

Critical Review

INTERACTIONS BETWEEN Bt CROPS AND AQUATIC ECOSYSTEMS: A REVIEW

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Abstract: The term *Bt crops* collectively refers to crops that have been genetically modified to include a gene (or genes) sourced from *Bacillus thuringiensis* (Bt) bacteria. These genes confer the ability to produce proteins toxic to certain insect pests. The interaction between Bt crops and adjacent aquatic ecosystems has received limited attention in research and risk assessment, despite the fact that some Bt crops have been in commercial use for 20 yr. Reports of effects on aquatic organisms such as *Daphnia magna*, *Elliptio complanata*, and *Chironomus dilutus* suggest that some aquatic species may be negatively affected, whereas other reports suggest that the decreased use of insecticides precipitated by Bt crops may benefit aquatic communities. The present study reviews the literature regarding entry routes and exposure pathways by which aquatic organisms may be exposed to Bt crop material, as well as feeding trials and field surveys that have investigated the effects of Bt-expressing plant material on such organisms. The present review also discusses how Bt crop development has moved past single-gene events, toward multigene stacked varieties that often contain herbicide resistance genes in addition to multiple Bt genes, and how their use (in conjunction with co-technology such as glyphosate/Roundup) may impact and interact with aquatic ecosystems. Lastly, suggestions for further research in this field are provided. *Environ Toxicol Chem* 2016;35:2891–2902. © 2016 SETAC

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BACKGROUND

Aquatic ecosystems are facing significant pressures that threaten natural dynamics, ecological integrity, and biodiversity [1]. Dominating stressors that reduce biodiversity include land use, homogenization of resources, eutrophication, and habitat destruction [2]. All these factors are intimately linked to modern agriculture. In fact, agriculture is highlighted as a key driver of environmental change in freshwater ecosystems [3].

Modern commercial agriculture is predominantly characterized by large-scale monoculture production. In the mid-1990s, transgenic crops with internally produced toxins to combat insect pests were introduced to the market. These crops, particularly for maize and cotton, now contribute significantly to the world markets, reflected in the large production volumes of the United States, Canada, Brazil, Argentina, Paraguay, India, China, Pakistan, and South Africa, among others [4].

The term *Bt crops* is the collective term for crops that have been genetically modified to include a gene (or genes) sourced from *Bacillus thuringiensis* (Bt) bacteria, which code for insecticidal proteins. These genes confer the ability to produce insecticidal proteins to the crop plants themselves, reducing the need to spray chemical insecticides to control pest insects [5,6]. The *B. thuringiensis* toxins that have been harnessed in Bt crops include parasporal crystal proteins, known as Cry proteins (Cyt proteins when they exhibit cytolytic activity), as well as vegetative insecticidal proteins (VIPs) [7–10]. The Bt toxin-producing crops have been used for controlling pests of Lepidoptera, Coleoptera, Diptera, and Hymenoptera, as well as nematodes [8]. Although *cry1Ab* is arguably the best studied of the Bt genes, the International Service for the Acquisition of the Agri-biotech Applications [11] currently lists 21 Bt genes in

commercial use (including truncations and other modified versions; for an overview, see Table 1), and hundreds of Bt toxins have been reported [12].

Single-transgene genetically modified crops are being replaced by varieties that combine or stack several transgenes—for example, incorporating multiple Bt toxins and/or other traits, such as herbicide tolerance (HT)—in the same plants. The genetic modifications of HT crops allow them to be used in tandem with specific herbicide co-technologies; such herbicides can be applied multiple times during a growing season for weed control without major damage to the HT crop plants [13]. Crops that stack Bt and HT traits are particularly popular and have overtaken single-transgene Bt crops in terms of area planted in recent years [4]. James [4] further reports that approximately 80 million ha of crops containing Bt genes were grown worldwide in 2014, with maize and cotton as the dominant crops. In addition to producing feed, food, and fiber, Bt crops produce a large amount of biomass (leaves, stalks, cobs, and roots that remain after harvest, as well as pollen) that enters into local food web interactions in soil and aquatic ecosystems.

The assessment of environmental safety is crucial and is a key element of transgenic crop technology [6]. Research on potential nontarget effects of Bt transgenic plants has focused on terrestrial ecosystems, and investigations have predominantly tested Cry1Ab-toxin and Cry1Ab transgenic crops, whereas other genes/toxins and stacked events (especially in conjunction with herbicide cotechnologies) have received less attention. Despite growing recognition that aquatic ecosystems near agricultural fields receive significant amounts of runoff and crop residues that contain these toxins [14,15], environmental risk assessments of transgenic crops tend to neglect aquatic ecosystems as a relevant context for testing.

The present study reviews literature related to exposure, spread, break-down rates, and effects of various types of Bt crop material on nontarget organisms and aquatic communities. Finally, we recommend research to fill existing knowledge gaps.

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Table 1. Crop plants modified to express Bt proteins, and target orders of each protein^a

Bt gene	Crops where present	Listed target organisms
<i>cryIA</i>	Cotton (1 event), maize (1 event)	Lepidoptera
<i>cryIA.105</i>	Maize (18 events), soybean (1 event)	Lepidoptera
<i>cryIAb</i>	Cotton (8 events), maize (53 events), rice (2 events)	Lepidoptera, particularly European corn borer, African corn borer
<i>cryIAb</i> (truncated)	Maize (1 event), rice (1 event)	Lepidoptera
<i>cryIAb-Ac</i> (synthetic fusion gene)	Cotton (2 events)	Lepidoptera
<i>cryIAC</i>	Cotton (28 events), eggplant (1 event), maize (1), poplar (2), rice (2), soybean (4), tomato (1)	Lepidoptera
<i>cryIC</i>	Cotton (1 event)	Lepidoptera, particularly Spodoptera
<i>cryIF</i>	Cotton (6 events), maize (4 events), soybean (2 event)	Lepidoptera
<i>cryIFa2</i> (synthetic form of <i>cryIF</i>)	Maize (45 events)	Lepidoptera
<i>moeryIF</i> (synthetic form of <i>cryIF</i>)	Maize (1 event)	Lepidoptera
<i>cry2Ab2</i>	Cotton (10 events), maize (20 events), soybean (1 event)	Lepidoptera
<i>cry2Ae</i>	Cotton (4), maize (1 event)	Lepidoptera
<i>cry3A</i>	Potato (30 events)	Coleoptera
<i>cry3Bb1</i>	Maize (18 events)	Coleopterans, particularly corn rootworm
<i>cry9C</i>	Maize (1 event)	Lepidoptera
<i>mcry3A</i>	Maize (30 events)	Coleoptera
<i>ecry3.1Ab</i>	Maize (5 events)	Coleoptera and Lepidoptera (multiple insect resistance)
<i>cry34Ab1</i>	Maize (35 events)	Coleoptera, particularly corn rootworm
<i>cry35Ab1</i>	Maize (35 events)	Coleoptera, particularly corn rootworm
<i>vip3A(a)</i>	Cotton (9 events)	Lepidoptera
<i>vip3Aa20</i>	Maize (22 events)	Lepidoptera

^aCompiled from information on the International Service for the Acquisition of Agri-biotech Applications GMO Approval Database [11].

