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Daphnia magna negatively affected by chronic exposure to purified Cry-toxins

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ABSTRACT

Cry-toxin genes originating from *Bacillus thuringiensis* are inserted into genetically modified (GM) plants, often called Bt-plants, to provide insect resistance to pests. Significant amounts of Bt-plant residues, and thus Cry-toxins, will be shed to soil and aquatic environments.

We exposed *Daphnia magna* to purified Cry1Ab and Cry2Aa toxins for the full life-span of the animals. We used single toxins in different doses and combinations of toxins and Roundup®, another potential stressor on the rise in agricultural ecosystems.

Animals exposed to 4.5 mg/L (ppm) of Cry1Ab, Cry2Aa and the combination of both showed markedly higher mortality, smaller body size and very low juvenile production compared to controls. Animals exposed to 0.75 mg/L also showed a tendency towards increased mortality but with increased early fecundity compared to the controls. Roundup® stimulated animals to strong early reproductive output at the cost of later rapid mortality.

We conclude that i) purified Cry-toxins in high concentrations are toxic to *D. magna*, indicating alternative modes-of-action for these Cry-toxins; ii) Cry-toxins act in combination, indicating that 'stacked events' may have stronger effects on non-target organisms; iii) further studies need to be done on combinatorial effects of multiple Cry-toxins and herbicides that co-occur in the environment.

1. Introduction

Most genetically modified (GM) crop plants grown on a commercial scale have two classes of traits built into their genome: (i) *cry* genes from the microorganism *Bacillus thuringiensis* (Bt crops) encoding for a range of Cry proteins, which are insecticides in theory targeting a narrow range of pest insects; (ii) *epsps* (and/or other) genes that make the plants herbicide tolerant (HT), to Roundup/glyphosate (and/or other) herbicides. Both classes of traits, insect resistance and herbicide tolerance, are increasingly used together, i.e. 'stacked' in the same plant. The commercial success and thus dominance of stacked events increases the runoff of both Cry-toxins and herbicides with unclear consequences for adjacent aquatic ecosystems.

Built-in insecticides may reduce the use of broad spectrum insecticides used in conventional industrial production, arguably resulting in a positive impact on the environment since internal insecticides target grazing organisms more specifically compared to spraying. Accordingly, higher abundance of non-target invertebrates have been observed in Bt-transgenic maize fields as compared to conventional fields sprayed with insecticides (Marvier et al., 2007; Naranjo, 2009). However, indications of some negative effects of the Cry1Ab toxin itself or of Cry1Ab maize plants, on non-target abundance were shown in the same meta-analyses: when conventional (non-GM) fields were not sprayed, the non-target abundance was significantly higher than in the Bt-fields. A similar result was reported on arthropod communities in sprayed and unsprayed Cry1Ac-transgenic and conventional cotton in Australia (Whitehouse et al., 2005).

Weed and pest resistance evolution to herbicides and built-in insecticides will arguably lead to increased doses/more applications of herbicides per season and a broader range of Cry-toxins in GM plants. Such development emphasizes the importance of potential environmental impact of these technologies. In addition,

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since these chemicals or 'traits' will meet and interact in stacked events as well as with other stressors in the environment, the *co-exposure* and potential *combinatorial effects* need to be studied (Bjergager et al., 2011; Nørgaard and Cedergreen, 2010; Then, 2009). Synergistic effects, i.e. stronger effects than expected from an additive model of toxicity, have been documented on target organisms, but there is a lack of knowledge of responses in non-target organisms (van der Hoeven, 2014). However, some studies on non-target insects document negative and synergistic effects among Cry-toxins, between Cry/Cyt-toxins, and between Cry-toxins and insect and plant-toxins (reviewed in Hilbeck and Otto, 2015). Direct and indirect potentially negative effects of Cry-toxins and agrochemicals are in addition relevant for a number of non-target species in the agroecosystem. This includes soil (Saxena and Stotzky, 2000; Saxena et al., 2002) and aquatic communities that receive runoff residues of plants, toxins and pesticides (Bøhn et al., 2012; Douville et al., 2009, 2007, 2005; Rosi-Marshall et al., 2007).

The water flea *Daphnia magna* is a key-stone species in aquatic ecosystems with a wide geographical range. Its ecology, life-history, genetics and responses to changes in the environment and to toxic effects of chemicals are understood to a very high degree, also with analyses of the genome and transcriptome (Asselman et al., 2012; Colbourne et al., 2011; Orsini et al., 2011). Because of its central position in aquatic food webs and its accessibility for field and laboratory testing, this species is invariably included in toxicological research for chemical effects on freshwater ecosystems.

In risk assessment, toxicological tests have often concentrated on a single acute effect like mortality or immobility after short-term exposure (in the 48 h range) and on adult or mature animals (Andow and Hilbeck, 2004). For example, no treatment-related adverse effects were observed in adult *D. magna* after exposure to 100–150 mg/L Cry1Ab or Cry1F maize pollen for 48 h (Mendelson et al., 2003). A ten days study exposing *D. magna* to high concentrations of the Cry-toxin Vip3A showed indications of negative effects as the size and body mass of animals exposed were significantly reduced compared to the control, although survival and reproduction were not affected (Raybould and Vlachos, 2011).

However, exposure to the relevant Cry-toxins in the field will last over time, eventually covering the whole life-cycle of the organisms living there. Direct, indirect and chronic effects (e.g. on survival, growth, reproduction) on non-target organisms are therefore all realistic and relevant to investigate.

Feeding studies using Cry1Ab maize have shown lethal and sub-lethal negative effects after long term exposure in *D. magna*, indicating allocation trade-offs based on weak toxic responses to Cry1Ab toxin itself, or to the Bt-maize plant material, tested as kernels (Bøhn et al., 2008) or leaves (Holderbaum et al., 2015), with the near-isogenic non-GM maize as comparator. Indications of a synergy between toxin/plant effects and another stressor (predation risk) were also reported, including sensitivity analyses for different age-classes of the test organism (Bøhn et al., 2010).

These observed adverse long-term effects of Cry1Ab-toxins or Bt-transgenic GM plants on non-target organisms (without the relevant receptors described in Lepidoptera) indicate that Cry-toxins may have alternative and more complex modes-of-(inter) actions (Vachon et al., 2012) that can harm non-target organisms. However, previous studies in *D. magna* have not been able to separate effects of the Cry1Ab toxin and other potential changes in the GM plant (Bøhn et al., 2008).

