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REVIEW

Quality of laboratory studies assessing effects of *Bt*-proteins on non-target organisms: minimal criteria for acceptability

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Abstract The potential risks that genetically modified plants may pose to non-target organisms and the ecosystem services they contribute to are assessed as part of pre-market risk assessments. This paper reviews the early tier studies testing the hypothesis whether exposure to plant-produced Cry34/35Ab1 proteins as a result of cultivation of maize 59122 is harmful to valued non-target organisms, in particular Arthropoda and Annelida. The available studies were assessed for their scientific quality by considering a set of criteria determining their relevance and reliability. As a case-study, this exercise revealed that when not all quality criteria are met, weighing the robustness of the study and its relevance for risk assessment is not obvious. Applying a worst-case expected environmental concentration of bioactive toxins equivalent to that present in the transgenic crop, confirming exposure of the test species to the test substance, and the use of a negative control were identified as minimum criteria to be met to guarantee sufficiently reliable data. This exercise stresses the importance of conducting studies meeting certain quality standards as this minimises the probability of erroneous or inconclusive results and increases confidence in the results and adds certainty to the conclusions drawn.

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Introduction

Western corn rootworm (WCR; *Diabrotica virgifera virgifera*) (Coleoptera: Chrysomelidae) is a major coleopteran maize pest and a serious threat to agriculture in North America (Tinsley et al. 2013) and Europe (FCEC 2009; Wesseler and Fall 2010). WCR larvae feed on the root system (Meinke et al. 2009), and negatively affect yield by decreasing nutrient and water uptake and plant stability. Pest management options for WCR consist of crop rotation, the use of maize seed coated with systemic insecticides and the application of soil insecticides (van Rozen and Ester 2010; Meissle et al. 2011). The genetically modified (GM) maize transformation event 59122, expressing the insecticidal binary Cry34Ab1 and Cry35Ab1 proteins encoded by genes isolated from *Bacillus thuringiensis* (*Bt*), offers an additional management tool for WCR. The Cry34/35Ab1 proteins (Cry34Ab1: 14 kDa; Cry35Ab1: 44 kDa) bind selectively to brush border membrane vesicles in the midgut of larvae of WCR (Li et al. 2013), leading to their death due to cell lysis and septicaemia. The Cry34/35Ab1 proteins are active in acidic environments (Masson et al. 2004), a characteristic of coleopteran guts, and are both required for optimal insecticidal activity (Ellis et al. 2002; Herman et al. 2002b; Schnepf et al. 2005). The activity of the Cry34Ab1 protein was shown to be potentiated by relatively small amounts of the Cry35Ab1 protein; for example, a 9:1 ratio of Cry34Ab1:Cry35Ab1 showed high larval mortality of southern corn rootworm (*D. undecimpunctata howardi*). However, an optimal ratio has not been defined (Herman et al. 2002b).

Maize 59122 can control western, northern (*D. barberi*) and Mexican corn rootworm (*D. virgifera zea*) (Dow AgroSciences 2007) and is approved for cultivation in North America (see ISAAA GM approval database; http://www.isaaa.org/gmapprovaldatabase/default.asp). At present, WCR is the only species of the corn rootworm complex present in Europe. The species was introduced into Europe from North America in 1992, and has since then spread across the continent, resulting in well-established populations in approximately 19 European countries (EC 2012). It is expected that this invasive pest species will expand further in Europe (Aragón and Lobo 2012). Maize 59122 is currently not authorised for cultivation in the European Union (EU), but the European Food Safety Authority (EFSA) has issued scientific opinions on the cultivation of this *Bt*-maize event (EFSA 2013a, b).

As part of the regulatory authorisation process, the potential risks that the cultivation of GM plants may pose to non-target organisms (NTOs) and the ecosystem services they provide are assessed in many jurisdictions. A typical risk hypothesis addressed during the environmental risk assessment (ERA) of *Bt*-plants is that the newly expressed *Bt*-proteins are not toxic to valued NTOs at concentrations present in the field (Romeis et al. 2008). Potential harmful effects on NTOs are evaluated within different tiers that progress from laboratory studies representing highly controlled, worst-case exposure conditions (Tier 1) to bio-assays with more realistic exposure to the toxin (Tier 2) and (semi-)field studies carried out under less controlled conditions (Garcia-Alonso et al. 2006; Rose 2007; Romeis et al. 2008). Moving to a higher tier is only considered relevant if adverse effects are detected at the lower tier, or if unacceptable scientific uncertainty remains. Because not all NTOs potentially at risk can be tested from a practical viewpoint, a representative subset of species is selected for assessment. The ERA focuses on species that play a role in ecosystem services (e.g., natural enemies for pest regulation, honeybees for pollination, springtails and earthworms for soil-related processes) or are of conservation concern (e.g., rare and protected species, or species of aesthetic or cultural value).

This paper reviews available laboratory (early tier) studies assessing the toxicity of Cry34/35Ab1 proteins to non-target terrestrial, soil and aquatic arthropods, and annelids. Both studies reported in documents submitted in the context of the regulatory authorisation process for cultivation of maize event 59122 and published in the scientific literature, are considered. Further, this paper evaluates the studies for their scientific quality and defines minimum criteria for their acceptability. The reviewed data and their

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relevance within an ERA context can be of interest to risk assessment bodies.

Expected environmental concentrations

As part of the ERA, an exposure characterisation is performed to determine to how much of the plant-produced *Bt*-proteins a particular organism can be exposed to under field conditions. This expected environmental concentration (EEC) is predicted from protein expression data in various plant tissues (Raybould et al. 2007; Rose 2007). Worst-case and more realistic, but still conservative, estimates of EEC can be calculated. In general, the worst-case EEC represents exposure via a test diet of 100 % of the relevant plant tissue (Raybould et al. 2007). The more realistic EECs are refinements of the worst-case exposure considering the diet of the NTO, including the dilutions of *Bt*-proteins in that diet. Typically, in Tier 1 studies, an additional safety factor is applied to the EEC to ensure an extreme exposure scenario. This factor, which is typically at least 10 times the EEC, accounts for inter-plant variation in insecticidal protein expression level, and intra- and inter-species variability in sensitivity to the toxin and thus adds certainty to a conclusion of no effect.

