RNA was mixed with 175 µL of RNA Lysis Buffer and 350 µL of RNA Dilution Buffer and loaded on a SV Total RNA column (Promega, France) to perform on-column DNAse digestion according to supplier's instruction. Then, 1 µg of total RNA was reversed-transcribed to cDNA using the iScript™ reverse transcription supermix for RT-qPCR (Biorad, France). Analyses were performed on a thermocycler (Step One Plus, Applied Biosystems, France) using Power SYBR Green from the same purchaser. The parameters used for the PCR were 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. The relative expression ratio (experimental/control) was normalized with *Danio rerio* HPRT1 (24) according to $2^{-\Delta\Delta Ct}$ method. Sequences of primers used in this study are listed in **Table 1**. RT-qPCR assays were performed in duplicates for each cDNA and each primer couple and the experiment repeated two times.

Statistical Analysis

Data obtained were expressed as mean ± SEM. Statistical differences among treatments were evaluated by Kruskall–Wallis method. *Post hoc* tests were used to identify statistical groups as described in each figure legend.

RESULTS

Manufacturer's instructions indicate that Stifenia has to be solubilized in water. However, Stifenia is neither fully soluble in water nor in other classical solvents such as 100% DMSO, acetone 60% in water (v/v), ethanol 100%, and RPMI medium (data not shown), because it is composed of crushed fenugreek seeds. To study its effect on human PBMCs or zebrafish larvae, we used an aqueous soluble extract obtained as described in Section "Materials and Methods." According to ANSES, the recommended use-concentration of Stifenia is 0.15–0.5% (m/v), which correspond to 1.5–5 mg mL^{-1} (ANSES 2012-1685, ANSES 2013-0227). In our study, we tested concentrations of Stifenia below these recommended use-concentrations.

TABLE 1 | List and sequences of primers used for RT-qPCR experiments.

Primer name	5′ → 3′ Oriented sequence
DrActin For	CCCAGACATCAGGGAGTGAT
DrActin Rev	CACAATACCGTGCTCAATGG
DrHPRT1 For	CAGCGATGAGGAGCAAGGTTATG
DrHPRT1 Rev	GTCCATGATGAGCCCGTGAGG
DrIL1B For	GTCCACGTATGCGTCGCCCA
DrIL1B Rev	GGGGCAACAGGCCAGGTACA
DrTNFa For	GTGCAATCCGCTCAATCTGCACG
DrTNFa Rev	AATGGAAGGCAGCGCCGAGG

Dr, Danio rerio; For, forward; Rev, reverse.

Stifenia Induces a Dose-Dependent Release of IL-1β in the Culture Medium

Different concentrations of Stifenia (0.03–1 mg mL^{-1}) were independently tested for 20 h on PBMC from nine different healthy human blood donors (**Figure 1A**; Figures S1 and S2 in Supplementary Material; **Table 2**). Cell MA was then measured using the XTT assay and IL-1β production was quantified in the culture medium using ELISA. A decrease of MA was observed from 0.1–1 mg mL^{-1} of Stifenia (**Figure 1A**). This decrease was observed with eight out of nine blood donors but to a different extend (Figure S1 in Supplementary Material). For these blood donors, it ranged from 9.5 to 33.2% when 0.3 mg mL^{-1} of Stifenia is used (**Table 2**).

In culture medium from unexposed cells, IL-1β was either not detected or very close to background level (**Figure 1A**). In this cell batch CHR026_91, the lowest Stifenia concentration used (0.03 mg mL^{-1}) did not induce IL-1β production. Higher concentrations progressively increased IL-1β release, which peaked at 3,240 pg mL^{-1} with 1 mg mL^{-1} of Stifenia. This quantity is equivalent to the one induced by 10 ng mL^{-1} of LPS. Although the pattern of IL-1β induction was always similar among the different PBMC batches tested, we noticed great variations in the quantities of IL-1β released (**Table 2**; Figure S1 in Supplementary Material). Thus, the lowest quantity found was 5 pg mL^{-1} after treatment with 0.3 mg mL^{-1} of Stifenia while the highest was 3,268 pg mL^{-1} (**Table 2**). The LPS-induced IL-1β production was also systematically measured and was also variable among the different blood donors. LPS (10 ng mL^{-1}) induced the release of 18–2,984 pg mL^{-1} of IL-1β in the culture media, depending on the donor. No clear relationship was found between LPS- and Stifenia-induced IL-1β productions (**Table 2**; Figure S1 in Supplementary Material). In an exploratory experiment, we evaluated the production of others cytokines in the culture medium of Stifenia-exposed PBMC using a Multiplex assay (Table S1 in Supplementary Material). As observed with ELISA, Stifenia induced a dose-dependent production of IL-1β. We also detected a slight induction of TNFα and of the anti-inflammatory cytokine IL-10. No modulation of IFNγ, IL2, or IL-12p70 concentrations was observed.

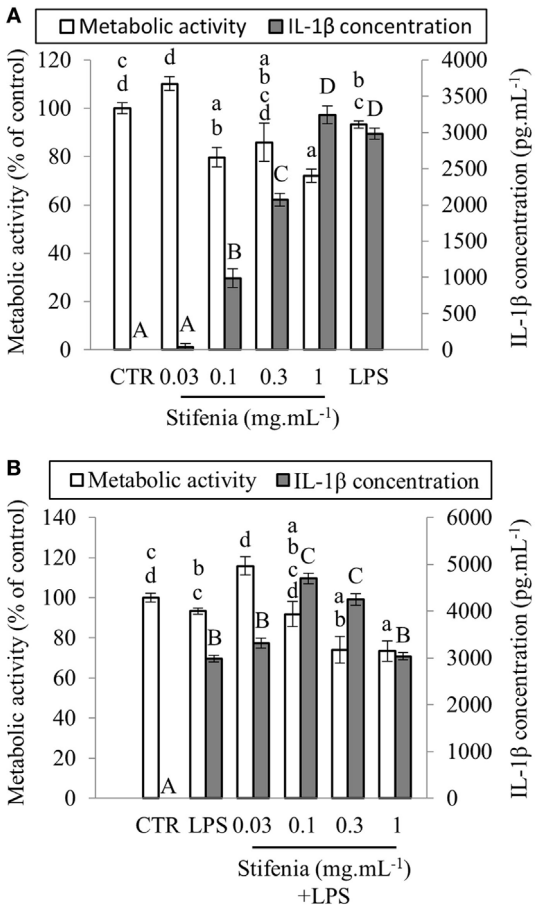

FIGURE 1 | Effect of Stifenia on human peripheral blood mononuclear cell (PBMC) metabolic activity (MA) and IL–1β production. PBMCs were stimulated by different concentrations of Stifenia added in the culture medium. Cell MA (white bars) was estimated by the XTT assay and IL-1β (gray bars) was measured in the culture medium. **(A)** PBMCs were exposed to Stifenia for 20 h. **(B)** PBMCs were stimulated by addition of 10 ng mL^{-1} of LPS 30 min after the beginning of Stifenia exposure. Results are obtained from the blood donor CHR026_91. Bars represent the mean of eight technical replicates. Different letters (lowercase for the XTT assay, capitals for IL-1β) indicate statistical differences between groups ($p < 0.05$). If two conditions share one or several letters, there are no statistical differences. Statistical differences were determined using a Kruskal–Wallis test followed by a comparison with the Steel–Dwass–Critchlow–Fligner method. CTR, control non-treated cells.

Stifenia Does Not Inhibit LPS-Induced Inflammation in PBMC

Literature frequently reported anti-inflammatory effects of fenugreek seed extracts (13, 25, 26). We thus tested the hypothesis that Stifenia could decrease the LPS-induced IL-1β production in PBMC. **Figure 1B** shows representative results of experiments conducted on PBMC isolated from six different blood donors. Cells were exposed with Stifenia as previously described and then stimulated 30 min later with 10 ng mL^{-1} LPS. IL-1β was measured 20 h later. Stifenia pretreatment did not reduce LPS-induced IL-1β production (**Figure 1B**; Figure S2 in Supplementary Material). For each Stifenia concentration tested, the IL-1β produced was either higher or equal to the LPS alone-induced IL-1β synthesis. Regarding MA, while LPS alone never reduced it, Stifenia pre-exposure (0.3–1 mg mL^{-1}) decreased the MA in a dose-dependent manner as observed when used alone (**Figure 1B**; Figure S2 in Supplementary Material).

Stifenia Does Not Contain Contaminating Microorganisms

In order to check if inflammatory activity of Stifenia was not due to contamination by microorganisms, we compared results obtained from a Stifenia preparation that was either directly used for cell treatment or previously filtered on 0.22 μm pore size membrane. In both cases, a dose-dependent induction of the cytokine production was found. IL-1β induction was not significantly different between filtered and non-filtered Stifenia although it seems that filtered Stifenia induce more IL-1β (**Figure 2**).

TABLE 2 | Comparison of Stifenia or LPS exposure on metabolic activity (MA) and induced IL–1β production of peripheral blood mononuclear cells from nine different healthy human blood donors.

Blood donor	CTR		Stifenia 0.3 mg mL^{-1}		LPS	
	MA	IL-1β (pg mL^{-1})	MA	IL-1β (pg mL^{-1})	MA	IL-1β (pg mL^{-1})
CHR026_62	100.00 ± 0.51	3.68 ± 1.24	83.18 ± 3.39	228.35 ± 12.99	97.15 ± 1.33	276.53 ± 8.98
CHR026_76	100.00 ± 1.67	0.00 ± 0.00	87.22 ± 4.13	563.99 ± 29.62	127.17 ± 3.12	517.39 ± 42.65
CHR026_91	100.00 ± 2.25	0.00 ± 0.00	85.86 ± 7.90	2,069.41 ± 86.84	93.34 ± 1.46	2,984.13 ± 75.03
CHR026_93	100.00 ± 3.27	4.44 ± 2.06	74.68 ± 5.45	2,276.04 ± 224.08	99.61 ± 1.08	1,403.12 ± 26.94
CHR026_95	100.00 ± 2.70	2.13 ± 2.13	76.47 ± 3.31	3,268.65 ± 62.54	90.23 ± 2.98	1,631.87 ± 52.96
CHR026_128	100.00 ± 2.41	3.90 ± 3.90	90.57 ± 3.39	5.09 ± 2.96	102.30 ± 2.39	718.22 ± 28.63
CHR026_135	100.00 ± 9.91	0.00 ± 0.00	69.71 ± 7.14	715.86 ± 47.95	108.68 ± 2.10	76.13 ± 7.72
CHR026_152	100.00 ± 10.37	0.00 ± 0.00	66.8 ± 1.82	788.61 ± 423.84	90.65 ± 11.20	18.24 ± 18.24
CHR026_153	100.00 ± 1.41	0.00 ± 0.00	110.34 ± 3.36	946.77 ± 84.61	119.91 ± 2.28	626.87 ± 13.94

Cell MA was estimated by the XTT assay, and IL-1β was measured in the culture medium 20 h after the addition of Stifenia or LPS. Results represent the mean between eight technical replicates.
CTR control non-treated cells.

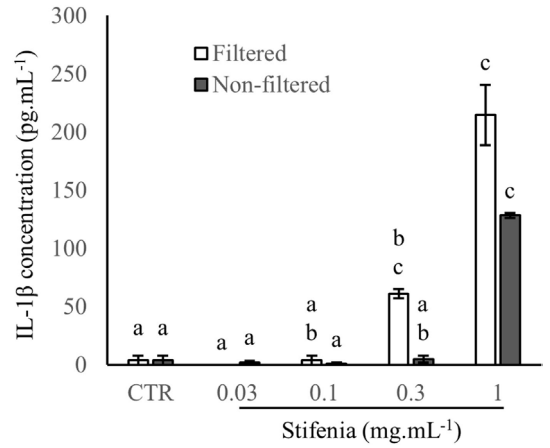

FIGURE 2 | Comparison of filtrated and non-filtrated Stifenia preparation on IL-1β production. Results are obtained from the blood donor CHR026_128. IL-1β was measured in the culture medium 20 h after the addition of filtered (white bars) and non-filtered (gray bars) Stifenia. Each bar corresponds to four technical replicates. Statistical differences were determined using a Kruskal–Wallis test followed by a comparison with the Conover–Iman method. Similar letters indicate no significant differences ($p < 0.05$). CTR, control non-treated cells. This experiment was repeated once with peripheral blood mononuclear cell from another blood donor (CHR026_135) with similar results (data not shown).

Inflammatory Effect of Stifenia Is Inhibited by Caspase Inhibitor

IL-1β is processed to its active released form by caspase-1 through the inflammasome multiprotein complex NLRP3 (7, 27). We used a pan-inhibitor of caspases in order to test whether IL-1β released upon Stifenia stimulation was generated *via* an inflammasome complex. Sixty per cent of the cytokine production induced by Stifenia was abolished by Z-VAD-FMK. As expected, Z-VAD-FMK also strongly reduced LPS-induced IL-1β production (**Figure 3A**; Figure S3 in Supplementary Material). We then checked the involvement of caspase-1 by ELISA on the production of IL-1β induced by Stifenia. In unexposed cells, detection of p-20 caspase-1 fragment in the culture medium was close to the detection threshold. Stifenia 0.3 mg mL^{-1} induced a huge release of extracellular p-20 Caspase-1 fragment that is much higher than with LPS exposure alone (**Figure 3B**).

Stifenia Induces Cell Death of PBMC

Using flow cytometry and PI, we checked if a 20-h Stifenia exposure can induce PBMC cell death (Figure S4 in Supplementary Material). In our experiments performed on two independent blood donors, the lowest concentrations of Stifenia tested (0.03 and 0.1 mg mL^{-1}) did not induced cell death. However, treatment of PBMC with 0.3 or 1 mg mL^{-1} induced cell death that ranged from 22.8 to 38.6% depending of the blood donor. LPS (10 ng mL^{-1}) alone did not significantly induced cell death as described by others. Furthermore, LPS treatment did not modify or potentiate the toxicity of Stifenia.

Stifenia Induces Cytokine Gene Expression in Zebrafish Larvae

To analyze the inflammatory effect of Stifenia on a whole organism, we used zebrafish larvae. Using RT-qPCR, we investigated the effect of Stifenia on IL-1β and TNFα gene expression. Twelve to fifteen zebrafish larvae were exposed to different concentrations of Stifenia for 20 h and then harvested for total RNA extraction. TNBS used as a reference inflammatory compound induced a slight IL-1β gene expression compared to unexposed fishes as previously described (24, 28). The lowest concentration of Stifenia induced a higher IL-1β gene expression than TNBS. Furthermore, we observed a dose-dependent effect of Stifenia on IL-1β gene expression. For TNFα gene expression, an increase was observed from 0.01 mg mL^{-1} of Stifenia exposure (**Figure 4**).

DISCUSSION

Inflammatory Effect of Stifenia

In this study, we demonstrated that the commercial plant protection product Stifenia decreased the MA of PBMC and affected cell viability. Stifenia also induced IL-1β production in all the PBMC batches tested, but to a different extent depending on

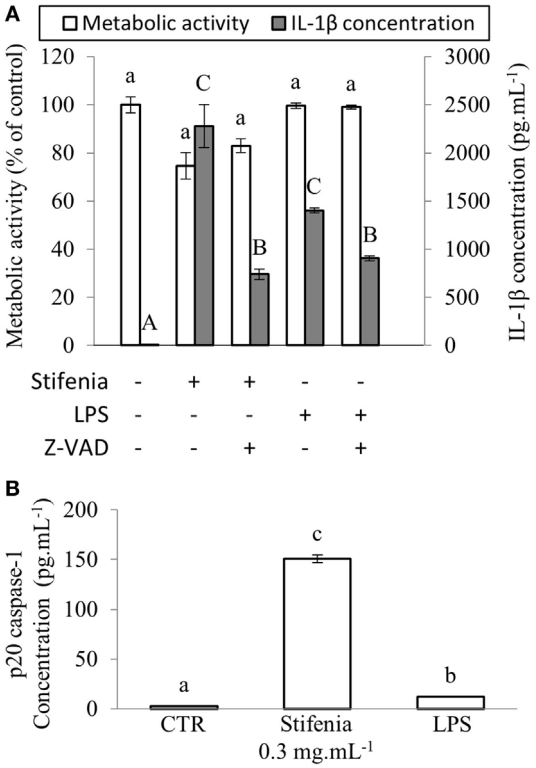

FIGURE 3 | Stifenia-induced IL-1β production is mediated by caspase1. **(A)** Peripheral blood mononuclear cells were stimulated by 0.3 mg mL^{-1} of Stifenia or 10 ng mL^{-1} of LPS. Cell metabolic activity (MA) (white bars) was estimated by the XTT assay and IL-1β (gray bars) was measured in the culture medium 20 h after treatment. Five micromolars Z-VAD-FMK (Z-VAD) were added simultaneously to Stifenia in the culture medium. LPS was added 30 min after Z-VAD-FMK treatment. The results showed are obtained from the blood donor CHR026_93. Bars represent the mean between eight technical replicates. Lowercase and capital letters indicate statistical groups for MA and IL-1β concentration, respectively. Different letters indicate statistical differences between groups ($p < 0.05$). If two conditions share one or several letters, there are no statistical differences. CTR, control non-treated cells. **(B)** Quantity of p-20 Caspase-1 fragment was measured using ELISA in the culture medium 20 h after the addition of 0.3 mg mL^{-1} of Stifenia or 10 ng mL^{-1} of LPS. Each bar corresponds to a mean of eight technical replicates. Results are obtained from the blood donor CHR026_93. This experiment was repeated once on another blood donor with similar results (data not shown). **(A,B)** Statistical differences were determined using a Kruskal–Wallis test followed by a comparison with the Steel–Dwass–Critchlow–Fligner method. Similar letters indicate no significant differences ($p < 0.05$). CTR, control non-treated cells.

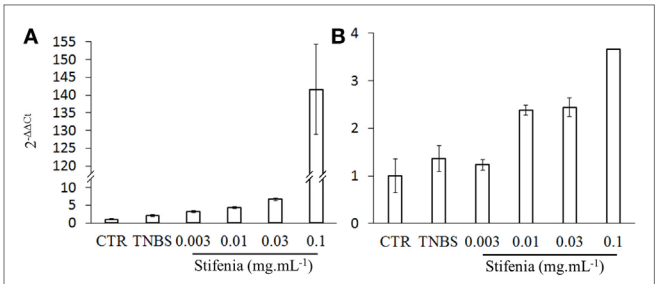

FIGURE 4 | Effect of Stifenia treatment on cytokine gene expression in zebrafish larvae. Induction of cytokine gene expression in zebrafish larvae exposed for 20 h to Stifenia aqueous extract. The transcript levels of IL-1β **(A)** and TNFα **(B)** were quantified by RT-qPCR using the *Danio rerio* HPRT1 gene as housekeeping gene and normalized to the transcript level of larvae that were not exposed (CTR) using $2^{-\Delta\Delta Ct}$ method. Data are the means of duplicates from one representative experiment out of two.

the blood donor. IL-1β is an inflammatory cytokine circulating at very low levels (0.5–10 pg mL^{-1}) in blood stream of healthy people (29, 30). In our culture conditions, unstimulated PBMCs produce similar concentration of IL-1β in the culture medium. When PBMCs were exposed to Stifenia 0.1 mg mL^{-1}, they released around 500 pg mL^{-1} of IL-1β, which corresponds to a 50- to 1,000-fold increase of IL-1β concentration compared either to control conditions or to the concentrations of IL-1β found in blood of healthy people. It could be hypothesized that such an increase of IL–1β concentration induced by Stifenia could have physiological effects.

The inflammatory effect of Stifenia contrasts with other published data indicating that fenugreek seed extracts exhibit anti-inflammatory properties [reviewed by Goyal et al. (13)]. We did not detect any anti-inflammatory properties when Stifenia was used as pretreatment before LPS stimulation. A similar protocol was, however, efficient when used with other natural or synthetic compounds (31, 32). The discrepancy between our data and published ones could be explained. First, we used an aqueous extract of Stifenia while other studies used organic extracts. For instance, Mandegary et al. (25) performed a methanol extraction of fenugreek seeds followed by a fractionation using water and different organic solvents. They showed that the aqueous fraction mostly contains flavonoids and inhibits carrageenan-induced paw edema in mice. Furthermore, ethanol extract of fenugreek seeds reduced Freund's complete adjuvant-induced arthritis in rats by lowering cytokine induction (26). We suspect that the anti-inflammatory effect found in these extracts (25, 26) could be hidden or lowered by other components present in our aqueous Stifenia extract. Second, the biological/experimental models used to demonstrate the anti-inflammatory effects were different from the human PBMC model used in this study. However, the use of an aqueous extract in our study is of relevance because it reflects what is done by farmers when Stifenia is prepared according to the manufacturer's instructions. In other words, the aqueous extract we tested is similar to what is sprayed by farmers on crops.

We showed that caspase-1 was involved in the inflammatory response induced by Stifenia suggesting a role for the inflammasome NLRP3. IL-1β is produced by immune cells as a response to NLRP3 inflammasome activation when cells are confronted to PAMP but also to different danger signals (DAMP) of metabolic origin (27, 33). It could be suspected that some compounds contained in the Stifenia extract could stimulate the NLRP3 inflammasome and lead to caspase-1 activation that is responsible for pro-IL-1β processing.

Because Stifenia induced IL-1β production in a caspase-1-dependant pathway, one may suggest that Stifenia effects are partly due to a contamination with LPS which induce pro-IL-1β gene transcription after its perception by TLR4, a phenomenon known as priming. Thus, the presence of LPS in Stifenia aqueous

extract has to be considered. To test this hypothesis, we checked for the presence of 3OH-fatty acids that compose LPS in the Stifenia aqueous extract using an HPLC/MSMS method (34). Interestingly, we detected 3OH-C10:0, 3OH-C12:0, 3OH-C14:0, and 3OH-C16:0 revealing a LPS contamination of Stifenia (data not shown). After filtration of the Stifenia aqueous extract on a poly-lysine column, LPS contamination was decreased by about 40%. However, this extract was still efficient in inducing IL-1β production on PBMC (data not shown). Furthermore, PBMC harvested from two blood donors, CHR026_135, and CHR026_152 (Figures S1D,E in Supplementary Material), are not or very slightly susceptible to LPS while they are highly reactive to Stifenia. We also showed that Stifenia exposure induced cell death of PBMC, whereas LPS did not. All these data suggest that Stifenia-induced IL-1β production and Stifenia-induced cell death are unlikely due to a direct effect of LPS, but we cannot rule out that LPS present in Stifenia aqueous extract could prime pro- IL-1β gene transcription.

Are Inflammatory Effects Relevant for Human Health?

The ANSES has assessed the acceptable exposure level of Stifenia for operators (0.3–2.9 mg kg^{-1} of body weight/day), workers (3–9 mg kg^{-1} of body weight/day), and neighborhood (0.01–0.09 mg kg^{-1} of body weight/5 min of continuous exposition) (ANSES 2012-1685 and 2013-0227). Even if these estimated contaminating doses seems to be low, recurrent or longer exposures have not been tested so far. Our results demonstrated some inflammatory properties and toxicity of Stifenia on blood mononuclear cells. Of importance, the potential toxicity of fenugreek was recently highlighted by some studies both in human and animals. Thus, a survey in Moroccan maternity hospital has linked consumption of fenugreek by pregnant women to congenital malformations (14, 35). In mice, feeding females during the entire period of pregnancy with a lyophilized aqueous extract from fenugreek seeds affects their reproduction and shows teratogenic and foetotoxic effects (4). Khalki et al. (15) also reported growth retardation and altered neurobehavioral performance of mice prenatally exposed to fenugreek seed extracts. Antifertility effects of fenugreek seed extract has also been reported in rabbits (17), and other toxic effects have been described in mice, rats and rabbits (16, 18). However, since fenugreek seeds have been used for centuries in traditional medicine or in food, many tests were not included in the risk assessment of Stifenia (36). Our results pointed out the possible danger of an extensive use of the plant defense stimulator Stifenia at the level of human health.

Environmental Toxicity

Our data demonstrated *in vivo* inflammatory effect of Stifenia on zebrafish larvae. We observed an induction of IL-1β and TNF-α gene expression that started at Stifenia concentration as low as 3 and 10 μg mL^{-1}, respectively. The predicted environmental concentration (PEC) of Stifenia in surface water was established by the ANSES at 0.160 μg mL^{-1} (ANSES 2012-1685). This is only 20-fold lower than the 3 μg mL^{-1} concentration that induced significant IL-1β gene expression in our experiments. This raises the issue of whether a longer exposure of zebrafish larvae to the PEC concentration could have similar inflammatory effects to those we observed. However, the methods used to predict PEC in surface water are controversial and could not reflect the actual concentrations of the molecule in the environment (37). Recently, it has been shown that the PEC calculated for several insecticides are underestimated by the procedure used by the European Union (38). Regarding the toxicity of fenugreek on animals, a study showed that topical application of 6 μg of fenugreek acetonic seed extract on two coleopteran species, *Acanthoscelides obtectus* and *Tribolium castaneum*, decreased their fertility and induced their mortality. These authors also showed that the presence of fenugreek seeds in the immediate environment of insects was sufficient to kill them (19). All these results suggest that the use of Stifenia could have adverse effects on non-target organisms.

CONCLUSION

Our results demonstrated unexpected effects of a plant protection product on human and animal health. As written by Burketova et al. (2), "although bio-based products are of natural origin, direct toxicity of these products to human, animals, insect, microbe communities, or even plants must be studied carefully to avoid toxicity as observed with classical pesticides." The human cell-based approach developed in this work revealed a high sensitivity concerning inflammatory properties of a plant protection product. These tests could be routinely used to screen the potential adverse effects of this type of compounds that can potentially cross-react with human innate immunity. This should be the first step before engaging more expensive studies on animal models and according to the European legislation, and this approach fits with the goal of reducing studies on animal models (No. 1107/2009).

ETHICS STATEMENT

For Zebrafish larvae, there is no need of ethics committee because they were euthanized at 120-h postfertilization [EU Directive 2010/63/EU, Strähle et al. (21) and Geisler et al. (22)]. For human PBMC, there is no need of ethics committee. PBMCs were obtained from Etablissement Français du Sang (EFS) according to agreement No. DECO-14-0124 and EFS do not indicate any information on PBMC donors.

AUTHOR CONTRIBUTIONS

LT, OL, and JLC designed this work. LT, JCo, OL, and JLC performed most of the experiments. JCh supervised zebrafish experiments. LT, JCo, SD, JCh, DW, OL, and JLC contributed substantially to the completion of this work. LT, OL, and JLC wrote the manuscript. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fpubh.2017.00074/full#supplementary-material.

TABLE S1 | Cytokine concentrations in culture medium of Stifenia-exposed peripheral blood mononuclear cells. Cytokines were measured in the culture medium 20 h after the addition of Stifenia at the indicated concentrations using a multiplex kit (ProcartaPlex human simplex, Affymetrix, France) according to the manufacturer's instructions on blood donor CHR026_91. C, concentration; IF, induction factor; CTR, control non-treated cells.

FIGURE S1 | Effect of Stifenia IL-1β production and metabolic activity (MA). Peripheral blood mononuclear cells were exposed to different concentrations of Stifenia added in the culture medium for 20 h. Cell MA (white bars) was estimated by the XTT assay, and IL-1β (gray bars) was measured in the culture medium. Results are obtained from six blood donors (**A**–**F**). Bars represent the mean of eight technical replicates. Different letters (lowercase for the XTT assay, capitals for IL-1β) indicate statistical differences between groups ($p < 0.05$). Statistical differences were determined using a Kruskal–Wallis test followed by a comparison with the Steel–Dwass–Critchlow–Fligner method. CTR, control non-treated cells.

FIGURE S2 | Effect of Stifenia on LPS-induced IL-1β production and metabolic activity (MA). Peripheral blood mononuclear cells were stimulated by addition of 10 ng mL^{-1} of LPS 30 min after the beginning of Stifenia exposure. Cell MA (white bars) was estimated by the XTT assay, and IL-1β (gray bars) was measured in the culture medium. Results are obtained from five blood donors (**A**–**E**). Bars represent the mean of eight technical replicates. Different letters (lowercase for the XTT assay, capitals for IL-1β) indicate statistical differences between groups ($p < 0.05$). Statistical differences were determined using a Kruskal–Wallis test followed by a comparison with the Steel–Dwass–Critchlow–Fligner method. CTR, control non-treated cells.

FIGURE S3 | Effect of ZVAD on Stifenia- or LPS-induced IL-1β production and metabolic activity (MA). Cell MA (white bars) was estimated by the XTT assay, and IL-1β (gray bars) was measured in the culture medium 20 h after treatment. Five micromolars Z-VAD-FMK (Z-VAD) were added simultaneously to Stifenia (0.3 mg mL^{-1}) in the culture medium or 30 min before the addition of LPS (10 ng mL^{-1}). Results are obtained from blood donors CHR026_95 (A) and CHR026_153 (B). Bars represent the mean between eight technical replicates. Different letters (lowercase for the XTT assay, capitals for IL-1β) indicate statistical differences between groups ($p < 0.05$). Statistical differences were determined using a Kruskal–Wallis test followed by a comparison with the Steel–Dwass–Critchlow–Fligner method. CTR, control non-treated cells.

FIGURE S4 | Peripheral blood mononuclear cell (PBMC) viability after Stifenia treatment. Cell viability was estimated by propidium iodide (PI) staining 20 h after PBMC treatments. The results shown are obtained from blood donors CHR026_153 **(A,B)** and CHR026_152 **(C,D)**. **(A,C)** Cell death was expressed as a percentage of PI-stained cells vs. total cells. Bars represent the mean between four technical replicates. Different letters indicate statistical differences between groups ($p < 0.05$). Statistical differences were determined using a Kruskal–Wallis test followed by a comparison with the Dunn's method. **(B,D)** Flow cytometry diagram of PI-stained cells 20 h after treatment. For each treatment, a representative diagram out of four technical replicates is shown for the blood donor CHR026_153 **(B)** or the blood donor CHR026_152 **(D)**.

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Complex Outcomes from Insect and Weed Control with Transgenic Plants: Ecological Surprises?

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Agriculture is fundamental for human survival through food production and is performed in ecosystems that, while simplified, still operate along ecological principles and retain complexity. Agricultural plants are thus part of ecological systems, and interact in complex ways with the surrounding terrestrial, soil, and aquatic habitats. We discuss three case studies that demonstrate how agricultural solutions to pest and weed control, if they overlook important ecological and evolutionary factors, cause "surprises": (i) the fast emergence of resistance against the crop-inserted Bt-toxin in South Africa, (ii) the ecological changes generated by Bt-cotton landscapes in China, and (iii) the decline of the monarch butterfly, *Danaus plexippus*, in North America. The recognition that we work with *complex systems* is in itself important, as it should limit the belief in *reductionist solutions.* Agricultural practices lacking eco-evolutionary understanding result in "surprises" like resistance evolution both in weeds and pest insects, risking the reappearance of the "pesticide treadmill"—with increased use of toxic pesticides as the follow-up. We recommend prioritization of research that counteracts the tendencies of reductionist approaches. These may be beneficial on a short term, but with trade-off costs on a medium- to long-term. Such costs include loss of biodiversity, ecosystem services, long-term soil productivity, pollution, and reduced food quality.

Keywords: Bt-toxins, ecology, herbicides/pesticides, glyphosate, GM crops, non-target organisms, resistance evolution

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THE USE OF PESTICIDES IN AGRICULTURAL PRACTICE WITH TRANSGENIC PLANTS

Genetic modifications of crop plants have great potential and promises, but current growing practices are overwhelmingly restricted to four crop species and two kinds of GM modifications. The four species are soybean (*Glycine max*), maize (*Zea mays*), oilseed rape (*Brassica oleracea*), and cotton (*Gossypium hirsutum*). The majority of these crops (except cotton) are grown on large-scale industrial–style farms, mostly in North and South America (James, 2016). The two dominant modifications, herbicide tolerance (HT) and insect resistance (IR), can make crops tolerant to selected herbicides, or toxic to specific groups of herbivorous insects, respectively. Most of the GM soybean and oilseed rape are HT, while transgenic maize and cotton are mostly IR. An increasing number of GM cultivars are "stacked" and/or "pyramided," containing both kinds of modifications, and several constructs.

HT GM plants open the formerly narrow time window when herbicides could be sprayed on crop fields without risk to the crop itself. Farmers planting HT crops can use herbicides, even at higher concentrations than previously, without damaging their crop. As a result, the global share of the few herbicides that can be used on such GM crops has increased dramatically. The commercially most successful application is linked to herbicides with glyphosate as the active ingredient (Roundup products), whose use only in the USA increased from 3.6 million kg in 1987 to 108 million kg in 2014 (Myers et al., 2016).

To make cultivated plants toxic to herbivores, the most commonly used method is the insertion of activated toxin genes from the soil-living bacterium *Bacillus thuringiensis* (Barton et al., 1987). Numerous strains of this bacterium have been isolated, characterized, manipulated and inserted into a variety of crop plants (Bravo et al., 2011), but the majority of field-grown insect resistant GM plants are maize and cotton. In the case of maize, the aim was to defend the plant from the attack of two pests that are important in the USA: the non-native European corn borer *Ostrinia nubilalis* (Lepidoptera: Pyralidae), and a native beetle, the corn rootworm (*Diabrotica virgifera*, Coleoptera: Chrysomelidae). In the case of cotton, the primary target pest was the cotton bollworm (*Helicoverpa armigera,* Lepidoptera: Noctuidae), an important pest in Eurasia, Africa, and the Americas. The presence of the bacterial toxin in the plant defends it from damage by these main pests. After the introduction of Bt-plants a reduction in the amount of insecticides sprayed was observed, especially on cotton, the most intensively insecticide-treated crop plant in the world (Deguine et al., 2008).

GM plants represent an important trajectory of modern agriculture, with a strong focus on weed and insect control. Moreover, in GM plants, whether HT or IR, permit new ways to use pesticides in agriculture. This has interesting practical as well as ecological and evolutionary consequences in comparison to other agricultural practices.

There is an ongoing and rapid change in GM crop plants. In 1996, the first year of commercial planting, GM crop plants predominantly expressed a single transgene of bacterial origins, either a *cry1Ab* Bt gene in IR crops, or a *cp4 epsps* gene (glyphosate tolerance) in HT plants. Twenty years later, an increasing number of GM crop plants can express up to 6 different Bt-toxins and up to 3 different herbicide tolerance traits (Hilbeck and Otto, 2015; Venter and Bøhn, 2016). We may even expect up to 14 different transgenes in a single GM plant by 2020 (Hakim, 2016). What is the driving force for "stacking" all these traits on top of each other?

In order to forecast the environmental consequences of field-growing GM plants, it is important to stress that genes alone do not determine the outcomes: the gene-organism-environment ("The Triple Helix") interactions are crucial for the understanding of any biological system (Lewontin, 2000). If we ignore the dynamic responses of nature, "surprises" and failures will be the order of the day. In contrast, by using ecological and evolutionary theory, we would have been able to foresee and possibly avoid many unwanted outcomes that we are experiencing today.

Pesticides are an integral, nearly unavoidable, part of the dominant current agricultural practices. Their dominance can be traced back to the period during and after the Second World War. Problems emerged gradually, and started to be voiced in the early 1960s with the "Silent Spring" of Rachel Carson (Carson, 2002). Since then, pesticides have been under tighter and tighter regulation, and were increasingly recognized as the mixed blessing they are. Notwithstanding the technological advances with pesticides and their applications, the serious global health effects caused by hazardous pesticides has recently made the UN formulate a new set of recommendations. These includes that (i) pesticide use must be closely monitored, regulated and reduced worldwide, and that (ii) non-chemical alternatives must be considered first, e.g., use (agro)ecological methods to naturally supress pests (United Nations, 2017).