In this study, in order to overcome some previous shortcomings in the study of potential non-target effects of Cry-toxins, we exposed *D. magna* over the entire life-span of the test group (78 days) to various combinations of purified Cry1Ab, Cry2Aa toxins and one formulation of Roundup, measuring a range of life-history traits. The experimental set-up also explored potential synergies of Cry-toxins and Roundup, which will co-occur in the environment.

Based on these earlier findings, we hypothesized that.

i) Cry1Ab, Cry2Aa and Roundup negatively affect survival, growth and reproduction of *D. magna*.
ii) Effects of Cry1Ab and Cry2Aa are dose-dependent.
iii) Exposure to both toxins simultaneously will cause additive effects.
iv) Exposure to Cry-toxins and Roundup will cause additive effects.
v) Sub-chronic effects of Cry-toxins/Roundup will lead to an allocation trade-off with priority of early reproduction at the cost of higher mortality in later life stages.

2. Methods

2.1. Experimental set up

All individuals of *D. magna* used in the experiments were born within 24 h from the third clutch of a single clonal population. In total, 120 juvenile individuals were randomly chosen and assigned to separate glasses with 50 mL M7 medium. Twenty animals were used as controls and ten animals were used for each of the other treatments with Cry1Ab, Cry2Aa and Roundup in different concentrations and combinations (summarized in Table 1). All animals were distributed on four trays that were given a new randomized position on the bench every third day to avoid potential bias. Variable *Tray* was later used as a co-variate in statistical models to test whether there had been any bias related to positioning of the animals on trays. As the effect of *Tray* was negligible throughout all analyses, variable *tray* was discarded from further analyses.

All individuals were fed *Desmodesmus subspicatus* green algae daily for the first 36 days, later every 3rd day. In the first 3 days, the feed concentration was 0.1 mg Carbon per day per animal. Thereafter, the feed concentration increased to 0.15 until day 23 and later 0.2 mg C per animal per day.

Every third day, we transferred each animals to a new glass with new medium that contained freshly made chemicals for each treatment, using a broad-tipped pipette. Thus, we had full control with the medium, including Cry1Ab and Cry2Aa toxin concentrations, Roundup, pH, oxygen and conductivity at the start of every three-day period, throughout the whole experiment. The experiment lasted for 78 days, at which the last animal from the control group died. Temperature was held constant at 22 + −1.5° C. Light regime was 16 h of light and 8 h darkness. The pH of the medium was 7.9 (range 7.7–8.1), oxygen saturation > 97% and conductivity 595 μs cm^{-1} (in control group, range for all treatments 594–608). Roundup at 1.35 mg/L reduced the pH with approximately 0.1 unit.

Table 1
Treatments design in the experimental set-up.

Treatment	Cry1Ab (mg/L)	Cry2Aa (mg/L)	Roundup (mg/L)	N=
Control				20
1	0.75			10
2		0.75		10
3	0.75	0.75		10
4	4.5			10
5		4.5		10
6	4.5	4.5		10
7			1.35	10
8	0.75		1.35	10
9		0.75	1.35	10
10	0.75	0.75	1.35	10

We measured oxygen concentration and pH to be stable through the three-day period of a given medium. The conductivity increased slightly (<10%) over the same three days, on average to 640 μs cm^{-1}. The clone of *D. magna* used was provided by Prof. Dag Hessen, University of Oslo and kept in the testing laboratory in Tromsø for many generations.

Cry1Ab and Cry2Aa toxins, sterile protein extracts produced in *E. coli*, were purchased from Dr. Marianne Carey, Department of Biochemistry, Case Western Reserve University, Cleveland, US. Cry-toxins were carefully weighed and dissolved in a small amount of buffer at pH 10.5 before pipetting into the relevant treatment. We consistently used low-bind pipettes to reduce binding and loss of cry-toxins during handling.

For the treatments with Roundup, we used a commonly used commercial formulation of Roundup (360 g/L) purchased in South Africa.

2.2. Measurements

Survival/mortality and juvenile counts were noted for each animal daily until day 36 and thereafter every third day until all animals were dead. Juveniles were removed, photographed until day 30, and discarded after each count. Size of adults was checked every 9 days for the first 36 days and later every 18 days by carefully taking them out of the glasses with pipettes and photographing them under a microscope with a high resolution digital camera (Nikon D300). After photographing, adults were put back into their respective glasses. The length was measured on digital images from the top of the head to the base of the caudal spine with the Image J software, calibrated with a micro-scale.

2.3. Statistical analyses

All statistical analyses were performed with the R software, version 3.1.1.

2.3.1. Survival

The binomial response variable "*survival*" (i.e. probability or proportion of survival) was analyzed with Cox's proportional hazard (Cox, 1972) test (Coxph function) from the *Survival* package in R. No censoring in the survival data-set was needed since we terminated the study when all individuals were dead. We estimated hazard ratios or risks of death and tested deviations in treatment groups from the control group.

In addition, to present the survival curves graphically, we constructed a generalized linear model with binomial distribution. While it leads to the same overall conclusion as the cox proportional hazard model, we will not discuss it further and use its estimates primarily for data visualization.

2.3.2. Body size

Body size, both of juveniles and adults, was analyzed with a linear model (lm function in R). To test if treatment effects changed over time, an interaction between treatment and day of experiment was used as predictor in the full model (lm (size ~ treatment * day)). Since measurements were only done on a few discreet points of times, day was defined as a factor rather than a continuous variable.

2.3.3. Reproduction

The binomial response variable "*maturation*" (immature, mature) was analyzed with Cox's proportional hazard test. Day of maturation was defined as the day when the first clutch of juveniles hatched. As for the survival data, we present the estimates of a generalized linear model with binomial distribution for data visualization.

The response variable "*juvenile counts*" was analyzed with a generalized additive model (*gam* function of the mgcv package) with an assumed Poisson distribution. Visual data exploration showed a distinct break point in the slope of juvenile production in some of the treatments (i.e. slope changes as the study progresses), which can be modeled with additive models. We assumed one break point (k = 3 term in the *gam* function) to avoid 'over-fitting' the model. Again, to test if the temporal development of juvenile productivity differed between treatments, an interaction of treatment and day of experiment was included in the full model (simplified gam (juvenile count ~ day * treatment, k = 3, family = quasipoisson)). This analysis models the average number of juveniles per adult which is still alive at any given point of time. Predicted values from this Poisson model were back transformed (log link) and presented as counts per living adult.