CERA (2013) reviewed the levels of Cry34Ab1 and Cry35Ab1 proteins in plant parts of maize 59122 obtained by enzyme-linked immunosorbent assay (ELISA) analyses of samples taken from field trials conducted on the American continent. In addition, data on protein concentrations have been published for maize 59122 grown in the EU (EFSA 2013a). Submissions and decision documents associated with regulatory authorisation processes indicated that the Cry34Ab1 and Cry35Ab1 proteins were detected in all parts examined. Samples were collected from different plant tissues at multiple growth stages, and from plants grown at different locations to produce representative data of the typical protein expression range of both *Bt*-proteins (Table 1). As the EECs reported in the various laboratory studies differ (e.g. because they are based on preliminary empirical data instead of data obtained from field trials), the highest concentration reported by CERA or EFSA was used to calculate the EECs for all the different groups of NTOs tested. A worst-case EEC was calculated for herbivores and pollinators, a realistic EEC for natural enemies, decomposers and aquatic species. This approach of using a base EEC allowed inter-comparability of the study outcomes at the level of exposure.

Laboratory studies assessing the impact of Cry34/35Ab1 proteins

Non-target terrestrial arthropods

Herbivores

Exposure

Herbivores can ingest *Bt*-proteins when feeding on *Bt*-plants, and transfer them to higher trophic levels (see section on Natural enemies below). Also larvae of non-herbivores of maize can be exposed to *Bt*-proteins when consuming *Bt*-maize pollen deposited on their host plants present in or near *Bt*-maize fields. Reported mean maize pollen densities during anthesis range from 150 to 500 pollen grains/cm^2 leaf surface in-field, depending on the host plant (as reviewed by Perry et al. 2013). For example, average densities found on milkweed (*Asclepias syriaca*) are lower (171 grains/cm^2 according to Pleasants et al. 2001) than those found on goosefoot (*Chenopodium album*) and mustard (*Sinapis alba*) leaves (250–500 grains/cm^2 according to Gathmann et al. 2006). In field margins, pollen densities decline rapidly, from 63 grains/cm^2 for milkweed (Pleasants et al. 2001) to less than 10 pollen grains/cm^2 at 4–5 m from the edge of the field for all host plants analysed so far (reviewed by Perry et al. 2013). Starting from the amount of pollen grains/cm^2 and assuming a mean pollen grain dry weight (dw) mass of 325 ng (Fonseca et al. 2003 reporting on dw ranging from 150 to 500 ng), 146 ng Cry34Ab1 per mg dw pollen (Table 1) and considering that 1 cm^2 of leaf weighs 14.5 mg, one can calculate the expected concentration of Cry34Ab1. For example, for Lepidoptera larvae feeding on mustard the calculated EEC in-field will be 1.6 ng Cry34Ab1 per mg diet. The concentration of Cry35Ab1 was not included in the calculation because of its low concentration in pollen (<0.3 ng/mg).

Laboratory (lower-tier) studies

Coleoptera In a laboratory study with the green dock leaf beetle *Gastrophysa viridula* (Chrysomelidae), an

Table 1 Levels of the Cry34Ab1 and Cry35Ab1 proteins in various parts of maize 59122 (μg/g dry weight) grown in the EU [Bulgaria (2003 and 2004) and Spain (2004); data retrieved from EFSA (2013a); ranges of concentrations are provided]; or in America [Canada (2003), Chile (2002 and 2003) and US (2003)]; data retrieved from CERA (2013; highest concentrations are provided). Values are combined data for the different locations in the EU and in the Americas

Plant parts	Cry34Ab1			Cry35Ab1		
	EU data		American data	EU data		American data
	Concentration μg/g dry weight	Plant stage		Concentration μg/g dry weight	Plant stage	
Leaf	<0.162[a]	R4	302	<0.162[a]	R4	126
	667			307		
Root	16.4–82.1	V6	102	1.12–26.1	R1	15.4
Pollen	45.4–146	R1	87.2	<0.324[a]	R1	0.15
Whole plant	49.0–89.0	R1	88	48.7–92.6	V9	18.1
Senescent plant	200	R6	88	47.5	R6	16.4

R, reproductive phase; R1, growth stage when silks become visible; R4, Growth stage when the material within the kernel produces a doughy consistency; R6, maturity, the typical harvest maturity for grain; V, vegetative phase, VX, stage where collar of Xth leave becomes visible

[a] These values are the LLOQ ("lower limits of quantification") reported for the corresponding proteins and tissue samples

oligophagous herbivore that shows a feeding preference for dock (*Rumex*) species, larvae were exposed to three different pollen concentrations: 50–100, 300–400 or 600–800 maize 59122 pollen grains/cm^2 *Rumex* leaf surface (Székács and Kong 2011), corresponding to 0.3–0.6, 1.7–2.3 and 3.5–4.6 ng Cry34Ab1/mg diet, respectively. These concentrations were selected to approximate the reported mean in-field pollen deposition rates and at least 10 times the pollen deposition rate at the edge of the field. EFSA (2013a) noted that the applied lyophilised pollen was stored at suboptimal conditions (at −10 °C for three months and subsequently at −20 °C for 12 months), which may have influenced the biological activity of the Cry34/35Ab1 proteins and food quality. The concentration of Cry34Ab1 protein in a retained subsample of thawed maize 59122 pollen after storage was found to be 160 ng/mg (dry weight), but the biological activity had not been quantified at diet administration. A prospective power analysis indicated that the sample size was sufficient to detect a 20 % mortality increase with a power of at least 70.4 %. and a 20 % weight change with a power of at least 86.0 %. No statistically significant differences in survival were observed at any concentration tested between the larvae fed maize 59122 pollen and those fed non-*Bt*-maize pollen. In addition, no reduction in mean body weight of the newly emerged adult beetles was found at 50–100 and 300–400 pollen grains/cm^2. However, the observed adult body weight of *G. viridula* fed 600–800 maize 59122 pollen grains/cm^2 as larvae, was lower (<10 %) than that of larvae fed non-*Bt*-maize pollen.