ENTRY ROUTES AND EXPOSURE PATHWAYS OF Bt TOXINS AND PLANT MATERIAL

Aquatic ecosystems receive much of their energy from terrestrial systems. Basic aquatic ecology (e.g., through the river continuum concept [16]) has shown that the energy input into a small stream can be significant—often larger than the local energy production within the stream in shaded areas—highlighting the role that allochthonous input can play in aquatic ecosystems. In agricultural settings, the source of allochthonous input is likely to be crop detritus from the surrounding farmland. Other natural links between terrestrial and aquatic systems are, for example, some insects that spend different life stages in aquatic and terrestrial environments and fish that feed on terrestrial insects.

Regarding such connection in terms of Bt crop fields and adjacent aquatic ecosystems, Carstens et al. [17] differentiate between *entry routes* and *exposure pathways*. The ways and means by which Bt crop materials (including plant material, Bt proteins, and transgenes) end up in aquatic ecosystems are the entry routes. Exposure pathways refer to the routes by which aquatic organisms may be in contact with Bt material and affected by it [17].

Entry routes

The main entry route of Bt material into aquatic systems seems to be the deposition of plant debris, including pollen, crop dust, leaves, stalks, and postharvest detritus, facilitated by wind, rain, and runoff [18–21]. A study conducted by Rosi-Marshall et al. [18] was among the first to investigate the fate of Cry1Ab/Bt corn by-products in 12 headwater streams of agricultural production areas in the Midwest of the United States. Following this work, 217 Indiana (USA) streams were sampled by Tank et al. in 2007 [21]. That study found that 6 mo after harvest was complete, 67% of the streams had maize leaves in the stream channel and 86% contained other maize detritus in addition to leaves, and that the average concentration of Cry1Ab in streams that tested positive for the protein was 14 ± 5 ng/L [21].

The amount of crop biomass that reaches aquatic ecosystems will be affected by the use of different agricultural and conservation practices, such as conservation tillage and the adoption of riparian buffers. Conservation tillage, which includes practices such as mulch-till, strip-till, and no-till, refers to production systems in which at least 30% of crop residues are left on the field to prevent soil erosion and water loss [22]. Other outcomes include improved soil structure and increased nutrient cycling, better drainage, and increased available crop material [23], some of which will enter into aquatic ecosystems [21]. Interestingly, a correlation has been shown between the use of HT crops and the adoption of conservation tillage practices. Taken together with the increasing use of Bt/HT crops, this may indicate an entry route for herbicides, in addition to Bt toxins, via herbicide-treated Bt plant material.

Riparian buffers, however, may help counter the entry of crop debris into aquatic environments [24,25]. Riparian buffers are zones of vegetation (such as grasses, shrubs, or trees) that are planted to form a barrier between the fields and streams, to reduce the amounts of sediment, nutrients (such as nitrates and phosphates), and runoff entering streams and thus improve water quality [26,27]. By impeding the flow of runoff, they may limit the transfer of crop material into streams, although this is likely to depend on the type and density of the vegetation making up the buffer. The degree to which riparian buffers impede the entry of Bt crop residues into adjacent streams has not (to our knowledge) been examined.

During storms or floods, the amount of crop plant material brought to a local stream or pond can be massive, although dilution as a result of a large volume of water should also be taken into account when considering Bt toxins in this context. Small streams or ponds may become densely packed with plant material, as can be seen in Figure 1. Conversely, some portion of the deposited crop material may be fine particles, such as those generated when whole maize plants are harvested for silage or methane production. These particles (coarse, >1 mm; fine <1 mm) may be a food source for aquatic invertebrates [19].



Figure 1. Runoff material of Bt-transgenic maize to a local stream after flood, South Africa. Photograph by Thomas Bøhn.

Furthermore, researchers in Canada found that rivers and streams could be implicated in spreading Bt material away from the immediate surroundings of maize fields, after they detected transgenic DNA from Bt maize several kilometers downstream from the fields where the maize was grown [28].

Entry routes for Bt proteins include transport from fields into aquatic ecosystems as part of erosion or runoff [29], or via drainage water and tile drains [21]. The Bt proteins are released into soil from living Bt crops via their roots and from dead plant tissues that remain on the field [30,31]. Soil properties will influence the amount of protein entering the aquatic system via this route. For example, clay particles appear to bind strongly to Cry1Ac, Cry1Aa, Cry1Ab, and Cry1Ab/1Ac fusion proteins and also reduce biodegradation of these proteins [15,30–32]. This has led some authors to theorize that soils with a high clay content may keep Bt toxins close to the soil surface and lead to a higher rate of bioactive Bt proteins in the runoff soil of these systems [29]. Recent studies, however, have found that Bt protein concentrations in the soil of no-tillage Bt maize fields tended to be low (averaging below 5 ng/g before pollination, peaking at 9–29 ng/g during pollination). Higher concentrations of the Cry1Ab protein were detected in surface water runoff and runoff sediment, which increased during the growing season, peaking during pollination at 130 ng/L and 143 ng/g dry weight for runoff water and sediment, respectively [20,33]. Interestingly, these studies also detected Cry1Ab protein in the runoff of a non-Bt field located close to the Bt field in question, at

an average concentration of 14 ng/L. Given that no Cry1Ab protein was detected in the soil of this field even during pollination, the authors speculated that its presence in the runoff was a result of the transfer of plant materials between fields after rain [20].

Subsurface drains (tile drains) may represent an alternative route of entry for Bt proteins that have been desorbed from soil particles, as was found by Tank et al. [21]. However, considering that only 2 of 120 groundwater and porewater samples drained from a Bt field analyzed by Strain and Lydy [20] contained detectable amounts of the protein (17.2 ng/L and 21.7 ng/L, respectively), this route may not contribute much Bt protein to aquatic systems.