Simplification of all models except the gam was performed with Akaike's information criterion (AIC) (Zuur et al., 2009) with a threshold of 2 after step wise removal of higher order predictor terms. The gam model on juvenile counts was over-dispersed, leading to the use of the quasi-poisson distribution, which do not allow the calculation of AIC. We used a p-value threshold of 0.05 of the interaction terms as the deciding factor. Only estimates of minimal models are used for the discussion. Significant differences between predictors can be observed when the estimated 95% confidence intervals (CIs) does not cross the mean value of another estimate and vice versa (Smith, 1997).

3. Results

3.1. Survival

D. magna exposed to 0.75 mg/L of Cry-toxins showed higher mortality, compared to controls, but differences were significant only for animals exposed to both Cry1Ab and Cry2Aa toxins simultaneously ($p = 0.0019$, Coxph test) (Fig. 1a and Fig. 2). Animals exposed to 4.5 mg/L of Cry1Ab, Cry2Aa and particularly the combination of both toxins, showed markedly higher and earlier onset of mortality compared to the control group ($p < 10^{-7}$ for all, Coxph test) (Figs. 1b and 2).

Animals exposed to Roundup alone showed high survival early in life, higher than controls, but rapid mortality after day 33 when all animals in that group died within 12 days (Fig. 1c). At that time (day 45), 70% of animals in the control group were still alive. Hence, overall mortality was higher in the Roundup only treatment than in controls (Figs. 2, $p = 0.0138$, Coxph). The effects of 0.75 mg/L Cry-toxins were stronger in combination with Roundup (Fig. 1c vs Figs. 1a, and Fig. 2) although survival in early life-stages was high. Animals exposed to Roundup plus 0.75 mg/L Cry1Ab, Cry2Aa or both toxins had significantly higher mortality than the controls ($p = 0.0003$, $p = 0.0034$ and $p < 10^{-4}$, respectively, Coxph).

3.2. Body size

At day 9 of the experiment, *D. magna* exposed to high concentrations (4.5 mg/L) of Cry1Ab, Cry2Aa and in particular, the combination of both toxins showed smaller body size than all other groups (Fig. 3). Animals exposed to Cry-toxins at 0.75 mg/L had larger body sizes than the controls, from day 18 and later, particularly in the Roundup groups (Fig. 3).

The body size of born juveniles closely mirrored the adult body size (c.f. Figs. 3 and 4). Mothers exposed to 4.5 mg/L of Cry1Ab, Cry2Aa, and in particular the combination of both toxins, produced smaller juveniles, whereas mothers exposed to 0.75 mg/L of Cry-toxins, without or with Roundup produced offspring with larger body sizes compared to controls (Fig. 4). The strongest stimulation

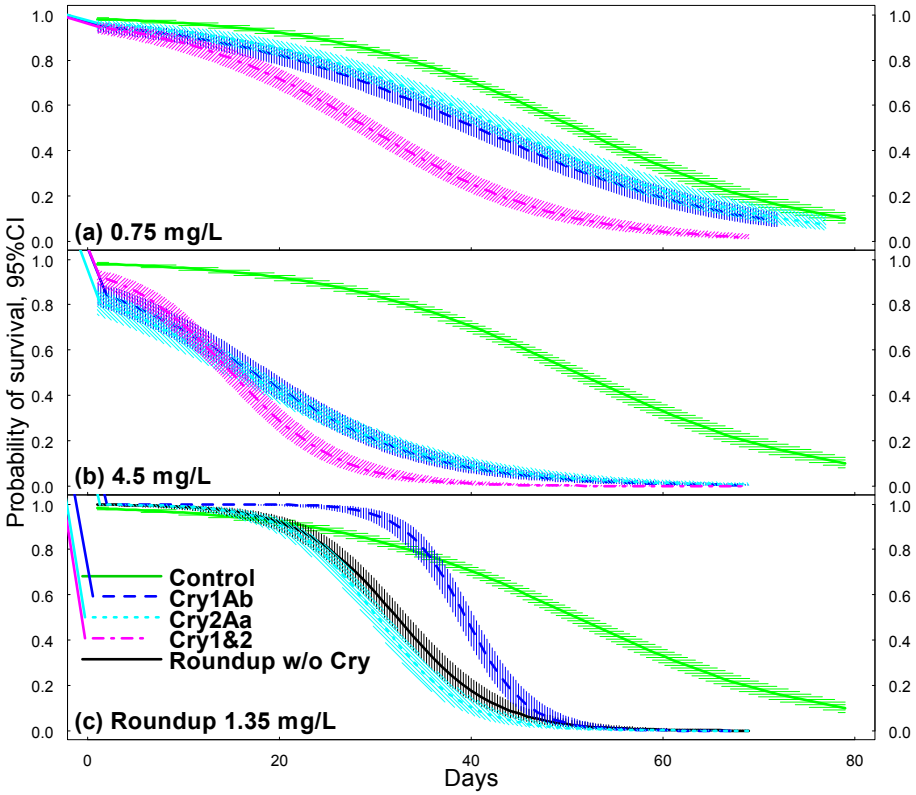

Fig. 1. Survival curves (based on a generalized linear model) for *D. magna* exposed to (a) 0.75 mg/L and (b) 4.5 mg/L of Cry1Ab, Cry2Aa toxins (as single toxins and combined), and (c) 0.75 mg/L of Cry1Ab and Cry2Aa (as single toxins and combined) co-exposed with 1.35 mg/L of Roundup. Black curve represents animals exposed to 1.35 mg/L of Roundup without Cry-toxins. Shaded bands show 95% confidence limits.

effect on juvenile body size was for the Roundup groups. The combination of Cry-toxins and Roundup however, reduced the juvenile body size compared to Roundup only, particularly when both toxins were present (Fig. 4).

3.3. Maturation

Whereas most animals in the control group matured between days 9 and 10, animals exposed to 0.75 mg/L of Cry1Ab or Cry2Aa matured between day 8 and 9. Animals exposed to both Cry-toxins at 0.75 mg/L showed however, a deviating pattern with some animals maturing much later (Fig. 5a).