Lepidoptera Larvae of the monarch butterfly *Danaus plexippus* (Nymphalidae) were not adversely affected when fed maize 59122 pollen. Sears and Rempel (2003) exposed *D. plexippus* larvae to seven concentrations of maize 59122 pollen or that of the near-isogenic line (50, 100, 200, 400, 800, 1600 and 3200 pollen grains/cm^2) applied evenly over entire leaves of milkweed. No significant differences between the monarch butterfly larvae that ingested maize 59122 pollen and those that ingested the pollen from the non-transformed near-isogenic line were observed in terms of survival, development (weight gain) and leaf area consumption after 9 days of exposure.

In laboratory experiments with larvae of two species potentially occurring on various host plants in field margins, i.e., the painted lady *Vanessa cardui* (Nymphalidae) and the small white *Pieris rapae* (Pieridae), pollen from maize 59122 or that of a control plant was incorporated into an artificial diet at

10 % of the final dry weight, which corresponds to 2 ng Cry34Ab1/mg diet (EFSA 2013a). No differences in mortality of *V. cardui* and *P. rapae* larvae exposed to maize 59122 pollen for seven days was observed between the *Bt*- and non-*Bt*-maize pollen treatments and the control diet without pollen. In addition, no statistically significant differences in larval weight between *V. cardui* larvae fed *Bt*- or non-*Bt*-maize were noted, but the average larval weight of *P. rapae* fed maize 59122 pollen was significantly lower (32 %) than that of larvae fed non-*Bt*-maize pollen. A prospective power analysis indicated that the sample size was sufficient to detect a 25 and 41 % weight decrease with a power of 80 %, for respectively *V. cardui* and *P. rapae* (authors' personal communication).

Natural enemies (predators and parasitoids)

Exposure

Predators and parasitoids are likely to be exposed to plant-produced *Bt*-proteins when feeding on herbivorous arthropods that contain *Bt*-proteins, or through the consumption of pollen or plant exudates. Several species of ground beetles, ladybirds, predatory bugs, and larvae of green lacewings found in maize (Meissle et al. 2012; Romeis et al. 2014) are predators of herbivores (including pest insects and mites). Many predatory arthropods are omnivores that also feed on pollen, nectar, plant juices and honeydew. Further, parasitic wasps attack a variety of herbivores occurring in maize ecosystems, using them as a host for oviposition. Adult hymenopteran parasitoids primarily feed on (extra-)floral nectaries or honeydew (Lundgren 2009). Despite the broader feeding habits of some species, the species for which the primary valued function is pest regulation are addressed in this paper as natural enemies. For example, the green lacewing *Chrysoperla carnea* (Neuroptera: Chrysopidae) is regarded as a predator despite the fact that only the larval stages are predacious, while the adults are prevalent pollen consumers in maize fields (Sheldon and MacLeod 1971; Li et al. 2010).

The level at which different prey/host organisms ingest *Bt*-proteins mainly depends on the expression pattern of the *Bt*-protein in the plant, and the mode of feeding of the herbivore. A precise realistic EEC is therefore difficult to set given the variety of food many natural enemies are likely to consume. Raybould et al. (2007) set the realistic EEC for natural enemies when feeding on prey at 0.2 × the highest *Bt*-protein concentration found in leaves. Applying the same standard here, the EEC would correspond to 60.4–133.4 ng/mg prey diet for the Cry34Ab1 protein and 25.2–61.4 ng/mg prey diet for the Cry35Ab1 protein on the basis of highest Cry34/35Ab1concentrations reported in leaf tissues of maize 59122 (Table 1).

Laboratory (lower-tier) studies

Coleoptera Vinall (2005) fed larvae of *Poecilus cupreus* (Carabidae) with blowfly (*Calliphora vomitoria*) pupae injected with a protein solution containing a mixture of the Cry34Ab1 and Cry35Ab1 proteins at nominal concentrations of 1000 and 333 ng/mg pupae diet, respectively. A negative control group of larvae was fed blowfly pupae injected with deionised water, and a positive control group was fed pupae injected with teflubenzuron. All treated pupae were stored in a freezer before being fed to the *P. cupreus* larvae. The Cry34/35Ab1concentrations in the thawed blowfly pupae were shown to be reduced to 350 ng/mg Cry34Ab1 and 203 ng/mg Cry35Ab1. Development of the ground beetle larvae was monitored until adult emergence. There was no statistically significant difference in pre-imaginal survival, mean development time, or mean adult weight of beetles fed Cry34/35Ab1 proteins, relative to the negative control.

A laboratory study with *Hippodamia convergens* (Coccinellidae) did not reveal adverse effects on survival of adult beetles exposed at the same time to a nominal Cry34/35Ab1 protein concentration of 280 µg (combined total of 160 µg Cry34Ab1 and 120 µg Cry35Ab1) per ml of sugar water (Bryan et al. 2000a), in addition to fresh water without *Bt*-proteins (authors' personal communication). The test was terminated on day 11, when 22 % mortality was observed in the negative control group fed non-treated sugar water.

Data on *Coleomegilla maculata* (Coccinellidae) have been reported by Higgins (2000, 2003). In a 7-day dietary bioassay, Higgins (2003) exposed larvae to the purified Cry34/35Ab1 proteins mixed in an artificial diet. The mixture of the Cry34/Cry35Ab1 proteins was administered at a nominal concentration of 901 µg/g diet. The Cry34Ab1 protein was incorporated at 900 µg/g and the Cry35Ab1 protein at 1 µg/g.

The stability of the binary toxin was not tested in this Tier 1 experiment and a positive control was lacking. No adverse effects on survival were observed after 7 days of feeding. However, after 7 days larval weight was reduced significantly for *C. maculata* larvae fed on the Cry34/Cry35Ab1-containing diet compared with those fed on the control diets, corresponding to a growth inhibition of 80 %. A retrospective power analysis was conducted and showed that the experiment was able to detect a minimum of 25 % increase in mortality at a level of 95 % power and a 25 % decrease in larval weight at a level of 99 % power. The observed sublethal effects on *C. maculata* at Cry34/35Ab1 dose levels exceeding the EEC by a factor of 5 (see Table 2 of supplementary material) triggered further investigations. Higgins (2003) then offered *C. maculata* larvae diets consisting of 50 % ground *H. zea* eggs and 50 % pollen of a homozygous maize 59122 inbred line. Neonate *C. maculata* larvae were placed in individual bioassay wells and allowed to feed ad libitum throughout the 14-day larval growth period. The maize pollen-egg mixture was ground completely to avoid selective feeding on lepidopteran eggs. The concentration of the Cry34Ab1 protein fell within the range reported for maize 59122 pollen collected in the EU (author's personal communication). There was no significant difference in larval survival and development time, or adult weight between ladybirds fed the control diet and those fed the *Bt*-maize diet. A retrospective power analysis was conducted showing that the test was able to detect a minimum of 30 % increase in mortality at a level of 89 % power and a 25 % decrease in larval weight at a level of 99 % power. Higgins (2000) reported results for *C. maculata* fed corn leaf aphids (*Rhopalosiphum maidis*) that were previously reared on the Cry34/35Ab1-expressing maize candidate event TC5638. No significant differences in adult mortality or weight monitored daily until day ten were found between ladybirds fed *Bt*-maize-reared aphids and those fed aphids reared on corresponding non-*Bt*-maize. Feeding on *Bt*-maize-reared aphids did also not affect the predator's development time. It was, however, not determined whether the aphids reared on the *Bt*-maize events TC5638 ingested any Cry34/35Ab1 protein.