Exposure pathways

Deposited plant material is itself available for consumption [19,34–36] and leaches Bt proteins into the water [37–39]. The proportion of Bt proteins that remains in the plant tissue versus the amount that leaches into the water and/or degrades after exposure to aquatic environments has been documented in a number of studies. Several complicating factors, including temperature, type of plant tissue, sediment composition, and influence of microbes, have been noted [15,20,38].

Bt toxin concentration of plant material in aquatic settings

During a field experiment in which rice stubble was left on the field after harvest, Li et al. [38] found that the Cry1Ac concentration of Bt rice stalks (originally 1501.3 ± 200.5 ng/g dry wt) decreased by 50% during the first month after harvest, but that the rate of degradation slowed after this. Seven months after harvest, 21.3% (319.8 ± 59.8 ng/g dry wt) of the original Cry1Ac toxin concentration was still present in stalk tissue. The concentration of Cry1Ac leaching from rice roots (original concentration 516.1 ± 86.4 ng/g dry wt) followed a different pattern, however, with initial release of the toxin being quite slow: 72.4% of the original concentration was present after 1 mo. However, the concentration decreased to almost undetectable levels by the end of the experiment, 7 mo post harvest. The authors also noticed that, for both stalks and roots, the winter months brought a reduction in the rate of Cry1Ac degradation. The field experiment did not include measurements of the amount of Cry1Ac present in the field soil or water.

Experiments that tracked the decrease in concentration of Cry1Ac protein in Bt rice plant residues in soil compared with an aquatic milieu have been performed under laboratory [38] and field conditions [40]. In both cases, degradation was found to be somewhat slower under aquatic conditions, at least initially. Under laboratory conditions, degradation of Cry1Ac protein in soil plateaued eventually (despite faster initial degradation), leaving 15.3% of the initial concentration in the leaf–soil mixture after 135 d, whereas none was detectable in water by this point [38]. In contrast, Xiao et al. [40] were unable to detect the protein in the soil surrounding the litterbags of plant materials in the field. This lack of consistency is thought to reflect different methodologies in terms of sample preparation [40].

In contrast to Cry1Ac rice, Cry1Ab concentrations in Cry1Ab maize plants decline more rapidly under aquatic conditions than in soil or aerobic conditions [14,41]. For example, it has been shown that 61% of the Cry1Ab toxin leached from Bt maize leaves within the first hour of aquatic exposure (the Cry1Ab concentration in the water was not determined, however) [37]. Strain and Lydy [20] similarly found that Cry1Ab had a half-life of approximately 2 h but

that the concentration of the protein in the water peaked at approximately 2 d after initial exposure.

The proportion of Cry1Ab reported to remain in Bt leaves over time varies between studies. It has been reported as 6% and 20% of the initial concentration after 21 d and 70 d of exposure to aquatic conditions, respectively [14]. Wandeler et al. [42] reported that after 20 d, 1 variety of Bt maize experienced a reduction in Cry1Ab concentration of 60%, while another decreased by only 21%. Although the plant material in the Wandeler et al. study [42] was not exposed to an aquatic environment, the study reflects the variation that differences in cultivar or environmental conditions can introduce.

Degradation of Bt proteins in aquatic settings

The Bt proteins that are leached into the water degrade over time, although there is great variation among the reports of how long this takes to happen. Strain and Lydy [20] found that the proportion of Cry1Ab protein in the water decreased to below reporting limits over approximately 2 wk. However, a study of Cry1Ac extracted from cotton seeds found that the Cry1Ac protein was still detectable in water and sediment after 60 d [15]. Prihoda and Coats [43] found that the half-life of Cry3Bb1 from MON863 Bt corn stalks, leaves, and roots was just under 3 d. They were also unable to detect Cry3Bb1 protein in the water or sediment of the microcosm treatments, which the authors attributed to rapid adsorption by organic particles, or swift dissipation. However, Strain et al. [44] suggest that this lack of detection may be because of the methodology used (i.e., not concentrating the water samples before determining concentration).

Differences in cultivar, as well as factors such as water chemistry and temperature, may account for differences in the rate of Bt protein loss [14,44]. Temperature in particular is an important factor for Bt protein longevity [15,20]. For example, when the temperature was kept at 4 °C, decline of Cry1Ab concentration in plant material and water was much slower than at warmer temperatures, with Cry1Ab concentrations in both matrices dropping to below reporting level in approximately 2 wk when incubation was at 37 °C. When temperature was held at 4 °C, the average concentration of Cry1Ab in the aquatic milieu after 2 mo was 300 ng L⁻¹ [20]. This indicates that the stability of Bt proteins could be extended during the cooler winter months (as was also noted for Bt rice by Li et al. [38]), which is significant because a great deal of plant material is present in aquatic environments during that time [20,21,25]. However, another factor to consider is whether the Bt proteins retain bioactivity after prolonged presence of plant materials in water.

Adsorption of Bt proteins

The Bt proteins that are leached from plant material may bind to sediment, especially sediment with a high clay and/or organic matter content [15,20]. In an experiment that took place over 2 mo, 20% to 40% of the total Cry1Ab protein present in a system of submerged Bt maize plant material was located in the sediment from the second week until the experiment was terminated 6 wk later [20]. Adsorption to sediment particles protects the Bt proteins from degradation and may also allow them to keep their toxic/insecticidal properties [45]. The Cry1Ac protein leached from cotton persisted in sediment longer than in soil, which the authors attributed to greater amounts of organic matter in sediment having reduced the bioavailability of Cry1Ac protein, and thereby reducing its degradation by microbes [15].

Also, leached Bt proteins may be adsorbed by algae: Cry1Ca protein was detected in cells of the green alga *Chlorella pyrenoidosa*, after it was cultured in media containing leachate from Cry1Ca-expressing rice [46]. The amount of Cry1Ca present in the algae cells increased with increasing concentration of the protein in the media but reached saturation at a concentration of 1000 µg/mL of the media. Interestingly, when the Cry1Ca protein concentration was too low to be detected in the culture medium, it could be detected in the algae [47]. Given the rapid adsorption of Bt protein reported in these studies, one may question whether adsorption by algae could affect the measurements of Bt proteins in aquatic field samples. Algae as a potential route of exposure for aquatic organisms has not yet been investigated.