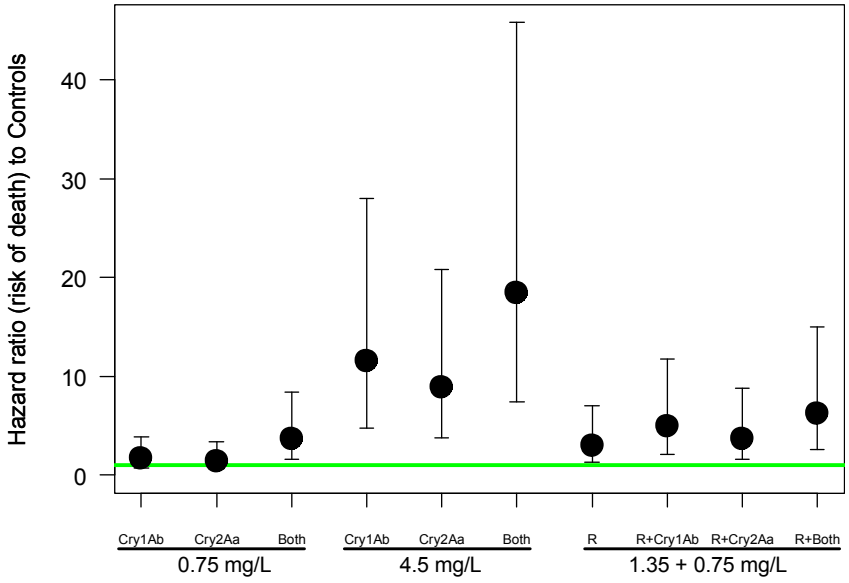

Fig. 2. Risk of death in treatment groups compared to controls, estimated by cox proportional hazards analysis. Horizontal line at 1 illustrates baseline control mortality. Values above 1 means higher risk. If 95% confidence intervals overlap 1, then difference is not significantly different.

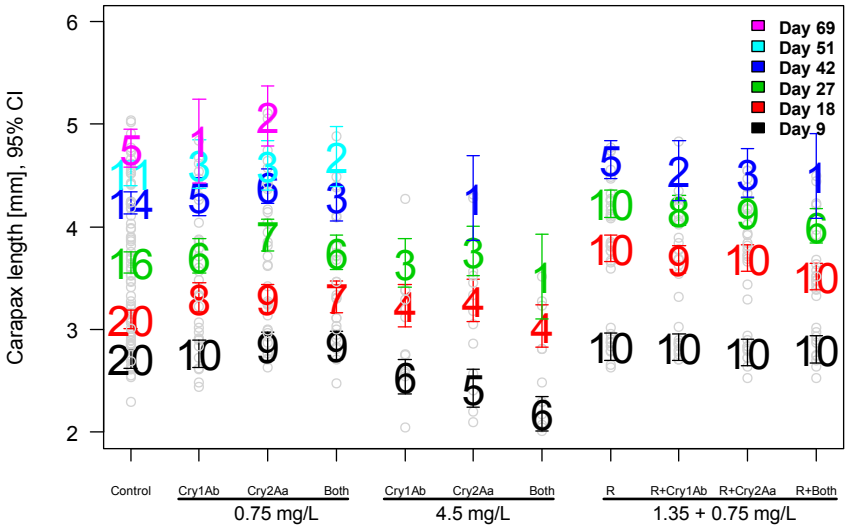

Fig. 3. Average body size of *D. magna* exposed to Cry-toxins and Roundup treatment combinations at different time points. Numbers in the plot are at position of estimated average body size and denote the amount of animals alive at that time point. Light grey circles show raw data. Error bars show 95% CI.

Animals exposed to 4.5 mg/L of Cry-toxins showed a markedly reduced ability to reproduce at all; none of the groups reached 50% maturation (Fig. 5b). Again, the combination of both toxins performed less well compared to the single toxins.

Groups exposed to Roundup and 0.75 mg/L of toxins matured consistently earlier than the controls. Cry-toxins in addition to Roundup stimulated early reproduction even further compared to Roundup only (Fig. 5c).

3.4. Fecundity

The fecundity of animals exposed to low concentrations (0.75 mg/L) of Cry 1Ab, Cry2Aa and the combination of both was somewhat higher than in the controls, particularly in early life stages (Fig. 6a).

Juvenile productivity was increasing with time for both control and low concentration groups. However, this increase was slower in the Cry1Ab and Cry2Aa groups ($p = 0.003$, $p = 0.029$, respectively; slopes from glm), while it was not different for the combination of both toxins.

Animals exposed to high concentrations (4.5 mg/L) of the toxins and their combination showed a strongly reduced fecundity throughout their lives (Fig. 6b); they had consistently lower accumulative (accum fig b) and daily juvenile productivity than the controls ($p < 10^{-14}$, $p = 0.0005$, $p = 0.009$ for Cry1Ab, Cry2Aa, and combination, respectively; slopes from glm).

The Roundup groups all showed a strong stimulation of fecundity in early life stages compared to controls, both with and without Cry-toxins ($p < 10^{-12}$ for all; intercepts from glm), but with stagnation and drop around day 35. In contrast, the control group continued to produce juveniles for about 50 days and had a significantly higher slope for accumulative fecundity than treatment groups ($p < 10^{-8}$ for Roundup alone, Roundup + Cry1Ab/Cry2Aa, and $p = 0.024$ for Roundup + combination; slopes from glm) (Fig. 6c).

4. Discussion

We demonstrate that exposure to purified Cry1Ab and Cry2Aa toxins, in high concentrations (lower ppm-levels), has dose-

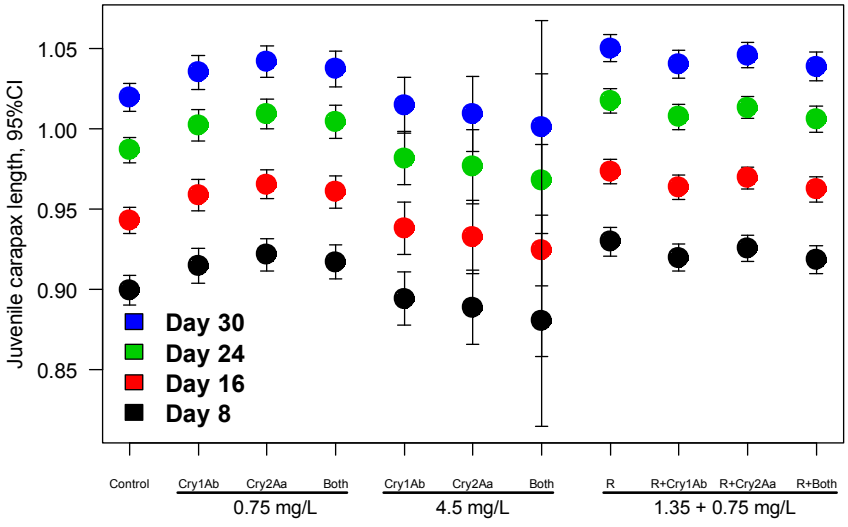

Fig. 4. Body size of juveniles at different time points and treatments. Error bars denote 95% CI.