In this article, we aim to illustrate the dynamics of ecological/evolutionary responses to field growing of transgenic plants, an important component of modern agriculture. We present three case studies and use these to discuss dynamic ecological and evolutionary responses related to insect and weed control with GM crop plants. Our center of attention concerns the sustainability of this agricultural practice, and whether the ignoring of ecological complexities may lead to new, mistaken technological solutions, resistance evolution and further pesticide use.

CASE STUDY 1. RESISTANCE EVOLUTION IN *BUSSEOLA FUSCA* TO CRY1AB TOXIN, SOUTH AFRICA

Several important insect pests of maize are internal feeders, and thus not easy to control by traditional pesticides. This was a strong motive to develop transgenic GM maize lines that can express an insect toxin *in planta*, thus presenting the potential to control such internal feeders. In South Africa, the main target insect pest, *Busseola fusca* (Lepidoptera: Noctuidae), is such a pest, whose larvae are boring inside the maize plant. They were successfully controlled by Bt-maize expressing the Cry1Ab-toxin (MON810) after its introduction in 1998, for a period of about 6 years. In the 2004/5 season, the first reports on resistant insects were coming in: *B. fusca* larvae could be found feeding on Bt maize plants (van Rensburg, 2007; **Figure 1A**). By 2010, the area where such resistant insects were found increased to cover most of the maize growing areas in the country (Kruger et al., 2012; Van den Berg et al., 2013). Where resistant insects appeared, farmers responded by re-starting the previous practice of spraying insecticides—now on the transgenic MON810 variety (**Figure 1B**).

This resistance evolution of *B. fusca* in South Africa triggered the replacement of the original, single toxin-expressing MON810 with MON89034, a plant that expresses two toxins: Cry1A.105 and Cry2Ab2 (Van den Berg et al., 2013). Thus, the emerging resistance in pest insects led to the stacking of two insect toxins in the same plant. This may resemble the start of the "pesticide treadmill," where the typical response to emerging resistance to an insecticide was to start using cocktails of various ones, with

FIGURE 1 | Resistance development to Cry1Ab in South Africa. After the development of resistance to Bt, the larvae of the target pest, *Busseola fusca*, could be observed in the field, feeding on MON810 maize cobs **(A)**. The response from the farmers was to spray insecticides, paid by Monsanto, on the "insect resistant" MON810 Bt-maize plants to reduce yield losses **(B)**. Photos taken in the Vaalharts region in 2012 by Thomas Bøhn.

the argument that these combinations will remain effective for longer (Nicholls and Altieri, 1997). For sprayed pesticides, this has not been a sustainable solution, because multiple resistances emerged, making the problem more severe.

CASE STUDY 2. *HELICOVERPA ARMIGERA* REPLACED BY MIRID BUGS AS MAJOR PESTS, CHINA

China is among the biggest cotton producers of the world. On five of the six major cotton-growing regions, mostly smallholders grow cotton, using it as a cash crop. Before 1997, the use of pesticides to control insect pests, the cotton bollworm *H. armigera* in particular, was very high and included 14–28 treatments per season (Pemsl and Waibel, 2007). Resistance was widespread, and the increased frequency of sprayings did not provide a lasting solution, even if the serious human safety issues are disregarded. The introduction of Bt-cotton after a government decision in 1996 resulted in quick take-up of the technology, and improved the conditions for the farmers considerably. Not only was there a decline in pest attacks on cotton, especially in the early season; other important crops harmed by the polyphagous *H. armigera* also started to show reduced pest densities (Wu et al., 2008). The reason was that the typically small cotton fields were dispersed in the landscape and cotton, the previously preferred crop for *H. armigera*, was replaced by Bt-cotton and suddenly became unusable as a habitat for that insect species. The efficient killing of the target pest had turned cotton plots *into a population sink* at the regional/landscape level (Lu et al., 2010). This shows the potential dynamics in source and sink populations, i.e., conditional sources and sinks (Loreau et al., 2013), altered by context dependent conditions, here the introduction of Bt-toxin in cotton, and the particular distribution of cotton plots in the overall cultivated landscape.

Had the story ended here, it would have been a great success story of the Bt-cotton (Wu et al., 2008). The fact that Bt-cotton led to a regional decline in a major pest species and therefore also diminished the damage caused by *H. armigera* on multiple crop plants also coincided with a reduced load of toxic pesticides (Pemsl and Waibel, 2007).

However, due to ecological responses and interactions, the full story is more interesting. As Bt-cotton became less suitable to *H. armigera*, large resources, both in terms of pesticide-free habitat and cotton plant biomass became available to species less susceptible to Bt-toxins. Additionally, the reduced pesticide pressure also, at least initially (Carrière et al., 2016), eased the pesticide pressure on other insect groups. A formerly secondary pest group, mirid bugs (Miridae, Heteroptera) now increased in numbers, and became an important pest on cotton (Lu et al., 2010). With the high densities of mirids on Bt-cotton, these plots now became *population sources* of mirids that subsequently migrated to other crops. The outcome was that the higher proportion of cotton was Bt-transgenic in the landscape, the higher became the densities of mirids on other crops in the region (Lu and Wu, 2011). Thus, the management regime of Bt-cotton, including the change in pesticide use triggered by the use of the GM cultivars, made the same cotton patches sources of another herbivorous pest, and caused the subsequent spread of a non-target, secondary pest at the landscape level (Lu et al., 2010).

Case study 2 illustrates how the single-species focus can backfire when a "technological solution" is employed against a single species, and ecological complexity gets ignored. Crops usually have numerous target pests, or potential target pests, some of which may be minor due to various reasons; this does not mean that they do not have the potential to cause large damage—their damage potential is only suppressed by the dominating pest species. Change the density of this major pest, and these other potential pests may quickly respond due to competitive release (Zeilinger et al., 2016).

CASE STUDY 3. THE MONARCH BUTTERFLY AND LANDSCAPE LEVEL EFFECTS OF HERBICIDE USE, NORTH AMERICA

One of the most fascinating insect migrations is performed by the North American monarch butterfly, *Danaus plexippus*

(Lepidoptera: Danaidae), that migrates from its summer distribution area, covering a large part of North America, to winter in a small part of central Mexico, in spectacular masses (Stenoien et al., 2016). For this reason, as well as for its spectacular coloration, the monarch has become a symbol of beauty and freedom—one of the conservation icons of North America. The monarch larvae feed on milkweed, *Asclepias syriaca*, which is a common weed, especially in maize and soybeans fields (Oberhauser et al., 2001). In 1999, an article in *Nature* reported that the larvae of the monarch butterfly suffered high mortality in laboratory trials where they fed on milkweed leaves dusted with a high density of transgenic Bt-maize pollen (Losey et al., 1999). This study generated heated discussions, but also triggered a large research project examining the possible consequences of planting transgenic maize over vast areas in the summer distribution range of the monarch butterfly. The summary conclusion was that the risk of being exposed to significant amounts of Bt-toxin from maize plants through pollen in the field was negligible (Sears et al., 2001). However, Oberhauser and co-workers cautioned that the issue needed to be looked at from a larger perspective, including weed control and the use of pesticides by farmers (Oberhauser et al., 2001).

By the mid-2010s it became obvious that the cautions were justified: the monarch population densities were dramatically reduced with a loss of an estimated 40 million individual butterflies per year (about 9% decline per year) after 1993/1994 (Williams and Brower, 2016; Oberhauser et al., 2017). The lowest recordings of the monarch densities on the wintering ground are from 2013 to 2015 (Rendón-Salinas and Tavera-Alonso, 2015), and the probability that the fascinating migration of the eastern monarch will go extinct within 20 years is estimated to be 11–57% (Semmens et al., 2016). Alarmed by these perspectives, initiatives across borders (US, Mexico, Canada) have been started to conserve the monarch butterfly. However, management action needs an understanding of the *causes* of the decline in order to improve the situation. So what are the key causal factors to explain the monarch decline?

The exponential growth of hectares with GM plants after 1996 in the US increased the acreage under Bt-transgenic plants, but even more those of glyphosate tolerant GM plants. From 2004 and until 2015, plants with "stacked" traits, i.e., both with Bt-toxins and herbicide tolerance increased from <10 million ha to about 60 million ha (James, 2015). As a result, non-target organisms including monarchs would interact, not only with Bt-toxins and herbicides directly, but also with the indirect effects of these factors at a landscape level.

A subtle but highly important indirect effect on the landscape level is related to herbicide tolerant GM crops and the massive use of broad-spectrum herbicides. The increased use of glyphosate products have caused a dramatic decline in the dominant host plant for the monarch. This is, seen from the monarch point of view, a serious habitat destruction of its highly specialized habitat. What is efficient weed management for the farmers can be fatal for a butterfly depending on a dominant host plant that grows between the rows of HT GM plants.

Monarch butterflies have experienced a dramatic decline in the availability of their host plant, the milkweed. In Iowa, milkweed was present in 51% of the fields in 1999, but only in 8% a decade later. In addition, even in the fields where still observed, milkweed density was reduced to about 10% of its original value (Hartzler, 2010). In sum, the decline in milkweed amounted to a near-complete elimination in the core of the breeding range of the monarchs (Pleasants, 2015). The maize and soybean fields were turned into milkweed deserts.

At the landscape level over the whole mid-western USA, the decline in milkweed has been almost 40%. However, since the monarch butterflies on average lay 3.9 times more eggs on milkweed stems in agricultural fields, where the reduction of milkweed is most severe, the capacity to support the monarch as a species is reduced by 71% (Pleasants, 2017).

The threat to the monarch triggered various conservation responses, including a restoration goal of reaching "six ha of overwintering monarchs" (i.e., six ha of trees covered by monarchs). To succeed, 1.6 billion additional milkweed plants would be needed, a number higher than the estimated current total population (1.34 billion plants) for the whole Midwest (Pleasants, 2017). Restoration of the milkweed seems to be crucial and the use of HT GM plants is identified as a key for the milkweed decline (Pleasants and Oberhauser, 2013; Zalucki et al., 2016).

DISCUSSION

The three case studies described above exemplify that agricultural ecosystems, even if arguably simplified, retain complexity, and that solutions to agricultural problems should be scrutinized from an ecological point of view. Ignoring ecological interactions tends to undermine the overly simple solutions, here exemplified with insect and weed control by the dominating GM plant traits and associated technology. When a pest insect or weed species overcomes a suggested "solution" to hold their density low, strong selective advantages will play out. Under such conditions, natural selection may be effective in a timespan of a few years, and threaten to undermine the efficiency of our weed and insect control, and thus also the goal of improved agricultural productivity.

When we look at the dominant technologies currently accompanying GM plants, there are particular challenges related to resistance evolution, both for herbicide tolerance traits and for insect resistance.

Herbicide Tolerant Crops and Weed Resistance Evolution

From 1995 to 2014, the global agricultural use of glyphosate rose 14.6-fold, from 51 million kg to 747 million kg and HT GM crops have been a major driver for this change. Moreover, by 2016, about 56% of the global use of glyphosate was connected to HT GM crops (Benbrook, 2016). Specific for the HT GM plants is that herbicides can be sprayed in higher doses and repeatedly during the growth season of the plants. The vast "experiment" that was initiated with HT GM crops and glyphosate as a stand-alone herbicide on millions of hectares of cropland, imparted tremendous selection pressure on the

weed populations. This has been a key factor for the resistance evolution, now documented for 37 species of weeds globally. Such development may lead to the familiar "treadmill" where resistance triggers more applications/higher doses, which leads to stronger selection pressure for resistance, etc. and eventually the use of additional herbicides like atrazine and 2,4D (Binimelis et al., 2009).

Unfortunately, the glyphosate resistant crops were not integrated into a total weed management program, rather it replaced all of the other programs (Shaner et al., 2012). In hindsight, this was not a wise move and showed us that no herbicide is invulnerable to resistance. At least three different mechanisms of resistance is identified: (i) alteration of the target site; (ii) changes in sequestration and/or translocation of the herbicide, and (iii) changes in the rates of metabolism of the herbicide (reviewed in Shaner et al., 2012).

For the farmers, resistant weeds are a difficult practical obstacle to handle. Although farmers often have a long-term perspective on their farming activity, they may also be attracted to quick-fix solutions, including pesticides and growing monocultures. Unfortunately, crop and herbicide monocultures create conditions for resistance development (Beckie, 2011).

The magnitude of resistance problems should be incentive enough to further explore the plurality of methods that can be used under integrated pest management, not only to delay resistance but to promote alternative and preferably non-toxic pest control systems (United Nations, 2017). Chemical treatments, coupled with the unavoidable resistance development are major blocking factors to a sustainable agriculture.

The use of herbicides like glyphosate also has the potential to affect ecosystem, animal and human health. The massive use of glyphosate, totaling 852 million kg globally by 2014 (Benbrook, 2016), which directly or indirectly will expose non-target biodiversity in terrestrial, soil and aquatic communities (Venter and Bøhn, 2016), represent a major source of environmental pollution. In addition, glyphosate is shown to accumulate in (i) soils that have a history of glyphosate use (Duke et al., 2017), and (ii) in HT soybeans (Bøhn et al., 2014), more when the plant is sprayed later in the season (Duke et al., 2003). This will bring glyphosate residues into the global food and feed chains (Bøhn et al., 2014).

The increased awareness of glyphosate toxicity, coupled with the increased volume used, should lead to stronger restrictions, for example lower acceptance level for glyphosate residues in food and feed (Cuhra et al., 2016). In this context it is perplexing why the maximum residue level (MRL) for glyphosate was raised 200-fold from 0.1 to 20 mg/kg in Europe, and to 40 mg/kg in the US (Cuhra, 2015). This set of events has been termed "The Glyphosate Paradox" (Cuhra et al., 2016). The WHO/IARC categorization of glyphosate as probably carcinogenic to humans (Guyton et al., 2015), although disputed by EFSA (EFSA, 2015), is underlining the significance of the controversy around the glyphosate-based herbicides.

Glyphosate is now also implicated in the decline of the monarch butterfly (Stenoien et al., 2016), further illustrating the various kinds of environmental damage that reliance on a few plant protection chemicals may bring. However, the monarch may not be the only species at risk for similar reasons—a total of 39 protected European lepidopteran species have maize weeds in their host plant range (Lövei et al., 2016).

Insect Resistant Crops and Resistance Evolution

GMO related "internal" pesticides such as Bt-toxins have a particular problem related to resistance evolution. The GM plants express Bt-toxins continuously, also when the "pests" are not a problem due to their low density. Pesticides expressed continuously, as in current insect resistant crops, simply raises the bar and offers continuous "trial and error testing" within potential pest populations, with a huge fitness reward on individuals that acquire resistance.

That evolution will eventually result in resistance developing in the target pest populations was foreseen before Bt-transgenic plants were grown commercially, and different strategies have been suggested and adopted to delay undesirable pest adaptation. For transgenic Bt- plants, the high-dose/refugia strategy is the most frequently recommended (Carrière et al., 2016). The role of the non-GM refugia is to secure the reproduction of susceptible insects and assure that the genes that make the target sensitive to the Bt-toxin do not disappear from the population. Thus, the high-dose Bt should remain effective, killing insects that have resistance alleles from one of the parents, and keeping the target population heterozygous.

The South African case with *B. fusca* showed that farmers initially did not follow the recommendation; only 8% of them established a refuge. By 2008, however, most or all farmers had established refugia (Kruger et al., 2009). This may have been too late, it seems likely that the initial non-compliance played a role in the fast appearance of the resistance (Van den Berg, 2016). The other key factor in promoting field-evolved resistance to Bt-toxin is that the high-dose standard is not met. The Cry1Ab maize used in South Africa in the relevant period did not fulfill this criteria for *B. fusca* (Van den Berg, 2016). Finally, the hypothesis of functionally recessive inheritance of resistance in the insect, meaning that when resistant and susceptible parents mate, the offspring will be susceptible, was rejected by experimental data in the South African *B. fusca* (Campagne et al., 2013).

The *B. fusca* case illustrates that the positive effect of reduced amounts of insecticides sprayed (e.g., Marvier et al., 2007; Osteen and Fernandez-Cornejo, 2013) may not last, or lead to the use of stacked events with multiple Cry toxins inserted. A recent review (Carrière et al., 2016) concluded that under the current way of growing, the pyramiding of Bt-toxins is not a stand-alone solution to the resistance development problem.

Another reason for pyramiding different Cry toxins in the same plant is to protect the plant from pest insects from different taxonomic groups, e.g., from both Lepidoptera (Cry1 and Cry2 toxins) and Coleoptera (Cry3 toxins). In maize, up to six different Cry toxins are combined in the same plant, as in hybrid MON 89034 × 1507 × MON 88017 × 59122, from Monsanto and Dow AgroSciences, which expresses *cry*1A.105, *cry*1F, *cry*2Ab2, *cry*3Bb1-, *cry*34Ab1, and *cry*35Ab1. Clearly, the added range of targeted pests is likely to produce stronger effects on non-target communities as well (Then, 2009).

When two toxins are active against the same insect species, resistance may be delayed. In North Carolina, transgenic cotton with two Cry-toxins resulted in much higher mortality (96 vs. 44%) in the pest *Helicoverpa zea* compared to cotton with a single Cry-toxin (Carrière et al., 2016). At the same time, using several toxins at the same time is analogous to the use of multiple antibiotics in the same treatment. The risk is that fewer agents are left unused when resistance appears. In a long-term perspective, resistance will inevitably develop, even if a range of pest management practices will delay the process. Reliance on pesticides, does not represent a sustainable agricultural practice. Therefore, the UN is recommending (i) proactive measures to reduce or eliminate harmful pesticides, and (ii) to consider non-chemical alternatives first (United Nations, 2017).

The risk that control measures against target pest insects are lost due to resistance evolution can be tracked back more than 100 years, and is particularly relevant if there is a continuous selection pressure for resistance (Andow, 2008). Nevertheless, most pest populations have remained susceptible to Bt toxins, but 5 out of 13 major pest species have already acquired field-based resistance (reviewed in Tabashnik et al., 2013).

A pesticide could best solve a pest problem if it was perfectly specific for the target species, and killed only that harmful organism. In that case, effects on other species would be limited to the altered ecological interactions in the ecosystem, most plausibly on species directly linked to the target species in the food web. However, known chemical and biological pesticides are either very broad, harming all insects with an exoskeleton (as for DDT), or are more specific because their action requires certain conditions (like high pH, cleavage by enzymes, etc. as with Bt-toxins). This may limit the range of species/taxa harmed, although there still may be several species in the harmed group. For example, the order Lepidoptera, the main target group of Cry1 and Cry2 toxins, contains 126 families and some 180,000 known species (Capinera, 2008). Moreover, a single plant species may be a host to numerous species. Maize and cotton are registered as hosts for 776 and 872 lepidopteran species, respectively (Robinson et al., 2010).

When the criteria of specificity to the pest species is not fulfilled, harmful effects on non-target species (biodiversity) can be expected. A range of factors contributes to potential negative effects on non-target species.

Firstly, the *toxicity* of the pesticides will be crucial, which is typically taxa/species/age- and context- dependent.

Secondly, the *break-down rate* will modify and reduce the toxicity over time. Pesticides that are decaying slowly may accumulate in the food web and have serious long-term effects, such as the PCBs (Gobas et al., 2016). Pesticides that do not break down may be a part of the food or feed produced and have further effects on humans or animals along the food/feed chain. The break-down rate of chemicals depends on environmental factors like pH, soil type, binding to other particles etc., which adds to the complexity.

Thirdly, the *timing and dosage* of applications are important for potential unwanted effects. Treatments with toxic pesticides are ideally precisely timed to hit when the problem is severe. The option to time the application of a pesticide can therefore be a good thing. With pesticides that are expressed continuously from the plant genome, as in Bt-plants, such flexibility is lost.

In the context of negative effects on biodiversity, the sensitivity of non-target organisms, most of the species in soil and aquatic communities have never been tested for their vulnerability to Bt-toxins. Several aspects of the fate of Bt-toxins are not well-known, including amounts, break-down rates and effects. Further, studies of tri-trophic relationships and food web interactions may provide insight to community responses (Yu et al., 2014).

Can Stacked Traits Act as Multiple Stressors?

The understanding of resistance evolution and stacking/pyramiding of traits must be linked to potential combinatorial effects on non-target organisms. The use of stacked events represents: (i) increased doses/more applications of herbicides per season, and (ii) a broader range of Cry toxins in insect resistant GM plants. Both these effects trigger positive feedback loops with stronger selection pressures and further resistance evolution. Since these toxins/chemicals/traits will meet and interact, also with other stressors in the environment, the co-exposure and potential combinatorial effects need to be studied (Nørgaard and Cedergreen, 2010; Bjergager et al., 2011). Combinatorial effects between Bt-toxins and herbicides may enhance toxicity (Then and Bauer-Panskus, 2017). For example, Bøhn and co-workers showed that Cry1Ab and Cry2Aa toxins act in combination (additively), indicating that "stacked events" may increase negative effects on non-target organisms (Bøhn et al., 2016). However, combinatorial effects represents a major knowledge gap in the scientific literature (Venter and Bøhn, 2016).

Sustainability of Agriculture

Agriculture has been fundamental for the rise of human civilization (Diamond, 1999) and continues to be vital for human survival through food production. However, many modern agricultural innovations relied on non-renewable resources that are not sustainable (Gliessman, 2015). We need a strong prioritization of resources for research to build knowledge to ensure that future food production is sustainable. In particular, there is a need to counteract agricultural practices that are beneficial on a short term, but with trade-off costs on a medium to long term scale. Such costs includes loss of biodiversity, ecosystem services, soil productivity, pollution and reduced food quality.

CONCLUSIONS

The currently dominant agricultural practice has changed the natural spatial distributions of plant species that provide food, fiber, and other important resources for us (classified as provisioning ecosystem services), and resulted in habitats that are less diverse than the original habitats that were converted to croplands. Nonetheless, these are biological entities, supporting and interacting with various plant, animal, fungal, and microbial communities in complex ways. The recognition that when trying to manage agricultural fields and landscapes,

we work with a *complex biological system* is in itself important, as adopting this view should limit the belief in *reductionist solutions*.

Moreover, from an evolutionary perspective on sustainable food production, chemical pesticides, both insecticides such as Bt-toxins and sprayed herbicides, carry problems that are hard to solve. Dominant technologies in transgenic plants rely on new ways of using pesticides. These practices does not utilize and often contradicts ecological understanding and are therefore likely to exacerbate current problems. It is not likely that pesticides can be eliminated from our dominant agricultural practices in the near future. Key factors to uphold or improve their efficiency would be to increase the precision (specificity, timing) while minimizing the amount used. This would reduce pollution, lower their accumulation in the environment as well as in food, both of which are positive outcomes for ecosystem and human health. We also have to place the analysis of possible environmental consequences into an agroecological context, because that approach inherently considers the possibility of multiple stressor interaction, sublethal effects, non-linear, and synergistic outcomes that are so characteristic of biological systems. The examples discussed in this article also underline the importance to incorporate landscape-level ecological knowledge into the evaluation practice, because spatially explicit analysis of potential impacts are important tools in making agricultural practice more sustainable.

In the three case studies discussed, the GM plants associated with simple pesticide-solutions were unable to solve complex agricultural problems. We argue that the resulting resistance development and increased use of herbicides arose because basic ecological and evolutionary theory was overlooked. Had such knowledge been included, we would have foreseen and possibly been able to avoid some of the unwanted outcomes we are experiencing today.

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TB and GL conceived the study and wrote the paper.

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REVIEW
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Specificity and Combinatorial Effects of *Bacillus Thuringiensis* Cry Toxins in the Context of GMO Environmental Risk Assessment

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Stacked GM crops expressing up to six Cry toxins from *Bacillus thuringiensis* (Bt) are today replacing the formerly grown single-transgene GM crop varieties. Stacking of multiple Cry toxins not only increase the environmental load of toxins but also raise the question on how possible interactions of the toxins can be assessed for risk assessment, which is mandatory for GM crops. However, no operational guidelines for a testing strategy or testing procedures exist. From the developers point of view, little data testing for combinatorial effects of Cry toxins is necessary as the range of possibly affected organisms focuses on pest species and no evidence is claimed to exist pointing to combinatorial effects on non-target organisms. We have examined this rationale critically using information reported in the scientific literature. To do so, we address the hypothesis of narrow specificity of Cry toxins subdivided into three underlying different conceptual conditions (i) "efficacy" in target pests as indicator for "narrow specificity," (ii) lack of reported adverse effects of Cry toxins on non-target organisms, and (iii) proposed modes of action of Cry toxins (or the lack thereof) as mechanisms underlying the reported activity/efficacy/specificity of Cry toxins. Complementary to this information, we evaluate reports about outcomes of combinatorial effect testing of Cry toxins in the scientific literature and relate those findings to the practice of environmental risk assessment of Bt-crops in general and of stacked Bt-events in particular.

Keywords: Bt toxins, non-target organisms, target organisms, synergistic effects, mode of action, adverse effects

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BACKGROUND

Today, many crop plants have been genetically modified (GM) to contain transgenic DNA sequences from the bacterium *Bacillus thuringiensis* (Bt) coding for the expression of so-called Cry toxins from 3 to 4 different classes (Cry1, Cry2, Cry3, and to a very limited extent Cry9 class) (Schnepf et al., 1998; Sanchis, 2011; Sansinenea, 2012; van Frankenhuyzen, 2013). These microbial toxins engineered into GM crops plants aim to control certain target pest species which differ depending on the regions where the crops are grown. In Bt plants, the Cry toxins are present persistently and usually in all plants parts from germination to harvest of the crops. Specifically, it is through genetic engineering that also the pollen of GM plants can express these bacterial toxins. Bt-toxins in pollen are not degraded by UV light and remain bioactive when, for example, deposited on host plants

(Ohlfrest et al., 2002). Toxic pollen is rare in nature as, typically, there is little selective advantage for such a trait to evolve.

When used as sprayable, externally applied *B. thuringiensis* based insecticides, the risk for non-target organisms to ingest the Cry toxins is low due to the limited persistence of these sprays in space and time as the Bt toxin is quickly degraded by UV light and removed from plants by rain (Behle et al., 1997). Risks to non-target insects are further reduced because sprayable *B. thuringiensis* products usually consist of Cry toxins in their inactive crystalline form (hence their abbreviation "Cry") that need to undergo a complex activation process before becoming active (see below for details). With the introduction of Cry toxin producing GM crop plants into industrial agricultural systems, a whole new dimension of spatio-temporal exposure to Cry toxins opened up involving a far broader range of non-target organisms below- and above ground (Hilbeck, 2001, 2002). Thus, the potential for chronic longterm effects became more likely to occur than with short-lived, inactive sprayable *B. thuringiensis* insecticides. Consequently, it was with the introduction of Cry toxin producing GM crops that the likelihood of potential adverse effects (i.e., risks) on non-target organisms, in particular beneficial insects like natural enemies of pests, pollinators, and species of conservation concern, came on the research agenda (Hilbeck, 2001, 2002).

Since about one decade, there is a marked increase in the commercial approval and adoption of GM plants carrying multiple transgenes coding for the simultaneous expression of several insecticidal Cry toxins (and also transgenes for herbicide resistance), so called "pyramided" or "stacked" events (Fernandez-Cornejo et al., 2014; United States Department of Agriculture and Economic Research Service (ERS), 2014). In 2014, stacked GM cotton reached almost 80% of cotton plantings up from around 25% 10 years earlier. Stacked GM maize made up 76% of the planted maize area in 2014 up from less than 10% a decade ago (Fernandez-Cornejo et al., 2014; United States Department of Agriculture and Economic Research Service (ERS), 2014). Stacking Bt transgenes was triggered by spreading resistance among target pest populations against GM crops expressing only single Cry toxins (Tabashnik et al., 2013). The underlying assumption is that pest species are less likely to develop resistance simultaneously against multiple Cry toxins because of their somewhat different modes of action. Whether or not this assumption will hold true for the currently employed Cry toxins that still share significant similarities in their modes of action (Hernández-Rodríguez et al., 2013) is not subject of our evaluation in this paper but deserves a separate analysis.

In this review, we are concerned with the drastically increased Cry toxin load in stacked or pyramided Bt crop varieties resulting in persistent exposure of a wide range of non-target organisms in terrestrial and aquatic ecosystems. In SmartStax® maize, for example, combining six Cry toxins, maximum amounts of >250 μg/g fresh weight in leaves, > 90 μg/g fresh weight in roots, and > 150 μg/g fresh weight in pollen was reported (**Table 1**; Stillwell and Silvanovich, 2007; Phillips, 2008). Potential combinatorial effects of these multiple Cry toxins have been recognized by the European regulatory authorities only few years ago (EFSA, 2010a), and are still intensely discussed by regulators and in the scientific community alike (de Schrijver et al., 2014). Yet, most regulators, including EFSA, still operate under the current controversial paradigm that limits the environmental risk assessment to focusing on the added novel substance only which is tested as single purified protein, produced by microbes, in isolation of the GM plants, following testing schemes developed for the regulatory approval of synthetic insecticides (for more details see Hilbeck et al., 2011). The results of these tests are then used in all risk assessments for Bt crops that express these Cry toxins but irrespective of the GM event (e.g., Garcia-Alonso et al., 2006; Romeis et al., 2008; Dolezel et al., 2011; Hilbeck et al., 2011). Consequently, for stacked GM crop plants combining multiple Cry toxins, developers of GM plants are arguing for minimal regulatory oversight of stacked events, if any at all, on the basis that *"previously approved GM events that have been combined by conventional plant breeding and contain GM traits that are not likely to interact in a manner affecting safety should be considered*

TABLE 1 | Maximum Cry toxin concentrations [μg/g] measured in field-grown SmartStax, several US locations in 2006*; FW, fresh weight; DW, dry weight.

Bt toxin	Leaves		Roots		Whole plant		Pollen		Kernels	
	DW	FW	DW	FW	DW	FW	DW	FW	DW	FW
Cry1A.105	210	34	100	12	86	10	21	16	4.9	4.3
Cry2Ab2	350	60	120	18	80	19	2.3	1.8	7.5	6.7
Cry1F	31	4.7	15	2.0	16	1.9	32	25	7.4	6.7
Total Lepidopteran-active Cry	591	99	235	32	182	31	55	43	20	18
Cry3Bb1	490	92	260	31	220	26	24	19	26	23
Cry34Ab1	279	42	150	19	196	23	117	90	94	85
Cry35Ab1	158	24	71	9.0	82	9.6	0.5	0.4	2.3	2.0
Total Coleopteran-active Cry	927	158	481	59	498	59	142	109	122	110
TOTAL	1518	257	716	91	680	90	197	152	142	138

Source: compilation based on Phillips (2008) and Stillwell and Silvanovich (2007).

to be as safe as their conventional counterparts" [Pilacinski et al., 2011, similar arguments by Raybould et al. (2012) and CropLife International (2015)]. EFSA (2010b) largely follows this view and has approved stacked events based on the above arguments—exemplary statements from an opinion for approval of a stacked event expressing three Cry toxins are as follows:

"*The safety of Cry1A.105 and Cry2Ab2 proteins expressed in maize MON 89034, the Cry1F and PAT proteins expressed in maize 1507, and the CP4 EPSPS and CP4 EPSPS L214P proteins expressed in maize NK603 have been assessed for their safety previously and no safety concerns were identified for humans and animals.... the EFSA GMO Panel considers it unlikely that interactions between the single maize events will occur that may impact on the food and feed safety and nutritional pro perties.*" [excerpts from EFSA (2010b)—similar justifications and wording has been used in other approvals of stacked events].

In this paper, we examine the two claims that serve as justification to minizime or omit the testing of combinatorial effects of multiple Cry toxins expressed in stacked GM Bt crop plants: (1) Due to narrow specificity of Cry toxins, no species outside of the primary class of target pest organisms are affected and (2) as long as single Cry toxins do not elicit adverse effects, they will not do so in combination with other Cry toxins or other naturally occurring compounds.

Lastly, we will reflect on the regulatory practice of risk assessment of stacked Bt-crops in the EU and will provide recommendations for improvements of the current testing practice.

DEFINITIONS AND DATA USED IN THIS REVIEW

We carried out a data base search of the Web of Science for peer reviewed international publications in English language for the following key word categories: Category I: *"Bacillus thuringiensis"* or "Bt" or "toxin" or "protein" combined with Category II: "synergistic" or "interaction" or "combined/combinatorial" "effects." We then selected those studies that reported about *in vivo* tests only, e.g., bioassays with target and non-target organisms. On occasion, these reports contained also data on *in-vitro* tests with cell lines that we disregarded for comparability reasons. We restricted our evaluation to studies that reported on combinatorial effects between Cry proteins and some naturally occuring plant-, bacteria-, or insect-compounds as they can be encountered by non-target organisms feeding on stacked GM Bt plants in their natural environment. Studies on combinatorial or complementary effects with herbicides and other pesticides, such as neonicotinoid residues (Douglas and Tooker, 2015), have been omitted and left for future evaluations. In **Table 2**, we compiled the selected key reports to allow for a quick overview of the involved compounds, test organisms, and proposed mechanisms.

We report our findings according to the claims outlined above. The definitions for various types of combinatorial effects followed those by Tabashnik (1992):

Synergistic effects in the context of this paper entails effects—e.g., mortality rates—of combined toxins exceeding those found for the individual toxin with the highest activity. If synergistic effects occur, this means that the toxicity of a mixture cannot be predicted from the individual ingredients.

Antagonistic effects in the context of this paper are effects contrasting synergistic effects: when a mixture of toxins leads to less toxicity than found for individual toxins with the lowest activity. If potency is less than expected.

Additive effects in the context of this paper entail effects—e.g., mortality rates—of combined toxins not exceeding those found for the individual toxin with the highest activity. If additive effects occur, this means that the toxicity of a mixture could be predicted from the individual ingredients.

RESULTS

The first claim of narrow specificity rests on two premises: (i) "efficacy" determined in economically important target pests is a reliable indicator for "narrow specificity" of Cry toxins, and (ii) the mode of action of Cry toxins supports the claim of narrow specificity. In the following, we will evaluate the data base for these two premises.

Furthermore, we will summarize the scientific literature documenting effects of single and multiple combined Cry toxins and their postulated mechanisms to examine whether the reported data and methodologies confirm the conclusion of predictability (likelihood) of combinatorial effects.

NARROW SPECIFICITY OF Bt PROTEINS

Since the claimed narrow specificity of Bt toxins serves as the first line of justification to forego the testing of non-target organisms, the premises this claim rests upon should be well-supported with scientific evidence. Below, we, firstly, scrutinize the current definition of specificity and its application within risk assessment of Bt-crops and non-target organisms. Secondly, we summarize the current state of knowledge regarding the proposed mode of action of Cry toxins.

Assumptions vs. Evidence

B. thuringiensis bacteria express and deposit a multitude of proteineous, insecticidal toxins in various crystalline forms in the bacterial mother cell (called Cry proteins). When the insecticidal properties of these deposited Cry proteins were discovered a century ago, very quickly their potential utility for pest control was recognized and research and development of *B. thuringiensis* based sprayable insecticides began. Today, several commercial *B. thuringiensis* based insecticide formulations are broadly available commercially and used in organic crop production as one of the few permitted sprayable insecticides. Hence, ever since their discovery, research and development efforts have focused almost exclusively on studying their efficacy on economically important, herbivorous pest species (e.g., Hoefte and Whiteley, 1989; Schnepf et al., 1998; Sanchis, 2011; van Frankenhuyzen, 2013). Naturally, for practical pest control purposes, the most efficacious *B. thuringiensis*-produced Cry proteins are considered to be those that induce maximum—if possible 100%—mortality in a given target pest population with the least amount of toxin

TABLE 2 | Some key publications highlighting different types of combinatorial effects on insects reported in the scientific literature.

