

# *Bt*-maize event MON 88017 expressing Cry3Bb1 does not cause harm to non-target organisms

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**Abstract** This review paper explores whether the cultivation of the genetically modified *Bt*-maize transformation event MON 88017, expressing the insecticidal Cry3Bb1 protein against corn rootworms (Coleoptera: Chrysomelidae), causes adverse effects to non-target organisms (NTOs) and the ecological and anthropocentric functions they provide. Available data do not reveal adverse effects of Cry3Bb1 on various NTOs that are representative of potentially exposed taxonomic and functional groups, confirming that the

insecticidal activity of the Cry3Bb1 protein is limited to species belonging to the coleopteran family of Chrysomelidae. The potential risk to non-target chrysomelid larvae ingesting maize MON 88017 pollen deposited on host plants is minimal, as their abundance in maize fields and the likelihood of encountering harmful amounts of pollen in and around maize MON 88017 fields are low. Non-target adult chrysomelids, which may occasionally feed on maize MON 88017 plants, are not expected to be affected due to the low activity of the Cry3Bb1 protein on adults. Impacts on NTOs caused by potential unintended changes in maize MON 88017 are not expected to occur, as no differences in composition, phenotypic characteristics and plant-NTO interactions were observed between maize MON 88017 and its near-isogenic line.

Yann Devos and Adinda De Schrijver contributed equally to the work and should be considered co-first authors.

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## Introduction

Western corn rootworm (*Diabrotica virgifera virgifera*; WCR) (Coleoptera: Chrysomelidae) is a major maize pest and a serious threat to agriculture in North America (Metcalf 1986) and the European Union (EU) (FCEC 2009; Wessler and Fall 2010). WCR larvae feed on maize roots, and negatively affect yield by decreasing nutrient and water uptake and plant stability. The bulk of plant damage is caused by larvae, but adults feeding on silk and grains can be particularly damaging in seed and sweet corn production (Meinke et al. 2009). WCR has been introduced accidentally into the EU from North America, where it is native and widespread (Miller et al. 2005; Ciosi et al. 2008). It was first detected in 1992, and has since then spread across the continent, resulting in well established pest populations in 19 European countries (<http://extension.entm.purdue.edu/wcr/>).

WCR larvae can be managed through crop rotation and chemically via the use of maize seed coated with systemic insecticides and/or soil insecticides applied at planting (reviewed by Boriani et al. 2006; van Rozen and Ester 2010; Meissle et al. 2011b). Foliar broad-spectrum insecticides are sometimes applied to suppress adult populations, especially in continuous maize, in order to decrease egg-laying by females, or to prevent silk clipping by adults in seed and sweet corn production, where high grain quality is essential for marketing. Crop rotation controls WCR effectively (Kiss et al. 2005), as hatched larvae in spring/early summer do not survive well on other crop roots. However, the evolution of resistance to several insecticides and the evolution of rotation-resistant pest populations in some maize growing regions in the USA have diminished the efficiency of these pest management practices in controlling WCR (Meinke et al. 2009). In EU regions where WCR populations have been detected, but are not yet established, mandatory eradication programs require the application of insecticides and planting restrictions of maize in buffer zones surrounding new introduction points (FCEC 2009; Carrasco et al. 2010).

Genetically modified (GM) *Bt*-maize transformation events expressing insecticidal Cry3 proteins against corn rootworms (*Diabrotica*) offer an additional means of control against WCR. The mode of action of Cry3 proteins is to bind selectively to specific receptors on the epithelial surface of the midgut of

larvae of susceptible insect species, leading to death of larvae through pore formation, cell burst and subsequently septicemia (reviewed by Sanahuja et al. 2011). Cry3 proteins have been characterised as having insecticidal activity against coleopteran species (Höfte and Whiteley 1989), and the insecticidal activity of Cry3Bb1 proteins has been shown to be restricted to certain species of the coleopteran Chrysomelidae family, including species of the corn rootworm complex (Donovan et al. 1992; Vaughn et al. 2005). The Cry3Bb1-expressing *Bt*-maize event MON 88017 is currently grown commercially in Argentina, Brazil, Canada and the USA (CERA 2012). Depending on the region, *Diabrotica*-resistant *Bt*-maize is used to control western (*D. v. virgifera*), northern (*D. barberi*) and Mexican corn rootworm (*D. v. zea*), and *D. speciosa*. In the EU, the authority responsible for providing advice on the safety of GM plants (European Food Safety Authority, EFSA) recently issued a scientific opinion on the cultivation of maize MON 88017 (EFSA 2011).