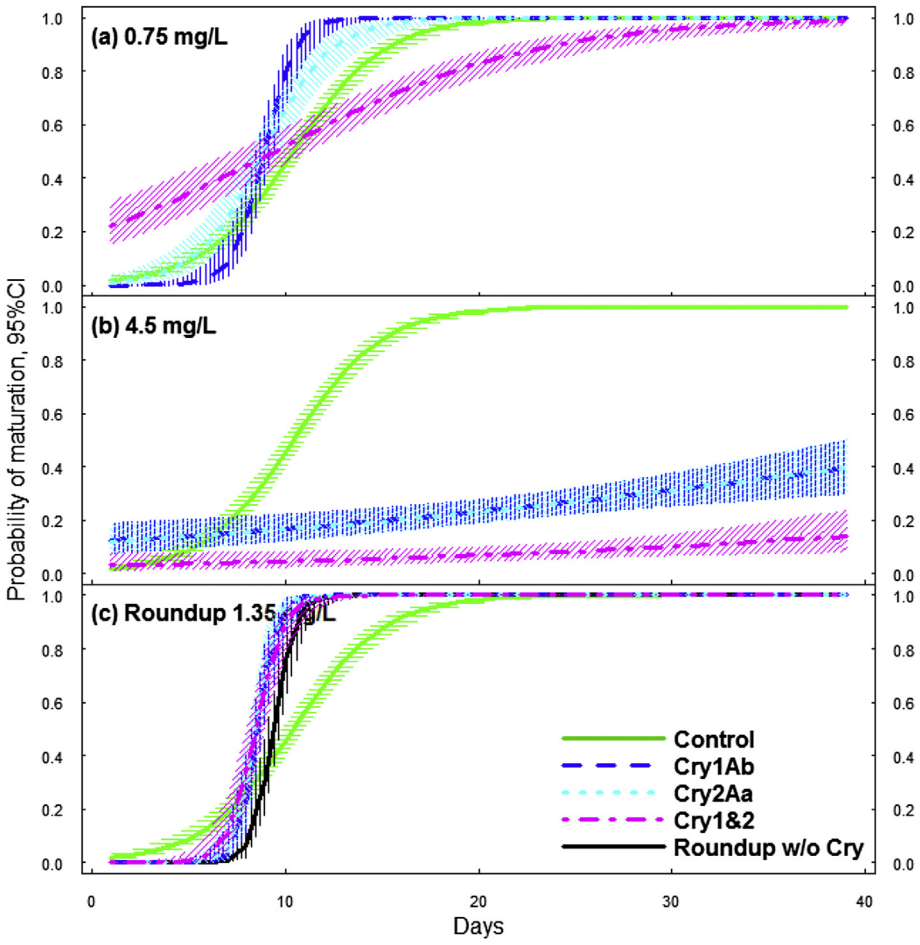

Fig. 5. Probability of maturation (based on a generalized linear model) for *D. magna* exposed to (a) 0.75 mg/L and (b) 4.5 mg/L of Cry1Ab, Cry2Aa toxins (as single toxins and combined), and (c) 0.75 mg/L of Cry1Ab and Cry2Aa (as single toxins and combined) co-exposed with 1.35 mg/L of Roundup. Black curve represents animals exposed to 1.35 mg/L of Roundup without Cry-toxins. Shaded bands show 95% confidence limits.

dependent lethal and sub-lethal effects on the aquatic water flea *D. magna*, supporting our two first hypotheses.

These results add to and build on previous findings where negative effects were documented in *D. magna* after feeding Cry1Ab maize material, both from kernels (Bøhn et al., 2008, 2010) and leaf material (Holderbaum et al., 2015). These previous studies can be criticized for the fact that it may be hard or impossible to single out whether the negative effects were caused by the transgenic product in the plant, i.e. the Cry1Ab toxin, or from other, confounding differences (e.g. from insertional effects) between the transgenic and the near isogenic control maize material (Bøhn et al., 2008). The data we present here, based on controlled toxin and feed conditions support the hypothesis that the transgenic toxins can play a role in the toxicity of whole plant material.

While in previous studies toxins were presented in the plant matrix, here we dissolved purified toxins in the medium. Thus, the exposure was *environmental and not by feeding*. These two different exposure pathways, as well as the total toxin concentrations present in the medium/feed, cannot be compared directly and need to be discussed in some detail.

Exposure by *feeding* on maize material containing Cry-toxins means to actively capture, swallow and digest the material. The toxins are actively brought to the gut system, following the intended exposure pathway in target species in terrestrial GM maize production systems. In contrast, *environmental exposure* represents unintentional release or leaching of toxins from transgenic plant material into surrounding ecosystems. The toxins used in our study were, similarly, dissolved and added to the water medium. Cry-toxins present in the environment can be taken up in various ways, e.g. through gills, skin, etc., or by feeding on green algae that has adsorbed Cry-toxins (see below). The exposure pathway by feeding, i.e. internal exposure, can be expected to be orders of magnitude more potent, in terms of effects, compared to external exposure to toxins dissolved in the surrounding environment, as we have tested in this article.

The distinction between exposure through feeding and indirect environmental exposure is not clear-cut. Cry-toxins from maize material in an aquatic ecosystem (pond or stream) can be directly taken up through feeding by non-target communities of grazers (leaves) (Rosi-Marshall et al., 2007) and filter-feeders (small particles) (Douville et al., 2009; Rosi-Marshall et al., 2007). Still, as the maize material gradually decomposes, Cry-toxins are released from the cells they were produced in and dissolve into the water. Aquatic ecosystems containing Bt-transgenic plant material may thus be exposed to both these pathways: feeding and environmental exposure. Moreover, the input of Bt-containing crop debris from harvest to planting may cause an accumulation of Bt-toxins in aquatic systems (Strain and Lydy, 2015). Measured concentrations of Cry-toxins in water bodies have however shown low concentrations, i.e. up to 0.032–1.9 ppb (parts per billion) and with a half-life in the range of days and longevity of Cry1Ab toxin for about two months (Douville et al., 2005; Strain and Lydy, 2015; Tank et al., 2010).