To summarize, organisms inhabiting aquatic environments adjacent to Bt crops will potentially be exposed to Bt-containing plant material and Bt toxins at varying concentrations, depending on their feeding habits, the type of crop and cultivar, the age and breakdown rates of the plant material, and the properties of the water and sediment of the aquatic environment. The timing of feeding in relation to when Bt crop material enters the system and how long it was exposed will also be important. Although some authors have suggested that the concentrations of Bt proteins to which aquatic organisms are exposed are too low to cause concern [17,48], others argue that the continuous input of crop debris, as well as runoff water and sediment, may lead to long-term exposure of aquatic organisms that may have chronic effects, warranting further investigation [14,19–21].

ACTIVATION AND SPECIFICITY OF Bt TOXINS

The toxicity of Bt proteins to target insects has been studied in some detail, and although different models exist for exactly how they cause harm to insects, all of the models agreed on the following: after ingestion, solubilization of the protoxin form of the Bt toxins in the alkaline midgut was required, proteolytically activating them to their smaller active toxin form [49–52]. However, recent studies of resistant target insects have found that protoxin activation was not necessary for an insecticidal effect to occur. In fact, in some cases, the protoxin was more effective than the activated toxin [51,53]. It is theorized that bacterial production of protoxins is a strategy to impede resistance development [51]. This pathway has been described only in resistant target orders so far; but considering that protoxin activation has been linked to Bt toxin specificity, one might enquire whether these findings might have implications for cross-activity within other orders [54].

Another important finding highlighted by Tabashnik et al. [51] and Gómez et al. [53] was that the use of mammalian trypsin or chymotrypsin proteases to activate the protoxins does not produce results exactly equivalent to protoxins activated by insect midgut juices, potentially leading to underestimations of toxicity. In addition, bacterially produced Bt protoxins may differ from those produced in some Bt crops. For example, MON810 and Bt 11 Bt maize events produce truncated (65-kDa) toxins, in contrast to the bacterially produced 135-kDa protoxin [41,55]. Furthermore, activation by plant proteases within Bt crops was reported by Li et al. [54], meaning that insects feeding on such plants are exposed to activated toxins. These points provide examples of mismatches between what is tested during risk assessment and what is found in the environment, since most safety testing of Bt toxins is performed on Bt protoxins produced by bacteria, typically in *E. coli* (not by the transgenic

crops), and if activation is done, it is usually with mammalian proteases.

Specificity of Bt toxins and sensitivity of aquatic nontarget organisms

The specificity of Bt toxins—that their effectivity is restricted to a limited range of target organisms (usually restricted to a specific order)—has been lauded as a major advantage for agricultural application, because unwanted negative effects on nontarget organisms can be minimized [56,57]. However, documented negative effects on nontarget organisms, such as *Daphnia magna* [58–62], which lack the relevant receptors, point to alternative modes of action for Bt toxins. van Frankenhuyzen [63] reviewed the subject of cross-activity of Bt toxins outside of their primary target orders. The study found that although 64% of the 148 Bt toxins considered were thought to be active within 1 order only, a large portion of these had in fact never been tested on organisms from different orders. Evidence of cross-activity was found in approximately 13% of the Bt toxins investigated [63].

Given the exposure of Bt toxin in aquatic systems and the uncertainty over alternative mode of actions or cross-reactivity of some Bt toxins, more testing of the sensitivity of aquatic organisms seems well justified. In addition, some of the aquatic insects exposed will belong, at some level, in the same taxonomic groups as the terrestrial target pest species. The main insect orders targeted by Bt toxins are Lepidoptera, Diptera, and Coleoptera (Table 1). These are biodiversity-rich groups with representatives found in aquatic environments. Aquatic stages of larval caddisflies, beetles, or midges, for example, may be vulnerable to Bt toxin exposure, depending on the toxin concentration, the feeding strategy, and the sensitivity of each individual species. Depending on the degree of relatedness, these groups may share physiological properties, receptors, and so forth, which may make them vulnerable to Bt toxins [18].

In terms of investigating nontarget effects, a few studies set out to characterize the degree to which arthropods were exposed to Bt proteins, regardless of whether they belonged to the target orders, by determining the amount of Bt protein present in arthropod specimens collected from fields of Bt crops [64–66]. What was fascinating about these studies was that, aside from providing baseline data for which species were potentially at risk because of Bt proteins, they were also able to provide data indicating how the concentration of Bt proteins present in the arthropods differed throughout the growing season, especially how these differed before and after anthesis. Furthermore, in some cases it was possible to detect the life stages during which the arthropods were most exposed (i.e., contained the highest levels of Bt protein). Exposure pathways of Bt proteins through the food chain were also illuminated, because Bt protein levels were measured in predator species as well [64]. Similar investigations of aquatic communities would help determine which species are the most exposed and which may be good candidates for further investigation.

EFFECTS OF Bt TOXINS ON AQUATIC ORGANISMS

Although effect studies testing Bt-expressing plant material and Bt toxins (i.e., toxicological testing), feeding trials, and field trials are being done with greater frequency with aquatic organisms, large knowledge gaps are still present. Most studies have been done using maize or rice producing Cry1Ab or Cry1Ac. Soy, rapeseed, cotton, and other Bt crops, as well as numerous Bt toxins, are meanwhile underrepresented in terms of investigations of potential effects on aquatic organisms.

Caddisflies

Caddisfly (Trichopteran) larvae have attracted attention as aquatic organisms that may be affected by Bt crops, because of the close relation of the Trichoptera to Lepidoptera, the target order of many Bt toxins. Even so, only 3 species appear to have been put through feeding trials [18,25,36]. The results of these studies have been at times contradictory, inconclusive, or controversial. Methodological issues have been at the root of most of these discrepancies.

The 3 caddisfly species that have been investigated to date are *Lepidostoma lima*, *Pycnopsyche scabripennis*, and *Helicopsyche borealis*. *Lepidostoma liba* experienced reduced daily growth rates of more than 50% ($p = 0.008$) when fed Bt corn litter compared with non-Bt litter. Another caddisfly, *H. borealis*, was shown to have increased mortality, but this response required exposure to a high concentration of pollen, (i.e., 2–3 times higher than the maximum pollen density observed in the field) [18]. The Rosi-Marshall et al. [18] study has been criticized for the lack of appropriate controls; for failing to quantify relevant properties of the crop material, especially the Bt toxin; and for overstating its conclusions [67,68]. Rosi-Marshall et al. matched the lignin content and the carbon-to-nitrogen ratio in the maize, rather than using isolines of Bt versus non-Bt treatments. They argued that the nutritional value would not be the same with the latter control because Bt maize contains markedly more lignin compared with its isogenic counterpart [69].