Cry × Cry	LE	Cry × (insect- or plant-) proteins	LE	Cry × cytolytic (Cyt) Bt proteins	LE
LEPIDOPTERA—Bt TOXINS FROM var. *kurstaki* (*Btk*)					
Species: *Helicoverpa armigera* *Toxin types:* Cry1Ac, Cry1Ab, Cry2Aa, Cry1F—trypsin activated toxins *Findings*: Single toxins: Cry1Ac, Cry1Ab, Cry2Aa Cry1F Cry1Ac × Cry1Ab Cry1Ac × Cry2Aa Cry1Ac × Cry1F Charkrabarti et al., 1998	 + 0 + + ++	*Species*: *Manduca sexta* (M.s.) *Heliothis virescens* (H.v.) *Helicoverpa zea* (H.z.) *Toxin and protein types*: Cry1Ab, Cry1Ac, Cry toxin-binding cadherin Bt-R1 peptide called CR12-MPED extracted from M.s. expressed in *E. coli* *Findings:* CR12-MPED synergizes low Cry1Ab toxin doses providing enhanced insecticidal activity in H.v. and H.z. Chen et al., 2007	 ++	*Species*: *Trichoplusia ni* cell lines, *Trichoplusia ni* *Toxin and protein types*: Cry1Ac toxin (from *B.t.kurstaki*), Cyt1A1 (from *B.t.israelensis*) engineered into bacteria (not stated which species) *Findings*: Cry1Ac Cyt1A1 Cry1Ac × Cyt1A1 Antagonistic effects between the two *in-vitro* and *in-vivo* Rincon-Castro et al., 1999	 + + –
Species: *Earias vitella* *Toxin types:* Cry1Ac, Cry2Aa—trypsin activated toxins *Findings*: Single toxins: Cry1Ac, Cry2Aa Cry1Ac × Cry2Aa—1:1, 1:2, 2:1—all Yunus et al., 2011	 +,– ++	*Species*: *Helicoverpa zea* *Heliothis virescens* *Spodoptera frugiperda* *Diatraea grandiosella* *Toxin and protein types:* Crystal protein: Cry2A—toxin (sublethal concentrations) Other protein: Plant cysteine protease Mir1- CP (a plant defensive compound accumulating at wound site after plant injury) *Findings*: Mir1-CP inhibits larval growth by attacking and permeabilizing insects peritrophic matrix (PM) Low doses of Mir1-CP synergized sublethal doses of Cry2A in all four species Mohan et al., 2008	 ++		
Species: *Bombyx mori* (B.m.) *Lymantria dispar* (L.d.) *Toxin types*: Cry1Aa, Cry1Ab, Cry1Ac—toxin *Findings*: Single toxin: Cry1Aa>Cry1Ab>Cry1Ac L.d.: 1Aa × 1Ac L.d.: 1Aa × 1Ab L.d.: 1Ab × 1Ac B.m.: all Lee et al., 1996	 ++ – 0 0	*Species:* *Spodoptera exigua* *Toxin and protein types:* Bt strains (70) not specified Other protein: Chitinases *Findings:* Chitinases produced by bacteria B.t. increased activity of Cry toxins more than two-fold Liu et al., 2002	 ++		
Species: *Chilo partellus* *Toxin types:* Cry1Aa, Cry1Ab, Cry1Ac—toxin		*Species:* *Spodoptera littoralis* (relatively insensitive to Cry1 toxins) *Toxin and protein types*: Cry1C, endochitinase ChiAII			

(Continued)

TABLE 2 | Continued

Cry × Cry	LE	Cry × (insect- or plant-) proteins	LE	Cry × cytolytic (Cyt) Bt proteins	LE
Findings: Single toxin: Cry1Ab>Cry1Ac>Cry1Aa L.d.: 1Ab × 1Ac L.d.: 1Ab × 1Aa L.d.: 1Aa × 1Ac Sharma et al., 2010	 ++ + +	*Findings*: ChiAII Cry1C (low, sublethal concentration) Cry1C × ChiAII Regev et al., 1996	 + + ++		
Species: *Choristoneura fumiferana, Ch. occidentalis, Ch. pinus, Lymantria dispar, Malacosoma disstria, Orygia leucostigma* (O.l.) *Toxin types:* CrylA crystal protoxins from cloned and native microbial strains *Findings:* All except O.l.: CrylA × CrylA O.l.: CrylA × CrylA Tabashnik, 1992	 0 –				
Species: *Helicoverpa armigera, Earias insulana* *Toxin types:* Cry1Ac, Cry2Ab, Cry1Fa—trypsin-activated toxins *Findings*: Cry1Ac—H.a., E.i. Cry2Ab—H.a., E.i. Cry1Fa—H.a., E.i. Cry1Ac × Cry2Ab—H.a., E.i. Cry1Ac × Cry1Fa—H.a., E.i. Ibargutxi et al., 2008	 +,+ +,+ 0,+ ++,+ +,+				
MOSQUITOS—BT TOXINS FROM var. *israelensis* (*Bti*)					
Species: *Anopheles stephensi* (A.s.); *Aedes aegypti* (A.a.), *Culex pipiens* (C.p.) *Toxin types:* Bti Crystal protein: CrylVA and CrylVB crystal protoxins *Findings:* CrylVA all species, CrylVB only to A.a. and A.s. CrylVA × CrylVB all species Delécluse et al., 1993	 +++ ++,0 +++			*Species:* *Anopheles albimanus* *Toxin and protein types:* Bti Crystal protein: Cry4Ba, Cry11Aa Cytolytic protein: Cyt1Aa All protoxins—spore × crystal combinations *Findings:* Cyt1Aa nontoxic Cry4Ba toxic at 43–360 ng Cry11Aa toxic at 90–360 ng Cry3Ba × Cyt1Aa Cry11Aa × Cyt1Aa* Cry4Ba × Cry11Aa × Cyt1Aa *remarkable because both were non-toxic when administered individually at that concentration—in combination they yield clear toxicity Fernández-Luna et al., 2010	 0 + + ++ + ++
Species: *Aedes aegypti, Anopheles stephensi, Culex pipens*				*Species:* *Culex quinquefasciatus, Aedes aegypti*	

(Continued)

TABLE 2 | Continued

Cry × Cry	LE	Cry × (insect- or plant-) proteins	LE	Cry × cytolytic (Cyt) Bt proteins	LE
Toxin types: CryIVA, CryIVB, CryIVD crystal protoxins *Findings:* CryIVA × CryIVB × CryIVD CryIVB × CryIVD Poncet et al., 1995	 ++ +			*Toxin and protein types*: Bti and *Bacillus sphaericus* (wild-type) producing Cry11a, Cry4A, Cry4B, and Cyt1Aa *Findings:* When Cry toxins from Bti were combined with *B. sphaericus*, in presence or absence of Cyt1Aa, synergistic increased toxicity and expanded host range were observed Wirth et al., 2004	 ++
				Species: *Culex quinquefasciatus* *Toxin and protein types:* CryIVD crystal protoxins CytA toxin Both co-transformed into *B. thuringiensis* bacteria *Findings:* CryIVD CytA CryIVD × CytA (co-expressed in Bt) Chang et al., 1993	 + + ++
				Species: *Culex quinquefasciatus* (C.q), *Aedes aegypti* (A.a.) *Toxin and protein types*: Bti and *Bacillus darmstadtiensis* (wild-type) expressed in *E. coli*; Cry4Ba and Cyt2Aa2 *Findings:* Cry4Ba (C.q., A.a.) Cyt2Aa2 (C.q., A.a.) Cry4Ba × Cyt2Aa2 (C.q., A.a.) Cry4Ba toxins were inactive as single toxin to *C. quinquefasciatus* but in combination with Cyt2Aa2 had strong effect Promdonkoy et al., 2005	 +,0 +,+ ++, ++
COLEOPTERA—Bt TOXINS FROM var. *tenebrionis* (*Btt*)					
		Species: *Diabrotica undecimpunctata howardi* (D.u.h.), *Diabrotica virgifera virgifera* (D.v.v.), *Leptinotarsa decemlineata* (L.d.) *Toxin and protein types:* Cry3Aa, Cry3Bb toxins toxin-binding fragment of cadherin receptor (CR 8 and 10) expressed in *E. coli* *Findings:* CR8 and 10 isolated from D.v.v. and expressed in *E. coli* binds activated Cry3Aa and Cry3Bb toxins and enhances toxicity of both toxins in L.d., D.u.h., and D.v.v. from 3- to 13-fold (synergistically). Indivdually, they did not elicit an effect and Cry3 toxins efficacy was lower and differed when administered alone Park et al., 2009	 ++		

LE, Level of effect; "–", antagonistic; "0", neutral; "+", additive (definitions see text).

ingested in the shortest period of time. We refer to this as the concept of "quick kill." Based on this economically motivated concept of "efficacy," most Cry toxins affect most efficaciously only a relatively narrow range of so called "target" pest species. However, this pest control-focused concept underlies the generalization of narrow specificity of all Cry toxins applied to all non-target species and the assumption that Cry toxins are unlikely to affect other species outside of their range of primary (target) organisms (e.g., Soberón et al., 2009; Sanchis, 2011; Pardo-López et al., 2013).

It was with the introduction of GM crops expressing activated Cry toxins constitutively, meaning in all tissues of the GM plants throughout their entire lifespan, that ecologists urged to revisit the validity of the economic definition of "specificity," or "quick kill," and began testing various Cry toxins on non-pest non-target organisms (Hilbeck, 2001, 2002). Today, with the vastly expanded spatio-temporal exposure of Cry toxins from GM crop plants, easily also reaching beyond the crop field (Hofmann et al., 2014), the need arose to investigate ecologically relevant adverse effects beyond the narrow scope of a small group of economically relevant herbivores considered pests in crop fields and also beyond the single economic parameter of "quick kill." Ecological parameters including cumulative lethal effects, i.e., "slow kill," and sublethal impacts (e.g., developmental time, weight gain, behavioral changes) have now gained importance. Such effects could cause as severe or even more severe ecological consequences for a terrestrial or aquatic ecosystem as a "quick kill" could.

The most comprehensive data source concerning the specificity of Bt proteins is the Bt Toxin Specificity Database (http://www.glfc.cfs.nrcan.gc.ca/bacillus/; van Frankenhuyzen and Nystrom, 2002). Analyses from this database have been published by van Frankenhuyzen in 2009 and 2013 with a recent update in de Schrijver et al. in 2014. While, originally, Cry toxin activity was assumed to be restricted to the insect order of Lepidoptera, this has successively been expanded to include today up to six arthropod orders for which so-called cross-active Bt toxins have been reported (van Frankenhuyzen, 2013). Notably, today, the most widely employed and studied Cry toxins, such as Cry1Ab or Cry1Ac, have been reported to affect species from different insect orders or even phyla (Cry1Ab: Coleoptera, Lepidoptera, Diptera, Hemiptera; Neuroptera, Trichoptera and Nematoda; Cry1Ac: Lepidoptera, Diptera, Hemiptera). But van Frankenhuyzen (2013) found that only a small fraction (17%) of Cry toxins have ever been tested with species from more than 1 or 2 insect orders. Despite this restriction to certain tested pest species, approximately 40% of all Bt toxins tested across two or more orders did show cross-activity (de Schrijver et al., 2014). Yet, even for the most tested lepidopteran-active Cry1 toxins, only a little more than one third has ever been experimentally tested outside of that order. In total, van Frankenhuyzen (2013) compiled evidence for cross-activity of 27 Bt toxins and 69 insect taxa. We expect that the number of reported cross-activities will likely rise as more experiments with non-target organisms emerge and the old definition of order-specifity of Bt-toxins (van Frankenhuyzen, 2013; de Schrijver et al., 2014) may no longer be regarded as a functional concept—cross-activity may actually become rather a common phenomenon than an execption, certainly under an ecological definition of "efficacy" or "specificity." Apart from few exceptions, the majority of available data from the van Frankenhuyzen-database rely on mortality as the measured endpoint. However, sub-lethal effects such as growth inhibition, changes in developmental time or other parameters which may affect fitness can be expected to occur at far lower effect-doses than those inducing a "quick kill."

Proposed Mode(s) of Action (Mechanism)

Much of the claimed specificity of Bt-toxins rests on what is known about the mode of action of Cry toxins from research with this narrow set of herbivorous target pest insects and from studying predominantly one Cry toxin class only, Cry 1. By comparison, modes of action of Cry 3 and Cry 2 toxins have received far less attention (Schnepf et al., 1998; Whalon and Wingerd, 2003; Vachon et al., 2012). Some authors think that specificity rests equally on the solubilization-activation process as on the receptor-binding and pore-formation process (Smouse and Nishiura, 1997), while others postulate specificity to rest mainly on (affinity to) specific receptors (Schnepf et al., 1998). Today, quite a controversy exists over the mode of action of Cry toxins which is owed to the fact that there is less scientific certainty about it today than there was when *cry* transgenes from *B. thuringiensis* were first engineered into GM plants roughly 30 years ago. Until about a decade ago, the predominant and most agreed model for the mode of action of Cry proteins (the "classical model") as produced by *B. thuringiensis* went as follows: ingested, inactive crystalline (Cry) proteins must be solubilized in an insect gut environment with a high pH (>10). The solubilization of crystalline proteins yields a still inactive so-called full-length protoxin (ca. 130 kDa Cry1 class and ca 73 kDa Cry3 class) that requires further biochemical cleavage to produce a small toxic fragment. This toxic fragment (ca. 65 kDa Cry1 class and 55 kDa Cry3 class) then must bind to certain receptors located in the midgut ephithelium and, thereby, induce pore formation (also called ion channel forming) and lysis of the gut resulting in septicemia subsequently killing the insect (Schnepf et al., 1998; Whalon and Wingerd, 2003; Vachon et al., 2012). Different sized fragments of the same Cry class have been shown to exhibit different activities in different ranges of affected insects (Haider and Ellar, 1987). A whole range of membrane binding proteins have been suggested as receptors, including numerous cadherins, aminopeptidases, and alkaline phosphatases as well as glycolipids (Pigott and Ellar, 2007; Sanchis, 2011; Vachon et al., 2012).

Today, additional models for Cry toxin mode of action have been suggested with supporting new data. In a recent review, Vachon et al. (2012) describe and critique three models: the "classical" model, the "sequential binding" model, and the "signaling pathway" model. The first two models have in common that the mode of action of activated Cry toxins hinges on the binding of activated (i.e., cleaved) toxic fragments of the original Cry protein to receptors in the midgut epithelium in insect larvae. The sequential binding model proposes a more complex sequence of events with

more binding steps involving more receptors and the removal of an alpha helix from the Cry toxin leading to a required oligomerization step of the Cry toxins before inserting into the gut membrane and inducing pore formation in the gut of the insect (Soberon et al., 2012). Consequently, in addition to the cadherin receptors of the "classical" model, which are now called to be "primary" receptors, so called "secondary" receptors, GPI (glycosylphosphatidyl-inositol)-anchored receptors, are suggested to have a significant role in pore formation (Soberón et al., 2009; Soberon et al., 2012; Pardo-López et al., 2013). While in the classical model it was believed that monomeric Cry1 toxins can bind to cadherin receptors and induce pore formation, in the sequential model it is proposed that cadherin-bound monomeric Cry1 toxins cause conformational changes favoring proteolytic cleavage that allows the rest of the toxin to oligomerize (Jiménez-Juárez et al., 2007; Soberon et al., 2012; Vachon et al., 2012). The Cry1 toxin oligomers subsequently bind to the GPI-anchored receptors and only then pore formation is induced. Mechanisms of resistance in target pests were found to be often associated with mutations affecting the binding to these cadherin receptors. Hence, a proposed solution to overcome such cadherin-based resistance was "*the rational design of improved toxins*" (Sóberon et al., 2007, 2009). This led to the development of modified Cry1 toxins, so called CryMod toxins, lacking an alpha-helix that circumvented the cadherin-binding step by oligomerizing simply in the presence of trypsin and, subsequently, continue to induce pore formation requiring only binding to "secondary" receptors (Sóberon et al., 2007). Vachon et al. (2012) challenge the validity of this model and also Pigott and Ellar (2007) pointed out that "*other explanations of the data are possible*." The credibility of the sequential binding model was further eroded because of later, admitted manipulations of images of the gels, including the removal of stains from the blot and shifting positions of the bands of the blot (e.g., Jimenez-Juarez et al., 2013), which were offered as evidence for the sequential binding model in a total of 11 publications. We conclude that the sequential binding model is, therefore, in need of independent validation and experimental reconciliation with the critique by Vachon et al. (2012) and Pigott and Ellar (2007).

Yet another model has been suggested by Zhang et al. (2005, 2006a) that differs from the two above in that pore formation is not an essential feature anymore in the cause of death of the insect. Instead the signal transduction model proposes that binding of Cry toxin monomers to cadherin activates an intracellular cell death mechanism (Smouse and Nishiura, 1997; Zhang et al., 2005, 2006a). This model also has been questioned by Vachon et al. (2012) and also by Soberón et al. (2009) who developed the competing "*sequential binding model*." In contrast, Jurat-Fuentes et al. (Jurat-Fuentes and Adang, 2006), suggested a combination of the Zhang et al. (2005, 2006a) and the "sequential binding" model to be at work, and Kumar and Kumari (2015) consider both modes of action to act in a complementary fashion. Most authors, however, seem to agree that much still needs to be learned about the modes of action of Cry proteins. Pigott and Ellar (2007) expect that *"as more toxin receptors are discovered and as our understanding of toxin-receptor interactions increases, it will be interesting to see the extent to which Cry toxins utilize a common mode of action."*

Further complexity has been added to the presented new proposals of modes of action of Cry toxins by research suggesting that Cry toxins require the interaction with gut microbes in order to exert their lethal effects in target pest organisms (Broderick et al., 2006, 2009). When highly susceptible lepidopteran larvae were fed with antibiotics prior to being offered Cry toxin-spiked diet, the Cry toxins lost entirely their activity and no adverse effects on survival could be observed. The hypothesis proposed is that the Cry toxin induced pore formation of the midgut of susceptible larvae allows pathogenic gut bacteria to enter the hemocoel, allowing the bacteria to multiply and kill the host larvae via septicemia. Without the presence of such bacteria Cry toxins alone do not kill the larva (Broderick et al., 2006, 2009). When *Enterococcus faecalis* bacteria were added again to the diet, susceptibility to the Cry toxins was restored and high mortality observed. Hence, *E. faeca*lis, which is a commensal bacteria in an intact gut, can become a pathogen when invading the hemocoel. A process the authors referred to as the "commensal-to-pathogen" switch (Mason et al., 2011). Similarly, Jung and Kim (2006) reported that while *B. thuringiensis* subsp. *aizawai* (Bta) did efficiently kill third instar *Spodoptera exigua* larvae, it did not cause high mortality of fifth instar larvae. But when adding nematodes to Bt fed fifth instar larvae, it resulted in significant synergistic effects. They also suggested that this was due to Bta damaging at least somewhat the midgut cells of the fifth instars allowing the nematodes to enter the hemocoel. While the controversy remains regarding whether or not midgut microbiota (bacteria or nematodes) is essential for Cry toxins to kill susceptible insect larvae (Johnston and Crickmore, 2009; Raymond et al., 2009), it does highlight that the modes of action of Cry toxins are far from conclusive to date (Graf, 2011), and that co-factors which naturally occur in the environment impact the efficacy and specificity of Cry toxins which may help explaining some of the effects of Cry toxins on non-target organismes reported in the literature.

SIGNIFICANT EFFECTS OF SINGLE CRY TOXINS ON NON-TARGET ORGANISMS

Interestingly, there are indeed many documented cases of cross-order activities of Cry toxins on non-target organisms including reports about a range of lethal and non-lethal developmental (e.g., Lövei and Arpaia, 2005; Hilbeck and Schmidt, 2006; Marvier et al., 2007; Lövei et al., 2009; van Frankenhuyzen, 2009, 2013) or behavioral effects (e.g., Meier and Hilbeck, 2001; Zemkova Rovenska et al., 2005) challenging the narrow specificity narrative of Cry toxins, in particular when expressed as activated Cry toxins in the GM plants (**Table 2**). These reports include significant adverse effects of Cry toxins on non-target coccinellid species, *Harmonia axyridis* and *Henosepilachna vigintioctomaculata* (Stephens et al., 2012; Song et al., 2012) with research groups reporting significant cross-order effects of lepidopteran active Cry1 proteins on coleopteran coccinellid predators both when administered directly (Dhillon and Sharma, 2009; Schmidt et al., 2009; Hilbeck et al., 2012a) and via unaffected and affected prey (Zhang et al., 2006a,b,c; **Table 2**). In some cases reports of adverse effects even on non-arthropod

species have been published. When studying Cry toxins expressed in GM maize material with the snail species *Cantareus aspersus*, the researchers found 25% lower growth rate than in the control treatment (Kramarz et al., 2009). Just recently Shu et al. (2015) reported about significant effects of Cry1Ab from Mon810 maize on compost worm *Eisenia fetida*. Furthermore, several researchers reported about adverse effects of Cry toxins from GM host plant material on several aquatic organisms. In laboratory feeding trials, Rosi-Marshall et al. (2007) and Chambers et al. (2010) showed that consumption of single Cry toxin maize plant material reduced growth and increased mortality of the non-target stream insects *Lepidostoma liba* and *Helicopsyche borealis,* respectively. Also Bøhn et al. (2008; Bøhn et al., 2010) reported that mortality was higher, a lower proportion of females reached sexual maturation, and the overall egg production was lower in *Daphnia magna* that were fed Cry1Ab toxin producing GM maize compared to *D. magna* fed control maize. The authors argued specifically that the combination of reduced fitness with earlier onset of reproduction of *D. magna* fed Cry toxin maize indicated a direct toxic effect. Other aquatic taxa for which negative effects on single species have been observed include crane flies (Isopods; 19% growth reduction; Jensen et al., 2010), Chironomids (Prihoda and Coats, 2008), and even crayfish (Linn and Moore, 2014).

The reported diversity of lethal and sublethal, chronic effects may sum up and lead to shifts in species composition at the community level. For example, Campos and Hernandez (2015) reported significant differences in dung beetle species composition—an important functional group—possibly leading to impaired ecosystems services such as feces removal, seed dispersal, edaphic aeration, and incorporation of organic matter. For aquatic habitats, Axelsson et al. (2011) reported that the composition of aquatic insect communities colonizing the litter from Cry3Aa expressing GM trees was significantly affected in unanticipated ways. Similarly, Rosi-Marshall et al. (2007) raised concerns that effects of Bt pollen and debris may negatively affect caddisflies and the food-web. Although this seems not to be the case for highly degraded industrial agricultural habitats (Chambers et al., 2010), the risk could not be clarified for more natural terrestrial and aquatic habitats which play an important role in ecosystem functioning.

In light of the above reports, it is clear that the claim of no reported adverse effects of single Cry toxins on cross-order non-target organisms is not supported by the scientific evidence in the scientific literature. In fact, there is an increasing body of evidence suggesting significant effects of Cry toxins far beyond the originally postulated primary taxa of herbivorous target pest organism are possible.

COMBINATORIAL EFFECTS OF CRY PROTEINS

Combinatorial (including synergistic) effects of Bt toxins were reported already decades ago (e.g., Wu et al., 1985). Best known are the synergistic effects of spores of *B. thuringiensis* subsp. *kurstaki* to increase toxicity of Cry toxins in susceptible and resistant larvae of the diamondblack moth, *Plutella xylostella* (Dubois and Dean, 1995; Tang et al., 1996; Liu et al., 1998). Cry toxins combined with spores *"can be toxic even though the toxins and spores have little or no independent toxicity"* (Liu et al., 1998). Tang et al. (1996) observed synergistic effects among spores and the three Cry toxins Cry1Aa, Cry1Ab, and Cry1Ac. They also reported about synergistic effects between spores and Cry1C toxins on *P. xylostella* but, interestingly, not between spores and Cry2A toxin. However, since spores play no role in stacked or pyramided Bt crops—although they may still be around naturally—combinatorial effects of Cry toxins with other compounds encountered in nature are at the center of this review. These include combinatorial effects with (a) other Cry toxins, (b) bacteria-derived compounds, (c) plant-derived compounds and (d) insect-derived compounds. In the following, we address the reported effects (phenomena) and the suggested modes of actions (mechanisms) separately. In **Table 2**, we compiled the data from some widely cited key reports to allow for a quick overview of reported combinatorial effects, the involved compounds, test organisms, and proposed mechanisms.

Combinatorial Effects

Combinatorial Effects of Different Cry Toxins

Of the eight studies listed in **Table 2** that tested various Cry toxin combinations, seven reported significant combinatorial effects involving lepidopteran and dipteran species. Lee et al. (1996) reported a synergistic effect for a combination of Cry1Aa with Cry1Ac but an antagonistic effect for Cry1Aa and Cry1Ab and no combinatorial effect for Cry1Ab and Cry1Ac in *Lymantria dispar*. Interestingly, when keeping Cry1Aa stable (at 1) but increasing Cry1Ac two-fold (1:2), susceptibility increased from 49.9 to 34.9 ng ID (growth inhibition dose). But when increasing Cry1Ac more (1:4, 1:6 up to 1:12), susceptibility dropped substantially. Yet, none of these combinatorial effects was observed in *Bombyx mori,* the other test organism (Lee et al., 1996). More recently, Sharma et al. (2010) reported synergistic effects of various Cry1A toxins in *Chilo partellus* larvae. Poncet et al. (1995) found synergistic and additive effects of combined Cry toxins in three different mosquito species. Both Ibargutxi et al. (2008) and Yunus et al. (2011) reported synergistic effects of Cry1Ac and Cry2A toxins on *Earias insulana*, the spotted bollworm. In contrast, in two studies involving *Helicoverpa armigera* larvae and testing a similar combination of Cry1Ac and Cry1F, Ibargutxi et al. (2008) found no synergistic interaction while Charkrabarti et al. (1998) did (**Table 2**).

Combinatorial Effects of Cry Toxins with Bacteria-derived Compounds [Cytolytic (Cyt) Toxins]

Rincon-Castro et al. (1999) tested Cry1Ac toxins and Cyt1A1 toxins from engineered bacteria on *Trichoplusia ni* cell lines and larvae and found antagonistic effects. In contrast, Cyt1A proteins were found to synergize toxicity of Cry4A and Cry4B toxins, Cry10Aa and Cry11Aa in mosquito larvae (Chang et al., 1993; Wu et al., 1994; Wirth et al., 2004; Fernández-Luna et al., 2010). In fact, Wirth et al. (2004) reported that when Cry toxins from Bti were combined with *B. sphaericus*, in the presence or absence of Cyt1Aa, synergistically increased

toxicity and an expanded host range were observed. Also Promdonkoy et al. (2005) reported that Cry4Ba toxins were toxic to *A. aegypti* larvae but virtually inactive to *C. quinquefasciatus* larvae. Cyt2Aa2 exhibited moderate activity against *A. aegypti* and *C. quinquefasciatus* larvae. But the combination of both toxins dramatically increased toxicity to both *A. aegypti* and *C. quinquefasciatus* larvae. Chitinases produced by bacterial *B. thuringiensis* increased activity of the produced Cry toxins more than two-fold in *Spodoptera exigua* larvae (Liu et al., 2002, **Table 2**). Chitinases are widely produced in many bacterial *B. thuringiensis* strains and in some cases enhanced the toxicity of the produced Cry toxins (Ramírez-Suero et al., 2011; Hu et al., 2013). It was proposed that they could be used to enhance efficacy of Bt toxins for pest control (Liu et al., 2002).

Combinatorial Effects of Cry Toxins with Insect-derived Compounds

Chen et al. (2007) reported that a peptide fragment of a toxin-binding cadherin isolated from *Manduca sexta* guts and expressed in *E. coli* synergistically enhanced toxicity of Cry1 toxins in other lepidoptera species (**Table 2**; Chen et al., 2007). Similarly, a fragment of a cadherin from *A. gambiae* was found to enhance the toxicity of Cry4Ba mosquitocidal toxins. For both types of co-factors, effects of individual proteins were often lower and non-lethal while in combination observed effects were stronger and lethal.

Combinatorial Effects of Cry Toxins with Plant-derived Compounds

Mohan et al. (2008) report synergistic effects of Cry2A toxin with plant defense compounds like Mir1-cysteine protease (**Table 2**) in maize varieties from Antiqua (Carribean). The combinatorial effects observed were lethal and, as in the example above, much stronger than the sub-lethal effects caused by Mir-CP alone. They were discovered in exotic maize varieties from Antiqua (Carribean) and bred conventionally into local varieties. Also here, effects of individual proteins were at best sublethal but when administered in combination effects were more dramatic and lethal (**Table 2**).

Proposed Mechanisms for Combinatorial Effects

Below, we summarize the diversity of possible mechanisms proposed for the various observed combinatorial effects as this information will contribute to understanding whether combinatorial effects can be predicted.

Combinatorial Effects of Different Cry Toxins

Three different hypotheses are proposed. One hypothesis suggests that individual pores are formed by each Cry1A toxin individually and may act cooperatively, together inducing higher toxicity. A second theory proposes the formation of additional hetero-oligomers which may have better insertion ability than a homo-oligomer complex (Charkrabarti et al., 1998). A third theory suggests that the toxin mix might enhance toxicity by preventing non-productive binding (Schnepf et al., 1998). However, all of these hypotheses presume that all toxin molecules interact similarly with the BBMVs following more or less the "classical model." Sharma et al. (2010) found that all three Cry toxin combinations showed increase in binding and direct positive correlation between increased binding and mortality. Many reports involve a great deal of speculation.

Combinatorial Effects of Cry Toxins with Cytolytic (Cyt) Toxins

As possible mechanism of the observed antagonistic effect between a Cry1 toxin and the cytolitic Cyt1A1, the forming of a complex blocking one or more binding sites or the competition for space instead of receptors was offered as explanation. However, none of this has been confirmed yet. For the observed synergistic effects between Cry11Aa and Cyt toxin, Pérez et al. (2005) suggest that the Cyt1Aa toxin acts as receptor for Cry11Aa. Wirth et al. (2004) propose an interplay between different affinities of the varying toxins for receptor binding sites as mechanism—either masking or enhancing toxicity through competition, blocking or preferential binding dynamics working in conjunction.

Combinatorial Effects of Cry Toxins with Insect-derived Compounds

For combinatorial effects with insect-derived compounds like various cadherin fragments or chitinolytic proteases different mechanisms have been suggested supported by data to some degree. Some researchers suggested that the presence of cadherin binding sites, i.e., fragments of cadherin receptors isolated from different target pest organisms allowed for increased oligomerization of activated, monomeric Cry toxins which in turn increased the ability of a Cry toxin-CR complex to insert into the midgut membrane and induce pore formation (Chen et al., 2007; Park et al., 2009). Chitinolytic proteases are known to affect the peritrophic matrix (PM) and, thus, like cysteine proteases, allow greater access for Cry toxins to epithelial cells where pore formation takes place. The PM is an extracellular matrix of chitin, glycoproteins and proteoglycans that lines and protects the midgut epithelium from damage and assists in nutrient uptake. Through greater (affinity) or faster access, their efficacy is likely enhanced and toxicity increased, meaning a smaller dose of Cry toxins can induce the formation of more pores quickly.

Combinatorial Effects of Cry Toxins with Plant-derived Compounds

For combinatorial effects with plant-derived compounds like the Mir-cysteine proteases (Mir-CP), it was suggested that they increase the permeability of the PM which in turn facilitates the movement of Cry toxins through the PM to allow greater access to epithelial cells where pore formation takes place.

DISCUSSION

The objective of this review was to evaluate the scientific basis of the claims serving as the rationale for minimizing or omitting the testing of combinatorial effects of multiple Cry toxins expressed in stacked GM crop plants. To do so we compiled and evaluated published experimental evidence.

Narrow Specificity Narrative Depends on Definition of Efficacy and Reference Systems

In our analysis, we observed that the prevailing narrative of specificity is based on a narrow economically motivated definition of efficacy. This definition of efficacy relies on the "quick kill" from experiments carried out with a narrow spectrum of focal—because economically important—pest species. In the context of ecological risk assessment, such a narrow definition is insufficient and non-precautionary. When extending the definition of efficacy beyond a "quick kill," thus, including ecologically relevant endpoints like sublethal effects that include developmental time and growth, or cumulative lethal effects over the entire juvenile life stage ("slow kill") or reproductive effects, we see little evidence to support the assumption of narrow specificity.

However, a current report commissioned by COGEM (de Schrijver et al., 2014) and van Frankenhuyzen (2013) use mortality under the "quick kill" definition as the sole meaningful indicator for specificity. From an ecotoxicological and agronomic pest control perspective, this may suffice in particular when the focus lays on short-lived *B. thuringiensis* based pesticides. However, it does not suffice from an ecological, longterm perspective resulting from year round large-scale industrial cultivation of Bt crops including soybeans, maize, and cotton. The latter produce and release Bt toxins at an unprecedented spatio-temporal magnitude in agroecosystems. We argue that this can and probably has already lead to shifts in community structures and alterations in ecosystem services that may become particularly noticable outside of highly disturbed industrial agricultural areas (Axelsson et al., 2011; Campos and Hernandez, 2015). Agroecosystems in industrial agricultural areas are highly degraded and subject to multiple, persistent anthropogenic stressors, like chemical fertilizer, and massive pesticide inputs (Benbrook, 2012; Douglas and Tooker, 2015). Thus, all invertebrate communities in such industrial agroecosystems—terrestrial and aquatic—are the survivors of these degraded conditions, and, therefore, the impact of a single stressor, such as Bt toxins, may not be readily discernable (Chambers et al., 2010). Massive areal applications of pesticides in addition to the ubiquitous routine treatment of seeds of industrial commodity crops with persistent neonicotinoids will likely mask any additional effect of the bacterial Cry toxins (Douglas and Tooker, 2015). But with Cry toxin coding transgenes and GM plants moving beyond the arable field and entering also aquatic ecosystems, longterm ecosystem services, and conservation issues should receive special attention.

Increasing Uncertainty on Modes of (Inter-)action of Cry Toxins

Over the past decade, substantially differing modes of action have been proposed, which all are contested to some degree. The classical model of mode of action has largely been studied with crystalline *B. thuringiensis* produced proteins which require a complex solubilization and activation process. These steps of activating the crystalline *B. thuringiensis* proteins have been shortcut in GM plants most of which express the already activated Cry toxins. Much of the complex proposed modes of action that determine their "specificity" has been eliminated in GM plants. Neither particular pH conditions nor cleavage enzymes are required for their activation. Hence, with Cry toxin producing GM plants, specificity would be determinded exclusively by receptor binding and pore formation. However, with the signaling pathway model, pore formation may be obsolete and most of the proposed receptors are not necessarily restricted to target organisms or target taxa (e.g., Watanabe et al., 1995; Luan and Xu, 2007; Hulpiau and van Roy, 2009) as are trypsin and other suggested enzymes necessary for Cry toxin activation. Because research has focused on herbivorous target pest species, hardly any knowledge about the presence or absence of midgut receptors required for Cry toxin activation in insects outside of the studied range of herbivorous pests exists.

Furthermore, most of the research into the modes of action of the past decade was driven by exploring the mechanism underlying the spreading resistance in some target pests in order to find ways to overcome resistance (e.g., Soberon et al., 2012; Storer et al., 2012). Consequently, an even more narrow subset of target pests namely those that have evolved resistance was studied. Notably, none of the newly discovered modes of action were discussed or investigated in the context of non-target organisms. Except for two studies (Rodrigo-Simón et al., 2006; Song et al., 2012), no efforts have been spent on understanding the mechanisms behind the reported adverse effects on non-target organisms despite the ensuing scientific dispute (Waltz, 2009a,b; Hilbeck et al., 2012b). In these disputes, the specifitiy of Bt-toxins are stressed in a paramount way. However, the narrow specificity narrative must be re-defined as more and more data on the cross-order acticivity are available (van Frankenhuyzen, 2009, 2013).