In a laboratory study with *Coccinella septempunctata* (Coccinellidae), larvae were fed a ground diet composed of lyophilised maize 59122 pollen with eggs of the lepidopteran *Ephestia kuehniella* at a 1:3 ratio by weight (Califf and Ostrem 2009; Hong 2009). The maize pollen-egg mixture was ground to a powdery consistency to avoid selective feeding on lepidopteran eggs. Lower concentrations of pollen were applied compared to the study with *C. maculata*, in order to allow a normal development of the less pollinivorous *C. septempunctata* larvae. Using ELISA the Cry34Ab1 protein level in the pollen was determined to be 2.8 ng/mg (dry weight), while the concentration of the Cry35Ab1 protein was below the limit of quantification. No statistically significant differences in larval survival and development time, or adult weight were identified when compared with the non-*Bt*-maize control.

In a tritrophic study, *C. septempunctata* larvae (first or second instars) were fed either a diet consisting of only aphids (*R. padi*) reared on maize (event 59122 or a control line), or a mixture of maize pollen and maize-reared aphids. Results from this study indicated no significant differences in survival and development of *C. septempunctata* larvae exposed to Cry34/35Ab1 or the control and both treatments of either aphid or aphid + maize pollen (Takács et al. 2010). In a follow-up study, larvae of *C. septempunctata* were simultaneously exposed to maize pollen containing 47.4 ng Cry34Ab1/mg fw (or 28.4 ng Cry34Ab1/mg dw considering pollen exists of 60 % moisture, see Luna et al. 2001) and maize-reared *R. padi* under semi-field conditions and their development was recorded from first instar to adult emergence. Still under the same field conditions, the emerged adults were allowed to prey on *R. padi* and to copulate for 2 weeks. Subsequently, fecundity and fertility of the emerged adults were compared in the laboratory. No difference in development time, fecundity and fertility between the *Bt*- and four non-*Bt*-treatments was evident (Takács et al. 2012). The only significant difference was a 10–15 % lower weight of newly emerged adult males in the *Bt*-maize treatment (Takács et al. 2012). In both studies the authors did not confirm whether the aphids actually had ingested the *Bt*-toxins when feeding on the *Bt*-maize, nor was the uptake of maize pollen by *C. septempunctata* in the presence of aphids confirmed.

Vinall (2011b) found that the survival and adult weight of *C. septempunctata* fed eggs of *E. kuehniella* and maize pollen from a stacked GM event containing 59122 in a 3:1 ratio, were not affected as compared to those fed a control diet. The concentration of the

Cry34Ab1 protein fell within the range reported for maize 59122 pollen collected in the EU (author's personal communication). The maize pollen-moth mixture was ground completely to avoid selective feeding on the eggs and a positive control group given eggs treated with teflubenzuron was included in the test (authors' personal communication).

Hemiptera Patnaude (2008) conducted four 10- to 14-days tests to assess the effects on survival of *Orius insidiosus* (Anthocoridae) nymphs when fed a combination of 750 µg Cry34Ab1 + 85 µg Cry35Ab1/g diet (mixed with bee pollen). Each test included a positive control group given potassium arsenate and a negative control group given purified water mixed with the diet. One test group was given heat-denatured Cry34Ab1/35Ab1. The survival of the nymphs was similar in the negative control and test groups while it was significantly reduced in the positive control. However, as the mortality of the negative control group in each test exceeded the allowed 20 % control mortality, an additional test with another *Orius* species was done. Vinall (2011a) fed *Orius laevigatus* (Anthocoridae) nymphs a meat-based diet containing Cry34/35Ab1 at two different concentrations, 82 µg Cry34Ab1/g + 2.5 µg Cry35Ab1/g diet, and 820 µg Cry34Ab1/g + 25 µg Cry35Ab1/g diet. The experiment included a group given teflubenzuron as a toxic reference, which caused adverse effects on both parameters recorded. No adverse effects on survival of *O. laevigatus* nymphs were observed at the two treatment rates compared to those fed a non-*Bt*-diet. The time required for the nymphs exposed to the highest concentration to develop into adults was significantly (15–19 h) shorter than that of nymphs fed the non-*Bt*-diet, while no effect was observed at the lower dose.

Hymenoptera The survival of adult *Nasonia vitripennis* (Pteromalidae), a pupal parasitoid of Diptera, was not adversely affected when exposed to sugar water containing the combined Cry34/35Ab1 proteins at a nominal concentration of 280 µg/ml (combined total of 160 µg Cry34Ab1/ml and 120 µg Cry35Ab1/ml) for 12 days (Porch and Krueger 2001). A post hoc statistical power calculation showed that a 30 % increase in mortality could be detected with a power of 98 %.

Neuroptera Larvae of *C. carnea* (Chrysopidae) were fed a diet containing lepidopteran (*S. cerealella*) eggs mixed with the Cry34/35Ab1 proteins at a nominal rate of 280 µg/ml (combined total of 160 µg Cry34Ab1/ml and 120 µg Cry35Ab1/ml) (Sindermann et al. 2001). No toxic reference was used, and exposure was thus not confirmed. The larvae were allowed ad libitum access to the Cry-coated eggs until the negative control mortality exceeded 20 % on day 10 of the test. Compared with the negative control, there was no significant increase in larval mortality in the Cry-protein treatment.