In a follow-up to the Rosi-Marshall et al. [18] study, Chambers et al. [36] increased the number of Bt and non-Bt varieties sampled and again found *L. liba* to be negatively affected by Bt maize in terms of growth. In contrast, Jensen et al. [25] did not find any significant differences between *L. liba* larvae fed Bt maize and those fed the non-Bt near isolate. It was noted by these authors [18,25,26] that obtaining a true isolate to use as a control can be problematic (for example, due to differences in lignin content mentioned in the previous paragraph), making causality difficult to establish. Considering the increased use of stacked events that contain multiple transgenes, the problem of availability of suitable reference material (controls) will become more difficult and complex.

Sedimentary midges

Chironomus dilutus, a filter-feeding aquatic midge frequently used for sediment toxicity testing [70], was known to be sensitive to Bt insecticidal formulations before the advent of genetically modified Bt crops [71,72]. The larvae of *C. dilutus* have since also been found to be sensitive to Cry3Bb1 [43] and Cry1Ac [15], extracted from Bt maize roots and cotton seeds, respectively. The 2 studies had very different approaches: Prihoda and Coats [43] exposed *C. dilutus* to Cry3Bb1 by adsorbing the protein to food particles, whereas Li et al. [15] spiked the sediment or water in which the organisms were placed with Cry1Ac extract.

A significant reduction in survival was observed in larvae exposed to nominal concentrations of 30 ng/mL to 48 ng/mL Cry3Bb1 (measured concentrations of Cry3Bb1 adsorbed to food were 19–27 ng/mL), although the amount consumed was not determined [43]. The LC50 of Cry1Ac cottonseed extract was determined to be 155 ng/g dry weight of spiked sediment and 201 ng/mL in spiked water [15]. Both studies [15,43] pointed out that the concentrations of Bt protein used in the experiments were far above what these larvae would likely be exposed to in nature. Although this is true, it should also be kept

in mind that empirical data on aquatic environmental concentrations of Cry3Bb1 and Cry1Ac (or any other Bt protein produced in cotton) are lacking, and the exposure regimes used in these studies do not reflect the pulsed, chronic exposure that is likely to occur in the field [20,43]. Furthermore, as filter feeders, *C. dilutus* may feed directly on Bt crop dust [19], which is an avenue of exposure that remains to be investigated.

Daphnia magna

The waterflea *D. magna*, a commonly used ecotoxicology model, fed powder from *cry1Ab*-expressing Bt maize (MON810) kernels over the whole life cycle showed early reproduction, but reduced survival and fecundity in later life stages compared with *D. magna* fed the near-isogenic maize, indicating a weak toxic effect of the Bt maize [58]. Exposure to the risk of a predator (smell of three-spined stickleback that had eaten *D. magna*) increased the differences in fitness and population growth rate between animals fed Bt maize and non-Bt maize [59]. A follow-up study showed that Bt transgenic leaves were also capable of producing negative effects in *D. magna* after chronic dietary exposure. Animals fed Bt maize leaves showed the typical stress response of producing more resting eggs [61].

Daphnia magna have also been used in testing nontarget effects of Bt rice. An experiment in which water from Bt and non-Bt rice paddies was used as an environment for culturing *D. magna* indicated that the water from Cry1Ab/1Ac- and Cry2A-expressing rice paddies was less toxic than water taken from the non-Bt control paddy. After 13 d, survival of *D. magna* in water from non-Bt water was 0%, whereas survival of those cultured in water from Cry1Ab/1Ac-expressing rice was 60%, and in water from Cry2A-expressing rice it was 52%. Notably, in this study, insecticides were sprayed on both Bt and non-Bt paddies, in a manner that was designed to make the yields of the different rice fields the same, which meant that the non-Bt field received more insecticide. Thus, the mortality in the non-Bt groups likely was because of the higher concentration of insecticides, potentially masking effects of the Bt toxin. The decreased use of insecticides is a frequently cited advantage of growing Bt crops, with environmental benefits, as shown in this study [73]. However, the normal amount of pesticide to use on a rice paddy is not given, nor what the yield of an alternative pest management strategy might have been under similar conditions (omitting the insecticide and the Bt). On the other hand, as the authors point out, insecticide use has led to a drastic reduction in rice paddy biodiversity, a situation that the use of Bt rice has been shown to alleviate [73].

A study from Syngenta showed a reduced growth rate for *D. magna* exposed to high doses (0.75 mg L^{-1}) of microbially produced Vip3A Bt toxin over 10 d [62], indicating weak toxicity or some other physical or chemical effect of the Vip3A test substance. Similarly, exposure to purified Cry1Ab, Cry2Aa toxins ($0.75\text{--}4.5 \text{ mg L}^{-1}$) resulted in higher mortality in a life cycle study by Bøhn et al. [60].

Aquatic vertebrates

In terms of fish, most studies have been done on the use of Bt crops in fish food for commercial species—namely, salmon and zebrafish—rather than fish likely to live in aquatic environments adjacent to Bt crops. Overall, consumption of Cry1Ab-expressing maize did not have a detrimental effect on the survival, growth, and development of either species, although some stress responses were observed in salmon [74–77]. These included decreased activity of certain digestive enzymes [75],

increased activity of superoxide dismutase, and changes in white blood cell population [74]. Conversely, zebrafish growth improved, transcription of superoxide dismutase decreased, and white blood cell populations were not affected by diet [76,77].

Frogs are a common feature of rice paddies and thus have received some attention as nontarget aquatic organisms. A feeding study with the model organisms *Xenopus laevis* [78] and a field study with *Rana nigromaculata* froglets [79] exposed to Cry1Ab/1Ac-producing rice indicated that neither frog species was significantly physically affected by the Bt protein. Some differences in feeding pattern were seen in *R. nigromaculata*, which is unsurprising considering that there was a substantial decrease in stem borers in the Bt rice paddies. Although Cry1Ab/1Ac-expressing rice did not affect tadpole densities, pesticides applied to non-Bt fields did elicit a decrease [79].