No Lack of Reported Cross-order Effects of Single Cry Toxins

From our analyses, we conclude that the claim of no reports of adverse effects of Cry toxins—directly or indirectly—on non-target organisms is invalid. In the scientific literature both can be found, reports from experimental studies that do find adverse effects of Cry toxins and those that do not and the outcome is very sensitive to the applied methodology including exposure schemes and measured endpoints and the author's interpretation of the data. As the range of organisms and the endpoints tested have been expanding, scientists began to find adverse effects of Cry toxins administered directly as microbially- or plant-produced compounds or indirectly via prey on a far broader range of organisms than previously assumed (van Frankenhuyzen, 2009, 2013; de Schrijver et al., 2014).

Because regulatory standards for GMO-testing are lacking—not only in the EU—the scientific interpretation of effect studies are subject of intense debate in the science and regulatory community. Studies pointing at potential negative effects are met by heavy criticism from developers and proponents of GM products (e.g., Waltz, 2009a,b). Dissenting interpretations and extrapolations are typically based on different conceptual approaches to (narrow vs. broad) risk assessment (e.g.,

Andow et al., 2006; Hilbeck et al., 2011, 2012b; Wickson et al., 2013 vs. Romeis et al., 2006) or are primarily concerned with, and triggered by, the policy responses the reported adverse effects on non-target organisms invoked (e.g., Ricroch et al., 2010; Kuntz et al., 2013; Romeis et al., 2013) rather than driven by scientific curiosity.

Different outcomes of experiments determining the sensitivity of testing organisms have been linked to differences in exposure length and intensity via the offered diets during the time period tested. While in many studies reporting significant effects, the tested non-target organisms were exposed to the test substance (Cry toxin containing diets or prey) continuously throughout most or all of their (susceptible) larval stage, this is often not the case in the studies not finding significant effects. For example, Hilbeck et al. (1998a,b, 1999, 2012b) Schmidt et al. (2009), Dutton et al. (2002), Stephens et al. (2012), Zhang et al. (2006a,b), Dhillon and Sharma (2009) did ensure exposure throughout the (almost) entire larval stage and, consequently, did observe effects. This was not the case in studies by Romeis et al. (2004), Rodrigo-Simón et al. (2006), Porcar et al. (2010), Zhang et al. (2006c,d). In other cases, exposure was ensured throughout the entire larval stage but with intermittent phases of recovery by offering optimal, non-Cry toxin diets (Alvarez-Alfageme et al., 2011) or by offering a Bt-laced suboptimal food in combination with a non-Bt optimal food (Zhang et al., 2014). The conclusions of Lövei et al. (2009) still hold today based on their meta-analysis: "*it is clear that conclusions that Bt... transgene products have "no harm" to natural enemies are currently overgeneralized and premature*."

Combinatorial Cry Toxin Effects Commonly Known

Also combinatorial effects of Cry toxins with other proteins or chemicals are actually widely recognized and reported in the literature. Combinatorial interactions of Cry toxins with each other or with other compounds enhancing their toxicity have been known and discussed in the scientific community since at least the 1980s (Wu et al., 1985; Schnepf et al., 1998). Already Schnepf et al. (1998) devoted a separate subchapter of this standard textbook on Bt toxins to unpredictable combinatorial interactions, mostly synergistic. They also pointed out the fact that *"little is known about the mechanism of this synergistic interaction or potentiating effects,"* keeping in mind that this knowledge is restricted to the target pests studied. Again, combinatorial effects have been recognized and discussed only under a utilitarian "quick kill" narrative, i.e., in the context of enhancing the pest control capacity either of GM crop plants expressing the Cry toxins or of sprayable Cry toxin formulations. Under this utilitarian narrative, combinatorial effects are explored also as a means to aid its application in pest control strategies. For example, Li and Yu (2012) are heading a section on combinatorial effects in their chapter with "*Utilizing the synergistic effect of helper proteins*." Such *"helper proteins"* are in fact nothing else but substances that exert combinatorial effects with Bt toxins. For example, "*chitinases for enhancing the entomotoxicity of engineered Bt strains*" are receiving considerable attention to develop *"new strategies"* for pest control (see Li and Yu, 2012 for references therein). Or as George and Crickmore (2012) put it "*to boost the efficacy of Bt insecticidal toxins and overcome resistance posed by insect pests, the use of other proteins like cadherin fragments have been shown to be a successful strategy*" or "*also combinations of Cry toxins have proven to be a very useful strategy employed in boosting efficacy and hting resistance*." For example, the secondary compound gossypol derived from the cotton has been applied in combination with Cry1Ac to boost its efficacy against a resistant population of *Helicoverpa zea* (Anilkumar et al., 2009). Why such previously unexpected and unpredictable combinatorial effects with co-factors—whether called "helpers" or otherwise—should be restricted only to those organisms that humans declare as target "pests" lacks a scientific hypothesis and certainly critical rigor (Then, 2010).

No Predictability of Combinatorial Effects

Many of the reported synergistic interactions in target organisms were entirely unpredictable and occured when their individual components did not elicit a response at all or only a sublethal response when tested in isolation. Liu et al. (1998) reported that spores and crystal toxins can act synergistically when administered together even if *"the toxins and spores have little or no independent toxicity."* Mohan et al. (2008) observed that low doses of Mir1-cysteine protease (a plant defense compound) "synergized sublethal doses of Cry2A" toxin. Similarly, it was shown that CR12-MPED peptide enhanced insecticidal activity of low Cry1Ab toxin doses (**Table 2**; Chen et al., 2007). Low (sublethal) doses of Mir1-CP synergized greatly sublethal doses of Cry2A. Both compounds hardly affected the lepidopterans when administered individually. Similarly for mosquito larvae, Promdonkoy et al. (2005) reported virtually no observable effect of a Cry4B toxin that in presence of a Cyt protein became deadly toxic to the larvae.

In some cases, cadherin receptor fragments increased toxicity of some Cry toxins but not of others (Lee et al., 1996). Masking effects by differential affinities to binding sites depending on kinetics of hetero-oligomer complex to receptors with higher or lower binding affinity or straightforward competing effects for binding sites were offered as explanation (Lee et al., 1996).

Chen et al. (2007) were surprised to find an enhancement of Cry1Ab toxicity. Because Cr12MPED peptide contains the critical Cry1Ab binding region, it was expected that Cry1Ab toxicity would be reduced in the presence of CR12 MPED peptides as they would bind the Cry toxin prior to their binding to the receptors in the midgut epithelium. Thus, the CR MPED bound Cry toxin would not be able anymore to induce pore formation. However, the opposite was true, also for Cry1Ac. In spectroscopy examinations, the authors found that CR12 MPED was present in an unfolded state which exposed more amino acid residues to the surrounding environment. It was speculated that this could modify interactions with Cry 1A toxins in the insect midgut and enhance toxicity. Also Park et al. (2009) suggested a similar mechanism as Chen et al. (2007) where Cry3 toxins are activated to a 55 kDa toxic fragment. This activated Cry3 toxic fragment binds to brush border membrane vesicles (BBMV) and recognizes a 144 kDa binding region in the BBMV. However, Cry3 toxins differ in capacity to oligomerize, solve and bind.

The suggested mechanism is presumed to be like CR12 MPED for lepidoptera. CR12 fragements induce the formation of a pre-pore Cry1Ab oligomer, a critical step in the intoxication of lepidopteran larvae, leading to an enhancement of Cry1Ab toxicity. A similar mechanism is expected also for Cry3 and CR8-10 fragments.

Additionally, adverse effects can arise from combining various biotic stressors. When studying the singular effect of microbially produced, activated Cry toxin Cry1Ab or via Cry1Ab producing GM maize in snails (*Helix aspera*), Kramarz et al. (2007a) found no negative effect on *H. aspersa* during the observed life stages. However, when snails were infected with nematodes, the growth of the snails was significantly slower than when fed control maize (Kramarz et al., 2007b). The authors concluded that *"long-term exposure is needed to reveal an effect of Bt maize."*

None of the observed combinatorial effects could have been predicted from the effects induced by their individual compounds. In a number of studies, researchers found that the synergistic lethal effects could be triggered in the absence of any effect when the toxins were administered individually or when administered at non-toxic doses or at low doses eliciting only sublethal effects (**Table 2**).

CONCLUSIONS FOR REGULATIONS AND ENVIRONMENTAL RISK ASSESSMENT

The regulatory requirements for the risk assessment of Bt-crops in the EU include testing combinatorial effects of different novel proteins such as Bt toxins expressed in the GMO. In practice, however, tests for combinatorial effects are carried out without plant material and with minimal effort. The rationale behind the test regime relies on the narrative of a narrow specificity of Bt toxins, which, from the developers perspective, backs their argument that relevant interactions between different Cry toxins should not be expected if the organisms are not known to be affected by single microbial Cry toxins. As a result, information on combinatorial effects is at best based on one laboratory study with a target pest species using a minimal combination of microbial Cry toxins.

Need to Re-define the Specificity of Bt Toxins for the Risk Assessment of GMO

We have argued here that the "narrow specificity" narrative of Cry toxins is based on an agro-economical perspective of specificity. However, in an environmental regulatory context, ecologically motivated studies (e.g., butterflies: Losey et al., 1999, lacewings: Hilbeck et al., 1998a, ladybird-beetles: Schmidt et al., 2009, daphnia: Bøhn et al., 2008, 2010; caddisflies Rosi-Marshall et al., 2007) are quite relevant as these must address protection goals and end-points that are different from and transcend those relevant from a narrow economically motivated pest-control perspective. Because of their relevance for environmental risk assessments in the European regulatory context, studies showing negative effects on groups of organisms which were not supposed to be sensitive to the Bt toxins in question and often occur outside of the agricultural field, were met with fierce criticism from circles favoring a narrow apporach to environmental risk assessment because they were pointing to existing uncertainties in these risk assessments and were not in line with the assumption of narrow specificity of Bt proteins. However, as these studies did deal with relevant questions for environmental risk assessments with a broader perspective, they were included in the evidence basis for policy decisions under the precautionary principle (e.g., German suspension for the cultivation of MON810, 2009; http://www.bvl.bund.de/SharedDocs/Downloads/08_PresseInfothek/mon_810_bescheid.pdf?__blob=publicationFile&v=2). As the available data show and the currently proposed modes of action of Bt-proteins are seemingly not exhaustive, the present definition of narrow specificity is of limited and, indeed, declining value for GMO environmental risk assessment in particular when operating under the precautionary principle.

Mode of Action not Conclusive for the Assessment of Non-target Effects

While not all non-target organisms will be adversely affected by Cry toxins, there is presently no way of predicting which species may or may not be affected based on the current state of understanding of the proposed modes of action of Cry toxins. The current knowledge on the modes of action for Bt toxins is clearly incomplete. All of the discussed requirements for activity of Cry toxins, suggested receptors or involved enzymes, occur in many organisms. Hence, more research into relating observed effects to possible mechanisms in non-target organisms going beyond the traditional narrow spectrum is urgently needed to better understand the likelihood and magnitude of non-target effects.

Sublethal and Chronic Effects Bound to be Overlooked

From our analyses, we conclude that relying on the narrow economic "quick kill" definition of efficacy, the risk assessment is bound to overlook sublethal, chronic, and cumulative adverse effects. Compared to acute lethal effects such effects are equally important for ecological functioning as they can trigger significant adverse effects on ecological processes. Sublethal effects in form of developmental delays or behavioral changes in host or prey preferences, for example, can lead to significant ecological consequences via disruption and altering of existing predator-prey relationships or synchronies within food webs. In an agroecosystem, such disruptions or shifts in preferences and behavior can cause significant shifts in arthropod community structures possibly favoring non-target pest species and giving rise to secondary pests (e.g., Lumbierres et al., 2004; Lu et al., 2010; Qui, 2010; Cantarino et al., 2015). Just recently, Campos and Hernandez (2015) observed adverse impacts of transgenic Cry toxin producing GM maize in Brazilian fields on the functional group dynamics within dung beetle communities. Furthermore, sublethal effects may substantiate only after several generations. For this reason, generational tests that provide the possibility to analyse important life-history parameters may improve the assessment of long-term effects on ecosystems.

However, generational tests with GMOs (Bøhn et al., 2010; Shu et al., 2015) are virtually absent to date.

Improvement of Regulatory Practice

To date, information on non-target effects of GMOs do not have to comply with a standardized and agreed methodology in terms of test protocols or test batteries. Likewise, no standards for the testing of combinatorial effects of Cry toxins or possible interactions with other bioactive plant compounds exist (Dolezel et al., 2011). Currently, regulatory dossiers in the EU include only a minimal data set on combinatorial Cry toxin effects, justified on the grounds discussed above. However, the regulatory importance of combinatorial Bt effects has recently been recognized by the European Food Safety Authority (EFSA) and national biosafety authorities. For example, The Netherlands Commission on Genetic Modification (COGEM) recently commissioned two reports, one on the mode of action (van der Hoeven, 2014) and the other on the predictability of combinatorial Bt effects (de Schrijver et al., 2014). The reports were complemented by a scientific workshop of EFSA and COGEM in October 2014. While the reports list many of the key literature of this review, the conclusions of the authors clearly reflect the utilitarian perspective of pest and resistance management at the expense of detecting and managing risks on biodiversity and ecosystem services. While, de Schrijver et al. (2014) acknowledge certain types of combinatorial effects (addition, synergism, antagonism) that may exhibit a high level of species-specificity and cannot be predicted they also argue that sufficient information is available to conclude that Cry toxins with different primary order of activity are not likely to interact.

In contrast to this interpretation, we argue that the concept of primary-order specificity of Cry toxins should be discarded as more and more evidence is being published that Cry toxins are cross-order active in quite unexpected ways (van Frankenhuyzen, 2009, 2013). Hence, even on grounds of the assumption that combinatorial effects can only arise if single toxins elicit effects on their own in isolation, combinatorial effects in a number of non-target insects should be expected. Together with the uncertainties regarding a multitude of possible modes of action of Cry toxins, the precautionary principle clearly applies. The first rational measure of precaution would be to require robust experimental testing of combinatorial effects of all Cry toxins as expressed in the stacked GM events on a broader taxonomic range of non-target organisms selected independent of their pest status. Such experiments require ecologically relevant and agreed protocols which are indeed available since many years (Birch et al., 2004; Andow et al., 2008; Hilbeck et al., 2012b).

For both the scientific community and regulators another political issue urgently needs to be resolved. Any experiments with GMOs and non-target organisms are in need of GMO plant material and/or synthetic variants of the Bt toxins. At present, technology agreements linked with the purchase of seeds prevent the use of GM seeds in the regulatory pipeline for biosafety research and, thus, are limiting industry-independent research on the activity spectrum, the mode of action and on the combinatorial effects of multiple Bt traits. To resolve this problem, policy action is required to allow independent biosafety research not to be restricted to GMOs that are already commercially available.

Inherent Biases and Gaps of Knowledge Unaccounted for in Current Regulatory Risk Assessments of GM Crops

The recent report commissioned by the Netherlands Commission on Genetic Modification (COGEM; de Schrijver et al., 2014) did include some—but not all—of the studies listed in **Table 2**. Both van Frankenhuyzen (2013) and the updated version in the COGEM report (de Schrijver et al., 2014) recognized that the published data depart from the dominant narrative of narrow specificity and lack of reported effects of single Cry toxins on non-target beneficial organisms. Yet, in their interpretation, most studies reporting non-target effects were dismissed on the following grounds: their evidence was (i) "*not established unequivocally*" in comparison to other studies, (ii) "*not confirmed*" by "*subsequent studies*" or (iii) "*at odds with other studies showing no effects*" (de Schrijver et al., 2014, p. 36; van Frankenhuyzen, 2013, pp. 80–81). van Frankenhuyzen (2013, p. 81) stated that since the contradictions in quantitative data could not be resolved they presented "*enough uncertainty to indicate lack of evidence for unequivocal cross-toxicity*" and, therefore, these cases were excluded in an effort to maintain the dominant narrative, reducing the number of reported cross-activities substantially from 27 proteins affecting 69 high ranking taxa to 19 proteins affecting 45 taxa. We argue that a balanced evaluation in light of the different narratives explained above, in particular when working under the precautionary principle, should include these peer-reviewed, independent reports and engage in a deeper analysis as to the underlying methodological commonalities and causes explaining the differences between the contradicting studies as we have attempted in this review.

Furthermore, another serious gap of knowledge exists regarding interaction effects with other chemical pollutants in particular the many chemicals that are integral components of the industrial agricultural system and of GM crops. The majority of GM crops are also resistant against herbicides. These are systemic chemicals that are taken up by plants and translocated into all tissues including pollen and seeds. This has lead to a substantial increase in chemical use as well as residual chemical loads in the harvested products (Aris and Leblanc, 2011; Benbrook, 2012; Then, 2013). Additionally, maize seeds, both GM and conventional varieties, are routinely coated with chemicals such as the neonicotinoid Clothianidin. Because of seed coating, Chlothianidin has recently been shown to be present in substantial concentrations (8 μg/ml) in guttation fluid (Reetz et al., 2011) and in maize pollen (Krupke et al., 2012). Both are preferred sources of food for a wide range of beneficial insects, honeybees, predators, butterflies, and many more.

Neonicotinoids are also systemic and therefore result in similar exposure pathways as herbicidal residues and Cry toxins in GM crops. Despite the fact that Cry toxins, synthetic pesticides such as glufosinate, glyphosate, 2,4D, Dicamba, and neonicotinoids may all be jointly present in GM crops, none

of the synthetic pesticides have been tested in combination with single or multiple Cry toxins. For "SmartStax®," the biggest stacked GM crop plant currently commercially produced combines 6 Cry toxins. From developers data, we calculated the total Cry toxin values (Stillwell and Silvanovich, 2007; Phillips, 2008; **Table 1**) and found that the toxin load, depending on the specific plant tissue, varies from 90 to 250 and from 140 to 1500 μg Bt toxin/g fresh and dry weight respectively. The toxin load from SmartStax introduced into the environment has been estimated to total 4.2 kg Bt/ha (Benbrook, 2012). With such unprecedented concentrations of potent bioactive bacterial toxins, we see a high probability that this increase of active ingredients will adversely affect the communities of organisms associated with these agroecosystems, alone and in conjunction with the likewise significant loads of herbicide and neonicotinoid residues. While such stacked varieties offer benefits to farmers for agronomic problems, these benefits may come with serious health and environmental risks that we find prudent to be experimentally studied prior to field release and market approval.

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PERSPECTIVE
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Cornell Alliance for Science Evaluation of Consensus on Genetically Modified Food Safety: Weaknesses in Study Design

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Cornell Alliance for Science has launched an initiative in which "citizen scientists" are called upon to evaluate studies on health risks of genetically modified (GM) crops and foods. The purpose is to establish whether the consensus on GM food safety claimed by the American Association for the Advancement of Science (AAAS) is supported by a review of the scientific literature. The Alliance's citizen scientists are examining more than 12,000 publication abstracts to quantify how far the scientific literature supports the AAAS's statement. However, we identify a number of fundamental weaknesses in the Alliance's study design, including: evaluation is based only on information provided in the publication abstract; there is a lack of clarity as to what material is included in the 12,000 study abstracts to be reviewed, since the number of appropriately designed investigations addressing GM food safety are few; there is uncertainty as to whether studies of toxic effects arising from GM crop-associated pesticides will be included; there is a lack of clarity regarding whether divergent yet equally valid interpretations of the same study will be taken into account; and there is no definition of the cutoff point for consensus or non-consensus on GM food safety. In addition, vital industry proprietary biosafety data on GM crops and associated pesticides are not publicly available and thus cannot inform this project. Based on these weaknesses in the study design, we believe it is questionable as to whether any objective or meaningful conclusion can be drawn from the Alliance's initiative.

Keywords: Cornell Alliance for Science, citizen science, genetically modified foods, glyphosate, scientific consensus, genetically modified food health risks

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CONSENSUS ON GENETICALLY MODIFIED (GM) FOOD SAFETY?

Cornell Alliance for Science has launched an initiative in which "citizen scientists" are called upon to evaluate studies on health risks of GM crops and foods (1).

The background to the initiative is that in 2012 the board of the American Association for the Advancement of Science (AAAS) issued the following statement:

> Consuming foods containing ingredients derived from GM crops is no riskier than consuming the same foods containing ingredients from crop plants modified by conventional plant improvement techniques.

However, as the Alliance explains, others have denied that any consensus on the safety of GM foods exists. In 2013, the European Network of Scientists for Social and Environmental Responsibility (ENSSER) issued a statement, which criticized the 2012 AAAS statement and asserted: "We strongly reject claims… that there is a 'scientific consensus' on GMO safety and that the debate on this topic is 'over'" (2).

The purpose of the Alliance's initiative is to establish whether the claimed consensus on GM food safety is supported by a review of the scientific literature. The project aims to perform this task using a similar methodology to that employed by Cook and colleagues in their 2013 study of the climate change literature. This study concluded that 97% of the peer-reviewed literature supported the consensus on the existence of human-caused climate change (3).

In order to address this question for the similarly contested proposed consensus on GM food safety, the Alliance is examining more than 12,000 publication abstracts (1996–2015) available from the Web of Science. The aim is to quantify, using these abstracts, how far the scientific literature supports or does not support the AAAS statement on the consensus on GM food safety.

In principle, this is a laudable initiative that aims to address an important public health question. However, in our view, some aspects of the methodology give rise to concerns that deserve to be addressed. If they are not addressed, we believe that the initiative risks failing to meet its stated objectives.

THE ALLIANCE'S METHODOLOGY

We have been informed by Jaron Porciello, Associate Director for Research Data and Engagement at the Alliance for Science, that the abstracts included in the citizen scientists' review were selected in the following way. A total of 12,000 abstracts were chosen from approximately 144,000 using the Web of Science database. After 6 months of testing, keywords were selected that were informed by exploring other meta-analyses (critical, supportive, and neutral regarding GM foods). Tests were run to analyze what was lost when using one word over another, and, where overlap exists, by consulting references such as the UN Food and Agriculture Organization's AGROVOC and MEDLINE in order to ensure that the broadest possible concepts were covered.

The website of the Alliance for Science further explains, "Each abstract will be rated twice, by two independent raters (and no rater will receive the same 'set' of abstracts to rate), and once again by the author of the abstract (pending their participation)."

CONCERNS ABOUT THE METHODOLOGY

We believe that there are a number of problems with the Alliance's approach.

First, each reviewed publication will only be judged as to its significance purely from the abstract. However, the message of a study lies in the fine detail of its results and their various interpretations. This is especially the case with many studies on GM food health risks. Frequently, the authors conclude that there were no treatment-related adverse effects in the GM-fed groups of animals, but a close reading of the detail of the study reveals indications of toxicity or signs of toxicity in the GM-fed animals.

For example, a Monsanto-sponsored 90-day rat feeding study with the company's GM Bt insecticidal maize MON863 concluded that it was as safe and nutritious as the non-GM control maize (4). However, a reanalysis of the full published results in combination with the complete raw dataset, undertaken by a team of academic scientists working independently of the industry, revealed adverse effects or signs of potential toxicity, especially pertaining to liver and kidney function, in the GM-fed animals (5).

Monsanto responded by dismissing these statistically significant and potentially adverse effects as "unrelated to treatment or of no biological or clinical importance because they failed to demonstrate a dose-response relationship, reproducibility over time, association with other relevant changes (e.g., histopathology), occurrence in both sexes, difference outside the normal range of variation, or biological plausibility with respect to cause-and-effect" (6).

This type of dismissal is contrary to normal scientific practice, which calls for statistically significant biological differences caused by an intervention to be followed up with further research in order to determine their long-term consequences with respect to health.

As another example, a three-generation feeding study in rats with GM Bt insecticidal maize reported in the abstract that there were "some minimal histopathological changes in liver and kidney" in the GM-fed animals (7). These changes were described as "minor" in a much-cited review of GM food safety studies by Snell and colleagues (8). Yet examination of the detail of the study reveals that the GM-fed rats suffered damage to their liver and kidneys and alterations in blood biochemistry, which some scientists may view as unresolved safety questions demanding further study.

These examples suggest that statistically significant changes in GM-fed animals can either be viewed as unimportant or as indications that further research is needed to understand their mechanism and significance, depending on the individual viewpoints of the authors and/or reviewers.

These examples also illustrate that it is necessary to have full access to (minimally) the full results section of a publication and that conclusions about the safety of a GM food cannot be derived purely from the information provided in the abstract.

FEW LONG-TERM STUDIES ON HEALTH IMPLICATIONS OF GM FOODS

The number of properly designed and executed long-term studies looking at health implications of GM foods are very few. A commercial lifespan feeding study in pigs under real farm conditions found that animals fed a mixture of commercialized GM crops (soy and maize) resulted in elevated levels of severe stomach inflammation and heavier uteri in females, compared with controls fed a non-GM diet (9).

In another example, in 2012, a study was published that found liver and kidney damage in rats fed glyphosate-tolerant GM

maize NK603 and low doses of its associated herbicide Roundup over a 2-year period (10).

The study gave rise to a great deal of controversy. In response, the French food safety agency ANSES conducted a search for other comparable long-term laboratory animal feeding studies on GM herbicide-tolerant crops. It found only two (11). One was a two-year study in mice by Malatesta and colleagues, which found more pronounced signs of liver aging in the GM soy-fed group (12). The other was a study that found "no apparent adverse effect in rats" fed GM soybeans (13). However, in this latter study, the fact that glyphosate was only detected at the level of quantification (0.1 ppm) in the GM soy implies that, contrary to usual farming practice, this crop was not sprayed with this herbicide during cultivation, since it is well established that relatively high residues of glyphosate are routinely found in US-grown soy (14, 15).

Given the results of ANSES's search, it is unclear how 12,000 study abstracts with direct relevance to health have been identified. This raises the question of which types of publication will be included in the review. Will only publications describing original research be evaluated, or will reviews of the literature also be included?

This is an essential consideration because it is important not to take at face value the conclusions of reviews of studies, but instead to examine the results of the original studies covered by the reviews. This is because the conclusions of reviews can be marred by bias and omissions.

For instance, Snell and colleagues published a review of animal feeding studies with GM foods (8). Some of these studies showed toxic effects in the GM-fed animals. This included Malatesta and colleagues' study showing more pronounced liver aging in the animals fed GM soy (12). However, Snell and colleagues dismissed these effects as being of "no biological or toxicological significance" on the grounds of various methodological weaknesses (8)—in spite of the fact that studies concluding safety for the GM food tested suffered from the same inadequacies in study design (16).

GM CROP-ASSOCIATED HERBICIDE RESIDUES AS A SOURCE OF TOXICITY

Multiple sources of potential harm from GM food consumption are acknowledged and covered in the scientific literature. Toxic effects in principle could arise directly from the GM transformation process, resulting from disturbed gene function leading to altered biochemistry. Alternatively, toxicity could arise from increased exposures to the pesticides that are used in GM crop cultivation. Around 85% of GM crops are engineered to withstand application of herbicides (17), which in the majority of cases are glyphosate-based products such as Roundup.

Thus the question arises as to whether studies that examine the toxic effects of glyphosate will also be included in the Alliance's review. This question is not addressed by the Alliance's website and was not answered by Jaron Porciello in his email communications with the authors. However, it is important as animals and humans will inevitably be exposed to high levels of residues in food made from glyphosate-tolerant GM crops (14, 15, 18, 19) and these may pose health risks in their own right (20–22).

By the same token, will studies looking at toxicity from Bt toxin in a non-GM-related context be included? Although these studies are not on GM crops, they are relevant to a discussion of GM crop toxicity since large numbers of these crops are engineered to systemically express this protein (17).

VARYING INTERPRETATIONS OF DATASETS

Experimental datasets are subject to more than one interpretation, that is, different and perhaps even divergent yet equally valid interpretations of results can be arrived at by different scientists. This is inherent in the nature of the scientific exercise and an essential driver of scientific discourse. To illustrate this point in the context of the Alliance's initiative, statistically significant differences in physiological parameters arising from the consumption of a GM food compared to its non-GM control can be viewed by some scientists as biologically not relevant/significant and thus an indication of safety, while other scientists may see such differences as signs of possible toxicity that need to be followed up with additional research. Thus conclusions of safety arrived at by the authors can frequently be open to challenge.

The only way that the Alliance's citizen scientist reviewers can confirm the validity of the authors' conclusions is to have access to the whole study dataset. Restricting the evaluation of a study simply to the scrutiny of a given publication's abstract does not meet this crucial requirement and thus introduces a high level of risk that the citizen scientists' exercise will fail to meet its stated objectives.

It is, therefore, open to question as to whether the Alliance can derive any meaningful conclusions by having the citizen scientists look only at the abstracts. It is unclear if the citizen scientists and the reading public will be made aware of these major limitations of the exercise.

TRANSPARENCY

We also have concerns about the transparency of the methodology. According to Jaron Porciello in email communications with the authors, the full dataset, including all the selected and tested keywords and search strings, will only be made available upon conclusion of the study. However, this is unacceptable as it denies observers the opportunity to constructively critique the methodology with the aim of ensuring scientific rigor. From an objective standpoint, it is a concern that the methodology has not been made fully available at the outset. This may raise suspicion among the skeptical public who form the target audience for this exercise that the criteria upon which the abstracts are evaluated might be retrospectively selected to fit a preordained conclusion.

MISSING INDUSTRY PROPRIETARY DATA

The study of GM food safety is undermined by the fact that the GM seed developer and pesticide companies own the biosafety studies that they conduct on their products to support regulatory approval. Frequently, the data from these investigations are kept hidden as commercial secrets and not published in the peer review literature. In addition, scientists working outside of the industry lack access to the necessary research materials, that is, the GM crop under examination and its non-GM isogenic closest relative, grown under the same conditions.

A review addressing these issues stated that confidential business information (CBI) is often claimed for documentation and materials supporting the biosafety assessments of GMOs intended for environmental release and food, and feed use, but "such claims oftentimes marginally serve their legitimate purpose to protect commercial interests and unnecessarily limit transparency and public peer review of data submitted to regulatory authorities." The author added that CBI and proprietary claims also restrict access to transgene sequence data, GM seeds, and other GMO materials, which "precludes the development of independent research and monitoring strategies."

The author concluded that such claims "hinder the accumulation of biosafety data in the open, peer-reviewed literature, which is needed for both public and scientific consensus-building on safety issues and for improvements to the risk-assessment procedure itself" (23).

These vital biosafety data thus are not available to inform projects such as the Alliance's initiative, which are designed to make judgments on GM food safety.

CUTOFF POINT FOR CONSENSUS

It is unclear at which cutoff point the organizers of the Alliance's initiative will conclude on a "consensus" on GM food safety. From the point of view of protecting public health, even if 90% of the studies reviewed conclude in favor of safety and 10% do not, this should be sufficient to prove a lack of consensus. By analogy, if a new aircraft type is tested and only 10% of the tests show a problem, it is clear that those 10% of test results should not be dismissed in favor of the 90% of results demonstrating safety.

CONCLUSION

In this commentary, we have highlighted weaknesses in the study design of the Cornell Alliance for Science's citizen scientist initiative to evaluate the scientific literature pertaining to GM food safety. Amongst these shortcomings are:

1. Evaluation is based only on information provided in the abstract of any given publication, even though the full impact of the GM diet is only revealed by a close reading of the study's complete dataset.
2. It is unclear what material is included in the 12,000 study abstracts to be reviewed by the citizen scientists, since the number of appropriately designed investigations directly addressing GM food safety are very few.
3. It is unclear whether studies of actual and potential toxic effects arising from GM crop-associated pesticides (for example, glyphosate and Bt toxin) will be included in the review.
4. Different scientists can interpret the same results in different yet equally valid ways, with some concluding safety while others see potential or actual harm. This again highlights the need to examine the full dataset of any given publication to arrive at a conclusion of either safety or harm. If such differing interpretations of the same dataset still exist, then this necessitates a conclusion of non-consensus on GM food safety.
5. The cutoff point for consensus or non-consensus on GM food safety has not been defined from the outset.

Based on the above weaknesses in the study design, it is questionable as to whether any objective or meaningful conclusion can be drawn from the Alliance's exercise.

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This article was conceived by MA. Both authors contributed equally to the drafting of the manuscript.

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REVIEW
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Re-registration Challenges of Glyphosate in the European Union

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One of the most controversial societal issues today, regarding pesticide registration in the European Union (EU) may be the case surrounding re-registration of the active herbicide ingredient glyphosate. Shortly before the announcement of the conflicting views regarding the carcinogenicity status of this regulated agrochemical by EU Agencies, the European Food Safety Authority (EFSA) and the European Chemicals Agency (ECHA) on the one hand, and the International Agency for Research on Cancer (IARC) on the other hand, the Cancer Assessment Review Committee of the US Environmental Protection Agency (US EPA) also published re-evaluations. The US EPA assessment classified glyphosate into Group E, "not likely to be carcinogenic to humans." Similar positions were reached by EFSA and ECHA, assessing glyphosate as "unlikely to pose a carcinogenic hazard to humans" and "not classified as a carcinogen," respectively. A strongly opposing evaluation has previously been reached by IARC by classifying glyphosate into Group 2A, "probably carcinogenic to humans." IARC identified potential cancer hazards in this case, but did not estimate the level of risk it may present, which was taken into consideration by opposing agencies. Multiple effects of glyphosate have been reported, of which carcinogenic effects are only one component. Formulated glyphosate products—especially with polyethoxylated tallowamine and related compounds—have been shown to cause stronger cytotoxic or endocrine disrupting effects than the active ingredient glyphosate alone. Questions related to hazards and corresponding risks identified in relation to this active ingredient and its formulated herbicide preparations divide scientific circles and official health and environmental authorities and organizations, and touch upon fundamental aspects of risk assessment and product regulation. The decision has to consider both hazard-based (IARC) and risk-based analysis (EFSA); the former may not be suitable to calculate practical significances, and the latter being challenged if exposure estimations are uncertain in light of new data on residue levels. The results of current analytical surveys on surface water are particularly worrisome. In turn, the precautionary principle appears to be the optimal approach in this case for regulation in the EU.

Keywords: glyphosate, plant protection products, formulating agents, polyethoxylated tallowamine, hazard identification, risk assessment

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INTRODUCTION

Since its introduction as an herbicide active ingredient in 1971 (Baird et al., 1971), glyphosate [*N*-(phosphonomethyl)glycine] became and remains the market leading herbicide active ingredient worldwide (Dill et al., 2010; Székács and Darvas, 2012; Benbrook, 2016). Its initial patent protection commenced in 1971 (Franz, 1974), and was renewed in the eighties on the basis of novel composition—through a process that involved property acquisitions among major pesticide companies. However, even this extended patent protection eventually expired, and glyphosate became a generic compound in 1991 in many parts of the world outside the United States (US), and even the US patent expired in 2000. The introduction of glyphosate-tolerant (GT), genetically modified (GM) crops, began in the US in 1996 and gave a further protected status and market boost to glyphosate, securing its market leading position ever since.

Nonetheless, not only the intellectual property rights, but also the legal authorization of any given pesticide active ingredient has to be periodically renewed by national or international authorities in different parts of the world, when the substance is intended to be applied in agriculture. In the European Union (EU), the re-registration of glyphosate was scheduled for 2013, and Germany was chosen as Rapporteur country, with Slovakia as co-Rapporteur. Re-registration of the compound received prominent attention, due to significant commercial interests and also environmental and health concerns.

The applied formulations may contain various additives (e.g., surfactants), besides the active ingredients, and these additives have long been classified as being inert or inactive components in relation to the main biological effects of the formulation. Such "inertness" is consequent by definition, as any component exerting the main biological effect would be considered an active ingredient, not an additive. However, these inert ingredients may be biologically or chemically active in their side-effect profile, which also has to be considered in risk assessment and policy-making.