Pollinators

Exposure

Honeybees can be exposed to plant-produced *Bt*-proteins as they collect and consume maize pollen, mainly when alternative pollen sources are scarce. It was reported that honeybee larvae consume 1720–2310 maize pollen grains under semi-field exposure conditions during their complete development, which corresponds to a worst-case exposure of 1.5–2.0 mg pollen (Babendreier et al. 2004) or 0.2–0.3 µg Cry34/35Ab1 (if one considers pollen contain 146.3 µg Cry34/35Ab1).

Laboratory (lower-tier) studies

Hymenoptera A 26-day laboratory study in which three to five-day-old larvae of the honeybee *Apis mellifera* (Apidae), were each fed 5.6 µg Cry34/35Ab1 (3.2 µg Cry34Ab1 and 2.4 µg Cry35Ab1) did not reveal adverse effects on larval survival (Maggi 2001). From the available data, however, it is not clear whether 'fed' implies that the larvae completely consumed the administered proteins. Maggi (2001) also performed a 26-day laboratory study providing pollen to 3- to 5-day-old honeybee larvae. Individual larvae were fed once with 2 mg pollen collected from either the Cry34/35Ab1-expressing maize candidate event TC5639, representing a consumption of 56 ng Cry34/35Ab1, or the near-isogenic counterpart. Larval survival was evaluated 6 and 12 days after treatment, and adult emergence was evaluated between 12 and 26 days after treatment. No statistical differences in larval mortality between honeybee larvae fed *Bt*-maize pollen or non-*Bt*-maize pollen were observed. Development rate and adult survival were not affected by exposure to *Bt*-maize pollen.

Decomposers

Exposure

Earthworms (Annelida) and non-target arthropods, such as springtails (Collembola), living on or in the soil can be exposed to plant-produced *Bt*-proteins introduced into the litter layer or soil via decomposing plant residues and via root exudates during plant growth. Several studies analysed Cry34/35Ab1 protein degradation under laboratory conditions or in the rhizosphere (Herman et al. 2002a; Shan and Embrey 2009a, b; Dunville et al. 2010). These studies suggest that Cry34/35Ab1 proteins degrade rapidly over time in several soil types. Further, based on the general knowledge of the degradation of plant-produced *Bt*-proteins, the Cry34/35Ab1 proteins are not expected to occur in soil at levels toxic to arthropods or annelids (Icoz and Stotzky 2008; Măruţescu 2012).

The realistic EEC for non-target soil organisms was determined to be 1.6 mg Cry34/35Ab1 per kg dry soil, assuming that a 1 ha field with 65,500 plants (with an average dry weight of 225 g/plant and the highest measured Cry34/35Ab1 concentration in senescent maize 59122 plant tissue to be 247.5 µg/g dw, as reported by EFSA 2013a) was incorporated in 2.25×10^9 g dry soil into the top 15 cm (see Raybould et al. 2007 for calculations). In the calculation we assumed senescent maize has a dry matter of 30 % (Swanckaert et al. 2016).

Laboratory (lower-tier) studies

Collembola Teixeira (2001) fed juveniles of *Folsomia candida* (Isotomidae) diets consisting of purified Cry34/35Ab1 proteins mixed in dry granulated brewer's yeast at a nominal rate of 12.7 mg/kg (3.2 mg/kg Cry34Ab1 and 9.5 mg/kg Cry35Ab1). Fresh diet was provided every third day. No adverse effects on the survival and reproduction of *F. candida* were observed after 28 days. A positive control treatment, i.e. test diet including thiodicarb demonstrated that the study design was able to detect toxic effects. Another 28-day laboratory study showed that a homogeneous mixture of dry yeast (95.8 %) and powdered 59122 maize (4.2 %) had no adverse effect on survival and reproduction of *F. candida* compared to individuals exposed to yeast only or yeast with 4.2 % non-*Bt*-maize (Teixeira 2006b). Again, thiodicarb added to dry yeast was used as a toxic reference.

Annelida Two laboratory studies with *Eisenia fetida* (Haplotaxida: Lumbricidae) are available. Exposing *E. fetida* adults to pure Cry34/35Ab1 proteins at a nominal rate of 25.4 mg/kg dry soil, gave no indications of adverse impacts to this earthworm species following a 14-day exposure (Bryan et al. 2000b). The Cry34/35Ab1 protein concentration was not monitored throughout the test period. Earthworm survival and changes in average body weights were not statistically different between the negative control and protein-amended soils. Earthworms exposed to the positive control treatment with chloroacetamide, however, were affected. Teixeira (2006a) conducted a 14-day laboratory study in which *E. fetida* adults were exposed to an initial concentration of 4.2 % lyophilised maize plant material (i.e. 2.8 mg Cry34/35Ab1 in senescent 59122 tissue per kg dry soil), or the toxic reference carbendazim. No mortality was observed during exposure to maize 59122, and no significant difference was found in development (weight change) between the *Bt*- and non-*Bt*-maize treatment.

Non-target aquatic arthropods

Exposure

Particulate organic matter from GM plants (e.g. pollen, crop dust, detritus) can be deposited in adjacent water bodies or transported in water courses to downstream water bodies thereby exposing non-target aquatic arthropods to the transgene product(s) (Rosi-Marshall et al. 2007; Chambers et al. 2010; Tank et al. 2010). There are two routes through which non-target aquatic arthropods may be exposed: (1) exposure to proteins in deposited plant material via direct feeding (see Table 1 for concentrations); or (2) exposure to freely soluble proteins (e.g. proteins that leach out of maize plant tissues into an adjacent water body) (Carstens et al. 2012). Owing to the rapid degradation of any freely soluble proteins, the concentration of freely soluble Cry34/35Ab1 proteins in an aquatic environment would be 2.0 mg/l assuming that all tissue from a 10 hectare maize field with 750,000 plants (with an average dry weight of 300 g/plant and a concentration of 181.6 µg Cry34/35Ab1 per g whole plant tissue) is deposited in a 1 hectare pond containing 20,000,000 L (see Carstens et al. 2012 for calculations).

Laboratory (lower-tier) studies

Cladocera Marino and Yaroch (2001) performed a 48-h laboratory study with *Daphnia magna* (Daphniidae). The test material consisted of purified Cry34/35Ab1 proteins added to water at a nominal concentration of 100 mg/l (combined total of 57 mg Cy34Ab1/l and 43 mg Cry35Ab1/l). No behavioural changes in terms of mobility were reported between the treated and control groups during the exposure period.