Field studies

Field studies have examined the potential influence of Bt crops on communities of organisms, as well as individual species, under realistic conditions. The cost of these realistic conditions, however, is that it is extremely difficult to exclude the possibility that other factors are not confounding. For example, Douville et al. [80] detected fragments of the *Cry1Ab* transgene in tissues of freshwater mussels (*E. complanata*) as well as waterways in proximity to Bt maize fields. At the most exposed site, the level of recombinant DNA in mussel organs was at its highest, and this coincided with a significantly reduced condition factor (weight/length) and oxidative stress in the mussels. Despite this correlation, the authors could not exclude the possibility of an influence from chemical pollutants as possible confounding factors for the decreased condition factor [80].

In 2 studies of overall invertebrate abundance and diversity, neither Swan et al. [81] nor Chambers et al. [36] reported significant effects on arthropod communities that could be explained by the Cry1Ab Bt maize. However, some taxon-specific effects on certain invertebrates were noted by Swan et al. [81]. *Pycnopsyche* sp. was 11 times more abundant on non-Bt maize compared with single-gene Bt maize litter, and *Caecidotea communis* was statistically less abundant on a stacked Bt event (containing both Cry1Ab and Cry3Bb1) than on the non-Bt and single-gene Bt treatments. Both of these studies also reported that overall degradation of the aquatic environment made the influence of a single potential stressor, such as a Bt toxin, difficult to discern.

An increase in abundance of aquatic invertebrates was noted on Bt poplar leaves (which expressed Cry3Aa), when leaves were submerged in the Tavelån stream in Sweden. Of the 2 Bt poplar lines tested, 1 displayed an increase in invertebrate abundance of 33%, whereas the other showed a 25% increase compared with the isogenic control. The community assemblages of the Bt lines were similar to each other, although both differed from the control. This indicates that Bt crops do have the potential to bring about changes in aquatic invertebrate community composition [34], although such changes need not necessarily be detrimental.

In the same vein, a much higher abundance and diversity of zooplankton (rotifers, cladocerans, and copepods) were found in paddies of Bt rice expressing Cry1Ab/1Ac or Cry2A, compared with non-Bt paddies [73]. As with the studies mentioned above involving *D. magna*, this positive effect was attributed to the difference in pesticide regime between Bt and non-Bt rice paddies, and not to the Bt rice as such. When no pesticides were

used, no significant differences in zooplankton density were observed between Bt and non-Bt paddies [39].

There is a need for more field-based studies, and especially for studies that consider food web interactions over time. This includes investigations of transfer of Bt toxins through the food web, as well as possible effects related to prey quality, diversity, and abundance. Interactions of Bt insecticidal sprays (produced using *B. thuringiensis* var. *israelensis* [Bti]), and widely used to control mosquitoes) were investigated in a multiyear study conducted in the wetlands of the Camargue area on the French Atlantic coast. Odonata (dragonfly) populations were reported to be negatively affected because of decreases in Chironomid populations in Bti-sprayed areas compared with control sites. After accounting for factors such as distance from the sea and land cover types between control and Bti-sprayed areas, the authors concluded that Bti use was a potential threat to Odonata [82]. Although the results of food web interaction studies of Bti sprays are mixed, and Bt crops and Bti sprays differ in several ways (limiting the usefulness of direct comparisons), further monitoring of aquatic ecosystems to determine the nontarget effects of Bt toxins is warranted [60,82,83].

Trophic perturbations reaching vertebrate (bird) populations were also indicated in the Camargue region by Poulin et al. [84]. They reported reduced clutch size and fledgling survival of house martins (*Delichon urbicum*), which they explained as an indirect effect of the use of Bti spray, correlating the use of the spray with decreased availability of certain house martin prey (Nematocera and the spiders and dragonflies that feed on them). This study was criticized for having its reference sites too far from its study sites, and a later study by Lagadic et al. [83] in a different area of France did not find any effects as a result of the application of Bt sprays, in agreement with a number of other studies [85–88].

Microbes and horizontal gene transfer

Few studies have been published on the effects that Bt crops may have on aquatic microbial communities, although data from studies on soil microbes suggest that effects will be slight and transient, if they manifest at all [89,90]. Studies on submerged soil communities in rice paddies have followed this pattern. Although changes in microbial phosphatase and dehydrogenase activity have been observed in flooded soils amended with Bt rice straw [91,92], a subsequent study (using the same Cry1Ab-producing rice) did not find this to be the case [93]. In-stream microbial respiration rates were shown to be the same for both Bt and non-Bt maize residues [37].

Considering the huge amount of attention that the possibility of horizontal gene transfer of transgenic fragments (especially antibiotic marker genes) to soil and gut microbes attracted when genetically modified crops were first released, it is surprising that this topic appears to have been almost entirely passed over in the context of aquatic bacteria. This is possibly because of the manifold challenges encountered when trying to detect such occurrences in soil environments (reviewed in Nielsen et al. [94]). That being said, Douville et al. [80] detected *Cry1Ab* transgene fragments in heterotrophic bacteria present in water samples taken from water sources near Bt corn fields. Freshwater mussels (*E. complanata*) taken from the same sites contained *Cry1Ab* transgene fragments in their gills, digestive glands, and gonads. Mussels may, however, also filter small-sized Bt maize particles directly; and although the presence of pollen particles was not indicated by the authors, this is difficult to entirely eliminate as a source. Given the filter-feeding habits of mussels, and the detection of transgene fragments in

heterotrophic bacteria, the authors speculated that bacteria transformed with transgenic DNA might be responsible for its presence in the mussels, and also that such bacteria acted to maintain and stabilize the presence of the *Cry1Ab* transgene in the aquatic environment [80].

ECOSYSTEM-WIDE EFFECTS: MIGHT Bt GENES HAVE COMMUNITY AND ECOSYSTEM PROPERTIES?

When genes have properties that may transform community structure and ecosystem processes, those genes contribute to community or ecosystem phenotypes [95,96]. For example, the variation in condensed tannins (which are genetically controlled) in poplar trees results in multiple effects on higher-level properties of the community and even on ecosystem processes [95]. This case illustrates how a single quantitative trait locus has significant effects on the entire ecosystem: on the community composition, endophyte community, related aquatic community, and also nitrogen mineralization and aquatic decomposition [96]. Poplar trees thus serve as a model system to study how a single but well-characterized gene may affect not only the individual and population, but also organisms that directly and indirectly interact with that gene [95].