A third exposure pathway in aquatic systems is the ingestion of

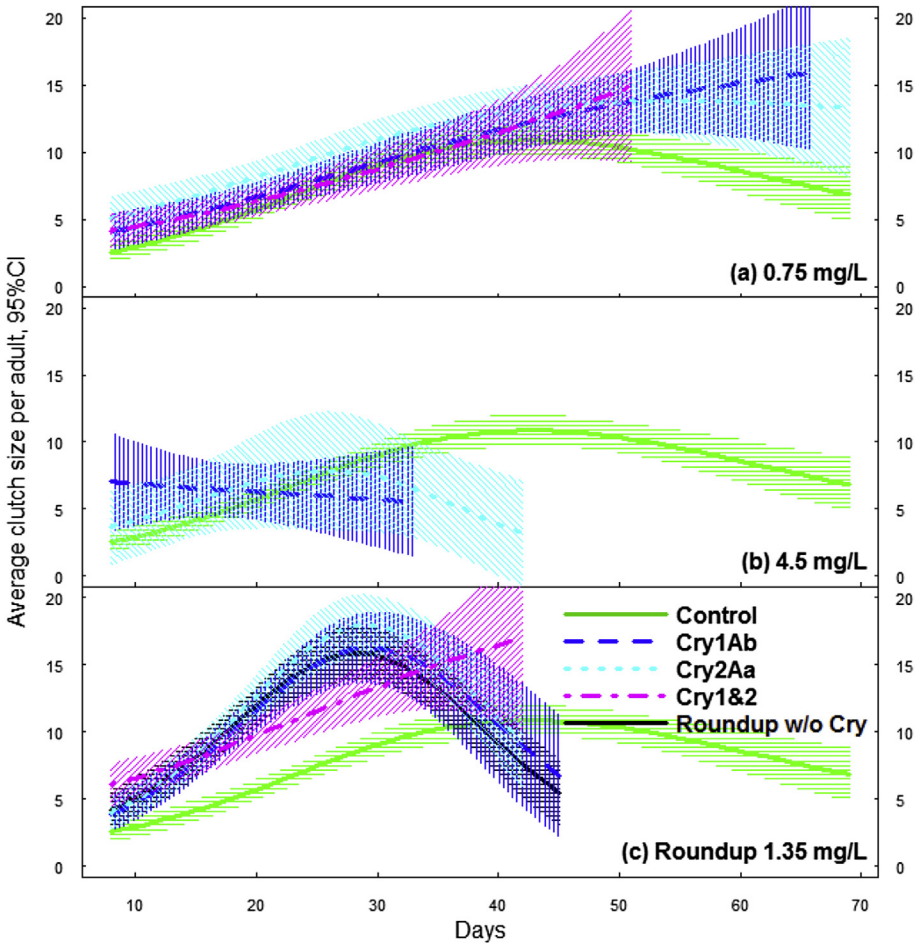

Fig. 6. Daily fecundity (based on a generalized additive model) at different ages for *D. magna* exposed to (a) 0.75 mg/L and (b) 4.5 mg/L of Cry1Ab, Cry2Aa toxins (as single toxins and combined) (4.5 mg/L of both toxins resulted in a single juvenile produced in total), and (c) 0.75 mg/L of Cry1Ab and Cry2Aa (as single toxins and combined) co-exposed with 1.35 mg/L of Roundup. Black curve represents animals exposed to 1.35 mg/L of Roundup without Cry-toxins. Shaded bands show 95% confidence limits.

Cry-toxins that have dissolved from its original plant context, but later binds to non-target animal feed. For instance, green algae are shown to adsorb Cry-toxins (Wang et al., 2014) and could as such serve as a vector transporting dissolved toxins to the gut of a non-target consumer organism. Accordingly, *D. magna* in our experiments may have been exposed internally by feeding on algae covered with toxins. However, this needs further investigation.

Depending on the type of plant material tested, Cry-toxin concentration will vary. For example, the feeding studies in *D. magna* mentioned above measured Cry1Ab concentrations of 67 (+- 27 SD) μg/kg in kernels (Bøhn et al., 2008) and 1880–2530 μg/kg in leaves (Holderbaum et al., 2015), respectively. This compares to a concentration of 0.067 and up to 2.53 mg/L = ppm in the pure maize material. Since the amount of maize feed given to the test animals in those studies were small (0.2 mg C per animal per day) compared to the volume of medium (50–100 mL), the maximum possible concentration of Cry1Ab toxins was 36.6 ng/L (Holderbaum et al., 2015) or 0.0000366 ppm = 0.0366 ppb (for leaves), i.e. about 4–5 orders of magnitude lower than the tested concentrations in the present study.

We used 0.75 and 4.5 mg/L (ppm) of purified Cry-toxins for this study, but we were not able to quantify how much toxin the experimental animals were ingesting. Further investigations should look into the fate of Cry-toxins in experimental as well as real-life environments, i.e. their distribution, break-down rates, interactions with other molecules and organisms, etc.

USEPA have calculated a worst case scenario for exposure of pesticides to aquatic invertebrates, based on 10% run-off from a 10 ha field of plants into a 1 ha × 2 m deep water body. Raybould et al. developed the calculation for a MIR162 GM maize plant producing the Cry-toxin Vip3A and ended up with a worst-case estimate of 0.75 mg toxin per litre (Raybould and Vlachos, 2011). Whereas this may represent a worst-case situation in some environmental contexts, we argue that agricultural run-off systems often are small creeks or ponds rather than 1 ha lakes with a depth of 2 m. These may be filled more or less completely with maize residues (Bøhn et al., 2012) and possibly end up with higher concentration of Cry-toxin than what was calculated by Raybould et al.

Nevertheless, using the 0.75 mg/L concentration of purified Vip3A toxin, dissolved in the medium, it was shown that *D. manga* responded with a significantly reduced growth rate after ten days of exposure (Raybould and Vlachos, 2011). This result supports our findings that Cry-toxins actually may cause harm to *D. magna*.