This survey attempts to summarize relevant data and information regarding decision-making in the re-registration process of glyphosate and its formulated herbicides, as well as main statements and events in evidence-based risk assessment that impacted it. It does not aim to justify or deny legislatory steps, but intends to reveal scientific data that had to be or should be considered in the corresponding decisions, with particular emphasis on results that have come to light since 2013, the preparation of the main risk assessment document on glyphosate, and with special attention to hazards identified in (eco)toxicity studies and to increased potential exposure levels corroborated by environmental monitoring of glyphosate residues.

THE WORLDWIDE MARKET OF GLYPHOSATE

Due to its patent protection, the market for glyphosate has been very favorable for the patent holder Monsanto Corporation for almost three decades. The leading glyphosate-based herbicide of Monsanto has been the Roundup group (Roundup Original®, Roundup Classic® Roundup UltraMAX®, Roundup WeatherMAX®), containing mostly isopropylammonium (IPA) or potassium salts of glyphosate having excellent water solubility. Other salts are also used, of which ammonium and sodium salts have less water solubility, while the trimesium (trimethylsulfonium) or IPA salts are almost twice as water soluble as the already highly soluble potassium salt—in fact this physicochemical feature has been used in formulations and claimed as an innovative novelty during patenting. Expiration of the patent protection outside the US in 1991 caused a 30, 40, and 50% drop in the market sales of Roundup within 1, 2, and 5 years, respectively. However, the introduction of GT GM crops has more than compensated Monsanto for initial market losses, as Roundup could then continue to be exclusively marketed as a product linked to Roundup Ready® (RR) crops, the first GT crop being RR soybean in 1996, followed by GT cotton, GT maize, GT canola, GT alfalfa, and GT sugar beet (Dill et al., 2010).

Regardless of the position of Monsanto in patenting and marketing glyphosate, the worldwide market for the active ingredient is continuously increasing as depicted in **Figure 1** on the basis of data reported (Bonny, 2011; Swanson et al., 2014; Benbrook, 2016). After average annual increases of 8% between 1982 and 1990, sales rose 16-fold in the 14 years between 1974 and 1990 (31% annual growth) and 26-fold in the 15 years between 1990 and 2005 (44% annual growth), and then maintained 8% annual growth between 2005 and 2014. The increasing boost after 1990 was clearly due to the worldwide introduction of GT crops, and this growth in consumption was further intensified with the expansion of the use of multiple trait (stacked genetic events) GM crops. Nonetheless, the use of glyphosate increased in regions without GM crop cultivation (due to pre-harvest or post-harvest chemical desiccation) as well: the overall consumption of glyphosate in Germany was boosted 5.7-fold between 1992 and 2012 (Berger et al., 2018). Thus, since

Abbreviations: AChE, acetylcholinesterase; ACS, American Chemical Society; ADI, acceptable daily intake; AMPA, aminomethyl phosphonic acid; ARfD, acute reference dose; BfR, German Federal Risk Assessment Institute, BVL, German Federal Office for Consumer Protection and Food Safety; CRP, Co-operative Research Programmes; DNA, desoxyribonucleic acid; EC, European Commission; ECHA, European Chemicals Agency; EEC, European Economic Community; EFSA, European Food Safety Authority; ELISA, enzyme-linked immunosorbent assay; ERD, enforcement residue definition; EU, European Union; FAO, Food and Agriculture Organization; GAT, glyphosate acetyltransferase; GM, genetically modified; GM38, human fibroblast cell line; GOX, glyphosate oxidoreductase; GT, glyphosate-tolerant; HEK293, human embryonic kidney cell line; HeLa, human cervical cancer cell line; HepG2, human hepatoma cell line; HT, herbicide-tolerant; HT1080, human fibrosarcoma cell line; HUVEC, primary neonate human umbilical vein endothelial cell line; IARC, International Agency for Research on Cancer; IPA salt, isopropylamine salt; IPA, isopropylamine, isopropylammonium; JEG3, human choriocarcinoma cell line; JAr, human chorioplacental cell line; JMPR, Joint Meeting on Pesticide Residues; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; MS, Member State; NE-4C, murine neuroectodermal stem cell-like cell line; NOAEL, no observable adverse effect level; OECD, Organisation for Economic Co-operation and Development; PAN, Pesticide Action Network; POEA, polyethoxylated tallowamine; PPP, plant protection product; RR, Roundup Ready®; T47D-KBluc, human transfected estrogen-dependent breast adenocarcinoma cell line; UF, uncertainty factor; US EPA, US Environmental Protection Agency; US, United States (of America); WHO, World Health Organization.

2012 glyphosate alone represented globally a stable 12% of the overall pesticide market and 13% of the market for synthetic pesticides (BCC Research, 2012; Transparency Market Research, 2014, 2016).

In 2014–2015, glyphosate accounted for 26% of maize, 43% of soybean and 45% of cotton herbicide applications. Considering oral rat LD_{50} or 24-month oral rat no observable adverse effect level (NOAEL) values for acute or chronic toxicity for all herbicide active ingredients used, glyphosate was estimated in a study (Kniss, 2017) to contribute only 0.1, 0.3, and 3.5% of the chronic toxicity hazard in those crops, respectively, on the basis of the hazard quotient approach weighting the hazard (toxicity) with the areas and dosages applied. Nonetheless, this estimation considered a factor termed "area-treatment" (instead of the absolute amounts applied), solely the average exposure (vs. exposure dynamics), and only of the active ingredients. Therefore, it did not take environmental fate, leaching toward drinking water supplies, ubiquitous exposures, side-effects by specific modes of action (genotoxic, hormonal, immunomodulant), as well as effects of the co-formulants into consideration. The study claims that increases in herbicide usage increased more rapidly on non-GM crops than on GM crops, and concludes that the replacement of glyphosate with other herbicides would be likely to result in increased chronic health risks to pesticide applicators. This strongly contradicts to earlier surveys (Heinemann et al., 2014; Benbrook, 2016; Perry et al., 2016), and is likely to be related to the fact that Kniss' study considered 159 herbicide formulations of 118 herbicide active ingredients, while herbicide-tolerant (HT) crops are designed against 8 herbicide active ingredients or active ingredient types (2,4-D, dicamba, glufosinate, glyphosate, oxynil type, sulfonylureas, imidazolinones, isoxaflutole), of which glyphosate by far is used most substantially in cultivation. Therefore, such an "overall" trend of all active ingredients considered, the vast majority of which not being related to HT crops is biased particularly for glyphosate, concealing the immense increases in glyphosate use in the grand average.

Overall production capacities have also risen over the decades. In 2012, the overall production capacity was 1.1 million tons/year which far exceeded the actual worldwide demand. Of the overall production, the Republic of China represents a substantial portion, and has increased its production capacity. Chinese production capacity was 323 thousand tons/year in 2007, but increased by 2.6-fold to 826 thousand tons/year in 2010, corresponding to a 37% annual increase rate. Statistics indicate that China alone is capable to meet the entire global glyphosate demand to date.

The success of glyphosate started with a predominant use of the active ingredient in the US in the seventies. Subsequently, the share of the US in the global annual turnover of glyphosate gradually decreased from 47% in 1974 to 15% in 2014, as seen in **Figure 2** on the basis of literature data (Benbrook, 2016). The decline of the US share has taken place more or less at a constant rate of −0.6%/year, except for the period of 1990–1995, when a steep decline of ∼-2.0%/year occurred, attributed to large increases in cultivation of GT crops in South America. The steep drop in 1991 also correlates with the expiration of the patent protection of glyphosate in different regions of the world (except for the US). The share of the US consumption may continue to decrease more rapidly as US consumption appeared to have leveled out after 2010 (reflected in a continuing decrease in the US share on the global consumption), while worldwide glyphosate consumption appeared to grow at an unchanged rate, partially due to GT crops gaining acreage in regions other than the US, and partially due to expanding glyphosate use in pre- or post-harvest crop desiccation. Globally, usage in GT crop cultivation and non-GT crop desiccation boosted the commercial success of glyphosate, while its manufacturing has shifted to Asia, resulting in a leading production role currently played by China.

REGISTRATION OF GLYPHOSATE IN THE EUROPEAN UNION

At the time of its introduction and following its approval in 1974, registration in 1983 and subsequent re-registration for use in cropland, forests, residential, and aquatic areas in 1993 by the US EPA (United States Environmental Protection Agency, 2016a), glyphosate had to be registered in Europe in each country, where it was intended to be marketed, and registration conditions and requirements varied by country. According to current patent laws, it was subjected to full product patent protection in Germany and other Common Market countries, while in the Soviet Bloc countries, where so-called "process patents" were in power, anyone could patent and register the active ingredient, who demonstrated by patent protection the invention of a novel chemical means for its synthesis.

A detailed and harmonized two-level registration system for plant protection products (PPPs) was introduced in the EU in 1991 with Council Directive 91/414/EEC (European Commission, 1991), specifying that pesticide active ingredients are regulated at EU level, managed by the European Commission (EC), while formulated pesticides are registered at Member State (MS) level. In addition, the new legal framework also requested re-registration of "old" active ingredients (already in use in the EU before 1991) (Klátyik et al., 2017a). Active ingredients subject to re-registration were specified in Annex I of the Directive, and the re-registration process was carried out in a four-stage work program completed by the end of 2010 (Anton et al., 2014). The first evaluation of glyphosate under Council Directive 91/414/EEC took place in 1995 within the first stage of the work program for existing active substances referred to in Article 8 (Dill et al., 2010). The basis of the evaluation was a joint dossier submitted by three industrial task forces, and Germany was designated as Rapporteur MS. Upon peer review of the documentation submitted, glyphosate was included in Annex I of Council Directive 91/414/EEC with Commission Directive 2001/99/EC (European Commission, 2001) coming into force in 2002. This authorization expired in 2012, when PPPs were already subject to Regulation 1107/2009 (European Commission, 2009) which came into force in 2011, and renewal of authorization of glyphosate under Regulation 540/2011 (European Commission, 2011b) was ordered.

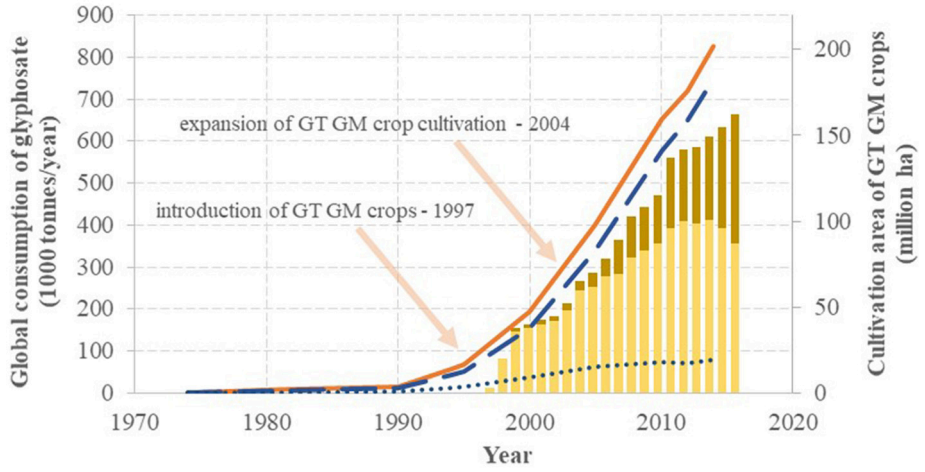

FIGURE 1 | The global annual turnover of glyphosate and the intensity of the cultivation of glyphosate-tolerant (GT) genetically modified (GM) crops: agricultural (*slashed line*), non-agricultural (*dotted line*) and overall (*solid line*) use of glyphosate, along with cultivation of single trait (*light columns*) and multiple traits (*dark columns*) GT GM crops. On the basis of Székács and Darvas (2012), Benbrook (2016), Cuhra et al. (2016), Myers et al. (2016) and the updated dataset of the International Service for the Acquisition of Agri-biotech Applications (James, 2015).

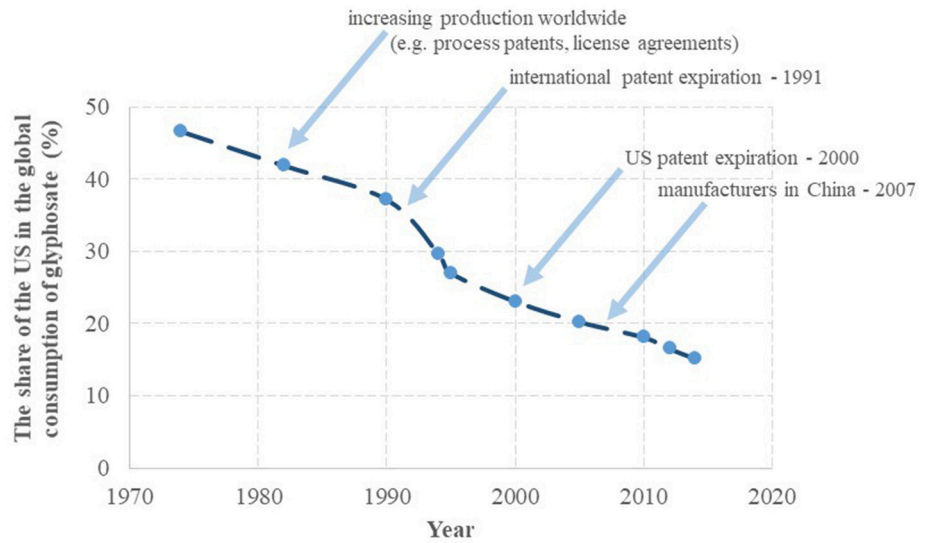

FIGURE 2 | The share of glyphosate uses in the United States from the global annual turnover. On the basis of Benbrook (2016).

The re-registration process took an unexpected turn that triggered wide public responses, when the renewal of the authorization of glyphosate, along with 38 other pesticide active ingredients, was postponed until 2015 (European Commission, 2010) and to be completed only in 2016. The reasoning for the postponement was related to delays in the overburdening task of pesticide authorization renewals. However, then more recent studies have indicated a range of potential harmful effects, including hepatotoxicity or hepatorenal effects on rats (Benedetti et al., 2004; Larsen et al., 2012), and the number of published studies increased by orders of magnitude e.g., publications related to glyphosate intoxication rose from 44 in 1978–1987 to 152 in 1996–2005 and to 875 in 2006–2015 (Zyoud et al., 2017). Although reviews of genotoxicity studies deemed DNA damage by glyphosate and glyphosate-based formulations secondary to cytotoxic effects (Kier and Kirkland, 2013; Kier, 2015), DNA-damaging effects and genotoxicity of glyphosate and particularly of its formulations (Roundup®, Glyfos®, Glyphogan®, Glyphosate-Biocarb®, etc.) on vertebrates (murine and human cells) (Bolognesi et al., 1997; Koller et al., 2012; Young et al., 2015; Townsend et al., 2017), cytotoxic effects of glyphosate-based herbicides on human embryonic and placental cells (Benachour et al., 2007; Benachour and Séralini, 2009; Gasnier et al., 2009, 2010; Mesnage et al., 2013a,b), indication

of endocrine disrupting effects by showing activity on estrogen receptors in human hormone-dependent breast cancer cells (Thongprakaisang et al., 2013; Mesnage et al., 2017a), inhibition of the biosynthesis of testosterone and estradiol (Romano et al., 2010) and progesterone (Young et al., 2015) or inhibitory effects on aromatase, a key enzyme in steroid hormone biosynthesis (Cassault-Meyer et al., 2014; Defarge et al., 2016), teratogenic effects on vertebrates by inhibiting the retinoic acid signaling pathway (Lajmanovich et al., 2003; Paganelli et al., 2010; Carrasco, 2013), birth defects in rats (Guerrero Schimpf et al., 2017), and nephrotoxic and hepatotoxic effects of Roundup® have been demonstrated in rats in connection to RR GM maize (originally published in the journal *Food and Chemical Toxicology* in September 2012, but retracted by the journal in November 2013 following an alleged intervention from the industry stakeholder (Foucart, 2016), and subsequently republished in another journal a year later) (Séralini et al., 2014). The analysis of kidney and liver tissues from the same rats by molecular profiling (transcriptomics, proteomics, metabolomics) confirmed pathology of these organs in the lowest dose Roundup® treatment group culminating in non-alcoholic fatty liver disease (Mesnage et al., 2015a,b, 2017a,b).

The *in vitro* data on the cytotoxicity of glyphosate on various cell lines, as determined in the corresponding effective concentration values causing 50% mortality (EC_{50}), are shown in **Figure 3**, and range over two orders of magnitude (between 0.1 and 10 mg/ml, corresponding to ∼0.5–50% of the dilution used in agricultural applications ∼2%), with a very broad range in sensitivity among various cell lines tested. In general, the most sensitive cell lines appeared to be the human hematopoietic Epstein-Barr virus transformed lymphocyte Raji cells (Townsend et al., 2017), regenerative fin cell lines of fish origin (Qin et al., 2017), human epithelial HaCaT keratinocyte cells (Elie-Caille et al., 2010; Heu et al., 2012a,b; Qin et al., 2017) and a murine neuroectodermal stem cell-like line, NE-4C (Székács et al., 2014). In contrast, cell types with the lowest apparent sensitivity were human choriocarcinoma cells (JEG3) (Benachour et al., 2007; Benachour and Séralini, 2009; Gasnier et al., 2009; Romano et al., 2010; Mesnage et al., 2013a), human chorioplacental cells (JAr) (Young et al., 2015), human hepatoma cells (HepG2) (Benachour et al., 2007; Benachour and Séralini, 2009; Gasnier et al., 2009, 2010), murine osteoblast precursor cells (MC3T3-E1) (Farkas et al., 2018), human embryonic kidney cells (HEK293) (Benachour et al., 2007; Benachour and Séralini, 2009; Gasnier et al., 2009; Mesnage et al., 2013a), and human primary neonate umbilical vein endothelial cells (HUVEC) (Benachour et al., 2007; Benachour and Séralini, 2009; Gasnier et al., 2009). Cytotoxicity has also been detected by other biochemical markers e.g., mitochondrial functions, release of lactate dehydrogenase, cell proliferation determined by the use of sulforhodamine B, or membrane integrity and lysosomal activities indicated by the uptake of neutral red dye (Koller et al., 2012; Defarge et al., 2016). The interaction of between glyphosate and mitochondrial succinate dehydrogenase has been verified by molecular modeling (Ugarte, 2014). The IPA salt of glyphosate was indicated to be genotoxic at concentrations of 0.16–1.6 μg/ml on human lymphocytes, fish erythrocytes and plant staminal nuclei *in vitro* and *in vivo* (Alvarez-Moya et al., 2014). Glyphosate has also been shown to be able to disrupt regenerative diploid (DIMF) and triploid fin cell lines from the Oriental weather loach (*Misgurnus anguillicaudatus*) with cytotoxicity of LC_{50} = 0.315 and 0.372 mg/ml, respectively. It also was found to induce DNA damage (micronucleus formation), cell damages (chromatin condensation, nucleus distortion, broken, and reduced endoplasmic reticulum, mitochondria and ribosomes) and apoptosis (Qin et al., 2017), intracellular oxidative cascade, morphological modifications, and apoptosis (Elie-Caille et al., 2010) caused by oxidative stress due to mitochondrial membrane potential disruption (Heu et al., 2012b) and cell morphological changes (Heu et al., 2012a). In addition to detection of decreased cell viability (tested in the above examples), cytotoxicity has been tested on other end points as well, including mutagenicity within the same range of toxicity (EC_{50} = 0.6–0.9 mg/ml) for human epithelial type 2 cells (Hep-2) as occurs for human cervical cancer cell (HeLa) contaminant (Mañas et al., 2009), human fibroblast cells (GM38) (Monroy et al., 2005), and human fibrosarcoma cells (HT1080) (Monroy et al., 2005). Exposure of hippocampal pyramidal cells from rats to glyphosate at 2–6 mg/ml caused impaired neuronal differentiation and development and axon growth (Coullery et al., 2016), and a glyphosate absorption study across epithelial tissues e.g., across Caco-2 cells revealed saturable glyphosate uptake through epithelial transporter enzyme activity in an ATP- and Na^+-independent manner, not competed by specific amino acids or transporter inhibitors. Enhanced uptake into the epithelial cells at barrier mucosae has been pointed out to potentially result in more significant local and systemic effects than predicted from the passive permeability of glyphosate, and may lead to neural disposition and risk for brain-related toxicities (Xu et al., 2016). It has been indicated that glyphosate at concentrations of 0.09–1.69 mg/ml may induce DNA damage in leucocytes such as human peripheral blood mononuclear cells, cause DNA damage (single and double strand-breaks by the comet assay) and DNA methylation (global DNA methylation and methylation of p16 (CDKN2A) and p53 (TP53) promoter regions), and trigger DNA methylation in human cells (Kwiatkowska et al., 2017). Correlations were less apparent for other biochemical end points e.g., endocrine disrupting effects. Glyphosate was found to inhibit aromatases in JEG3 cells with EC_{50} values of 7 mg/ml (Richard et al., 2005), but causing ∼10% inhibition only at 0.024 mg/ml (Defarge et al., 2016). It has not been reported to exert estrogen agonist effects in the estrogen receptor activation-reporter assay on JEG3 cells, but was proven to be anti-androgenic at sub-agricultural and non-cytotoxic dilutions (Gasnier et al., 2009). In contrast, it has been indicated to exert estrogen receptor activation on human transfected estrogen-dependent breast adenocarcinoma cells (T47D-KBluc) with an EC_{50} value of 0.005 ng/ml (Thongprakaisang et al., 2013) or in a later study 0.002 mg/ml (Mesnage et al., 2017a), i.e., two orders of magnitude below concentrations causing cell mortality. Glyphosate-based herbicide preparations, containing polyethoxylated tallowamine (POEA) as formulating surfactant, showed a somewhat similar pattern at dilutions corresponding to two orders of magnitude lower glyphosate concentrations, (between 0.001 and 0.1 mg/ml,

corresponding to ∼0.005–5% of the dilution used in agricultural applications), with notable outstanding sensitivities for cell lines NE-4C and JAr. A study of exposure to glyphosate on human HepG2 cells using biomarkers of oxidative stress found prompt (upon 4 h) elevated levels of permanent DNA damage (micronucleus formation) in cytokinesis-block micronucleus cytome assay and in alkaline comet assay (indicating a possible aneugenic effect), as well as decreases in lipid peroxidation, glutathione peroxidase activity and total antioxidant capacity at occupational exposure level (0.0035 mg/ml) revealing oxidative damage. In contrast, no significant effects remained upon 24 h of exposure in the levels of reactive oxygen species, glutathione and lipid peroxidation, indicating a certain ability of the cells to cope with prolonged exposure (Kašuba et al., 2017). Supported by an optical biosensor method and holographic microscopy, Roundup Classic® and glyphosate have recently been shown to inhibit normal cell adhesion of MC3T3-E1cells with IC_{50} values upon 1 h of exposure of 0.086 and 0.59 mg/ml in serum-containing medium and 0.10 and 1.97 mg/ml in serum-free conditions, respectively; and the approximately one order of magnitude higher inhibitory potency of Roundup Classic® was proven to be attributed to POEA (Farkas et al., 2018).

Substantially higher cytotoxicities recorded for glyphosate-based herbicide preparations at given dilutions than those seen for the corresponding glyphosate concentrations indicate that the excessive toxicity is clearly due either to component(s) in the formulation, or to their interaction with the active ingredient (see below). A problem occurring frequently in the scientific literature is, however, that reports do not always accurately specify the actual glyphosate formulation used, and often attribute the observed effect to the active ingredient, glyphosate. This is, in several cases, a wrong assumption, which can be verified only with the use of pure glyphosate. For this reason, comparative studies with glyphosate, co-formulants and formulations involved are of increasing significance (Klátyik et al., 2017a; Székács, 2017; Defarge et al., 2018; Mesnage and Antoniou, 2018).

Three glyphosate-based formulations, Roundup Express®, Roundup Bioforce®, Roundup GT® and Roundup GT Plus® at 5% dilutions corresponding to 0.04–2.3 mg/ml concentrations of glyphosate showed 22–97% inhibition of the mitochondial activity and activation of caspase 3/7 enzymes of HepG2 cells, while such levels of inhibition with glyphosate alone could be achieved only at or above 20 mg/ml concentration (Benachour et al., 2007; Benachour and Séralini, 2009; Gasnier et al., 2009; Mesnage et al., 2013a). Effects were similar, but even somewhat stronger on the more sensitive human Hep3G hepatic cell line (Gasnier et al., 2010). Roundup Bioforce®, but not glyphosate, caused cytotoxicity through caspase 3/7 inhibition on testicular Leydig, Sertoli, and germ cells from rat and through adenylate cyclase activation on Leydig cells (IC_{50} = 0.36–0.9 mg/ml; 0.1–0.25%) (Clair et al., 2012). Roundup Original® similarly induced calcium-mediated cell death in rat testis and Sertoli cells (de Liz Oliveira Cavalli et al., 2013). A study using a glyphosate-based herbicide formulation Glifosato Atanor® and spray adjuvant alkylphenol ethoxylate (Impacto®) on the human Hep-2 cell line after 24 h of exposure indicated cytotoxicity due to oxidative damage by increased levels of reactive oxygen species with an IC_{50} value of 0.38 mg/ml (corresponding to glyphosate concentration of 0.14 mg/ml) (Coalova et al., 2014), while glyphosate or its primary metabolite did not exert observable cytotoxicity in the test at concentrations up to 1 mg/ml (Chaufan et al., 2014). Addition of the spray adjuvant further reduced the IC_{50} value to 0.18 mg/ml (corresponding to glyphosate concentration of 0.064 mg/ml). Vice versa, the glyphosate formulation also increased the toxicity of the spray formulant; i.e., the two substances showed synergistic cytotoxicity. The same formulation was shown to inhibit proliferation and differentiation of adipocyte 3T3-L1 fibroblasts (Martini et al., 2012) and to increase lipid peroxidation and antioxidant enzyme activity by oxidative stress, and to inhibit the expression of genes normally up-regulated during adipogenesis, e.g., master gene PPAR gamma (Martini et al., 2016a). Three glyphosate-based formulations showed cytotoxicity on adipocyte 3T3-L1 fibroblasts in the order of Roundup FG® > Glifosato Atanor® > Glifogran® in the range of IC_{50} values corresponding to glyphosate concentrations of 2.5–63 μg/ml, while glyphosate itself exerted an IC_{50} value of 3.5 mg/ml (Martini et al., 2016b). In addition, Glifosato Atanor® was found genotoxic, correlated with lipid peroxidation and DNA fragmentation effects, at concentrations of 9–26% (corresponding to glyphosate concentrations of 0.038–0.113 mg/ml) on human peripheral blood leukocyte cells (Barbosa et al., 2017). Roundup Original® was found cytotoxic to human adipose-derived mesenchymal stem cells with an IC_{50} value corresponding to glyphosate concentration of 43.0 ± 1.7 μg/ml, induced death by apoptosis and necrosis upon 24 h of exposure, and caused reduced alkaline phosphatase activity in cells induced to osteogenic differentiation (de Melo et al., 2018). Formulations containing glyphosate IPA salt, Roundup 3 Plus®, Roundup Biovert®, Amega®, Cargly® and Cosmic®, unlike glyphosate alone, were shown to affect cell proliferation in embryonic cells 360 min upon fertilization of the sea urchin *Sphaerechinus granularis* inducing a delay in entry into the M-phase in the cell cycle (Marc et al., 2002, 2004). A Roundup® formulation, but again not glyphosate, was shown to affect cell proliferation and steroid production, as dramatically decreased cell numbers, as well as estradiol and progesterone production were recorded in granulosa cells from beef heifer ovaries upon exposure to Roundup® at 0.01–0.30 mg/ml (corresponding to glyphosate concentrations of 0.0018–0.054 mg/ml) (Perego et al., 2017). Roundup® inhibited the survival of human L-02 hepatocytes (IC_{50} = 0.15 mg/ml, corresponding to glyphosate concentration of 0.062 mg/ml) by inducing mitochondrial and DNA damage, changes in membrane integrity and permeability, inhibition of the antioxidant system, and thus, apoptosis (Luo et al., 2017). Roundup Transorb® exerted cytotoxicity on a zebrafish (*Danio rerio*) hepatocyte cell line ZF-L at concentrations as low as 0.068–0.27 μg/ml (corresponding to glyphosate concentrations of 0.033–0.13 μg/ml), due mostly to lysosomal instability and inhibition of mitochondrial function, and slightly to impaired cell membrane integrity. Synergistic detrimental effects were observed when Roundup Transorb® was applied with an insecticide formulation (Furadan 350

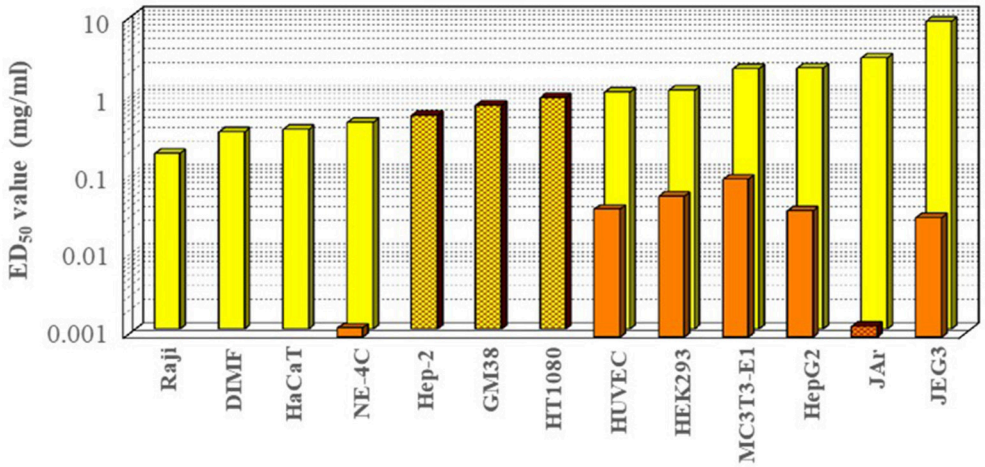

FIGURE 3 | *In vitro* cytotoxicity of glyphosate (*light columns*) and its formulated preparation Roundup® (*dark columns*) on various cell lines Raji: human hematopoietic Raji (Epstein-Barr virus transformed human lymphocyte) cells (Townsend et al., 2017), DIMF, diploid fin cell line from the Oriental weather loach *Misgurnus anguillicaudatus* (Qin et al., 2017); HaCaT, human epithelial keratinocyte cells (Elie-Caille et al., 2010); NE-4C, murine stem cell-like neuroectodermal cells (Székács et al., 2014); Hep-2, human epithelial type 2 (HeLa contaminant) cells (Mañas et al., 2009); GM38, human fibroblast cells (Monroy et al., 2005); HT1080, human fibrosarcoma cells (Monroy et al., 2005); HUVEC, primary neonate human umbilical vein endothelial cells (Benachour et al., 2007; Benachour and Séralini, 2009; Gasnier et al., 2009); HEK293, embryonic kidney cells (Benachour et al., 2007; Benachour and Séralini, 2009; Gasnier et al., 2009; Mesnage et al., 2013a); MC3T3-E1, murine osteoblast precursor cells (Farkas et al., 2018); HepG2, human hepatoma cells (Benachour et al., 2007; Benachour and Séralini, 2009; Gasnier et al., 2009); JAr, human chorioplacental cells (Young et al., 2015); JEG3, human choriocarcinoma cells (Benachour et al., 2007; Benachour and Séralini, 2009; Gasnier et al., 2009; Mesnage et al., 2013a, 2017a). Plain and grid column patterns indicate cytotoxicity detected by MTT test and mutagenicity tests, respectively.

SC®) of no known interaction between the active ingredients, indicating that toxicity was likely to be due to the surfactants in the commercial formulations (Goulart et al., 2015). Moreover, Roundup Full II® exerted acute toxicity (LC_{50} = 0.009 mg/ml upon 96 h of exposure) and genotoxic effects in blood, gill, and liver cells of the pacu fish (*Piaractus mesopotamicus*) determined in comet micronucleus and nuclear abnormalities assays (Leveroni et al., 2017). Roundup Transorb® was found mutagenic and genotoxic on gill erythrocyte cells of the guppy *Poecilia reticulata* (De Souza Filho et al., 2013), and a glyphosate-based herbicide at dilutions corresponding to glyphosate concentrations above 1.7 ng/ml caused significant decreases in the numbers of differentiated neuronal clusters and myotubes on primary embryonic stem cells from *Drosophila melanogaster*, being indicative of potential teratogenic effects (Argueta and Torres, 2017).

Upon clinical observations that surfactants used in glyphosate-based formulations substantially contributed to development of symptoms e.g., hypotension, mental deterioration, respiratory failure, acute kidney injury, and arrhythmia in intoxication cases by those formulations (Seok et al., 2011), targeted studies on surfactant-induced cellular effects found that cytotoxicity via apoptosis and necrosis caused by mitochondrial damage by surfactant POEA (TN-20) on mouse alveolar epithelial, fibroblast-like, and heart cell lines was reduced in the presence of glyphosate, while that of a corresponding polyoxyethylene lauryl amine ether (LN-10) surfactant was unaffected on alveolar cells, but increased on fibroblast-like and heart cell lines in the presence of glyphosate. Glyphosate alone did not exert cytotoxicity at up to 0.17 mg/ml (100 μM) (Song et al., 2012; Kim et al., 2013).

Moreover, the primary metabolite of glyphosate, aminomethyl phosphonic acid (AMPA) was found to be genotoxic on human and murine cell lines using the comet assay, the chromosome aberration test and the micronucleus test (Mañas et al., 2009). Assessing cytotoxicity of glyphosate, its metabolite AMPA and impurities (N-(phosphonomethyl) iminodiacetic acid, N-methylglyphosate, hydroxymethylphosphonic acid and bis-(phosphonomethyl)amine) on human peripheral blood mononuclear cells, found statistically significant decreases in their viability and ATP levels. Thus, N-methylglyphosate and bis-(phosphonomethyl)amine exerted cytotoxicity upon 24 h of exposure with IC_{50} values of 1.8 mg/ml, AMPA showed significant but minor inhibitory effects at concentrations of 0.06–1.1 mg/ml, while the others affected viability only slightly at concentrations of 0.48–1.7 mg/ml, glyphosate itself being the least cytotoxic (Kwiatkowska et al., 2014, 2016). AMPA as photodegradation product of glyphosate was shown to cause high genotoxicity on Chinese hamster ovary cells (CHO-K1) (Roustan et al., 2014). In addition, the side-product of AMPA formation, glyoxylate was shown by activity-based protein profiling to be capable to react with cysteines across many proteins in mouse liver, inhibiting fatty acid oxidation and thus, increasing liver fat (Ford et al., 2017).