Whitham et al. [96] argue that genetically modified organisms with traits, such as insect resistance and herbicide tolerance in genetically modified plants or enhanced growth in genetically modified fish, represent a special case of exotic introductions of ecological novelty with several uncertain effects on community and ecosystem phenotypes. Stacked events with more than 1 *cry* gene will strengthen the plant protection, but at the same time widen the spectrum of potential nontarget and cascading effects in the food web. Moreover, adding herbicide co-technology on top of insect resistance in the same plant will have an increased potential for ecosystem impact.

Unintended effects

Occasionally, changes are wrought in transgenic crops that do not directly relate to the intended activity of the inserted transgene [97]. For example, increased lignin content of Bt maize has been reported [69]. Pleiotropy, positional effects, and insertional effects may bring about unintended changes in gene expression and regulation [98]. In some cases, organisms feeding on Bt crop material have shown negative effects, despite inactivity of the Bt protein (determined through assays with susceptible organisms), leading to questions about unintended effects in these plants.

For example, Jensen et al. [25] observed negative effects on 2 aquatic species: the isopod *Caecidotea communis* and the crane fly larvae *Tippula abdominalis*. This study included single-event Cry1Ab maize as well as a stacked event that contained Cry3Bb1 in addition to Cry1Ab. Crane fly larvae experienced 19.6% less growth as well as lower mass when fed Bt maize compared with non-Bt maize. *Caecidotea communis* fared worse under Bt feeding regimes (both single-gene and stacked events) compared with non-Bt isolines. Negative effects were noted for body length (49.7% less), final mass (50% less), and survival (43.3% decrease). Considering that a sensitivity assay performed with *Ostrinia nubilalis* on the preconditioned maize leaves found no bioactivity of the Bt toxins past 2 wk of environmental exposure, the authors suggested that the effects seen were the result of tissue-mediated factors or micronutrient differences between the near-isolines, rather than the Bt toxins themselves.

Li et al. [99] tested the effects of Cry1C- and Cry2A-producing rice lines on *Propylea japonica* and noted that larval development was significantly longer when Bt pollen was fed compared with control pollen. However, when the toxins were provided in their pure forms (mixed with rapeseed pollen) at more than 10 times the concentration in the rice pollen, *P. japonica* was not affected. This suggests that unintended changes were induced in the rice genomes when the transgenes were inserted, which caused the negative effects on the phenotype observed.

Stacked events and resistance evolution

A challenge for the use of Bt transgenic crops is to ensure that their solution to insect attacks is sustainable (i.e., that the solution will last over time). The development of resistance to Bt toxins in pest insects, for example, in South Africa, India, China, South America, and the United States [100–103], has shown that pest insects may evolve quickly to regain food resources protected by Bt toxins. Resistance development has led to a gradual replacement of the first-generation Bt crops that had single *cry* genes only. A growing majority of Bt crops now contains 2 to 6 *cry* toxin genes at the same time [104,105]. This means that the total expression of Bt toxin per plant can be expected to multiply by a factor of 2 to 6, which may lead to increased negative effects on a range of nontarget organisms. In addition to the higher total expression of Bt toxins in stacked events, the fact that different Bt toxins aim at target pest species from different taxa is likely to expand the range of sensitive nontarget organisms. Industry data show that stacked Bt-expressing plants may express higher levels of Bt toxins compared with the mother lines. For example, Smartstacks maize expresses on average Cry1A.105 at levels 54% and 97% higher for grains and pollen, respectively, than the parent line MON89034 [106].

Mass-sequencing programs may offer huge potential for the discovery of new Bt toxin sequences in future [107]. This may help to manage the increasing occurrence of resistance to Bt-based insecticides or transgenic Bt crops [108].

Added or combinatorial effects of stacked events?

The general trend is that Bt crops with only insect protection seem to be on their way out, replaced by stacked events with HT as well. For example, by 2012, nearly twice as large an area of genetically modified crops with both insect protection and HT traits (mainly for glyphosate) were grown, compared with plants only expressing Bt toxins. In South Africa, 64% of the production of Bt maize in 2014 was also HT [4]. In the United States, in 2015, approximately 90% of the maize grown carried HT traits [109]. This means that herbicide co-technology regularly co-occurs with Bt toxins in the environment. Glyphosate-based herbicides are the most common herbicide cotechnologies used in conjunction with HT crops.

Herbicide-tolerant crops will always be sprayed by the farmer, as they are specifically designed to be used in combination with herbicides and cannot fulfil their function without them. Because of the development of glyphosate resistance in weeds, the amount of active ingredient used has increased both per hectare and in total. A 15-fold rise in glyphosate use has been documented globally since the introduction of Roundup Ready HT crops in 1996 [110]. Surprisingly, co-technology herbicides are usually not tested as a part of the risk-based science and assessment of HT crops. For example, in 13 of 16 published feeding studies with such crops, test material had not been sprayed with the relevant

herbicide, which represents a serious flaw in the testing procedures [111]. This may lead to underestimated effects of such material on nontarget species, not least in aquatic ecosystems (e.g., exposed to runoff crop residues), where it is documented that glyphosate/Roundup is more toxic than previously reported [112,113]. Furthermore, safety testing (both environmental and health) of the agrochemicals themselves tends to focus on the active ingredient of the herbicides (e.g., glyphosate), and not the commercial formulations (e.g., Roundup), resulting in unrealistic and misleading safety assessments because the influence of adjuvants is excluded from the testing regime [114,115].

Stacked events are relatively easy to produce, as conventional breeding is used to combine 2 or more events that are already approved singly for the market. For example, the maize hybrid MON 89034 × 1507 × MON 88017 × 59122, from Monsanto and Dow AgroSciences, is broadly resistant to insects with 6 different *cry* genes (*cry1A.105*, *cry1F*, *cry2Ab2*, *cry3Bb1*-, *cry34Ab1*, and *cry35Ab1*) and is tolerant to both glyphosate and glufosinate ammonium herbicides. Multiple-trait stacked crops may be expected to have a wider range of effects [116], both on target pest insects (as intended) and on nontarget organisms (unintended and unwanted) from their multiple Bt toxins. However, very few studies have tested plant material from multi-Bt crops.

The genetically modified crops recently approved for the market in some countries have tolerance to several herbicides, such as different combinations of glyphosate, glufosinate ammonium, 2,4-D, and dicamba. For example, the DAS-44406 soybean is tolerant to glyphosate, glufosinate, and 2,4-D [117]. Such tolerance to multiple herbicides is already combined with multiple Bt toxins—up to 6 Cry toxins in the case of the Smartstacks maize from Monsanto. Thus, multiple Bt toxins will co-occur with 1 or several cotechnology herbicides in the same environment. Research on interactions and potential synergies between these toxins is crucial [116].