Diptera Fisher et al. (2012) reported on a 48-h laboratory study with *Culex quinquefasciatus* (Culicidae) larvae. A prospective power analysis indicated that 48 larvae per treatment were sufficient to detect a 20 % mortality increase with a power of at least 80 %. There was 100 % mortality among larvae treated with Spheratax (*Bacillus sphaericus*), the positive control substance. No statistically significant difference in survival of *C. quinquefasciatus* larvae exposed to 82 µg Cry34Ab1/ml + 2.5 µg Cry35Ab1/ml test solution was observed compared with the negative control treatment.

Scientific quality of the laboratory studies

To ensure that robust and reliable data on NTOs are generated in early tier studies, any study should be reproducible and be carried out in such a way that it minimises the probability of erroneous or inconclusive results (Rose 2007; WHO 2009; Romeis et al. 2011). This increases confidence in the results and adds certainty to the conclusions drawn. It is therefore important to assess the quality of evidence used to support the ERA. Romeis et al. (2011) indicated that the quality of laboratory test systems for the assessment of effects of GM plants on non-target arthropods is optimised if certain conditions are met. These conditions include (1) biochemical and functional equivalence of the test substance to the proteins produced in the GM plant (see also Raybould et al. 2013); (2) ensuring worst-case exposure conditions; (3) confirmation of exposure of the test organisms to the test substance; (4) inclusion of negative control treatments (buffer/diet only) to assess the suitability of the test system; (5) confirmation of the stability and biological activity of the test substance during the testing period; (6) usage of a number of replicates so that defined effect sizes can be detected with sufficient statistical power; (7) the use of suitable measurement endpoints allowing to indicate the possibility of adverse effects and (8) representativeness of the selected species (see also Romeis et al. 2013; Carstens et al. 2014). Whether these conditions are met for the previously presented laboratory studies is discussed below and summarised in Table 2 of supplementary material for the non-pest species tested.

1. Equivalence of the test substance with the proteins produced in the GM plant

For the ERA of *Bt*-crops, large quantities of toxins are needed to conduct tests and are therefore often produced in microbes. The microbial produced proteins can then be used in safety tests as a surrogate for the plant-produced proteins provided that they are biochemically and functionally equivalent (Raybould et al. 2013). The biochemical equivalence of the *Pseudomonas*-produced Cry34/35Ab1 proteins with the Cry toxins isolated from maize event 59122 was demonstrated by comparing the molecular size, immuno-recognition and *N*-terminal amino acid sequence, and by confirming the lack of glycosylation of the plant-produced protein (Schafer 2002; USDA 2004). Further, the bioactivity of maize event 59122 against a range of pest species was shown to be similar with the profile reported for the bacterial-produced Cry34/35Ab1 proteins (US EPA 2010). Therefore, the outcomes of the laboratory studies with NTOs fed a diet containing microbe-produced Cry34/35Ab1 proteins were considered informative to the ERA of maize 59122 (EFSA 2007; US EPA 2010). In two studies (Higgins 2000; Maggi 2001), different Cry34/35Ab1-expressing maize candidate events (i.e., TC5638, TC5639) were used, containing a transformation cassette other than that of maize 59122 (Gao et al. 2004). The biochemical and functional equivalence of their expressed *Bt*-proteins with the microbial-produced Cry34/35Ab1 proteins was demonstrated (Gao et al. 2004).

2. Exposure of the test organisms to sufficiently high concentrations of the test substance

In the laboratory studies assessing the impact on NTOs, often 1 X the EEC was applied, particularly when plant material was used; a safety factor (ranging from 1 up to 51 X EEC) was obtained in approximately 70 % the studies. In all the studies, except three, the

test organisms were initially exposed to at least the EEC. In the *C. septempunctata* study by Califf and Ostrem (2009) the concentration of purified Cry34Ab1 applied to the pollen/egg diet (2.8 ng/mg dw) was far below the reported values in maize 59122 pollen [approximately 30× (for America) and 60× (for the EU) below the EEC; see Table 1]. Therefore, this study was considered of limited value for risk assessment (COGEM 2008; EFSA 2013b). In the study where *A. mellifera* larvae were exposed to 2 mg TC5639 pollen (Maggi 2001), the concentration of Cry34Ab1 in pollen was estimated to be lower than that of 2 mg 59122 pollen and thus below the EEC. For the study by Teixeira (2006b), where *F. candida* was exposed to 4.2 % maize material in a yeast diet, it was concluded that the concentration chosen was too low and did not reflect worst-case exposure (EFSA 2013a).

3. Confirmation of intake of the test substance by the test organisms

Plant-produced *Bt*-proteins have no contact toxicity and must be ingested by a susceptible organism to be effective. Thus, direct dietary intake is required to evaluate the toxicity of *Bt*-proteins (Rose 2007). In ten laboratory studies (summarised in Table 2 of supplementary material), a positive control (toxic/reference) substance was used to (indirectly) demonstrate exposure to the *Bt*-proteins. However, in three of the ten studies this goal was not achieved. As the toxic reference used in the study with *E. fetida* (Teixeira 2006a) did not include maize tissue, it did not prove effective exposure to the Cry proteins. Moreover, the carbendazim used as a toxic reference may also have acted by contact. The only indication that the worms fed on the maize tissue is that the weight loss in the assay control was greater (8–10 %) than in the *Bt*- and non-*Bt*-treatments (authors' personal communication). Similarly, the toxic reference used in the study with *F. candida* (Teixeira 2006b) did not include maize powder. The control therefore only shows that the collembolans ingested yeast and yields no evidence that the test was able to detect toxic effects of the *Bt*-containing maize tissue. In addition, the thiodicarb may also have acted on the collembolans by contact. Contact toxicity caused by the use of thiodicarb may also have occurred in an earlier study by Teixeira (2001) with *F. candida*. As in the latter study purified protein was mixed in the yeast diet, ingestion is to be expected (Yang et al. 2015).