To ensure that *Diabrotica*-resistant *Bt*-maize cultivation does not harm other organisms than the pests it targets, termed non-target organisms (NTOs), an environmental risk assessment (ERA) needs to be carried out as part of the regulatory approval process for commercial cultivation of GM plants. In particular, the ERA needs to assess whether NTOs and the ecological and anthropocentric functions (hereafter ecosystem services) they provide, might be adversely affected. Valued ecosystem services to preserve in an agricultural context are pest regulation, pollination, decomposition of organic matter, soil nutrient cycling, soil structure, water regulation and purification, and cultural services (such as aesthetic value) (Moonen and Bàrberi 2008; Sanvido et al. 2009, 2012). Because from a practical viewpoint not all NTO species can be tested, the toxicity of Cry proteins is generally tested on a representative subset of species that are exposed to the toxin (Garcia-Alonso et al. 2006; Rose 2007; Romeis et al. 2008). Focus is put on species that are representative for specific ecosystem services (e.g., natural enemies for pest regulation, honeybees for pollination, springtails and earthworms for soil-related processes) and species of conservation concern (e.g., rare and protected species, or species of aesthetic or cultural value). Potential harmful effects on NTOs are evaluated within different tiers that progress from highly controlled worst-case exposure lower-tier

studies in the laboratory over greenhouse studies to more realistic but less controlled higher-tier studies in the field. Moving to a higher-tier is only considered relevant if adverse effects are detected, or if unacceptable scientific uncertainty remains (Garcia-Alonso et al. 2006; Rose 2007; Romeis et al. 2008). In some cases, higher-tier studies may be conducted at an initial stage when lower-tier studies are not possible or meaningful. Besides the assessment of potential adverse effects on NTOs due to the intended genetic modification (i.e., the expression of the Cry protein), the ERA includes those arising from potential unintended changes in the GM plant, which go beyond the primary objectives of the genetic modification (EFSA 2010; Raybould et al. 2010; Wolt et al. 2010).

The present review paper explores whether the Cry3Bb1-expressing *Bt*-maize event MON 88017 causes adverse effects to NTOs. It considers peer-reviewed studies published in the scientific literature, as well as studies submitted to and subsequently reviewed by risk assessment bodies as part of the GM plant market approval process (US EPA 2000, 2002a, b, c, 2007, 2010; BAC 2010; EFSA 2011). Available lower- and higher-tier studies on NTOs, assessing the target specificity and toxicity of the Cry3Bb1 protein of maize MON 88017, are reviewed for non-target terrestrial, soil and aquatic arthropods, and non-arthropod NTOs. Studies conducted with the Cry3Bb1 protein variants of the maize events MON 863 and MON 853, or with plant material from these events are also considered as the amino acid sequence of the Cry3Bb1 protein variants of maize MON 88017, MON 863 and MON 853 are similar and their biological activity equivalent (Box 1). Few studies with other Cry3Bb1-expressing maize lines (MON 858 and MON 859) are mentioned, but are not used to draw conclusions on the safety of maize MON 88017, as the degree of similarity of the Cry3Bb1 protein variants expressed in these lines to that of maize MON 88017 is not known. Exposure data are included where considered relevant for ERA. Further, this paper puts the data into an ERA context taking into account the ecological relevance of the tested species and their likely exposure to maize MON 88017. The reviewed data and the discussion of the data within an ERA context can be relevant to other risk assessment bodies.

Maize MON 88017 also provides tolerance to the herbicidal active substance glyphosate by the introduction of a gene coding for the enzyme

5-enolpyruvylshikimate-3-phosphate synthase (EP-SPS) from *Rhizobium radiobacter* (formerly named as *Agrobacterium tumefaciens*) strain CP4 (CP4 EPSPS). This review paper does not address potential risks due to the novel herbicide regimes applied on maize MON 88017.

## Studies assessing the impact of Cry3Bb1 proteins on NTOs

Non-target terrestrial (plant- and ground-dwelling) arthropods

### *Herbivores*

*Lower-tier studies* Lower-tier studies with several herbivores (Table S1 in Online Supporting Material) have been conducted to determine the target specificity and hence the spectrum of activity of Cry3Bb1 proteins. Herbivores (prey/host organisms) are an important food source for other species of the food web, can ingest plant-produced Cry proteins when feeding on *Bt*-plants, and can transfer Cry proteins to higher trophic levels. The level at which different herbivores ingest Cry proteins depends on the site and time of protein expression in the plant, the mode of feeding of the herbivore, and the amount of plant material they ingest (Romeis et al. 2009).

*Acari: Tetranychidae (spider mites)* The lower-tier study by Li and Romeis (2010) indicated that the spider mite *Tetranychus urticae* is not affected by the Cry3Bb1 protein. Development time to the adult stage, immature survival, female longevity, as well as reproduction parameters (pre-oviposition period, fecundity and fertility) did not differ for spider mites fed maize MON 88017 or non-*Bt*-maize.

*Coleoptera: Chrysomelidae (leaf beetles)* No adverse effects on larval survival of the chrysomelid *Callosobruchus maculatus* were observed in bioassays after exposure to the Cry3Bb1 protein up to 200 ppm (Head et al. 2001).