As innovation in agriculture moves toward more complex stacked transgene combinations, with multiple insect toxins and tolerance to multiple cotechnology herbicides/pesticides within the same plant, there is an increasing need to test their potential environmental consequences, both as single compounds and as combinations, and to assess their risks. One assumption, endorsed by the Food and Agriculture Organization, World Health Organization, the Organisation for Economic Co-operation and Development, the International Seed Federation, and Crop Life International, is that stacked transgenic events will function as a sum of their separate parts [118]. Accordingly, the safety of stacked transgenic events can be based on previous safety assessments of the single-transgene parental events; that is, no relevant interactions between the stacked traits are expected. This is controversial, and it is increasingly acknowledged that studies of combinatorial effects of multiple stressors are missing, for example, by the European Food Safety Authority (GMO Panel Working Group on Animal Feeding Trials [119]) and elsewhere [120]. Bøhn et al. [60] showed that coexposure to Cry1Ab and Cry2Aa resulted in higher mortality in the aquatic waterflea *D. magna*, supporting the hypothesis that stacked events may cause stronger effects on nontarget organisms.

In addition to the potential additive (or combinatorial) effect of multi-Bt crops, the spraying of agricultural fields throughout the growing season with 1 or several herbicides on stacked genetically modified crops (i.e., Bt/HT) will add stress for

aquatic organisms that live in nearby aquatic ecosystems. Bioactive herbicides ultimately enter into water courses directly or indirectly through processes such as drifting, leaching, and surface runoff [121]. Negative effects of herbicides are documented for a number of aquatic species related to the most relevant of the herbicides, glyphosate-based formulations (Roundup), including for amphibians [112,122–124], shrimps [121,125], and waterfleas [113].

CONCLUSIONS AND RECOMMENDATIONS

The Bt and HT crops are in many ways the pioneers of the genetically modified crop movement. The reasons for highlighting the gaps in monitoring and risk assessment are not simply to determine whether these specific crops themselves are problematic, but to consider gaps in the risk assessment of genetically modified crops generally. Almost 10 yr of planting Bt crops had passed before aquatic ecosystems were seriously considered within risk assessment, and another decade has passed since then with limited improvement. The decline in aquatic biodiversity attributable to pesticides and agrochemicals is well documented [123,126,127]; but even so, approximately 90% of major agricultural areas worldwide have not been included in investigations of pesticide concentrations in surface water [128], and the possible contribution of Bt crops to this situation has not been considered. There are significant knowledge gaps about the fate of Bt crops and their potential effects in aquatic systems. We recommend that the following issues be further investigated.

Other Bt genes/proteins

The research relating to aquatic organisms that has been done up to now has focused primarily on single-gene events (mostly *Cry1Ab*) in maize or rice. Other crops and Bt genes/toxins should also be investigated. This includes the amount of plant material that reaches the aquatic system, the rates of toxin release and breakdown, and the influence of factors such as temperature, biotic and abiotic factors in the environment, and potential effects on aquatic organisms.

Effects of stacked events

Risk assessment is currently operating under the assumption that a stacked event is no more than the sum of the parts of the single genes that were combined to make the stack, and that there are no interactions between the components. However, this has been disputed [129]. Instead of relying on assumptions, the possible effects of stacked events (some of which have yet to be investigated as single events; see *Other Bt genes/proteins*) should be researched.

Bt expression and unintended effects

Patterns and concentration levels of both endogenous proteins and Bt toxins in Bt crops, especially stacked events, merit further investigation. Variability in expression may be altered by the event, the environmental conditions, cotechnologies used (e.g., herbicides), climate, stress, and so forth [130–132]. Cases of adverse effects when the Bt toxin was determined to be inactive may indicate possible unintended (pleiotropic, insertion and position) effects in genetically modified Bt crops [25]. High-throughput sequencing, proteomics, and metabolomics may all shed light on such effects [129,133], which are of interest not only in terms of environmental effects but also for the refinement of the genetic engineering process.

Combinatorial effects of Bt and herbicide cotechnology

Although the present study has focused on Bt crops, in many cases the stacked nature of Bt/HT crops makes the issue of their potential environmental effects inseparable. The potential additive or synergistic effects of Bt and herbicide cotechnologies should be investigated.

Determining which aquatic species are relevant to test

Several perspectives exist on which organisms to test, but what is still missing for aquatic ecosystems is exposure surveys such as those performed by Yu et al. [64], which measured the amount of Bt protein present in terrestrial arthropods in Bt soybean fields. The advantage of this approach is that it provides baseline data on which organisms are exposed, illuminates possible tri-trophic relationships and food web interactions, and may also indicate community-level responses. Investigations of the levels of co-technology herbicides present would be an interesting expansion of such studies.

Acute dose–response toxicity testing of Bt on relevant aquatic species

Dose–response experiments, including additional control groups fed nontransgenic material with purified Bt toxin added, would assist in distinguishing the effects of Bt toxin from other effects of the genetic modification process (see Li et al. [99]). This may also assist in recognition of potential alternative modes of action of Bt toxins. Modes of action of Bt toxins in adversely affected nontarget organisms outside the target order are largely undescribed [8,63,134].

Pulsed, chronic exposure studies

For crops such as maize, the entry of plant material and Bt protein into the aquatic system is likely to take place fairly consistently, with spikes at times of pollen shed and harvest, or after rain [20]. Studies that reflect this pattern are more likely to reflect natural conditions than single-exposure regimes. The consideration of plant material as a delivery system for herbicides and pesticides (in addition to Bt toxins) to aquatic ecosystems, and the role of riparian buffers in limiting this, should also be investigated.

Field studies

In nature, individuals and populations often live under stress (e.g., from competitors, predators, parasites), so that even a small additional stressor may turn out to be critical for their survival. Amphibians die from much lower levels of certain pesticides when they are under stress (predator present) than without the predator [122,135,136]. Laboratory experiments are thus not representing worst-case-scenarios [137], because the natural stress factors and variation in the environment is changed to stable and mostly favorable conditions in the laboratory. Therefore, an integrated approach based on test protocols in the laboratory should be linked to food web characteristics and trophic roles as well as early warning systems and computer simulation models [138]. Field studies under realistic conditions are difficult but necessary.

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