Given that the non-target organism *D. magna* may be harmed by Cry-toxins, we may speculate whether the specific receptors described for target insect pests, which are described to trigger the toxin specific response, may be present in the *D. magna* gut, or if alternative modes-of-actions are at play in the case of absence of these receptors. The latter seems more likely, since daphnids are phylogenetically far away from the order of lepidopterans (moths and butterflies), the main target group of Cry1 and Cry2 class toxins (Ferre et al., 2008). Distant phylogenetic relationship reduces the

likelihood of sharing biological traits like receptors for particular toxins. Hence, our findings in this and other studies (Bøhn et al., 2008, 2010; Holderbaum et al., 2015; Raybould and Vlachos, 2011) indicate that i) Cry-toxins may be less specific than previously believed, and ii) Cry-toxins have alternative modes of action(s) in non-target organisms. Taken together, Cry-toxins may unintentionally harm non-target species and communities.

The importance of the specificity (or lack of specificity) of Cry-toxins is proportional to their use, the amount of run-off plant material, the leakage of toxins into the environment and finally the exposure to, or ingestion of, toxins by non-target organisms. Further studies should investigate what concentration level of Cry-toxins that may be harmful to *D. magna* as well as in other non-target organisms in soil and aquatic ecosystems. We argue that such studies should test chronic exposure over long-term rather than acute studies. The calculation of NOEC and LOEC (No Observed and Lowest Observed Effect Concentrations, respectively) on a range of life-history traits would be indicative for community and ecosystem relevant effects.

Our results support our third hypothesis that Cry1Ab and Cry2Aa act in combination and give stronger (seemingly additive) effects on *D. magna* as compared to the single toxins. Such test resembles a comparison of GM single and double stack Bt-transgenic plants, and indicate that stacked events may cause stronger effects on non-target organisms. The relevance of these results can be illustrated by the development In South Africa. There, resistance evolution in the main target organism *Buseola fusca*, against Cry1Ab toxin (Kruger et al., 2009, 2012; Van den Berg et al., 2013; Van den Berg, 2013b) has led to the replacement of MON810 maize with the MON89034, a plant that produces both *cry1A.105* and *cry2Ab2* toxins (Van den Berg, 2013b). One factor that may contribute to rapid resistance evolution is that in-plant pesticides represents continuous exposure to the toxins for the target pest species. A second factor is if resistance development comes without an associated fitness cost for the target insect, as shown for *B. fusca* and Cry1Ab toxin in South Africa (Kruger et al., 2014).

The use of Cry-toxins is increasing in several dimensions. First, the area where Cry-toxin producing plants are cultivated has increased steadily since their introduction in 1996 (James, 2014). This comprises key crop plants like maize, rapeseed, soy and cotton for use as food, feed and fiber. Secondly, the development of resistance to Cry-toxins in pest insects, e.g. in South Africa, India, China and the US (Tabashnik et al., 2009; Van den Berg, 2013a; Van den Berg, 2013b) has led to a gradual replacement of the first GM plants that had single *cry*-toxins genes only. These are replaced by 'stacked events' that produce multiple Cry-toxins in the same plant.

In the US, the proportion of stacked GM maize plants has increased from below 10% to 76% the last decade (Fernandez-Cornejo et al., 2014). A similar development can be found elsewhere. Thus, a growing proportion of Bt-plants now contain two to six *cry*-toxin genes at the same time (Niu et al., 2013; Raybould et al., 2012). This means that the total amount of Cry-toxin per *plant* will roughly multiply with a factor of 2–6, given that the expression of each Cry-toxin is similar between single events and stacked events. However, data from the producers indicate that the expression in multistack plants can be much higher than in the parent plants, even for the single toxins. For example, the measured expression of Cry1A.105 in Monsanto's Smartstax maize (that contains six different Cry-toxins) is on average 54% higher in grains and 97% higher in pollen compared to the parent line MON89034 (Stillwell and Silvanovich, 2007). Moreover, the expression of Cry1A.105 and Cry2Ab2 in Smartstacks (Stillwell and Silvanovich, 2007) show on average 50–100 times higher expression in leaf and kernels, for each of the toxins, compared to the Cry1Ab expression that we previously measured in MON810 and tested in *D. magna* (cf. Bøhn et al., 2008; Holderbaum et al., 2015). In addition to these lepidopteran-active toxins, Smartstacks also express three coleopteran-active toxins in even higher concentrations (Hilbeck and Otto, 2015; Phillips, 2008; Stillwell and Silvanovich, 2007).

Given such order of magnitude increase in toxin-load, the range of non-target organisms affected may also expand because multistack GM plants combines Cry-toxins with different mode-of-action, giving a higher likelihood of affecting more non-target species. Evidence of cross-activity between Cry-toxins is available and document that Cry-toxins are active across many non-target taxa, i.e. outside their intended range of target insects (de Schrijver et al., 2014; Van Frankenhuyzen, 2013).

Such cross-reactions highlight the relevance of combinatorial effects of stacked Cry-toxins. The scientific literature indicates that synergism, i.e. stronger total effect than additive effects, is relatively common among Cry-toxins on target species. However, there is lack of data for responses in non-target organisms (van der Hoeven, 2014).

Since stacked events typically contain Cry-toxins and herbicide tolerance traits simultaneously, Cry-toxins and herbicides will co-occur in the environment. Aquatic systems must be expected to receive increasing concentrations of glyphosate-based (and/or other) agrochemicals in combination with Cry-toxins since GM plants have stacked Bt and HT traits (Benbrook, 2012a; James, 2014). Further and more detailed investigations need to study *combinatorial effects* (Hilbeck and Otto 2015), for example how LOEC of one stressor may be affected by another stressor. One example from our data-set is that the effects of Roundup were interacting with the effects of Cry-toxins. The mortality from 0.75 mg/L of Cry-toxins was higher in the presence of Roundup, particularly from Cry1Ab and the combination of both toxins (Figs. 1 and 2). In other words, the NOEC of 0.75 mg/L Cry1Ab on survival, shifted to a significant effect in the presence of Roundup. This partly supports our fourth hypothesis. However, as the Roundup stimulated the animals early in life, the overall response was more complex and therefore not fully covered by the hypothesis.