Consequently, reported *in vivo* effects of glyphosate and its formulated herbicide preparations are far more scattered than data from *in vitro* assays (Mesnage et al., 2015b). *In vivo* toxicity has been reported for a wide range of organisms of

various phylogeny and with diverse symptoms (Gill et al., 2018). Glyphosate and even more its commercial formulations were indicated to induce DNA damage (micronucleus formation) in a wide range of animal species by numerous studies and a meta-analysis (Ghisi et al., 2016), moreover, the active ingredient was found to be genotoxic also in plants (Nardemir et al., 2015) and to induce oxidative stress and catalase activity in submerged macrophytes (Zhong et al., 2018). It has also been shown to serve as a source of phosphorous for algae at low concentrations (Klátyik et al., 2017b; Wang et al., 2017), and therefore, to potentially induce algal bloom (Drzyzga and Lipok, 2017). Formulated glyphosate-based herbicides, Touchdown® and Roundup® caused neurotoxicity (McVey et al., 2016) as well as locomotion and fertility inhibition (García-Espiñeira et al., 2018) on the nematode *Caenorhabditis elegans*. Following the results of the late Andrés Carrasco and his research group (Paganelli et al., 2010; Carrasco, 2013), recent findings in environmental toxicology of glyphosate and its formulated products include its revealed toxicity on amphibian species (Mann et al., 2009; Relyea and Jones, 2009; Meza-Joya et al., 2013; Wagner et al., 2013; Henao Muñoz et al., 2015; Baier et al., 2016a,b), on mollusks (Conners and Black, 2004) and on earthworms (Zaller et al., 2014; Gaupp-Berghausen et al., 2015). It affected hemocyte parameters and acetylcholinesterase (AChE) activity, but not antioxidant enzyme activities in mussel *Mytilus galloprovincialis* (Matozzo et al., 2018) with disruption of key biological processes including energy metabolism, Ca^{2+} homeostasis and endoplasmic reticulum stress response, as well as cell signaling identified by transcriptome analysis (Milan et al., 2018). Glyphosate exerted acute toxicity on the invasive snail *Pomacea canaliculata* with a 96-h LC_{50} value of 175 mg/l, overly high for control purposes, but indicating oxidative stress, enhanced overall metabolic rate and altered catabolism from protein to carbohydrate/lipid mode (Xu et al., 2017).

Among organisms with life cycle related to water bodies, a glyphosate-based herbicide Factor 540® at exposures corresponding to glyphosate concentrations of 1–1000 μg/l modified structural and functional properties of freshwater phytoplankton communities (6 algal and 3 cyanobacterial species/strains) living in streams located in agricultural areas, causing a concentration-dependent reduction in chlorophyll-a and carotenoid levels, changes in the algal community structure, reduced diversity, as well as biochemical, and physiological parameters (shikimate content, lipid peroxidation, antioxidant activity of superoxide dismutase, catalase, and ascorbate peroxidase) (Smedbol et al., 2017, 2018). Roundup® was shown to be toxic to the food-borne trematode *Echinostoma paraensei* developing in given life stages in aquatic hosts (Monte et al., 2016). Roundup Express® and POEA were detected to exert toxicity on juveniles of the Pacific oyster *Crassostrea gigas* upon sub-chronic (35-day) exposure at concentrations of 0.1, 1 and 100 ng/ml (Mottier et al., 2013, 2014; Séguin et al., 2017). Roundup Original® exerted lethal and sub-lethal effects on the widely distributed in dipteran freshwater nematoceran fly *Chironomus xanthus* with a 48 h LC_{50} corresponding to glyphosate concentration of 251.5 mg/l, as well as reduced larval growth of and disturbed emergence of adults at lower concentrations (Ferreira-Junior et al., 2017), and it reduced the growth rate, the escape swimming speed and the fat storage also on the endangered damselfly *Coenagrion pulchellum* at a level corresponding to a glyphosate concentration of 2 mg/l, which is likely to lead to negatively influence fitness, mortality by predation and population dynamics (Janssens and Stoks, 2017). As in the latter test Roundup systematically resulted in 25–100% higher effects than glyphosate at equivalent concentrations, the enhanced effect was attributed to POEA. Toxicity to the water flea (*Daphnia magna*) is of special importance, and not only formulated glyphosate-based herbicides (Cuhra et al., 2013; Ørsted and Roslev, 2015), but also glyphosate residues in GT soybean (Cuhra et al., 2015) were shown to exert toxicity on this standard ecotoxicity indicator organism. Effects of glyphosate on somatic and ovarian growth, as well as of glyphosate-based formulations on ovarian growth and immune status of freshwater and estuarine crab species were reported (Hong et al., 2017; Avigliano et al., 2018; Canosa et al., 2018). Toxicities have extensively been reported for fish, including the zebrafish *D. rerio*, on which both glyphosate (Armiliato et al., 2014; Lopes et al., 2014; Uren Webster et al., 2014) and its formulated products (Bridi et al., 2017; Sulukan et al., 2017) were found to exert acute toxicity through inhibited carbonic anhydrase activity due to oxidative stress (production of reactive oxygen species), cellular apoptosis, effects on locomotor activity and aversive behavior, as well as reproduction disorders, deteriorating sperm quality, embryotoxicity malformations, and other endocrine disruption symptoms. Another study found carp (*Cyprinus carpio*) more sensitive to embryotoxicity of glyphosate at high concentrations (50 mg/l) than zebrafish (Fiorino et al., 2018). Glyphosate and AMPA were found toxic to guppies *Poecilia reticulata* (Antunes et al., 2017) with median lethal concentrations (LC_{50}) for 96-h exposure of 0.07 and 0.16–0.18 mg/ml, respectively, with tissue- and gender-specific histopathological responses at sublethal concentrations. Formulated glyphosate preparations (Roundup®, Roundup Transorb®) exerted even stronger toxicity to this and related guppy species (De Souza Filho et al., 2013; Harayashiki et al., 2013; Rocha et al., 2015; dos Santos et al., 2017) with exposure-time dependent hepatic histopathological damage (dos Santos et al., 2017) or genotoxicity (De Souza Filho et al., 2013). Roundup® was shown to affect hematological response and tissue AChE activity in carp (*C. carpio*) (Gholami-Seyedkolaei et al., 2013; Kondera et al., 2018), increased glycogen and triacylglycerol consumption and lipid deposition in the liver, as well as changes in muscle glycogen in catfish (*Rhamdia quelen*) (Persch et al., 2018), DNA strand breaks in the goldfish (*Carassius auratus*) (Çavas and Könen, 2007; Lushchak et al., 2009; Li et al., 2017). Cytotoxicity by oxidative stress by Roundup® has been shown by transcriptomic profiling in carp and zebrafish (Uren Webster and Santos, 2015; Sulukan et al., 2017). Acute toxicity, harmful physiological effects including hepatotoxicity, neurotoxicity, deteriorated sperm counts, early life stage development and DNA-damaging effects have been reported for numerous other fish species as well for sublethal exposures to Roundup® products including Roundup Original®, Roundup Transorb® and Roundup WG®, Garlon®, and other glyphosate-based herbicides (Soso et al., 2007; Cavalcante et al., 2008; Guilherme et al., 2010, 2014a,b;

Modesto and Martinez, 2010; Shiogiri et al., 2012; Ghisi and Cestari, 2013; Nwani et al., 2013; Marques et al., 2014; Moreno et al., 2014; Navarro and Martinez, 2014; Richard et al., 2014; Sinhorin et al., 2014; Braz-Mota et al., 2015; Menéndez-Helman et al., 2015; Li et al., 2016; de Moura et al., 2017; Sánchez et al., 2017; Gonçalves et al., 2018; Zebral et al., 2018) or POEA (Yusof et al., 2014). Roundup® was found to disrupt 17β-estradiol and reduce glutathione concentration in the liver of the endangered fish species delta smelt (*Hypomesus transpacificus*) upon 6 h of exposure at levels corresponding to 78 μg/l glyphosate concentrations and above (Jin et al., 2018). As described earlier, effects of glyphosate and its formulated products on amphibians and mollusks (Conners and Black, 2004; Mann et al., 2009; Relyea and Jones, 2009; Paganelli et al., 2010; Carrasco, 2013; Meza-Joya et al., 2013; Wagner et al., 2013; Henao Muñoz et al., 2015; Baier et al., 2016a,b) received particular attention due to their known hormonal sensitivity. Differential toxicity of glyphosate and its formulated PPPs have been also considered in the official scientific opinion by EFSA on pesticide risk assessment for amphibians and reptiles (Ockleford et al., 2018). Identification of acute lethal, physiological and genotoxic effects of glyphosate-based herbicides, including Roundup Original® and Roundup Transorb®, have continued on amphibians (Lajmanovich et al., 2011, 2013, 2014; Yadav et al., 2013; Bellantuono et al., 2014; Levis and Johnson, 2015; Gandhi and Cecala, 2016; Rissoli et al., 2016; Soloneski et al., 2016) and reptiles (Latorre et al., 2013; Siroski et al., 2016). Teratogenic effects were reported in a treefrog (*Scinax nasicus*) (Lajmanovich et al., 2003) and in the embryo of the African clawed frog (*Xenopus laevis*) (Paganelli et al., 2010) in response to exposure to Glyfos® or Roundup Classic® at levels corresponding to 3–7.5 and 96–160 mg/l glyphosate concentrations, respectively. Hepatotoxicity of glyphosate and Roundup Ultramax® was observed in tadpoles of the neotropical frog *Leptodactylus latrans* (Bach et al., 2018), but the formulated herbicide product was found 10-fold more toxic than glyphosate, leading to histopathologic lesions at a level corresponding to a glyphosate concentration of 0.37 mg/l. Teratogenicity is not exclusively related to POEA, as a POEA-free micro-emulsion formulation containing polyethoxylated isotridecyletherpropylamine as a surfactant, Roundup® Power 2.0 has been found to exert embryotoxicty on *X. laevis* in the frog embryo teratogenesis assay–*Xenopus* (FETAX) with a 96-h EC_{50} value of 7.8 mg/l, while glyphosate was not found to be embryolethal, only causing edemas at the highest concentration tested, 50 mg/l (Bonfanti et al., 2018). Toxic effects were correlated with the inhibition of degradative enzymes (esterases and glutathione S-transferase) (Lajmanovich et al., 2011, 2013, 2014), while teratogenic effects and malformations have been linked to inhibition of the retinoic acid signaling pathway (Paganelli et al., 2010; Carrasco, 2013). Other studies on amphibians indicated increased excretion of defensive chemicals in the common toads (*Bufo bufo*) upon exposure throughout larval development to Glyphogan Classic® (360 g/l glyphosate—the same composition as Roundup Classic®, 41.5 w/w% glyphosate and 15.5 w/w% POEA) at a level corresponding to 4 mg/l glyphosate concentration (Bókony et al., 2017; Miko Z. S et al., 2017a) and behavioral changes of adult newts (*Lissotriton vulgaris*) in response to exposure to Glyphogan Classic® at levels corresponding to 2 and 6.5 mg/l glyphosate concentration (Mikó Z. et al., 2017b), even though the same research group previously reported no observable effects on *L. vulgaris* with Glyphogan Classic® at a final glyphosate concentration of 6.5 mg/l (Ujszegi et al., 2015, 2016). In artificial pond mesocosm experiments exposure to the generic glyphosate-based herbicide GLY-4 Plus affected mortality, body size, cellular immune response and tail morphology of the larvae of the spotted salamander *Ambystoma maculatum* in an UV-B radiation dependent manner (Levis and Johnson, 2015), and similar effects were observed on the blue ridge two-lined salamander *Eurycea wilderae* as well (Gandhi and Cecala, 2016). The exposure of wild-living amphibians present in agricultural fields was assessed to be increased in parallel to the 5.7-fold increase of the overall consumption of glyphosate in German agriculture between 1992 and 2012 (Transparency Market Research, 2016). The formulated glyphosate-based herbicide Clinic® at a level corresponding to a glyphosate concentration of 30 mg/l at 96-h exposure caused significant increases in the gene expression and activities of catalase, superoxide dismutase, and AChE in the freshwater turtle, the red-eared slider *Trachemys scripta elegans* and the Mediterranean pond turtle *Mauremys leprosa*, on the basis of which the herbicide is considered a threat to these turtle species (Héritier et al., 2017).

Among mammals, the highest toxicity has been reported on rats during a near life-long exposure (Séralini et al., 2014). A feeding experiment was carried out with Sprague-Dawley rats (55-day old at the beginning of the experiment) and substance (Roundup GT Plus® containing 450 g/l glyphosate IPA salt) administration *ad libitum* in drinking water through 2 years. The final three groups were fed with the control diet and had access to water supplemented with, respectively, 1.1×10^{-8}% of Roundup GT Plus® (100 ng/l, corresponding to ∼50 ng/l of glyphosate—a common contamination level of regular tap waters), 0.09% of Roundup GT Plus® [corresponding to ∼400 mg/kg of glyphosate—the US maximal residue limit (MRL) of glyphosate in GM feed], and 0.5% of Roundup GT Plus® (corresponding to 2.25 g/l of glyphosate—half of the minimal agricultural working dilution). The highest tumorogenic activity was noted on day 600 in female rats treated at the lowest dose, i.e., the effect did not appear to increase with dose. As the numbers of rats used in the experiment were too few to constitute a definitive carcinogenicity study, it is only suggestive of a trend and possible outcome that needs to be repeated with a greater cohort of animals. In a study on Wistar rats treatment with glyphosate at 0.7 or 7 mg/l *ad libitum* in the drinking water for 30 and 90 days, respectively, resulted in reduced glutathione and enhanced glutathione peroxidase levels in the liver, kidney and gut of the treated animals (Larsen et al., 2012), and similarly, when Wistar rats treated with 4.87, 48.7, or 487 mg/kg of Roundup (commercialized under the name Glyphosate-Biocarb® in Brazil), reduced alanine aminotransferase and aspartate aminotransferase levels were recorded in their liver (Benedetti et al., 2004). Roundup Transorb® administered to Wistar rats at 50 mg/kg body weight (b.w.) *ad libitum* in

their drinking water given at 0.25 ml/100 g of b.w. caused a testosterone-disruptor effect (Romano et al., 2012). In addition, nephrotoxicity (Hamdaoui et al., 2016), hepatotoxicity (El-Shenawy, 2009; Haskovic et al., 2016; Tang et al., 2017; Lozano et al., 2018), neurotoxicity on dopaminergic markers (Hernández-Plata et al., 2015; Martínez et al., 2018), and on the immature rat hippocampus (Cattani et al., 2014), effects on intestine peristalsis (Chłopecka et al., 2014, 2017), sperm quality (Abarikwu et al., 2015; Dai et al., 2016) and reproductive toxicity (Owagboriaye et al., 2017), estrogenic effects (Vandenberg et al., 2012; Varayoud et al., 2017) and the effect of neonatal exposure to female adult reproductive performance (Ingaramo et al., 2016, 2017) have been demonstrated. Liver dysfunction observed in rats correlated with gut microbiome disturbances identified in a recent study (Lozano et al., 2018): long-term effects of Roundup Grand Travaux Plus® at 3 doses (corresponding to glyphosate concentrations of 50 ng/l, 100 μg/l, and 2.25 g/l) on the gut microbiota in Sprague-Dawley rats were observed by determining 141 bacteria families by high-throughput sequencing, of which alteration of the *Firmicutes* to *Bacteroidetes* ratio was recorded at different levels in females (but not in males). In contrast, another recent study using Glyfonova® 450 Plus at doses corresponding to glyphosate concentrations of the established European Acceptable Daily Intake (ADI), 0.5 mg/kg body weight, found only limited short-term effects on the gut bacterial community in Sprague Dawley rats (Nielsen et al., 2018), but warned that the effects can be more pronounced under malnutrition, when aromatic amino acids are less available. Repeated 4-week intranasal administration of Glifoglex® in male CF-1 mice (∼2 mg/nostrils/day) affected the central nervous system (probably by altering neurotransmission pathways), caused neurobehavioral effects (by decreasing the ambulatory activity and increase in thigmotaxis, indicating higher anxiety levels), and impaired recognition memory as early as after 6 h (Baier et al., 2017). Results on hormonal effects of glyphosate-based herbicides on rats indicate modulation of the expression of estrogen-sensitive genes in the exposed animals with non-monotonic dose-response curves (Varayoud et al., 2017), indicating the need for hazard-based considerations in risk assessment (Vandenberg et al., 2012; Varayoud et al., 2017). A recent meta-analysis of eight previous studies on reproductive toxicity on males, carried out between 1992 and 2016, on sperm counts in rodents (Kunming and B6C3F mice and Sprague Dawley, Wistar and Fischer F344 rats) upon glyphosate administration at 40–50,000 mg/kg resulted in decreased sperm concentrations showing that glyphosate-based herbicides exerts reprotoxicity to male rodents. The effect, however, has erroneously attributed to glyphosate (Cai et al., 2017). Among mammals, significant increases in chromosome aberration (including chromatid breaks) frequencies and sister chromatid exchanges per cell were seen in large hairy armadillo *Chaetophractus villosus* peripheral blood lymphocytes upon treatment with Roundup Full II® at doses corresponding to glyphosate concentrations of 0.065–0.26 mg/ml, confirming genotoxicity of the formulated glyphosate-based herbicide, evaluated by cellular and genetic biomarkers e.g., the mitotic index, cell proliferation kinetics, as well as frequencies of chromosome aberrations and sister chromatid exchanges (Luaces et al., 2017; Rossi et al., 2018).

Exposure to glyphosate during pregnancy has been indicated to significantly correlate with shortened pregnancy lengths in a cohort study in the US (Parvez et al., 2018). In addition, glyphosate has been potentially correlated with disruption of glycine homeostasis (Pérez-Torres et al., 2017) and pathological conditions e.g., autism (Nevison, 2014), fatal chronic kidney disease (in regions with heavy metal contamination in water) (Jayasumana et al., 2014, 2015), bronchial inflammation (Kumar et al., 2014), cardiovascular diseases (Gress et al., 2015), and cancer (Paumgartten, 2017). Dermatology problems upon skin contact with glyphosate-based herbicides have also been reported (Amerio et al., 2004; Heras-Mendaza et al., 2008; de Ávila et al., 2017; Elsner et al., 2018). A recent clinical study on the severity of cardiovascular effects due to poisoning with glyphosate-based herbicides attributed differential cardiovascular toxicity to the salt form (ammonium or IPA) of glyphosate used in the formulation (Moon et al., 2018), which in light of the toxicity data of the formulants used, may not be fully justified, as could easily be caused by differences in the formulant. Among environmental factors, glyphosate or its formulated products have been indicated to be linked with increased incidence of and mortality by multiple diseases (including cataract related to subsequent breast carcinoma) and cancer (Swanson et al., 2014; Singh et al., 2017). In the latter category, associations were found with non-Hodgkin lymphoma or multiple myeloma incidence (Hardell and Eriksson, 1999; McDuffie et al., 2001; Hardell et al., 2002; De Roos et al., 2003, 2005; Eriksson et al., 2008; Schinasi and Leon, 2014; Mesnage et al., 2015b), although other reviews claimed no causal relationship between exposure to glyphosate and lymphohematopoietic cancers (Acquavella et al., 2016; Chang and Delzell, 2016; Williams et al., 2016a). Some of these findings have been questioned by Monsanto (Acquavella et al., 1999) and a recent cohort prospective epidemiology study carried out in the US on 57310 licensed pesticide applicators and 32347 spouses in Iowa or North Carolina States found no apparent correlation between glyphosate use and solid tumor or lymphoid malignancies, including non-Hodgkin lymphoma, except for increased risk of acute myeloid leukemia in case of high exposure (Andreotti et al., 2018). Alleged attribution of certain chronic diseases (e.g., diabetes, neuropathies, obesity, asthma, infections, osteoporosis, infertility) to long-term exposure to glyphosate were judged unreasonable (Mesnage and Antoniou, 2017), but extremely low levels of a glyphosate-based herbicide (Roundup®) have been shown in a multiomics study to possibly correlate to the development of metabolic syndrome, causing marked alterations of the liver proteome and metabolome revealing the presence of non-alcoholic fatty liver disease and its progression to non-alcoholic steatohepatosis (Mesnage et al., 2017b).

As glyphosate-based herbicides have been indicated to be associated with birth defects in the exposed population (Antoniou et al., 2011, 2012), the Pesticides Action Network (PAN) Europe and Greenpeace brought forward a lawsuit against the EC in connection to the postponement of authorization of glyphosate until 2015, based on the claim that they failed

to properly consider the teratogenicity and cytotoxicity data in the risk assessment provided by the German Federal Office for Consumer Protection and Food Safety (BVL). Although the legal case has not been finalized to date, it has led to a decision by the Court of Justice of the EU that the EC has to make information on the release of PPPs into the environment accessible to any applicant requesting it, including the "identity" and the quantity of all of the impurities contained in the active substance, as well as the composition of the PPPs (InfoCuria, 2017). This is of particular importance, as in the previous policy the manufacturer had only to account for the technical purity of the active ingredient (i.e., unidentified technical impurities could be present in the product up to 5%), and the exact chemical identity of the formulating agents could be handled as proprietary information.

The German Federal Risk Assessment Institute (BfR) compiled its Renewal Assessment Report on glyphosate at the end of 2013 (German Federal Institute for Risk Assessment, 2013), and expanded it in its voluminous (4,322 pages!) addendum (German Federal Institute for Risk Assessment, 2015) by 2015, on the basis of which the European Food Safety Authority published its peer review report (European Food Safety Authority, 2015b) and statement (European Food Safety Authority, 2015a). This report concluded that glyphosate is "unlikely to pose a carcinogenic hazard to humans." If accepted by the EU MSs, this verdict would allow immediate renewal of the authorization. However, in the meantime, after the BfR report, but prior to the EFSA statement, the International Agency for Research on Cancer (IARC), an expert organization of the World Health Organization (WHO), announced its evaluation on glyphosate (along with four other organophosphate active ingredients, diazinon, malathion, parathion and tetrachlorvinphos) in its periodical *IARC Monographs* (International Agency for Research on Cancer, 2015), and classified glyphosate in its Group 2A carcinogenicity category, "probably carcinogenic to humans." This classification has also been published in a leading medical periodical on this subject, *Lancet Oncology* (Guyton et al., 2015), and the full monograph was published recently (International Agency for Research on Cancer, 2017). The IARC classification is of particular importance for policy-making, because Regulation 1107/2009 on PPPs (European Commission, 2009) specifies strict conditions and restrictions for known or presumed human carcinogens and partly for suspected human carcinogens.

The diverging opinions among international risk assessment agencies EFSA and IARC has triggered a fierce debate in the scientific literature. A large team of researchers including 96 research professors from 22 countries worldwide (Portier et al., 2016) analyzed the data. Taking a contrary position were a set of six studies published in the journal *Critical Reviews in Toxicology* (Acquavella et al., 2016; Brusick et al., 2016; McClellan, 2016; Solomon, 2016; Williams et al., 2016a,b), sponsored by Monsanto and other members of the pesticide industry, as declared in the papers themselves. These reviews focused on the carcinogenicity, genotoxicity of glyphosate and exposures to it, but considered partly the same data and followed the same concept published in a previous risk assessment by the same lead author (Williams et al., 2000). Subsequently in 2016, the expert body Joint Meeting on Pesticide Residues (JMPR) of the Food and Agriculture Organization (FAO) and the WHO of the United Nations, including the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues, discussed re-evaluation of diazinon, glyphosate and malathion in the light of new studies that had become available since their last full assessments. The meeting concluded that glyphosate is "unlikely to pose a carcinogenic risk to humans from exposure through the diet" (Joint FAO/WHO Meeting on Pesticide Residues, 2016)—a conclusion quite similar to the opinion of EFSA. To provide openness and transparency in the risk assessment process and to facilitate the public debate, as a part of its "Open EFSA" policy, EFSA has shared the raw data used in the EU safety evaluation of glyphosate (European Food Safety Authority, 2016). Moreover, in its additional position regarding risk assessment on the potential endocrine activity of glyphosate as a follow-up assessment to its previous conclusion in 2015, EFSA stated that glyphosate does not have endocrine disrupting properties through estrogen, androgen, thyroid, or steroidogenesis mode of action, based on a comprehensive toxicology database (European Food Safety Authority, 2017a). In connection to hazard identification, an IARC expert has stressed that causal relationships need to be empirically tested; prior assumptions may affect conclusions; and conflicts of interest have to be avoided (Saracci, 2016), another statement claimed that the IARC classification of glyphosate as a probable human carcinogen was the result of a flawed and incomplete summary of the experimental evidence (Tarone, 2018), while others voice the opinion that such accusations against IARC and its Monographs Program evaluation process are driven by economic interests, and are intimidating to IARC (Infante et al., 2018). The debate over glyphosate even caused the Netherlands to ban non-agricultural uses of glyphosate as of November, 2015, as well as a conflict between the EC and the European Parliament. The scientific background behind the risk assessment by EFSA and BfR was published in a research paper in 2017 (Tarazona et al., 2017a), and was immediately challenged and discussed in the same periodical (Portier et al., 2017; Tarazona et al., 2017b).

In June 2016, the EC extended the registration of glyphosate for 18 months. For this decision, an important factor, besides the ones discussed above, has been that the draft assessment by the European Chemicals Agency (ECHA) published in the meantime also concluded that there was no sufficient evidence to support a carcinogenicity hazard classification of glyphosate (European Chemicals Agency, 2017b). Public consultations were held over the summer of 2016, and ECHA, on the basis of the Harmonized Classification and Labeling Report (BAuA Federal Institute for Occupational Safety and Health, 2016) submitted by the German competent authority and other comments received during the public consultation, according to its statement in March 2017, endorsed that glyphosate was capable of causing serious eye damage and exerted toxicity to aquatic life with long-lasting effects, but concluded that it cannot be classified as a carcinogen, a mutagen or a reprotoxic compound on the basis of currently available scientific evidence (European Chemicals Agency, 2017a). Just as before regarding the opinions

by EFSA or JMPR (AgroNews Scientists challenge EFSA claim of glyphosate safety, 2015; Nelsen, 2016), members of the Risk Assessment Committee of ECHA have also been accused of having competing financial interests leading them to a biased decision in favor of the re-registration of glyphosate (Johnston, 2017). In July 2017, the EC restarted negotiations with the MSs on the renewal of the approval of glyphosate with specific provisions regarding (a) protection of groundwater, as well as terrestrial animals and non-target plants; and (b) a ban of POEA as a formulating agent for glyphosate (see below). The aggregated economic impacts of a possible ban on glyphosate were assessed to be relatively small by a highly detailed, spatially explicit bio-economic model of silage maize production in Germany (Böcker et al., 2018). The debate remained unsuccessful until November 2017, when, upon the fourth revision of the EC proposal and Germany unexpectedly changing its position, the EC Appeal Committee reached a qualified majority in favor of the renewal the approval of glyphosate for 5 years (European Commission, 2011a) adopted by the EC in December 2017 (Erickson, 2017; European Commission, 2017). In retrospect, the Executive Director of EFSA warns not to entangle broader societal issues e.g., the role of modern agricultural practices and multinational biotech firms in our food supply with evidence-based risk assessment of regulated products (Url, 2018). Others express concern regarding the expertise used by the regulatory agencies to evaluate the safety of glyphosate and point out that toxicologists at Monsanto Corp. anticipated the carcinogenicity classification of glyphosate by IARC (Foucart and Horel, 2018; Infante et al., 2018).

In the meantime, focus has been gradually put on the issue of differential toxicity of glyphosate and its formulated herbicide preparations. It has been previously shown that pesticide formulations exert higher toxicity than their active ingredients alone. In a study on formulated herbicide, insecticide, and fungicide preparations (three PPPs in each group), French researchers (Mesnage et al., 2014) demonstrated an increased cytotoxicity of the formulations of herbicides Roundup GT Plus® (glyphosate), Matin EL® (isoproturon), and Starane 200® (fluroxypyr-meptyl), insecticides Pirimor G® (pirimicarb), Confidor® (imidacloprid), and Polysect Ultra® (acetamiprid), as well as fungicides Maronee® (tebuconazole), Opus® (epoxiconazole), and Eyetak® (prochloraz). These formulations were shown to be 2–2,000 times more toxic on human cell lines (HepG2, HEK293, and JEG3), than their active ingredients (indicated in parentheses). The effects of elevated cytotoxicity were attributed to formulating surfactants POEA, alkyl-aryl sulfonates, docusate sodium, N,N-dimethyldecanamide, 1,2-benzisothiazoline-3-one, and benzenesulfonic acid, as well as solvents naphtha, 1-methyl-2-pyrrolidinone, xylene, isobutanol, and ethanol. POEA derivatives refer to non-ionic surfactants as mixtures differing in their ethoxylation rate, originating from animal fats. These substances are principally used as formulation agents (Castro et al., 2014; Klátyik et al., 2017a) both as built-in and tank-mix adjuvants, for herbicides, especially for glyphosate, and previously for diquat. POEA is added to glyphosate to allow uptake of the water-soluble active ingredient across plant cells, affecting membrane transport and to reduce the wash-off effect after spray application. Less ethoxylated POEA products are used for emulsifying mineral oils, and as dispersants, stabilizers, sanitizers, and defoaming agents, industrial detergents, metal cleaners, textile dye-leveling agents, paper de-inking reagents, and drilling lubricants. Among a number of surfactants used in the formulation of PPPs (**Table 1**), POEA appears to be associated with highest toxicity concerns, particularly in of formulated glyphosate-based PPPs (Romano et al., 2010; Mesnage et al., 2013a; Székács et al., 2014; Farkas et al., 2018). Considering this unfavorable toxicity profile, particularly as related to aquatic toxicity (Prosser et al., 2017; Rodriguez-Gil et al., 2017a,b), cytotoxicity and also the assessment by EFSA (European Food Safety Authority, 2015c), the EC has recommended EU MSs in September, 2016 to exclude POEA as a co-formulant from the use in PPPs containing glyphosate (European Commission, 2016a,b). As a result, a number of glyphosate-based herbicides containing POEA as a formulant have been banned for use in Hungary (**Table 2**) (National Food Chain Safety Office, 2016) and in other EU MSs. A yet unrefined issue regarding the EC recommendation is, however, why the use of POEA is proposed to be restricted only in conjunction with glyphosate. If the toxicity parameters of a substance justify restrictions, those should apply to all uses of the given substance in formulations of any pesticide active ingredient (e.g., diquat, nicosulfuron, or others) or should be considered for other industrial applications as well.

As seen in **Table 2**, glyphosate-based PPPs containing POEA as a formulant can be found in all three approval categories, original, derived, or parallel trade authorization. As specified by EU Regulation 1107/2009 (European Commission, 2009) at an EU level and in a Ministerial Decree 89/2004 (Ministry of Agriculture Rural Development Hungary, 2009) in Hungary, original authorization is based on full documentation of the PPP, derived authorization applied to PPPs distributed under names other than trade names on the original authorization, and parallel trade permits to be obtained to PPPs authorized in other MSs. As seen, 11, 7, and 6 PPPs with original authorization, derived authorization, and parallel trade permits have been banned due to their POEA content. (Note that one product, Roundup Classic®, has been authorized both under original authorization and parallel trade permit).

EFSA VS. IARC

Shortly before the announcement of the conflicting views by EFSA and IARC (WHO) about glyphosate, the Cancer Assessment Review Committee of the US EPA also published a re-evaluation in 2015 (United States Environmental Protection Agency, 2015), followed by a more detailed assessment topic a year later (United States Environmental Protection Agency, 2016b). Their assessment classified glyphosate into Group E, not likely to be carcinogenic to humans. The Agrochemical Division of the American Chemical Society (ACS) dedicated an entire symposium to glyphosate during the 252nd Annual ACS Meeting in 2016, the presentations of which having been published recently (Duke, 2018). The 23 papers presented covered the history of glyphosate, plant glyphosate resistance management, its plant biology and societal issues, but not the toxicology

TABLE 1 | Various types of surfactants used in pesticide formulations.

Surfactant product	Manufacturer	Chemical class	CAS No[a]	Conc. (%)[b]	Use[c]
ANIONIC					
Agrosurf WP85	Lankem Ltd	Sodium dodecyl benzene sulfonate	25155-30-0	75–90	SL, EW
Eucarol Age SS	Lamberti SpA	Sodium alkyl polyglucoside sulfosuccinate	151911-53-5	45	SL, EW, ME
Eucarol Age 91/S K	Lamberti SpA	Sodium alkyl polyglucoside sulfosuccinate	151911-53-5	45	SL, EW, ME
Eucarol Age EC	Lamberti SpA	Sodium alkyl polyglucoside citrate	151911-51-2	30	SL, EW, ME
Imbriol OT/NA/70	Lamberti SpA	Dioctyl sulfosuccinate sodium salt	577-11-7	70	SL
Kemgluco CLM	Kemcare Ltd	Lauryl glucoside	110615-47-9	45–60	SL
Plantapon LGC	BASF	Lauryl glucose carboxylate, lauryl glucoside	383178-66-3 and 110615-47-9	28.5–34	SL
Rolfen Bio	Lamberti SpA	Polyethoxylated alkyl phosphate ester	68130-47-2 and 50769-39-6	70	SL, EW, ME
CATIONIC					
Emulson AG CB 30	Lamberti SpA	Quaternary ammonium compound	66455-29-6	30	SL
NON-IONIC					
Emulson AG GPE 3SS	Lamberti SpA	POEA[d]	61791-26-2	100	SL
Emulson AG GPE 3/SSM	Lamberti SpA	POEA[d]	61791-26-2	70	SL
Tergitol 15-S-9	Dow Chemicals	Secondary alcohol ethoxylate	68131-40-8	100	SL, EW
Triton N-57	Dow Chemicals	Nonylphenol polyethylene glycol ether	127087-87-0	100	SL, EW

[a] *Chemical Abstracts Registry Number.*
[b] *Percentage concentration (w/w).*
[c] *SL: soluble liquid, EW: emulsion (oil in water), ME: microemulsion.*
[d] *POEA: polyethoxylated tallowamine.*

of the compound, reflecting the favorable evaluation by the US EPA. In contrast, agreeing with the IARC classification, California listed glyphosate as a known carcinogen on July 7, 2017, under Proposition 65 law, which would require indicating this carcinogenicity hazard on the product label of glyphosate-based herbicide products. However, in response to a legal claim by an agricultural coalition including the National Association of Wheat Growers, Monsanto Corporation and farmer groups the U.S. District Court issued a preliminary injunction against this evaluation on the basis that the classification by IARC claims glyphosate only probably carcinogenic, while apparently all other regulatory and governmental bodies found the opposite, including the US EPA (Erickson, 2018). The positions of BfR, EFSA, JMPR, and ECHA were in accordance with the US EPA opinion, but the IARC evaluation (that followed the BfR statement, but preceded both EU Agency and FAO/WHO Agency statements, in chronology) was strongly opposed to it. It is worth noting that this has not been the only dramatic difference between classifications by IARC and the US EPA (see classification of lindane, 2,4-D and chlorothalonil).

Why is there a striking difference between statements by the US EPA, EFSA, ECHA, and JMPR on the one hand and IARC on the other hand? Why is it that while the formers concluded that glyphosate is unlikely to cause cancer, and suggested to increase its ADI value from 0.3 to 0.5 mg/kg b.w./day within the EU, the latter have classified it as probably being carcinogenic to humans on the basis of limited evidence on humans and sufficient evidence on animals? Factors that resulted in these substantial differences between the opinions as stated by EFSA and IARC include: (a) The IARC evaluation is hazard-based, while EFSA is committed, by its legal mandate (European Parliament Council, 2002) to risk-based assessment. Risk-based assessment does not disclaim possible hazards, but assesses the likelihood of their actual occurrence under realistic scenarios. (b) The EFSA statement is restricted to the assessment of the active ingredient glyphosate, while IARC also considered reported effects of formulated herbicide preparations of practical importance. Within the latter approach, toxicity of tallowamine substances (e.g., POEA) used as formulants in PPPs has been well demonstrated and cannot be disregarded from the toxicology evaluation. (c) IARC based its assessment on peer reviewed publications in the scientific literature, while EFSA based its assessment also on non-public data from industry documentations submitted for the product approval into consideration. An analysis by PAN found that peer reviewed studies were dismissed by BfR and thus were not included in the EFSA assessment (Pesticide Action Network and Use of science in EU risk assessment, 2018). Indeed, the EFSA opinion does not cite any peer reviewed studies (European Food Safety Authority, 2015a). As a follow-up, a recent evaluation publication condemns the BfR, EFSA, and ECHA of violating current risk assessment guidelines, when dismissing 11 statistically significant cases of increased tumor incidences in two rat and five mouse studies, and claims that glyphosate should have been classified in the EU category 1B,

TABLE 2 | Glyphosate-based herbicide formulations banned n Hungary, as of November 30, 2016, due to their content of polyethoxylated tallowamine (POEA).