Besides the study by Teixeira (2006b), also for other laboratory studies it is uncertain that the test organisms have actually ingested sufficient amounts of the test substance. These include (1) tri-trophic studies where *Bt*-maize fed aphids were used to expose predatory Coccinellidae larvae to the *Bt*-proteins (Higgins 2000; Takács et al. 2010, 2012) since there is strong evidence that aphids do not (or at very low levels) ingest Cry proteins when feeding on *Bt*-transgenic plants (Romeis and Meissle 2011); (2) studies where the predatory insects were fed a mixture of a preferred prey that did not contain the Cry toxins (i.e., aphids) with an alternative food containing the Cry proteins (i.e., *Bt*-maize pollen) (Takács et al. 2010); (3) studies in which adult test specimens were simultaneously exposed to the test treatment and an alternative water source on which they can survive (Bryan et al. 2000a) and (4) studies using insects with piercing-sucking mouthparts such as *C. carnea* and *O. insidiosus* where the test compounds were provided coated to moth eggs or pollen (Sindermann et al. 2001; Patnaude 2008). Predatory insects with piercing-sucking mouthparts do not consume the external surface of insect eggs (Rose 2007; US EPA 2010).

For several studies, exposure can be deduced based on the feeding habits of the test species and their development during the course of the test. The development of *G. viridula* (Székács and Kong 2011) and *D. plexippus* (Sears and Rempel 2003) on leaves of their host plant dusted with pollen confirmed exposure. Monarch larvae do not avoid maize pollen grains even up to a very high density of >1000 grains/cm^2 (Hellmich et al. 2001). Also, the similar development of honeybees solely fed *Bt*-pollen compared to bees fed conventional pollen (Maggi 2001), confirms exposure.

4. Inclusion of negative control treatments

All studies conducted included an appropriate negative control treatment consisting of pure diet (artificial diet or untreated prey or pollen). One study by Patnaude (2008) even added heat-denatured Cry proteins to the negative control. The observation of lethal or sublethal effects in the negative control treatment group is a strong indicator of an inappropriate study design. In the study by Patnaude (2008),

the high control mortality indicated that the diet was not optimal for the development of *O. insidiosus* nymphs and the study was therefore replaced by one with a more appropriate diet (Vinall 2011a). Vice versa, the absence of (sub)lethal effects in the negative control treatment, points to a good study design. Further, the negative control is also an indicator of when the validity of a test ends. The laboratory studies with the ladybird *H. convergens* (Bryan et al. 2000a) and the lacewing *C. carnea* (Sindermann et al. 2001) were terminated at day 11 and 10, respectively, when the control mortalities rose above 20 %. This is because control mortalities exceeding 20 % raise concerns over the test results (Vogt et al. 2000; Rose 2007).

5. Confirmation of the stability and bioactivity of the test substance during the testing period

Over the course of the laboratory study, consistent exposure to the test substance is preferable. The stability of the *Bt*-proteins (total protein concentration) over the test duration was ensured in 12 laboratory studies by recording the test substance concentration or replacing it at regular intervals (Table 2 of supplementary material). For the other studies, it is unclear whether the test organisms were constantly exposed to the Cry proteins. Only for one study with earthworms (Bryan et al. 2000b) the Cry34/35Ab1 protein concentrations were reported not to be monitored throughout the test period (EFSA 2013a). For the latter study, it therefore remains unclear for which period of time the earthworms were exposed to the *Bt*-proteins.

Information on whether the test substance remained bioactive during the study period was not provided in the majority of studies. If optimal storage conditions are used for the test substance, one can presume that it remains stable and active (Nguyen and Jehle 2009). When suboptimal conditions are used, like in the study of Székács and Kong (2011), it is advisable to confirm bioactivity. In this study, the harvested pollen was stored at −10 °C for three months, and subsequently lyophilised and stored at −20 °C for approximately 12 months (EFSA 2013a), instead of at −80 °C. The bioactivity of the Cry34/35Ab1 proteins was, however, not quantified at diet administering in the latter study and therefore it is uncertain whether the test species were exposed to fully bio-active Cry proteins.

6. Measurement endpoints

In the laboratory studies, typical measurement endpoints to detect lethal and sublethal effects were considered such as survival, development or weight gain, the percentage of individuals that reach a certain life stage, and to a lesser extent reproduction and mobility. Appropriate measurement endpoints are those that are easy to evaluate and likely to indicate the possibility of adverse effects (Romeis et al. 2011). For all studies, the endpoints measured were in accordance with international (e.g. OECD) standards and were considered appropriate.

7. Statistical power analysis

Each experiment should be sufficiently replicated to detect a defined effect size (i.e., 20 % is suggested by EFSA 2010; 50 % is used by US EPA; Rose 2007) with an acceptable statistical power. A level of 80 % power at an alpha level of 0.05 is usually considered acceptable (see Romeis et al. 2011). For several laboratory studies, either a prospective or retrospective power analysis was performed to demonstrate that the studies had acceptable statistical power (>80 %) to detect an effect size that ranges from 20 to 40 % (Table 2 of supplementary material). As effect sizes to be detected vary from jurisdiction to jurisdiction, the studies may be judged differently at the level of statistical power.

8. Representativeness of the tested species

Bt-proteins were tested against a range of NTOs. These species are usually selected because they are either of conservation concern or represent taxonomic or functional groups that contribute to ecosystem services. Given the activity of the Cry34/35Ab1 proteins towards corn rootworm beetles, several in vivo laboratory studies considered non-target beetle species. The green dock leaf beetle, a herbivorous chrysomelid, was tested as well as predators from the Carabidae (ground beetles, *P. cupreus*) and Coccinellidae families (ladybirds, *C. maculata*, *C. septempunctata*, *H. convergens*). Other species studied providing ecosystem services were *N. vitripennis* (parasitic wasps), *Orius* spp. (predatory flower bugs) and *C. carnea* (predatory lacewings). Further, pollinators (i.e., the honeybee *A. mellifera*) and decomposers including soil-dwelling ones (i.e., the

springtail, *F. candida,* and the compost worm, *E. fetida*) were examined, as well as some species of conservation concern (i.e., different species of Lepidoptera), and a non-target aquatic organism *D. magna*, a food source of fish that may occur in water bodies near maize fields.