Resistance development is also a key issue for HT traits. Worldwide, 32 species of weeds are documented to be resistant to glyphosate, some of these have also acquired resistance to other/multiple sites of herbicide action (Heap, 2014, 2015). Resistance in weeds may lead to accelerated use which again promotes further resistance development (Binimelis et al., 2009). Glyphosate tolerant GM plants have contributed to the expanding use of Roundup/glyphosate (Benbrook, 2012b).

Bio-active herbicides ultimately get into soil and water systems through processes such as drifting, leaching and surface runoff (Mensah et al., 2012). For instance, glyphosate is recently documented to be present even in ground water, urine of animals and humans and in women breast milk (Borggaard and imsing, 2008; Honeycutt and Rowlands, 2014; Kruger et al., 2013; Niemann et al., 2015). Such development emphasizes the importance of potential wider impact of these technologies. Since Cry-toxins and herbicides will meet and interact, also with other stressors in the environment, the co-exposure and potential combinatorial effects need to be studied in more detail, in the laboratory as well as under more realistic conditions (Bjergager et al., 2011; Nørgaard and Cedergreen, 2010; Then, 2009).

Glyphosate, the active compound in Roundup products, has been described as an ideal herbicide with low toxicity for operators, consumers and the environment surrounding agricultural fields (Duke and Powles, 2008). However, recently glyphosate and Roundup have received more risk-related attention due to its documented negative effects on both aquatic and terrestrial ecosystems. Studies on non-target organisms indicate that glyphosate-

based herbicides in fresh-water and marine ecosystems have significant negative effects on for instance aquatic microbial communities (Perez et al., 2007), macrophytes (Lockhart et al., 1989; Simenstad et al., 1996), cnidaria (Demetrio et al., 2012), sea-urchin embryogenesis (Marc et al., 2004), fish (Servizi et al., 1987), amphibians (Mann et al., 2009; Relyea, 2005) and planktonic algae (Perez et al., 2007). A recent review of glyphosate herbicide effects in aquatic ecosystems gives a comprehensive overview of individual studies for most investigated taxonomic groups (Perez et al., 2011). Recently, glyphosate was classified as "probably carcinogenic to humans" by a working group from the International Agency for Research on Cancer. This classification was primarily based on evidence from animal studies, but also with independent support from mechanistic studies (Fritschi et al., 2015).

A previous study from our group showed that *D. magna* exposed to environmental concentrations of 0.05, 0.45 and 1.35 mg/L (active ingredient of glyphosate) of Roundup were negatively affecting offspring size, reproduction and survival (Cuhra et al., 2013). This shows that negative effects on aquatic animals can be found even below the accepted environmental concentrations in the US, i.e. at 0.7 mg/L.

In the present study, however, we found a lower toxicity of Roundup to *D. magna*. The test animals were even stimulated by 1.35 mg/L (active ingredient) of Roundup to grow fast, reproduce early and with large sized offspring. Nevertheless, after about 35 days all animals exposed to Roundup died abruptly in contrast to the control animals. The difference between our two studies with Roundup was unexpected but may have been caused by several differences in the experimental setup: i) The ADAM medium in the first experiment (see (Bøhn et al., 2008) for details) was replaced with the more complex M7 medium; ii) the pH was 7.5 in the first and 7.9 in the second experiment; iii) it was different formulations of Roundup that were tested in the two experiments; and iv) the basic feed was composed of *Selenastrum* sp. and *Desmodesmus acutus*, in the first and second experiment, respectively. We argue, therefore, that the effects of glyphosate or Roundup may be highly variable depending on the environmental context, such as water chemistry, pH, etc. Further research is needed in that direction.

Animals exposed to Roundup and Cry-toxins matured extremely early at the cost of later survival. This result support our fifth hypothesis that animals display an allocation trade-off with priority of early reproduction at the cost of higher mortality in later life stages, as shown earlier for *D. magna* fed Cry1Ab-transgenic maize material (Bøhn et al., 2008, 2010).

From our results, we argue that introductions of stacked events have a much higher potential to pose an environmental risk on aquatic ecosystems surrounding transgenic fields than single events have. The scientific literature is very limited with regard to studies testing plant material from 'stacked traits'. A couple of studies from industry sources show no effects on European corn borer/Colorado potato beetle (Raybould et al., 2012) and in rats (Appenzeller et al., 2009). Schuppener et al. showed that feeding activity and survival were negatively affected by a stacked GM-maize trial in a non-target Lepidoptera, *Aglais urticae*, but only at concentrations of pollen that were higher than found in the field (Schuppener et al., 2012).

5. Conclusions and further recommendations

Our new results support and complements earlier feeding studies with whole plant material (Bøhn et al., 2008, 2010; Holderbaum et al., 2015) and leads to the conclusion that both purified and plant produced Cry-toxins are negatively affecting survival and fitness of *D. magna*, an important filter-feeder in aquatic ecosystems worldwide. Further, our results confirm that high concentrations of Cry-toxins are able to harm the non-target organism *D. magna*, through environmental exposure. This indicates that: i) Cry-toxins have alternative modes-of-action than previously described, and ii) also other non-target organisms in relevant, i.e. exposed, aquatic ecosystems may be negatively affected by Cry-toxins. Both points warrant further investigations. We also conclude that potential negative effects on non-target organisms from stacked events that co-produce several Cry-toxins, and/or herbicide tolerance traits, likely will be stronger than from single event plants.

We need to improve the understanding of the fate of residues of transgenic material and Cry-toxins in run-off aquatic environments. We recommend more detailed studies under ecologically more realistic conditions, such as in mesocosm or field studies. The species pool for testing should include key species from different functional groups, and should represent the actual agricultural region for cultivation of the plants (Andow and Hilbeck, 2004; Gillund et al., 2013; Hilbeck et al., 2014). We also suggest further studies on the physiological effects of different Cry-toxins to gain a mechanistic understanding of their function to be better able to assess the risk of releasing them into the environment.

The lack of detailed studies on exposure and uptake pathways and modes-of-action of Cry-toxins, also in combination with agrochemicals like Roundup/glyphosate, are needed to understand observed negative effects of these key agricultural toxins. Further research efforts can lead to a development of improved management practices that will conserve species diversity and ecosystem functioning and services for the future.

Conflicts of interest

All authors declare that they have no conflict of interest.

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Transparency document

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