PPP[a]	Manufacturer	a.i.[b]	CAS No.[c]	Conc. of a.i.[d]	Conc. of POEA[d,e]	HUN/EPA Reg. No.[f]
ORIGINAL AUTHORIZATION						
Clinic 480 SL®	Nufarm GmbH and Co KG	g IPA	38641-94-0	41.5% (486 g/l)	8.1%	02.5/10717-2/2010
Dominator®	Dow AgroSciences	p IPA	38641-94-0	41.5% (480 g/l)	10-20% (150 g/l)	02.5/10718-2/2010
Glialka 480 Plus®	Monsanto Europe	g IPA	38641-94-0	41.5% (485.8 g/l)	15.5%	02.5/968/1/2010
Glyfos®	Cheminova A/S	g IPA	38641-94-0	42% (480 g/l)	9% (150 g/l)	02.5/12019-2/2010; EPA 67760-49
Glyphogan 480 SL®	Agan Chemical Manufacturers Ltd.	g IPA	38641-94-0	41.5% (485.8 g/l)	15.5%	04.2/829-1/2011; EPA 66222-105
Roundup Classic®	Monsanto Europe	g IPA	38641-94-0	41.5%	15.5%	02.5/915/2/2010
Roundup Classic Plus®	Monsanto Europe	g K	70901-12-1	35.5%	7% (surfactant)	02.5/118/1/2009
Roundup Forte®	Monsanto Europe	g K	70901-12-1	49% (540 g/l)	5-6%	02.5/10505-1/2010
NASA®	Agria S.A.	g IPA	38641-94-0	41%	12%	02.5/2575/2/2009; EPA 87845-2
Nufozát®	Nufarm GmbH and Co KG	g IPA	38641-94-0	41.5% (480 g/l)	8.1%	02.5/422/1/2010
Taifun 360®	Adama Deutschland GmbH	g IPA	38641-94-0	480 g/l	10-25%	02.5/1625/1/2009
DERIVED AUTHORIZATION						
Amega®	Nufarm GmbH and Co KG	g IPA	38641-94-0	41.5%	8.1%	04.2/1285-1/2011
Figaro®	Monsanto Europe	g IPA	38641-94-0	41.5%	15.5%	04.2/254-3/2011
Gladiator 480 SL®	Agan Chemical Manufacturers Ltd.	g IPA	38641-94-0	39-43% (480 g/l)	13-18%	04.2/4501/1/2011
Glyphogan Classic®	Monsanto Europe	g IPA	38641-94-0	41.5%	15.5%	04.2/176-3/2011
Hardflex 480 SL®	Adama Agan Ltd	g IPA	38641-94-0	41.5% (485.8 g/l)	15.5%	04.2/4468/1/2011
Rodeo®	Monsanto Europe	g K	70901-12-1	35.5%	6%	04.2/93-1/2016
Vesuvius®	Ventura Agroscience Ltd.	g IPA	38641-94-0	41.1% (480 g/l)	15.5%	04.2/4184-2015
PARALLEL TRADE PERMIT						
Agria Glypho®	Monsanto Europe	g IPA	38641-94-0	41.5% (485.8 g/l)	15.5%	02.5/1393/2/2010
Glifostar 480 SL®	Monsanto Europe	g IPA	38641-94-0	41.5%	15.5%	04.2/4185-4/2015
Glyfogan®	Agan Chemical Manufacturers Ltd.	g IPA	38641-94-0	41.5% (485.8 g/l)	15.5%	04.2/3069-1/2016
Roundup Classic® [g]	Monsanto Europe	g IPA	38641-94-0	41.5%	15.5%	02.5/10576-1/2010
Sherif 480 SL®	Monsanto Europe	g IPA	38641-94-0	41.5%	15.5%	04.2/64-2/2011
Uyuni®	Monsanto Europe	g IPA	38641-94-0	41.5%	15.5%	04.2/1207-1/2012

[a] *PPP: plant protection product.*
[b] *a.i.: active ingredient; g IPA: glyphosate isopropylammonium salt; g K: glyphosate potassium salt.*
[c] *Chemical Abstracts Registry Number.*
[d] *Percentage concentration (w/w), in some cases specified as g/l as well.*
[e] *POEA: polyethoxylated tallowamine.*
[f] *Hungarian Registration Number; US Environmental protection Agency (EPA) Registration Number.*
[g] *Identical to Roundup Classic® approved under original authorization, also permitted for parallel trade.*

"presumed human carcinogen" (Clausing et al., 2018). EFSA, in the meantime, published its guidance document on uncertainty analysis (Benford et al., 2018) that considers possible omission of carcinogenicity data on the basis of genotoxicity/carcinogenicity margins of exposure, differences in the benchmark dose level due to unquantified uncertainties, the relevance and adverseness of the effects seen animals to humans, or misinterpretation of the probability of a given chemical having a carcinogenic mode of action as the probability cancer caused in an individual. It has to be noted that not only the German authority study (BAuA Federal Institute for Occupational Safety and Health, 2016), but also the evaluation by US EPA (United States Environmental Protection Agency, 2016b) covered these studies, and although admitting the occurrence of statistically significant differences, also disregarded them due to various considered reasons, including non-monotonic of flat dose response, effects deemed due to unusually low negative controls, no statistical significance in pairwise analyses or in multiple comparisons, effects concluded to be not compound-related, not always clearly apparent adenoma/carcinoma effects or no evidence of progression from adenomas to carcinomas, overly high dosages, or the presence of a viral infection within the colony tested.

Risks related to glyphosate may originate from increasing residue levels and incidence due to increased usage; and from modified residue composition due to the use of GT crops. Increasing use of glyphosate on GT crops and also as a crop desiccant on non-GT crops, and its subsequent release into the environment is seen both in increased residue levels found in environmental matrices, first of all drinking water (see below, Environmental and food analysis of glyphosate) and in more frequent occurrence reported, on the basis of which glyphosate and AMPA have been considered as ubiquitous surface water

contaminants (Villeneuve et al., 2011; Székács and Darvas, 2012). These trends both increase risk through exposure. Thus, large increases in use in the EU, and even larger increase in exposures for citizens due to uses of GT crops have been evidenced (Myers et al., 2016). In addition, expanding applications of GT crops have modified residue composition: while a type of GT crops modified with a *cp4-epsps* gene achieves tolerance to glyphosate by expressing enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme derived from *Agrobacterium* sp. strain CP4 not inhibited by glyphosate, other types of these GT plants modified with *gox* or *gat* genes provide tolerance to the compound by its enhanced degradation by transgenic metabolic enzymes, glyphosate oxidoreductase (GOX), or glyphosate acetyltransferase (GAT) expressed in the plant, leading to increased levels of the main metabolite AMPA (Székács and Darvas, 2012; Myers et al., 2016). Therefore, risk assessment has to consider such modified exposures to glyphosate and AMPA (Vandenberg et al., 2017) and other metabolites. Moreover, increasing occurrence of new metabolites has to be taken into account in the enforcement residue definiton (ERD) used for setting MRLs in various commodities and food products.

As risk assessments undertaken by government or government-related agencies concluded rather favorably for the re-registration of glyphosate, the ADI of glyphosate has been recommended by the BfR and endorsed by EFSA to be raised from 0.3 to 0.5 mg/kg b.w./day in the EU (European Food Safety Authority, 2015a). The new proposed ADI value was established on the basis of maternal and developmental NOAEL values of 50 mg/kg b.w./day from a developmental toxicity study in rabbits, considering the standard uncertainty factor (UF) of 100. If approved, the recommendation has several serious implications. On the one hand, it represents a significant (66%) rise, which raises questions. Have our previous estimations on human exposure been improper to such extent, that the ADI can now be raised in spite of the increasing occurrence of glyphosate and AMPA in food, feed, drinking water, and the environment? Or is this rise simply a consequence of a technological issue: the increase of glyphosate residues due to excessive use on GM crops or as a pre-harvest desiccant? On the other hand, and probably even more importantly, the increase in ADI has consequences on future regulatory assessment. ADI is a crucial value in policy-making being a threshold level, below which no adverse effects are anticipated, and which already contains a 100-fold UF relative to the NOAEL. In consequence, doses below the ADI level are considered safe, and are not considered in regulatory assessments. However, several studies have indicated a range of toxic effects (hepatorenal and chronic toxicity below the ADI), which in this way skip the attention of the regulatory decision-maker. It is worth noting that while proposing an alleviation of the ADI as a part of the update of the toxicological profile of glyphosate, EFSA also proposed new, modified MRLs and ERD (see see below, Exposure to glyphosate – environmental and food analysis, human biomonitoring), and increased severity of the toxicity indices by setting, for the first time, an acute reference dose (ARfD) for the compound (European Food Safety Authority, 2015a). The reason for the ARfD has been severe toxicity and mortality seen in pregnant females in seven rabbit developmental toxicity studies, as well as post-implantation losses observed in two of those studies. It is noted that JMPR established an ADI of 1.0 mg/kg b.w./day for the sum of glyphosate, N-acetylglyphosate, AMPA and N-acetyl-AMPA, and considered as unnecessary to set an ARfD (Food and Agriculture Organization of the United Nations, 2011).

It should also be noted that glyphosate and AMPA were proposed as lead compounds in the design of possible new anticancer drugs as well, due to their reported inhibitory effect on the proliferation and induction of apoptosis of cancer cells (while not affecting non-cancerous cells) (Li et al., 2013). The public debate is evidenced by a degree of outrage on both sides claiming short-sitedness, ignorance on one side (Zaruk, 2016) or conflicting interests or corruption of the evaluators on the other side (Burtscher-Schaden et al., 2017).

A recent GM technology development has been that Monsanto is extending the range of its Roundup Ready® crops toward older, potentially more toxic active ingredients e.g., dicamba (Roundup Ready 2 Xtend® crops) (Monsanto Corp, 2016). The prime driver of the technology is the wide scale presence of glyphosate-resistant weed species infesting agricultural fields in the US, and glyphosate by itself has failed as a stand-alone weed control agent. The Xtend® crops are not only glyphosate-tolerant, but also tolerant to dicamba. Thus glyphosate is not being replaced but being added to by systems such as Xtend®.

EXPOSURE TO GLYPHOSATE—ENVIRONMENTAL AND FOOD ANALYSIS, HUMAN BIOMONITORING

Glyphosate is a globally occurring pollutant in surface water due to its widespread use, good solubility (11.6 g/l at 25°C) and degradation (half-life time (DT_{50}) = 28–91 days, if photodegradation is excluded) in water (MacBean, 2012). Numerous surveys indicated residue levels between the limit of detection (LOD) of the analytical method used (e.g., 0.01 μg/l) and substantial concentrations (**Table 3**), with a striking difference between the Americas and Europe.

As pointed out earlier (Székács and Darvas, 2012), glyphosate and AMPA used to be detected in environmental studies previously less frequently due to the limited sensitivity of the traditional analytical methods based on gas chromatography. This has created an advantageous reputation for glyphosate of being environmentally benign. With the development of novel methods of increased sensitivity e.g., hyphenated techniques, such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) or immunoanalytical methods, such as enzyme-linked immunosorbent assays (ELISAs), and their subsequent wide availability for routine analysis, glyphosate residues have been detected at lower concentrations more frequently during the last two decades, than before, as also seen in the data listed in **Table 3**. Nonetheless, increasing research needs demand the development of advanced chemical

TABLE 3 | Glyphosate residues found in surface and ground water in selected environmental monitoring studies.

Location	Residue level found (μg/l)	Comment	Year	References
NORTH AMERICA				
USA (North Appalache)	up to 5200	Leaching from agricultural areas to watersheds, influenced by application rates and time of run-off event after application	1973–1975	Edwards et al., 1980
Canada (British Columbia)	3.2–162	Leaching from agricultural areas, primarily associated with bottom sediments	1987	Feng et al., 1990
Canada (British Columbia)	0.15–1.8	25–75 m Leaching after silvicultural applications	1987	Payne et al., 1990; Payne, 1992
USA (Washington DC, Maryland, Missouri, Wyoming)	Glyphosate: up to 8.7 (in 35–40% of samples) AMPA: up to 3.6 (in 53–83% of samples)	Residue level in surface water depended on pre- and post-emergence applications	2002	Battaglin et al., 2005
USA (Arizona, Colorado, Georgia, Iowa, Kansas, Minnesota, Nevada, New Jersey, New York, South Dakota)	Glyphosate: 0.1–2.2 (in 17.5% of samples) AMPA: 0.1–3.9 (in 67.5% of samples)	US Geological Survey, stream samples collected upstream and downstream of waste water treatment plants	2002	Kolpin et al., 2006
USA	up to 887	Run-off events in 7 small watersheds sampled for run-off from agricultural fields of maize or soybean with different tillage practices; increased glyphosate run-off associated with conservation tillage (no-till)	2002–2004	Shipitalo and Owens, 2011
Canada (Southern Ontario)	Glyphosate: 1.2–40.8 (in 21% of samples) AMPA: 0–48.4	Through binding to soil particles, glyphosate is likely to enter surface waters sorbed onto water-borne particles during run-off events	2004–2005	Struger et al., 2008
Canada (Pacific, Prairie, Ontario, Quebec, Atlantic)	Glyphosate: up to 11.8 AMPA: up to 2	glyphosate and AMPA occurred as frequent contaminants in urban rivers across Canada, mainly in Prairie Province, with concentrations greater after rainfall events	2004, 2007	Glozier et al., 2012
USA (Washington, DC, Maryland, Iowa, Wyoming)	Glyphosate: 0.1–328 AMPA: 3.0–15	Leaching into vernal pools and adjacent flowing waters in protected areas from agricultural areas or from control of nonindigenous plants	2005–2006	Battaglin et al., 2009
USA (Mississippi, Iowa, Indiana)	Glyphosate: 0.02–430 AMPA: 0–400	Common contaminants in basin rivers, levels dependent on application (source strength), rainfall run-off and flow route	2007–2008	Coupe et al., 2012
USA (34 States and the District of Columbia)	Glyphosate: 0–476 (in 39.4% of samples) AMPA: 0–397 (in 55% of samples)	US Geological Survey, found as common contaminants in streams, groundwater, ditches and drains, large rivers, ground water, lakes, ponds wetlands, precipitation, soil and sediment and waste water treatment plant effluents	2001–2010	Battaglin et al., 2014
Canada (Ontario)	Glyphosate: up to 0.66 (in 10.5% of samples) AMPA: up to 0.70 (in 5.0% of samples)	Residues found persistent enough to allow groundwater to store and transmit glyphosate residues to surface waters, also supported by atmospheric transport occurrence in precipitation	2010–2011, 2013	Van Stempvoort et al., 2016
USA (South Dakota, Nebraska, Kansas, Minnesota, Iowa, Missouri, Wisconsin, Illinois, Michigan, Indiana, Ohio, Kentucky	up to 27.8 (median 1.68)	US Geological Survey, 100 streams in the Midwestern US, median AMPA/glyphosate ratio at agricultural sites 3.31, residue occurrence differing by land use	2013	Mahler et al., 2017
USA (New York State)	up to 90	Rainfall-triggered occurrence in the outflow of agricultural fields (run-off and shallow drainage) right after controlled spray applications of glyphosate	2015–2017	Richards et al., 2018
Mexico	up to 36.7	Rain facilitates the mobility and leaching of glyphosate from agricultural fields to water bodies, but also reduces the final environmental concentration by dilution	2014	Ruiz-Toledo et al., 2014

(Continued)

TABLE 3 | Continued

Location	Residue level found (μg/l)	Comment	Year	References
Mexico (Yucatan Peninsula)	up to 1.4	Glyphosate found in 90% of groundwater samples evaluated	2016	Rendón-von Osten and Dzul-Caamal, 2017
Belize	0.22–1.7	Residues found in phytotelmic water at seven sites near Maya Mountain Protected Areas	2006–2007	Kaiser, 2011
SOUTH AMERICA				
Argentina (Buenos Aires Province)	100–700	Flow increased by rain caused the transport of the herbicide from the direct area of influence to downstream sites	2004	Peruzzo et al., 2008
Argentina (Buenos Aires Province)	Glyphosate: up to 298 AMPA: up to 235	Glyphosate and AMPA are present in the soil of the agricultural basin (35–1502 and 299–2256 μg/kg, respectively), and reach surface water via surface run-off of soil particles	2012	Aparicio et al., 2013
Argentina (Buenos Aires Province)	Glyphosate: up to 258 (in 69% of samples) AMPA: up to 5865 (in 69% of samples)	Surface stream, ground water sampled; the sampling site under urban-industrial land use had high concentrations in the spring (attributed to point pollution),	2010–2013	Caprile et al., 2017
Argentina (Formosa, Chaco, Santa Fé, Buenos Aires, Entre Rios Provinces)	Glyphosate: 0.2–1.8 (Galeguay River) up to 0.7 (in 15% of samples) (Paraná River) AMPA: 0.1–1.9 (Galeguay River) <0.3 (Paraná River)	Higher levels in the middle- and lower-course tributaries of Paraná River in accordance with the intensive agriculture in those regions; pollutant adsorption on suspended matter	2011–2012	Ronco et al., 2016
Argentina (Buenos Aires Province)	Glyphosate and AMPA: up to 0.5 (in 33 and 20% of samples, respectively) (Quequén Grande River)	Glyphosate and AMPA were registered in almost all matrices at different sampling times (pre- and post-application events).	2012–2013	Lupi et al., 2015
Argentina (Buenos Aires Province)	Glyphosate: up to 18.5 (in 78.9% of samples) AMPA: up to 47.5 (in 96.5% of samples)	Glyphosate and AMPA predominated in surface water and sediment samples in the El Crespo stream	2014–2015	Pérez et al., 2017
Argentina (Buenos Aires Province)	Glyphosate: up to 4.5 (in >40% of samples) AMPA: up to 0.9	In shallow lakes in the Pampa region	2015	Castro Berman et al., 2018
Brazil (Rio de Janeiro region)	Glyphosate: 2.6–10.1, AMPA < 0.1 (LOD) in surface water glyphosate < 0.35 (LOD), AMPA < 0.1 (LOD) in ground water	Surface and ground water used for irrigation from the region of Rio de Janeiro tested	2017	Pinto et al., 2018
ASIA				
Malaysia	Glyphosate: 0–6.23 AMPA: 0.34–3.76	Higher glyphosate and AMPA concentrations detected in surface water near oil palm plantation area	2011–2012	Mardiana-Jansar and Ismail, 2014
AUSTRALIA				
Australia (Western Australia)	380	Glyphosate is by far the most widely used pesticide in Australia; it is considered as a pesticide active ingredient with intermediate persistence; the use of Roundup Ready® crops may result in substitution of low volumes of sulfonyl urea and other herbicides with high volumes of Glyphosate	1995	Australian Academy of Technological Sciences, and Engineering, 2002
Australia (Queensland)	up to 54	Substantial off-site herbicide movement from irrigated sugarcane farms	2005–2010	Davis et al., 2013
EUROPE				
Germany (Northern Rhine-Vestphalia, Ruhr)	Glyphosate: 0–0.59 AMPA: 0–0.07	Glyphosate occurred in surface water due to weed control application in rail tracks as one of the main sources	1995–1996	Skark et al., 1998

(Continued)

TABLE 3 | Continued

Location	Residue level found (μg/l)	Comment	Year	References
Mediterranean region	–	Not commonly detected	1997	Barceló and Hennion, 1997
UK (East Midlands)	50–650	Rainfall intensity after herbicide application may increase total herbicide concentrations discharging from the treated area	1997	Ramwell et al., 2002
Norway	Glyphosate: 0.01–0.93 AMPA: 0.01-0.2	12 stream and river locations sampled, 86% of 49 samples analyzed found contaminated	1995–1999	Ludvigsen and Lode, 2001
Norway	–	6 small catchment areas sampled, 91% of 57 samples analyzed contaminated	1996–2000	Ludvigsen and Lode, 2002
Denmark	Glyphosate: 0.54–4.7 or 0.01–0.03 AMPA: 0.17–0.73 or 0.05–0.15	Glyphosate and AMPA can leach from agricultural fields through structured soils posing a potential risk to the aquatic environment	2000–2002	Kjaer et al., 2005
France (Burgundy)	Glyphosate: up to 17 AMPA: 0.2–9.4	Glyphosate, and to a greater extent, AMPA, leach through the soils; thus, both may be potential contaminants of groundwater	2001–2002	Landry et al., 2005
France	Glyphosate: up to 90 AMPA: up to 3.6	Glyphosate detected in 99.7% of 303 surface water samples	2003–2006	Coupe et al., 2012
Switzerland	Glyphosate: up to 28 (93% occurrence in river water) AMPA: up to 8.8 (95% occurrence in river water)	Monitored in groundwater, in rivers and streams, and in waste water treatment plants effluents	2006–2013	Poiger et al., 2017
France	Glyphosate: 0.2–1.0 AMPA: 0.2–0.6	Peak glyphosate contamination due to urban run-off	2007–2008	Botta et al., 2009
Spain (Valencian Mediterranean region)	0.10–0.85	92% of 13 surface and ground water samples were found contaminated with glyphosate	2005	Ibánez et al., 2006
Austria	Glyphosate: up to 0.67 (0.5–2.0 in waste water treatment plant effluents) AMPA: up to 2.8 (4–14 in waste water treatment plant effluents)	Elimination of glyphosate and AMPA from waste water at the present concentration levels is not straightforward	2008	Popp et al., 2008
Spain	0.1–2.6	47% of 139 ground water samples were found contaminated with glyphosate	2007–2010	Sanchís et al., 2012
UK (York)	Glyphosate: up to 9.0 AMPA: up to 1.2	Mitigation against glyphosate inputs to surface waters are targeted at the appropriate source of emission	2009	Ramwell et al., 2014
France (Maine-et-Loire)	Glyphosate: up to 386.9 AMPA: up to 47	Maximum concentrations in 20 rainfall-run-off events in this vineyard catchment area were over one order of magnitude higher than those reported in French vineyards	2009–2012	Lefrancq et al., 2017
Switzerland	Glyphosate: 0.05–4.2 AMPA: 0.04-1.1	The occurrence of glyphosate in surface waters could not be explained by agricultural use only; more than half of the load during selected rain events originated from urban areas via drainage and effluents from waste water treatment plants	2010	Hanke et al., 2010
Switzerland	Glyphosate: up to 12 AMPA: up to 6.5	Moisture increases downhill transport of glyphosate and AMPA by surface run-off, in a dissolved state or bound to small colloids	2010–2011	Daouk et al., 2013

(Continued)

TABLE 3 | Continued

Location	Residue level found (μg/l)	Comment	Year	References
Hungary	0.04–0.98	50% of 42 surface water samples were found contaminated with glyphosate at significant concentrations after a rainy period	2010–2011	Mörtl et al., 2013
Hungary	0.13–0.46	Varying leaching or run-off of glyphosate to surface water	2012	Székács et al., 2015
France (Auvergne-Rhône-Alpes, Provence-Alpes-Côte d'Azur))	Glyphosate: 0.05–0.81 AMPA: 0–05–0.09	Quantified in the low μg/l range in Rhône River and its tributaries	2013	Slomberg et al., 2017
Switzerland	Glyphosate: 0.15 (up to 1.43 in tributaries, 0.018–0.35 in waste water treatment plant effluents) AMPA: 0.13 (0.024–0.42 in tributaries, up to 1.7 in waste water treatment plant effluents)	Seasonal occurrence in Lake Greifensee in July (below concentrations in the two main tributaries) and rapid dissipation of glyphosate, but not AMPA	2013	Huntscha et al., 2018
Hungary	–	Slowreed dissipation of glyphosate in formulation and in the presence of algal biofilms	2017	Zhong et al., 2018
Italy (Lombardy Region)	up to 96	Sampling intensity increased due to more concern about glyphosate residues during 2012–2014 than previously, 2008–2011	2008–2014	Di Guardo and Finizio, 2018

analysis methods of better sensitivity and accuracy (Huhn, 2018). Glyphosate and AMPA were found to emerge in surface water by leaching from agricultural areas in the US and Canada (Edwards et al., 1980; Feng et al., 1990; Payne et al., 1990; Payne, 1992; Battaglin et al., 2005, 2009; Kolpin et al., 2006; Struger et al., 2008; Shipitalo and Owens, 2011; Coupe et al., 2012; Glozier et al., 2012) among others by the US Geological Survey (Kolpin et al., 2006), at concentrations up to 5,200 and 400 ng/l, respectively in the US North Appalache and the Midwest (Battaglin et al., 2005, 2009) in regions, where the use of glyphosate-based pesticide formulations is substantial e.g., due to the cultivation of GT (RR) crops (Cuhra, 2015). Glyphosate has also been indicated as a significant water pollutant from intensive agriculture in Mexico (Ruiz-Toledo et al., 2014). The concentrations of glyphosate in surface waters in the EU appears to be lower, but consistently occurring e.g., Germany (Skark et al., 1998), the Mediterranean (Barceló and Hennion, 1997), the Northern region (Ludvigsen and Lode, 2001, 2002; Kjaer et al., 2005), in France (Botta et al., 2009; Van Stempvoort et al., 2016; Clausing et al., 2018) and elsewhere, and its dissipation has been found to be slowed down in formulation and in the presence of algal biofilms (Klátyik et al., 2017b). Thus, glyphosate residues have been deemed to be worldwide the most common pesticide contaminant in freshwater ecosystems, AMPA being the most frequent, glyphosate being the third most frequent contaminant in France (Villeneuve et al., 2011). The geographical distribution of peak glyphosate residue concentrations in surface and drinking water, reported worldwide, are depicted in **Figure 4**. As reference values, maximal permitted concentrations of glyphosate residues in drinking water in given regions are also indicated, revealing that maximum allowed pesticide residue levels in drinking water are 7,000-fold higher in the US than in the EU, and Australia is even more permissive (Note, that concentrations depicted worldwide, being peak values, do not reflect real life, everyday situations, rather correspond to worst cases).

It has been concluded that glyphosate and AMPA often occur as run-off from fields originating from glyphosate-based herbicide application. These residues not only became ubiquitous or "pseudo-persistent" contaminants in surface water, in periods with increasing concentrations over the years (McKnight et al., 2015; Carvalho, 2017; Primost et al., 2017), but through surface waters they were shown to be able to reach the seas as well, as documented in Germany in the estuaries of the Baltic Sea (Skeff et al., 2015). Glyphosate and AMPA were also found at up to 2.5 and 0.48 μg/l in rain and up to 9.1 and 0.97 ng/m^3 in air, respectively in the USA in Mississippi, Iowa and Indiana States in 2004 and 2007–2008 (Chang et al., 2011), where both have been identified in the same period as common surface water contaminants near agricultural fields (Majewski et al., 2014). Glyphosate and AMPA were detected both in rain and air near agricultural fields in the estuarine region of the Mississippi River in 2007, while such residues were not detected (possibly due to less sensitive analytical methods available at the time) in 1995 (Maqueda et al., 2017).

Glyphosate residue contamination has been demonstrated to correlate with sources of agricultural applications (Payne, 1992; Coupe et al., 2012) corresponding to GM crop cultivation (Barceló and Hennion, 1997; Skark et al., 1998; Australian Academy of Technological Sciences, and Engineering, 2002; Ramwell et al., 2002; Peruzzo et al., 2008; Aparicio et al., 2013; Davis et al., 2013; Mardiana-Jansar and Ismail, 2014; Lupi et al., 2015; Ronco et al., 2016; Caprile et al., 2017; Pérez et al., 2017), post-harvest chemical desiccation (Shipitalo and Owens, 2011; Soracco et al., 2018) or other technologies in intensive agriculture e.g., biomass production of industrial crops (Mardiana-Jansar and Ismail, 2014). Moreover, non-agricultural or urban uses of

FIGURE 4 | Maximal residue levels (µg/l) of glyphosate (*black*) and AMPA (*gray in parentheses*) in surface and ground water reported worldwide. Corresponding permitted levels in drinking water are indicated in red e.g., maximum admissible concentration (MAC) in the European Union, maximum contaminant level (MCL) in the US and health-based guideline value (HBGV) in Australia.

glyphosate have also been indicated (Kolpin et al., 2006; Botta et al., 2009; Hanke et al., 2010; Glozier et al., 2012) to contribute to environmental contamination. Soil contamination appears to show a closer correlation with agricultural usage intensity, while water contamination results from run-off events by precipitation (Landry et al., 2005; Maqueda et al., 2017; Poiger et al., 2017). Thus, glyphosate and AMPA were detected at concentrations 4–17 and 4–9 times greater than the sources of emission, respectively from agricultural soil and emitted respirable dust, 12 months after glyphosate application, in a central semiarid region of Argentina, indicating that these compounds are accumulated in the respirable dust and can potentially be a source of air contamination (Mendez et al., 2017). Moreover, glyphosate residues have often been detected in soil and sediments (Peruzzo et al., 2008; Aparicio et al., 2013; Battaglin et al., 2014; Mardiana-Jansar and Ismail, 2014), and it has been shown by numerous studies that glyphosate reaches surface waters via dispersed small soil particles or colloids (Struger et al., 2008; Slomberg et al., 2017). A recent study provides a Europe-wide assessment of the dispersal of glyphosate and AMPA in EU agricultural topsoils, being present in 45% of the topsoils collected, originating from 11 countries and six crop systems, with a maximum concentration of 2 mg/kg, as well as their potential spreading by wind and water erosion (Silva et al., 2018), further affected by small-scale sediment transport in water erosion (Bento et al., 2018), persisting under low bacterial activity in limited aerobic conditions or non-neutral pH (la Cecilia and Maggi, 2018), and adversely affecting soil microbial and nematodal diversity (Dennis et al., 2018). From the soil glyphosate can be translocated by plant roots; and it can affect non-target plants near agricultural ditches (Saunders and Pezeshki, 2015), and affect soil rhizosphere-associated bacterial communities (Newman et al., 2016) and soil *Pseudomonas* species (Aristilde et al., 2017). Toxicity of Roundup® to the soil filamentous fungus *Aspergillus nidulans* was reported with a median lethal dose (LD_{50}) corresponding to glyphosate concentrations of 90–112 mg/l, ∼100-fold below agricultural application levels (Nicolas et al., 2016). A proteomic analysis indicated protein expression modulation and possible metabolic disturbances (Poirier et al., 2017). Similar effects were not observed for *Aspergillus* section *Flavi* strains and *A. niger* (Carranza et al., 2017). Negative effects of glyphosate and/or N fertilization on soil enzymes and arbuscular mycorrhizal fungi were reported (Nivelle et al., 2018). A recent concern is that glyphosate (along with other herbicide active ingredients dicamba and 2,4-D), as well as common surfactants (Tween80, carboxymethyl cellulose) at or below recommended application concentrations can change the susceptibility of bacteria to a diverse range of antibiotics (ampicillin, chloramphenicol, ciprofloxacin, kanamycin, tetracycline) upon concurrent exposure, and thus, glyphosate may serve as one of the drivers for antibiotic resistance (Kurenbach et al., 2017; Van Bruggen et al., 2018). As indicated, through water pollution its formulations can disturb aquatic ecosystems (Vera et al., 2010; Perez et al., 2011) including fish (Jofré et al., 2013). Removal or degradation of glyphosate residues from raw drinking water by bank filtration may not be efficient, but oxidants used in water treatment (e.g., Cl_2 or O_3) were shown to be effective in degrading their

concentration below the EU drinking water threshold level of 0.1 μg/l (Jönsson et al., 2013). Nonetheless, glyphosate residues were reported in bottled drinking water (Rendón-von Osten and Dzul-Caamal, 2017). Glyphosate residues were detected in honey and soy sauce (Rubio et al., 2014), in produce (Bøhn et al., 2014), processed food products, even in human specimens (blood, urine, mother's milk) (Knudsen et al., 2017; Rendón-von Osten and Dzul-Caamal, 2017). It should be noted, however, that measurements of glyphosate in complex biological matrices e.g., blood and breast milk, that led to positive scores used ELISA methods of questionable accuracy, and instrumental analysis by LC-MS/MS did not find glyphosate above its limit of detection in human breast milk (McGuire et al., 2016; Steinborn et al., 2016). As a result of its expanding release into the environment, increased residue levels have been detected in crops, 0.3–5.2 mg/kg glyphosate, 0.3–5.7 mg/kg AMPA (Arregui et al., 2004).

In the US, the accepted MRL for glyphosate residues in drinking water is 700 μg/l (United States Environmental Protection Agency, 2003), while 0.1 μg/l in the EU under the Drinking Water Directive (European Parliament Council, 1998). The MRL of glyphosate (among all pesticide residues) in the aquatic environment used to be 1.0 μg/l in the EU (European Parliament Council, 1976). Currently, there are no MRLs defined for surface water according to the Water Framework Directive (European Commission, 2000), as glyphosate or AMPA are not listed among priority substances for which environmental quality standards have been set. However, both compounds are likely to be re-considered as priority substances (European Parliament Council, 2006; European Parliament, 2008).

Most recent results in residue analysis in food in the EU indicated a more favorable picture. Analyzed in 22 countries in EU MSs (predominantly in Germany) in raw and processed food products (mainly fruits, nuts, vegetables and cereals, yet in limited numbers in oilseeds and soybeans, and none reported in animal products) in 2014, detectable levels of glyphosate residues were found in 4% of the samples, but at levels all below the MRL with the exception of a dry bean sample, where the residue level, 2.3 mg/kg was 15% above the MRL. The highest incidence of glyphosate residue levels was detected in sunflower seeds, dry lentils and peas, mustard and linseeds, soybeans, as well as barley, wheat, oats and rye among cereals (European Food Safety Authority, 2017b). Characterizing and quantifying glyphosate residues in food and feed of plant and animal origin, considering their stability in those matrices, estimating dietary exposure of consumers and comparing it to EFSA reference toxicity values for glyphosate and AMPA published in 2015 (European Food Safety Authority, 2015a) and for N-acetyl-glyphosate and N-acetyl-AMPA published in 2018 (European Food Safety Authority, 2018a), without their any further assessment, EFSA proposed 207 new MRLs with proposed ERD (glyphosate plus AMPA and N-acetyl-glyphosate) to replace the existing ERD (glyphosate) (European Food Safety Authority, 2018b), and considered them to pose no apparent risk to consumers (European Food Safety Authority, 2018a,b). These proposed MRLs are in 153 cases more restricting (lower than before) than the existing ones, being set at an improved LOD (0.05 mg/kg) made possible by advanced analytical methods, but are more permissive (higher than before) for main commodity crops e.g., potatoes, dried commodities (beans, lentils, peas, lupine beans and linseeds), olives for oil production, grains (barley, buckwheat and other pseudo-cereals, millet, oat, rye, sorghum, wheat), seeds (borage, rape/canola, cotton, maize), soybeans and sugarcanes, as well as food of animal origin (swine, bovine, sheep, bovine, equine and poultry muscle and tissues, milk and bird eggs). The stricter MRLs represent 2- to 10-fold (in the case of wild fungi 1,000-fold) lower values, than the existing ones, while proposed alleviations correspond to 1.5- to 300-fold increase in other MRLs. However, while lowered MRLs apply mostly to non-problematic commodities, higher values are proposed for commodities, where common glyphosate contamination has been reported, or where high glyphosate metabolite levels are endogenous (GT crops with transgenic GOX or GAT enzymes). Such mitigation in the requirements is consonant with the increase proposed also in the ADI value for glyphosate, on the one hand, yet appears to reflect as if regulatory rigor yielded to technology, as limits previously considered necessary and accepted by industry are proposed to be replaced with more permissive ones, on the other hand. As for compliance of MRL, it is worth mentioning that negative health outcomes have been observed in laboratory animal studies showing toxicity at levels of exposure below regulatory set safety limits (Mesnage et al., 2015b, 2017b; Defarge et al., 2016).