Most of the selected non-pest species were considered representatives of important arthropod and annelid species in maize ecosystems (Meissle et al. 2012). Exceptions are *N. vitripennis* (COGEM 2008; EFSA 2013a; US EPA 2010) and *C. quinquefasciatus* (EFSA 2013a). The parasitic wasp *N. vitripennis*, chosen as a surrogate for hymenopteran parasitoids, parasitises fly pupae in bird's nests (Abraham 1985). It was therefore suggested to use a more ecologically relevant parasitoid like *Macrocentrus grandii* (Hymenoptera: Braconidae), a parasitoid of the European corn borer (*Ostrinia nubilalis*; Lepidoptera: Crambidae) (US EPA 2010). Because there is some evolutionary relatedness between the Cry35Ab1 protein and the mosquitocidal binary toxins from *Lysinibacillus (Bacillus) sphaericus* and *Bacillus cereus* (reviewed by Federici et al. 2003; Krauss 2011) the southern house mosquito *C. quinquefasciatus* was selected as a surrogate for potentially exposed Diptera. As mosquitoes are not species typically occurring in maize, other Diptera, such as hoverflies or saprophytic dipteran larvae, occurring in maize fields (Meissle et al. 2012; Romeis et al. 2014) were considered to be more ecologically relevant (EFSA 2013a). Nonetheless, studies as the one with *N. vitripennis* and *C. quinquefasciatus* add confidence to the risk assessment that the Cry34/35Ab1 proteins are unlikely to affect Hymenoptera and Diptera.

Conclusion

A typical risk hypothesis addressed during the ERA of *Bt*-plants is that the newly expressed *Bt*-proteins are not toxic to valued NTOs at concentrations present in the field. Twenty-five laboratory studies with Arthropoda and Annelida testing this hypothesis for Cry34/35Ab1 proteins expressed in *Bt*-maize were reviewed and assessed for their scientific quality using the recommendations on experimental design put forward by Romeis et al. (2011). Maize event 59122 was selected as an example to verify in how far these recommendations are met and to assess the importance of adhering to these recommendations.

As information was not always reported for each criterion in every study, we experienced some limitations in the appraisal of the experimental design. In particular, the statistical analysis criterion could often not be judged due to the lack of information. In order to allow appraisal of the overall study design, we presumed that the power of those studies that lacked the information was sufficient to detect a predefined effect size. Further, in order to place the diverse studies within a more uniform framework for comparison at the level of exposure, a base EEC was calculated on the basis of highest reported dry weight expression levels in field trials, where possible. This base EEC could be helpful in weighing the studies for their relevance and thus in understanding how much reliance can be placed on single studies by risk assessment bodies to assess impacts on non-target organisms.

An evaluation of the early tier studies with NTOs revealed that in order to have key studies for ERA, all good design recommendations are to be met. Not adhering to (one of) the criteria, increases the probability of erroneous results and decreases confidence in the test results. A review of the conducted tests for maize event 59122 showed that they fulfilled most of the scientific quality criteria set, but not all. On the condition that the defined effect size was detected, only a restricted set of studies (4) can be considered as key studies (study 5, 13, 15, 18 in Table 2 of supplementary material).

For those studies that do not fulfil all recommendations, the exercise in judging their quality and weighing their usefulness in the ERA becomes more challenging. Clearly some minimum criteria should be met to consider a study sufficiently robust to have relevance for ERA. The equivalence of the test substance with the one produced by the GM crop is considered a prerequisite for the study to be informative for ERA. If the test substance is not equivalent at the biochemical and functional level (i.e. less active), deviating results might be obtained. Further, if the test substance provided is not bioactive or if the test organism is not exposed to the Cry protein(s) through oral ingestion, then a study is not suitable for ERA. Another criterion considered of major importance to judge the robustness of the experiments, is the use of an appropriate negative control. Without such a

control, it is impossible to evaluate the appropriateness of the diet used and thus to interpret the effects observed. As pointed out by Romeis et al. (2011), rearing species on a sub-optimal medium may in itself cause unforeseen side-effects on the measurement endpoints. Studies that do not fulfil these minimum criteria (study 1, 6, 9, 11, 12, 14, 17, 21) ought to be discarded.

Besides the four key studies, an additional thirteen studies met the minimum set of recommendations. As for these studies either exposure was low (below the EEC), intake could be better confirmed (e.g. by use of a positive control) or a less representative species was used, we would not consider them as key studies to come to a risk assessment conclusion on adverse effects to NTOs. However, they could still provide some supportive information to the risk assessment.

On the basis of studies, including early tier studies, to asses impacts on NTOs, different jurisdictions concluded that the risk to non-target terrestrial (plant- and ground-dwelling), soil and aquatic arthropod NTOs and annelids resulting from the exposure to the Cry34/35Ab1 proteins is negligible (CFIA 2005; EFSA 2013a; US EPA 2010). These conclusions were drawn using a weight of evidence approach considering not only the outcomes of the laboratory studies, but also those of higher tier studies, i.e. field trials. EFSA recently revised its initial evaluation by stating that there remains scientific uncertainty for ladybirds as a.o. the worst-case EEC conditions tested were not sufficiently conservative for spider mite-consuming ladybirds (EFSA 2013b). Spider mites (*Tetranychus urticae*) have been shown to contain bio-active plant-produced Cry protein concentrations that can be similar or even higher (fourfold) to those measured in leaves of *Bt*-maize on which they have fed (Obrist et al. 2006; Álvarez-Alfageme et al. 2008; Meissle and Romeis 2009). As a study is lacking exposing a ladybird to (extreme) worst-case EEC conditions resembling the high concentrations that may occur in spider mites, it was not considered recommendable to extrapolate the results of the well-conducted studies with (mainly aphidophagous) coccinellids to all types of coccinellids occurring in maize ecosystems. In particular, this example stresses the importance of conducting key studies with high enough amounts of the toxins, preferably with concentrations exceeding the mean EEC observed and even including a safety factor (usually 10). This should prevent uncertainty resulting from gaps in knowledge about the expression levels that exist at the time of conduct of the studies.

In conclusion, this review highlights the necessity to carry out studies meeting a minimum number of the quality criteria set by Romeis et al. (2011) in order to be of any relevance for ERA. This exercise also showed that adhering to the study design recommendations will ease the work of risk assessors in judging the reliability of the laboratory studies. We envisage that with the 2011 recommendations on study design and the knowledge gathered over the years with conducting early tier studies, the usefulness of these studies for risk assessment will be further enhanced.

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