Although the number of results available in the scientific literature on glyphosate residue levels in human tissues is limited, human biomonitoring is of prominent importance as its results serve as primary end-point indicators of exposure. Biomonitoring of glyphosate residues in human urine have been carried out in the USA (Acquavella et al., 2003; Curwin et al., 2007; Mills et al., 2017), Europe (Mesnage et al., 2012; Krüger et al., 2014; Connolly et al., 2017; Conrad et al., 2017) and Sri Lanka (Jayasumana et al., 2015), and indicated maximal concentrations of 0.45–233 ng/ml. Within these studies, one report compared glyphosate levels in the urine of humans and livestock, and found over one order of magnitude higher levels in the latter (Krüger et al., 2014). A survey of the human biomonitoring studies argued that the results posed no health concerns as corresponding exposures were estimated to be magnitudes below the ADI or Acceptable Operator Exposure Level values, but conceded characteristic differences between exposure levels in Europe and North America with substantially higher maximum levels in the latter region (Niemann et al., 2015), levels being 0.65–5 ng/ml in Europe and 18–233 in the US. A systematic study carried out in Southern California found that the mean glyphosate and AMPA levels in human urine increased between 1993 and 2016, and reached 0.449 and 0.401 ng/ml, respectively (Mills et al., 2017). It has to be noted that levels of 3.3–73.5 ng/ml have been reported in a non-peer reviewed report in Germany (Connolly et al., 2017).

A wide range of ecotoxicological and human health problems related to glyphosate and its formulated PPPs have been indicated (Székács and Darvas, 2012; Mensah et al., 2015; Kurenbach et al., 2017), partially related to the chelating properties of glyphosate (Mertens et al., 2018). Moreover, ecotoxicological and resistance-related consequences of the extended use of glyphosate (Schütte et al., 2017) or of the use of GT GM

crops (Pandolfo et al., 2018) have been emphasized, along with effects non-target terrestrial plants as well (Cederlund, 2017). As for emerging plant resistance against glyphosate, glyphosate itself and glyphosate-based herbicides have been shown to affect the disease resistance and health of plants by undermining their innate physiological defenses in mechanisms related to the mode of action of glyphosate, even in crops engineered for glyphosate-tolerance, and by interferences with the local microbial ecology in the rhizosphere (Martinez et al., 2018). The evolution of resistance was shown to occur due to gene amplification (Chen et al., 2017; Dolatabadian et al., 2017; Fernandez-Escalada et al., 2017; Han et al., 2017; Jugulam and Gill, 2018). Epigenetic alterations through increased levels of DNA and histone methylation were identified in response to exposure to glyphosate (Nardemir et al., 2015; Kim et al., 2017; Margaritopoulou et al., 2018; Markus et al., 2018). The results suggest that epigenetic pathways may influence the regulation of genes important for herbicide detoxification (Markus et al., 2018). As for ecotoxicity, more research on the effects of exposure, particularly at sub-lethal levels, using appropriate target biomonitor species (Kissane and Shephard, 2017) and a corresponding new concept in agriculture are urged (Nicolopoulou-Stamati et al., 2016; Torretta et al., 2018). Taking these indications into consideration, and also considering the wide occurrence of glyphosate residues in environmental matrices, foodstuff and biological matrices, including the urine of livestock and humans, our current estimations of dietary exposure (Stephenson et al., 2018) may need to be reconsidered, which could substantially modify risk assessment. It has to be noted that the ecotoxicological profile of replacement herbicide active ingredients proposed as alternatives to glyphosate appear to be similar or worse than that of glyphosate itself, both in the area of conventional treatment and desiccant technology (e.g., bromoxynil, diquat or other total herbicides) or on HT GM crops (e.g., 2,4–D, oxynil, dicamba, glufosinate). Nonetheless, this does not constitute a part of risk assessment of glyphosate, but is to be considered by risk managers in their decision-making. Moreover, practically any xenobiotic substance is prone to lead to adverse effects when used in such excessive quantities as glyphosate.

CONCLUSION

The issue of re-registration of glyphosate in the EU and corresponding evaluation reports clearly show a fundamental difference between risk- and hazard-based assessments of regulated products. Considering acute toxicity, legislatory decision-makers focus on risks as a product of hazard and exposure, and weigh the subsequently identified hazards based on their likelihood of occurrence through real exposures. In contrast to acute toxicity, however, EU pesticide regulation is hazard-based (and not risk-based) for carcinogenicity, reproductive/developmental toxicity, neurotoxicity and endocrine effects. The main problem, where the re-registration routine of pesticides may become seriously perplexed, is that at EU level, the authorities, following their legal mandate, focus on the toxicology of the active ingredient(s), while in real life situations subjects are exposed to the formulated, complex products, reflected in the IARC evaluation, as that agency is not legally bound to EU authorization policies. Moreover, government or government-related agencies have to consider all stakeholders, including patent holders and industry, in their assessment. An international expert agency in public health, however, may focus on hazards in its assessment, particularly if novel hazards have been identified in relation to the target substance, and also if concentration- or dose-dependence of the health effects are questionable or do not exist, as often seen for endocrine disruption effects (Vandenberg et al., 2012). A particular issue in the EU is that active ingredients used in PPPs are regulated at the EU level, while formulated pesticide products are governed at MS level. In other words, the responsibility of sound toxicological evaluation of the formulated products lies in the EU at MS level. Therefore, re-assessment of glyphosate can indeed focus on the parent compound itself, as possible biological and health effects of other formulants (e.g., surfactant and other additives) will be considered during the registration of the formulated products at MS level. The withdrawal of glyphosate-based herbicides containing formulating agent POEA and the ban of the use of glyphosate as a post-harvest chemical desiccant at MS level are effective means to reduce environmental contamination and to mitigate environmental and human health consequences. Nonetheless, the wide occurrence of glyphosate and its residues as a ubiquitous contaminant in environmental matrices, feed and food, and even in livestock and human samples indicates that our exposure to this substance, boosted in use by expansion of GM crops worldwide and the use of pre- or post-harvest chemical desiccation in agriculture, may be substantially higher than predicted from dietary exposure models, which may therefore cause our current position in risk assessment to be re-assessed.

Data on occupational or community exposure to glyphosate residues have been shown to be limited (International Agency for Research on Cancer, 2017), and thus, expected exposure levels need to be updated, based not only on earlier estimations on the total food basket, but also on recent environmental and biological monitoring data indicating increased levels or more wide-spread occurrence of glyphosate residues. Critical gaps in the re-registration of glyphosate, including the EU re-registration process itself, have been addressed (European Parliament Council, 2002; Myers et al., 2016), particularly those considered more pressing by recent scientific findings. These include: (a) increasing exposures of EU citizens to glyphosate residues, supported by human and environmental biomonitoring data in limited number (Curwin et al., 2007; Mesnage et al., 2012; Krüger et al., 2014; Niemann et al., 2015; Connolly et al., 2017; Conrad et al., 2017; Mills et al., 2017; Vandenberg et al., 2017), but identifying a clearly rising trend; (b) carcinogenicity classification by IARC, evidence of linkages of glyphosate or its formulated products to non-Hodgkin's lymphoma (Hardell et al., 2002; De Roos et al., 2003, 2005; Eriksson et al., 2008; Schinasi and Leon, 2014; Mesnage et al., 2015b), and effective dose levels indicated in rodent oncogenicity studies being 1–2 orders of magnitude lower when formulated glyphosate-based herbicides were used compared to those obtained with the pure

active ingredient; (c) evidence of contributions to fatal chronic kidney disease by glyphosate in areas with heavy metals in water (Jayasumana et al., 2014, 2015) and the finding of non-alcoholic fatty liver disease upon exposure to a glyphosate-based herbicide (Roundup®) (Mesnage et al., 2017b), coupled with the powerful animal metabolism data embedded within the re-registration document appendices (showing glyphosate and AMPA levels higher in kidney than in liver, and much higher than in muscle tissue); as well as (d) problems (e.g., risk assessment studies for regulatory purposes of re-registration of glyphosate being carried out with pure glyphosate) arising from the dual character of pesticide registration in the EU with active ingredients authorized at EU and formulated products at MS level (Klátyik et al., 2017a). In light of these findings, earlier risk assessment statements (Williams et al., 2000) are untenable for both hazard and exposure levels.

In summary, the "glyphosate debate" among agencies is mostly confined to carcinogenicity, while a variety of other effects (e.g., non-alcoholic fatty liver disease, endocrine disruption) have also been found. The *in vitro* sensitivity assays on a variety of cell lines indicate that glyphosate is less toxic than its common co-formulants e.g., POEA. Moreover, synergistic effects between glyphosate and its co-formulants cannot be ruled out. POEA has been banned in glyphosate-based preparations in various MSs in hope to solve the „glyphosate case." Nevertheless, inhibition of aromatases has been demonstrated at very low concentrations, implying hormonal disrupting effects, and the estrogenic potential of glyphosate (and its formulated products) have been indicated by their estrogen receptor activation.

AUTHOR CONTRIBUTIONS

AS conceived the concept of the review. AS and BD wrote the manuscript together, edited the manuscript, and took responsibility for the integrity of the data.

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Enhancements Needed in GE Crop and Food Regulation in the U.S.

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Genetically engineered (GE) crops, multi-ingredient foods derived from one or more GE ingredients, and GE agricultural inputs are regulated in the United States under a "Coordinated Framework" that was literally cobbled together in the early 1990s. *Via* this Framework, responsibility is spread across three federal agencies for the assessment and management of potential risks arising from the planting of GE crops, the raising of GE animals, or uses of GE inputs. The Framework was incomplete and conceptually flawed from the beginning. Despite multiple, piecemeal efforts to update aspects of GE risk assessment and regulatory policy, the Coordinated Framework survives to this day largely unchanged. Its shortcomings are recognized in both the scientific and legal communities, but meaningful reforms thus far remain out of reach, blocked by the intense controversy now surrounding all things biotech. Five generic reforms and another five specific initiatives are described to create a more robust, science-driven GE regulatory infrastructure in the U.S.

Keywords: Coordinated Framework, substantial equivalence, resistance management, gene editing, scientific integrity, resistant weeds, labeling GE food

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Over most of the last 20 years, the limitations of the Coordinated Framework (1), and the U.S. government's lax approach to genetically engineered (GE) risk assessment, triggered deep-set concern and scrutiny among some stakeholders and consumers, food companies, and organizations but not much beyond that. In the last 5–10 years though, the slice of U.S. and global markets responsive to concerns regarding the safety, environmental impacts, and/or the socioeconomic consequences of GE crops and inputs has grown, and is now driving economically meaningful shifts in market share (2).

Given that GE applications are now spreading to fresh fruits and vegetables and animals, the range of potential risks and gaps in risk-assessment science are likely to become both more acute and undeniable. At some point, U.S. Ag Inc., and especially those companies and growers significantly dependent on exports, will no longer accept the collateral market damage caused by the shortcomings of the Coordinated Framework and the corollary erosion of confidence in the science supporting the regulation of agricultural biotechnology in the U.S.

Recognizing the growing demand for constructive change, the Obama Administration announced in 2015 that it would undertake a long-overdue review of the Coordinated Framework (3, 4). Their goal is to identify at least some improvements that would garner widespread support and could be implemented *via* Executive Orders and/or regulatory policy changes prior to the transition to a new Administration in January 2017. As part of this ongoing process, the Food and Drug Administration (FDA) issued a notice calling for public comments under the ponderous title: "*Clarifying Current Roles and Responsibilities Described in the Coordinated Framework for the Regulation of Biotechnology and Developing a Long-Term Strategy for the Regulation of the Products of Biotechnology*" (5).

Such an Executive Branch review will hopefully guide the actions of this and the next Administration, as well as Congress, in providing federal agencies a clear mandate and stronger authority to conduct state-of-the-art risk assessments on GE plants and foods, animals, and microbes. Herein, I describe current agency roles and the most important reforms that are needed if this effort is to bear fruit worth harvesting.

AGENCY ROLES AND RESPONSIBILITIES

The Food and Drug Administration

The FDA was given responsibility for assessment of food safety risks and most aspects of food labeling, drawing primarily on the Food, Drug, and Cosmetic Act (FDCA). Agency regulations, data requirements, and decision criteria are, in turn, grounded in legislation crafted and passed years before the first applications of genetic engineering in the food industry and agricultural sector. While the FDA's role within the Coordinated Framework is arguably the most important in terms of protecting public health, its role and actions have for the most part flown below the radar.

The FDA regulates GE animals as new animal drugs, for which there is a mandatory, FDCA requirement for a safety assessment. For GE plants, FDA regulates them under a 1992 Statement of Policy that asserts that GE (a) is just an extension of conventional breeding, (b) does not raise new health risks, and (c) does not need any special safety assessments once nutritional and compositional "substantial equivalence" is demonstrated (6).

There is only a cursory agency review of industry-submitted documents over the course of a "voluntary consultation" (7, 8). The FDA neither conducts research, review experimental designs, and statistical analyses nor reaches independent conclusions about the safety of a proposed GE trait or plant. In essence, FDA has allowed companies to assert that new, "substantially equivalent" GE crops are "generally recognized as safe" (GRAS) (8).

Once so designated, officially or in practice, there is little or no justification for any ongoing, food safety-focused regulatory scrutiny, or need for federal investment in research on possible food safety risks. In short, the FDA's process and actions suggest that the science is settled, despite the lack of modern, well-designed studies of the sort needed to detect subtle cellular, metabolic, genetic, and epigenetic impacts that do not substantially change the nutrient composition of harvested foodstuffs.

The Environmental Protection Agency

The Environmental Protection Agency (EPA) was, and remains, responsible for the assessment and approval of GE applications accompanied by pest management-related claims. EPA science reviews and actions evolve in accord with the detailed requirements and regulations put in place over decades in administering the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), an Act addressing chemical and botanical pesticides. The EPA's GE-related responsibilities include

- characterizing and quantifying exposures to novel proteins or other toxins produced by GE crops;
- assessing the need for new or altered tolerance levels for GE plant proteins and/or pesticides used in conjunction with GE crops;
- determining whether a new GE application poses any new or worrisome worker or applicator risk, or environmental risks; and
- addressing the risk of resistance, and whether and how steps should be taken *via* mandatory label directions to mitigate the risk of resistance.

The EPA regulates GE microorganisms under the Toxic Substance Control Act, despite the indisputable fact that the risks stemming from release of GE microorganisms that can reproduce and spread are very different than the risks posed by toxic chemicals, which cannot reproduce.

United States Department of Agriculture

Ironically, the United States Department of Agriculture (USDA)'s role in GE regulation is the least important relative to risk identification and prevention, but has triggered the most extensive delays, as well as the most intensely contested litigation and public controversy. The USDA regulates the agronomic and some environmental impacts of GE plants under the Plant Protection Act (PPA), a statute that limits the purview of USDA assessments to whether GE plants might act as "plant pests" (e.g., as a weed or virus) (8). Thus, if a plant is GE but does not contain genetic material from a known, plant pest, the plant is typically not considered a "regulated article" (9).

The USDA regulates GE insects under the Animal Plant Health Protection Act, which was designed to protect livestock and poultry, including farmed fish from animal diseases. Thus, for GE insects, USDA only considers whether the GE insect has an impact on communicable diseases of livestock and poultry, rather than broader environmental or ecological impacts.

CRITICAL CHALLENGES CONFRONTING THE COORDINATED FRAMEWORK

No one expects the Coordinated Framework review process started by the Obama Administration to quickly solve any of the foundational problems with biotechnology regulation in the U.S. (4). But it will hopefully clarify the major issues and challenges, and bring new players and ideas into the ongoing policy-reform process.

I suspect that eventually the U.S. will be forced to upgrade the science supporting the assessment and management of risks arising from agricultural biotechnologies. The now-heavy dose of wishful thinking embedded in GE risk assessments will hopefully be replaced with hard science. Progress is especially needed in five areas in order to create a biotechnology regulatory framework that is as dynamic as the science and technology it seeks to help manage.

Focus on Fetal and Child Development

To date, there has been little serious research on the impact of GE crops and technology on human reproductive performance and childhood development, despite wide recognition that untimely, very low dose pesticide and toxin exposures can trigger endocrine system and epigenetic effects of lasting consequence (10).

For this reason, it is indeed unfortunate that EPA has failed to invoke the historic, health-promoting provisions of the 1996

Food Quality Protection Act (FQPA) in its assessments of the acceptability of GE technology. The FQPA calls for an added 10-fold safety factor in regulating pesticides, and indeed any crop protection technology, when there is (a) uncertainty regarding risks to pregnant women, infants, and children or (b) inadequate data to characterize exposure levels (11).

On both of these counts, several GE technologies and their associated pesticides should have triggered the FQPA's added safety factor. This is an area ripe for litigation.

Gene Editing Technologies

The high-priority issues throughout the review of the Coordinated Framework will surely include how to deal with gene editing technologies, such as RNAi, and other new gene editing technologies (e.g., CRISPR-cas9, TALEN, ZNF, and meganucleases) (4, 12). Many of these gene editing techniques will presumably not entail movement of foreign deoxyribonucleic acid (DNA) into crops.

Under current policy, companies or teams using RNAi and gene editing tools can simply write to the USDA and request a letter from the Department acknowledging that the resulting GE plants are not "regulated articles." To date, USDA has sent letters exempting over 30 GE plants from USDA reviews – including multiple glyphosate-tolerant crops and Loblolly pine trees with increased wood density.

If these new gene editing technologies are deemed exempt from U.S. regulatory reviews, as many in the GE industry have requested, these presumably safer technologies will invite intense scrutiny and likely create a new wave of litigation, market disruption, and labeling confusion.

New Tools to Manage Adoption

The revised Framework must recognize that the scale of adoption of any GE crop technology will drive the nature and magnitude of possible adverse environmental, public health, or marketplace consequences.

Under current law and regulations, federal agencies assess the risks and benefits of a new GE technology when planted or adopted on a given field. It is assumed that the risks arising from the planting of any particular field to a GE crop will be determined solely by what happens in that field. Current risk assessments do not take into account whether a given GE technology is likely to be adopted on 1%, or 10%, or nearly 100% of the cropland planted to a specific crop.

Current policy and risk assessments also fail to consider incremental and cumulative adverse impacts that can worsen over time, such as the rise in the costs of weed management (13, 14), loss of biodiversity, and increases in the volume and number of herbicide applications that invariably follow the emergence and spread of herbicide-resistant weeds (15–17).

For technologies that depend on biological and ecological impacts and interactions to work as intended (e.g., essentially all GE crop technologies), wider and more frequent use will generally result in additional, and/or potentially more severe, unintended consequences. This general rule applies to all biologically based technologies and has stood the test of time.

Going forward, the revised Coordinated Framework must grapple with the challenge of calibrating the sophistication and sensitivity of risk assessments, and risk mitigation interventions, to the scope of adoption and the magnitude of possible and actual adverse impacts. Fortunately, there are already accepted regulatory strategies and tools in place to do so in the U.S., and several have already been invoked in approvals of GE crop technology [e.g., refuge requirements, limiting deregulation decisions (approvals) to specific geographic areas or fixed time periods, and mandatory monitoring of target insects for resistance].

The revised Framework should work toward calibrating the risk assessment process to the scope of adoption by seeking from technology developers an estimate of the expected degree of market penetration in specific regions, within say 5 years of approval. Agencies could then focus risk assessments on high-adoption regions, and if deemed necessary, limit approvals or impose targeted monitoring or risk mitigation measures. After 5 years, the agency and technology developers could then re-assess estimates of adoption, actual experiences in the field, and the need for any further efforts to better characterize or mitigate risk.

Such new tools and authority is badly needed to avoid the proliferation of collateral damage to farmers and the food industry (e.g., failing and increasingly expensive pest management systems; loss of markets) and/or the environment and public health.

Dealing with Risks Arising from the Emergence and Spread of Resistant Organisms

The failure of the Coordinated Framework to address the risk and consequences of resistance is a serious deficiency. In fairness to the agencies implementing the Coordinated Framework in the early years, several constructive steps were taken to build resistance management into the *Bt*-transgenic corn- and cotton-approval processes. These included sizable, mandatory refuges planted to non-GE-*Bt* seeds and rigorous, annual resistance monitoring of insect populations.

After about a decade of largely successful prevention, GE technology developers pressured the EPA to relax *Bt*-crop refuge requirements, despite warnings from many independent entomologists. The consequences, and price tag, associated with this regrettable error in judgment are steadily rising and will continue rising for years to come.

To prevent resistance from eroding the benefits of GE crop technology and GE-based animal health and microbial products, the revised Framework should direct all federal agencies to take a variety of steps. The most important include

- sponsoring competitive research grant programs on the genetic mechanisms triggering resistance and/or the spread of GE technology-induced resistance;
- phasing out the use of antibiotic-related marker genes;
- requiring resistance risk assessments and management plans as a routine part of applications for approval and evaluating such plans *via* an independent review panel;
- post-approval resistance-monitoring provisions, including how ongoing resistance management testing will be paid for; and
- establishing resistance thresholds when exceeded will quickly trigger a second tier of resistance risk prevention strategies.

Need for Independent Science

Most people expressing a view on how the Coordinated Framework needs to be updated agree on one thing – poor and inadequate science has become an endemic problem in the GE risk assessment and regulatory processes (4). The revised Coordinated Framework must broaden and deepen the science base supporting GE regulation in order to enhance confidence in the scientific judgments supporting government decisions on GE technology.

Another step is equally important in convincing those skeptical of current GE crop safety assessments, within and outside the scientific community. The majority of the new, more sophisticated risk-assessment science should be conducted by scientific teams with no ties to the companies developing and marketing GE crop technology. In addition, institutions funding and carrying out this work should take proactive steps to insulate the individuals conducting the work from non-scientific criticisms, personal and professional attacks, and initiated or supported by GE companies and their surrogates. Regrettably, such unprecedented measures are now needed to slow the erosion of scientific integrity in this economically important, fast-moving area of technology.

Several practical, low-cost steps can be taken immediately. Federal agencies should require, as part of the application process, a guarantee from technology developers that requests for isolines and/or genetic markers and probes, or other technical information necessary to conduct risks assessments will be provided to federal agency scientists and independent researchers, and without imposition of restrictions on what non-commercial research can be conducted with them, or when and how results may be reported.

The FDA should publicly disclose the data provided by GE technology developers and allow for public comments on these data as well as on the adequacy of risk assessments. Both steps should be completed before the GE organisms are allowed on the market. Approvals can then incorporate any needed actions and requirements, such as post-approval surveillance and additional testing for applications in areas outside those studied prior to commercial launch.

Before any field trial of a new GE trait, USDA should require and disclose the exact sequence information of the inserted genetic material so that USDA, the grain trade, and food companies can detect possible contamination. Presently, USDA does not require detailed sequence information and therefore has no way to detect contamination. In addition, the locations of GE field trials should be disclosed, so that neighboring farmers and the food industry can guard against genetic contamination.

Independent scientists should be awarded funding and granted both the needed time and data/tools necessary to conduct state-of-the-art, GE technology risk assessments. They must be free to raise questions and reach independent conclusions without fear of personal or professional retaliation. Efforts to elucidate the metabolic-breakdown pathways of novel proteins in the edible portions of GE plants should receive special focus and dedicated funding, now that some widely consumed, GE fresh fruits (e.g., Artic apple) and vegetables (Innate potatoes, and *Bt* and Roundup Ready sweetcorn) have been approved and are in the food supply in several countries.

TOWARD A BRIGHTER FUTURE OF BIOTECH REGULATION IN THE U.S.

Currently in the U.S., the trigger for GE regulatory oversight is based on the attributes of GE organisms not the process used to create them (e.g., GE *via* a gene gun). This is conceptually flawed and leads to all sorts of problems: the USDA exempts GE plants produced without plant pest DNA from its admittedly limited purview; the FDA allows GE plants to be treated as GRAS if deemed substantially equivalent, with little or no focus on novel risks that are outside the parameters considered in judging substantial equivalence. It also means that some of the risks associated with GE organism (genetic contamination, resistant pests) are simply neither assessed nor addressed.

Five steps discussed below address specific, concrete steps the U.S. federal government could take to improve the GE risk assessment and risk mitigation processes. Steps 3.1 and 3.5 could be adopted relatively quickly, while more time to craft and implement solutions will be necessary in the case of the other three.

Adopt the Internationally Accepted Definition of Biotechnology

The U.S. should adopt the definition of "modern biotechnology" set forth by the Codex Alimentarius in the *Principles for the Risk Analysis of Foods Derived from Modern Biotechnology* (18):

> 'Modern biotechnology' entails the application of:
> (i) *In vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or (ii) Fusion of cells beyond the taxonomic family, that overcome natural physiological, reproductive, or recombinant barriers and that are not techniques used in traditional breeding and selection.

Assess All Aspects of GE Applications

The new Coordinated Framework should direct federal agencies to take into account both the novel proteins and other compounds produced by a GE plant, as well as any other related chemicals that must be, or typically will be used in conjunction with the GE crop technology. These will, of course, include all herbicides associated with a herbicide-tolerant crop variety, as well as seed treatments marketed as important in order for farmers to bring a GE crop to harvest.

Restore Scientific Integrity in Judging Substantial Equivalence

Despite its flaws, "substantial equivalence" is likely to remain part of the GE regulatory process. Accordingly, the erosion of scientific integrity in the assessment of GE crop equivalence must be reversed by

- Assuring that the protein, trait, or plant under investigation in risk-assessment studies is identical to those from the GE plant under evaluation;

- No longer considering the range of "natural variation" in nutrient and phytochemical levels in a GE crop versus its isoline, when both are grown in properly designed side-by-side trails;
- Requiring that the diet fed to control animals consists of the isoline of the GE crop being tested, and the GE crop and its isoline should be grown in the same environment; and
- The diet of the control animals should be tested for the presence of contamination from other GE crops and pesticides typically used in conjunction with GE crops.

Acknowledge That Stacked Varieties May Pose Unique Risks

The new Framework should require agencies to develop new test requirements for stacked varieties, acknowledging that multiple traits and regulatory sequences can lead to unexpected interactions and possibly adverse outcomes, just as treatment with multiple medications can lead to drug interactions and contraindications.

Require Labeling of GE Products

All products from GE crops, animals, and microorganisms should be accurately labeled, both to ensure consumer choice and to enhance the odds that public health officials, doctors, and scientists will quickly recognize unexpected problems, if and when they arise. In addition, companies that develop GE organisms should be required to disclose any GE trait, marker genes, or other genetic constructs in commercial, GE seed products, including traits and genes from obsolete and no longer-marketed traits.

Today, FDA requires labeling on food products when there has been a change in a "material fact," such as a food product's nutritional value, organoleptic properties, or functional characteristics. But several times in the past, and for good reason, the FDA has required labeling under the "material fact" construct in the absence of a change in nutritional value, organoleptic properties, or functional characteristics.

For example, in the final food irradiation rule, the FDA acknowledged that the large number of respondents who asked for labeling of irradiated retail products was evidence that irradiation was, indeed, a "material fact" (19). In its decision, the FDA wrote "Whether information is material under section 201(n) of the act depends not on the abstract worth of the information but on whether consumers view such information as important and whether the omission of label information may mislead a consumer. *The large number of consumer comments requesting retail labeling attest to the significance placed on such labeling by consumers*" (Emphasis added) (19).

Clearly, state and national polling, and the near 50–50% split in the voting on several state GE food ballot initiatives, is evidence of the significant consumer interest in whether a food product contains GE ingredients.

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The author confirms being the sole contributor of this work and approved it for publication.

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OPINION
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Scientists and Civil Society Must Move Together toward a New Science

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In the current context of research and innovation that are increasingly driven by short-term industrial interests, science and technology require thorough social, political, ethical, and legal changes leading to better democratic control. A huge gap has opened between citizens and scientists, with the latter sometimes inspiring more mistrust than trust. Major health and environmental scandals of past years (for example, asbestos, Bovine Spongiform Encephalopathy, PCBs, and nuclear disasters) may be related to this situation.

To restore the links between science, policy makers, and civil society is a difficult task with many challenges. This involves (a) substituting a research approach strictly entrusted to the scientific community, with approaches based on a willingness to access and respect various forms of knowledge; (b) taking into account, at a very early stage in public research policy, the societal challenges of science and the tools for its democratic orientation; (c) expanding access to scientific knowledge in society, allowing those that are often wrongly called "ignorant" to interact with researchers in a balanced dialog and a co-construction of knowledge. How is it conceivable, for instance, to develop an agricultural research project without a close exchange and collaboration with those people who invented agriculture – not the researchers, or even the agronomists, but farmers? Moreover, in a knowledge society, in which innovation does not necessarily mean "progress," citizens may be especially willing to participate in choosing scientific and technological orientations.

Such a task implies in particular the setting up of systems enabling civil society to access opportunities to develop scientific knowledge, as well as for innovation and expertise (1). Participatory research, which is joint research work with equal partnerships between non-profit organizations from civil society or groups of citizens and academic researchers (from universities or major research organizations), is an integral part of this process of democratization of science. Several public programs successfully promote participatory research. Examples include the Canadian program of *Community-University Research Alliances* (ARUC)[1]; several regional research programs in France, such as *Partnerships between Institutions and Citizens for Research and Innovation* (PICRI),[2] set up by the Region Ile-de-France under the leadership of the *Fondation Sciences Citoyennes* organization[3]; and the *Social Appropriation of Sciences* (ASOSC),[4] developed by the Brittany Region.

A project resulting from collaboration between researchers and actors of civil society often addresses a societal issue. Thus, participatory research involves mainly applied research projects and projects that fall within the field of expertise (health, environmental, ethical, etc.). For basic research, i.e., research that is conducted solely for the sake of increasing human knowledge, such collaborations are more difficult to consider, since this research generally falls into skills that are specifically those of scientists. However, citizens can participate in some basic research projects, by collecting data on

[1] http://www.sshrc-crsh.gc.ca/funding-financement/programs-programmes/cura-aruc-fra.aspx
[2] http://www.iledefrance.fr/competence/picri
[3] http://sciencescitoyennes.org
[4] http://xnet1.region-bretagne.fr/Recherche

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a large scale (for example, in the field of biodiversity). In this case, participants train themselves with the files and protocols that are given beforehand. Besides, citizens could be consulted about the fundamental questions that they are concerned with and that they would like to be addressed by scientists.

The requirement for openness of science to civil society is particularly striking in the area of technologies and the products of technology. The phenomenon of lucrative research driven by industrial interests that require rapid returns on investment leads to negative consequences in terms the quality and transparency of health and environmental assessment. The time required to conduct these assessments with proper rigor is not compatible with the urgency of patents and profits, and commercial confidentiality is used to justify the failure to communicate raw data from regulatory tests.

Many civil society organizations have emerged to oppose the possible assessment deficiencies of new products placed on the market. These organizations play an important role at the interface between the assessment authorities and civil society as a whole, not as mediators, but as shields designed to protect citizens from potential hazards resulting from inadequate assessments. Participatory research projects provide an opportunity for civil society organizations to intervene as interlocutors and collaborators with scientists who are engaged in research on assessment issues. These organizations are then able to relay the results to the general public and decision makers and develop arguments for possible revision of assessment regulations by public authorities.

An example of this was provided by the international conference on "Assessment and Regulation of Genetically Modified Organisms (GMOs) and Pesticides,"[5] held in France at the Orsay Scientific Centre (University of Paris-Sud) on 12 and 13 November 2015.

The originality of this symposium was the fact that it was open to civil society (with French/English simultaneous translation), organized as part of a participatory research (PICRI project, funded by the Île-de-France Region), and managed collaboratively between University Paris-Sud and two associations: *Générations Futures*[6] and the *Committee for Research and Independent Information on Genetic Engineering (CRIIGEN)*.[7] This project[8] focuses on the study of the "substantial equivalence" principle (i.e., close nutritional and element similarity between two crop-derived foods), which has been used as the basis to allow the commercial approval of all agricultural GMOs cultivated across the world. This concept, adopted by the Organization for Economic Co-operation and Development (OECD) in 1993 and endorsed by the Food and Agriculture Organization (FAO) and World Health Organization (WHO) in 1996, is registered in the Food and Drug Administration (FDA) regulation (*Part IX: Foods derived from New Plant Varieties*) and was used to claim that GM crops are as safe and nutritious as currently consumed plan-derived foods (2). Since this concept applies at the chain end (i.e., to the food from these plants), it should consider the context of the growing crops. Precisely, in the case of herbicide-tolerant GM crops, this context is not the same as for their conventional counterparts since the former are sprayed with the herbicide. Surprisingly, during substantial equivalence studies, either the tested plants are not sprayed with the recommended herbicide (3) or the herbicide residues are not measured anyway (4).

The international dimension of the conference was not only due to the panel of speakers but also to those who attended. Among the 140 participants, many came from different countries (not only in Europe but also in America and Africa).

According to the spirit of participatory research, this conference allowed the creation of a bridge between academic research and the "scientific third sector" (citizens, associations, NGOs, policy makers), with presentations of experimental scientific data, made accessible to the general public, as well as round tables bringing together civil society stakeholders. Both were followed by long interactions with the public. This spirit was also the reason why we chose to organize this conference at the Orsay Scientific Centre: because companies are allowed to sit on university boards of directors, it was important for my colleagues and myself, concerned about the democratization of science, to offer the opportunity for citizens to penetrate inside the walls of the university.

Together with results from Brazilian (5) and Norwegian (6) research groups, some of the results of the PICRI project (7) questioned the relevance of the substantial equivalence principle, especially when used to approve the commercialization of herbicide-tolerant GM crops. The second scientific session offered a state-of-the-art review of experimental data showing the insufficiencies of regulatory toxicity tests of GMOs and pesticides (8, 9). It was emphasized that the duration of feeding trials is insufficient to detect potential chronic (long-term) toxic effects and that contamination of laboratory rodent diets by toxic environmental pollutants is a confounding factor in regulatory tests. Some of the results presented at this conference showed that commercial formulations of pesticides are always more toxic than their so-called "active principles." Yet, the latter are tested alone to calculate safety thresholds, even though they are never used in isolation, but always mixed with toxic adjuvants. Last but not least, it was explained that regulatory tests are unable to detect endocrine disrupting effects, a common toxic mechanism shared by many pesticides. During the third scientific session, the panel of speakers showed the detrimental effects of pesticides and GMOs on soil ecosystems, for example, on rhizosphere microflora and earthworms (10), and on food microorganisms (11).

Round tables allowed exchanges on various models of participatory research and highlighted the need to involve civil society in research programs and in the choice of major research directions. The regulation of pesticides and GMOs (including those resulting from the use of new genetic engineering technologies other than transgenesis) at national and European levels was also widely discussed. There was a particular emphasis on the questions of data transparency, conflicts of interests in assessment committees, and the responsibility of experts and policy makers. Finally, a farmer, an agronomist, a physician, and a company manager producing flour and animal feed explained and exchanged their views on

[5] http://picriogm.weebly.com/colloque.html
[6] http://www.generations-futures.fr
[7] http://www.criigen.org
[8] http://www.picri-ogm.fr

the question of agricultural sustainability and food choices in the future.

These 2 days of exchanges, where multiple participants from different backgrounds contributed to a unified collective intelligence, were particularly rich and intense. They confirmed the need to open up science to the public, not only to share results but also to build on each other's knowledge.

To this end, and in line with proposals made by *Fondation Sciences Citoyennes*,[9] the following arrangements must be implemented:

(a) integrating participatory research programs in all public research policies;
(b) taking into consideration the value of the participation of civil society (non-profit) to research;
(c) setting up evaluation criteria for researchers involved in participatory research projects;
(d) supporting the mobility of researchers toward civil society organizations;
(e) expanding communication on participatory research to researchers, students, and civil society.

There are professional scientists – the researchers – and professional politicians, including elected officials. But the scientific approach, like political action, belongs to everyone. It is time to move toward a new science, considering the "substantial equivalence" between professional scientists and citizens.

[9]http://sciencescitoyennes.org/rubrique/tiers-secteur-de-la-connaissance/recherche-participative/

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