Shirai (2006) reported that survival and development rate of larvae of *Galerucella vittaticollis* to the third instar were not adversely affected when exposed to pollen from maize MON 863 at doses of 500, 1,000 and 2,000 grains/cm<sup>2</sup> for 10 days after hatching. Densities of maize pollen deposited on host plants in

**Box 1** Equivalence of Cry3Bb1 protein variants

The wild type *cry3Bb1* gene from *Bacillus thuringiensis* (*Bt*) subsp. *kumamotoensis* has been modified to enhance protein activity resulting in different Cry3Bb1 protein variants (see Table B1 below). The amino acid sequences of the Cry3Bb1 protein variants present in maize MON 863 and MON 853 share a high identity with that of MON 88017: they differ by one amino acid of the 653 amino acid residues, respectively at positions 166 (G-D substitution) and 349 (Q-R substitution). The MON 863 and MON 853 Cry3Bb1 protein variants differ from each other by two amino acids: a G-D substitution at position 166 and an R-Q substitution at position 349. Tertiary modelling data comparing the MON 863 and MON 853 Cry3Bb1 protein variants indicated that the 2 amino acid substitutions do not alter their 3-dimensional structure (Astwood et al. 2001). Further, the LC<sub>50</sub> values of the MON 853 and MON 863 Cry3Bb1 protein variants for Colorado potato beetle larvae (Astwood et al. 2001), as well as the LC<sub>50</sub> values of the MON 863 and MON 88017 Cry3Bb1 variants for Colorado potato beetle and WCR larvae (Duan et al. 2003) were shown to overlap. On the basis of this dataset, the MON 88017, MON 863 and MON 853 Cry3Bb1 protein variants were considered equivalent in their physicochemical characteristics and biological activity by CFIA (2006), US EPA (2007, 2010), BAC (2010) and EFSA (2011)

Table B1 Cry3Bb1 protein variants: amino acid position differences and LC<sub>50</sub> values

Cry3Bb1 protein variant	Amino acid positions*		Tests of equivalence	Reference
	165/166	348/349		
MON 853/Cry3Bb1.11231	Aspartic acid (D)	Glutamine (Q)	Primary structure analyses, tertiary structure modelling & insect bioassays	Astwood et al. (2001)
MON 863/Cry3Bb1.11098 (Q349R)	Glycine (G)	Arginine (R)		
MON 863/Cry3Bb1.11098 (Q349R)	Glycine (G)	Arginine (R)	Primary structure analyses & insect bioassays	Duan et al. (2003)
MON 88017/Cry3Bb1.pvzmir39	Aspartic acid (D)	Arginine (R)		

\* The *Bt*-produced Cry3Bb1 protein variants contain 652 amino acids, and differ at positions 165 and 348. The *Escherichia coli*- and plant-derived Cry3Bb1 protein variants contain 653 amino acids (due to an extra Alanine at position 2) and differ at positions 166 and 349

maize fields during anthesis are rarely as high as 1,000 grains/cm<sup>2</sup>; mean maize pollen densities reported range from 250 to 500 pollen grains/cm<sup>2</sup> in-field and are less than 100 pollen grains/cm<sup>2</sup> in the field margin (Pleasants et al. 2001; Gathmann et al. 2006).

For two other chrysomelids, namely *Gastrophysa viridula* and *Phaedon cochleariae*, higher mortality, lower growth (biomass) and, in the case of the *G. viridula*, a lower development rate to pupae was observed after exposure to diets containing different concentrations of the purified Cry3Bb1 protein up to 0.68 µg per larva (Huber and Langenbruch 2008). However, exposure to 470 and 750 maize MON 88017 pollen grains did not adversely affect larval survival, development rate to pupae and weight of *G. viridula* and *P. cochleariae*.

Several studies showed that the Cry3Bb1 protein has adverse effects on the larvae of the Colorado potato beetle *Leptinotarsa decemlineata*, a key pest of potato. Donovan et al. (1992) showed that the Cry3Bb

protein increased larval mortality of the Colorado potato beetle. Higher mortality and slower growth for *L. decemlineata* larvae were observed when fed diets, containing either purified Cry3Bb1 protein, or maize MON 88017 pollen, silk, leaves or roots, in 7-days assays, as compared to the respective control treatment (Huber and Langenbruch 2008; Meissle and Romeis 2009a). LC<sub>50</sub> values of 1–7 µg Cry3Bb1/g of diet (Park et al. 2009), 0.52–0.86 µg Cry3Bb1/ml of diet (Meissle and Romeis 2009a), or 1.35 ng Cry3Bb1/mm<sup>2</sup> of diet surface (Donovan et al. 1992) were reported. Exposure to 470 and 750 maize MON 88017 pollen grains, however, did not adversely affect survival, development rate to pupae and weight of the Colorado potato beetle larvae (Huber and Langenbruch 2008).

Larvae of *Oulema melanopus* fed maize MON 88017 leaf material (containing 28 µg Cry3Bb1/g dry weight [DW]) had lower survival compared to those fed non-*Bt*-maize leaf material, but similar development time to the

prepupal stage and prepupal weight (Meissle et al. 2012). No effect on adult survival and weight was observed after 21 days of exposure to maize MON 88017 leaf material.

**Coleoptera: Coccinellidae (ladybirds)** Survival and development rate to third instars of *Epilachna vigintioctopunctata* larvae reared on leaf discs dusted with 250, 500, 1,000 and 2,000 maize MON 863 pollen grains/cm<sup>2</sup> for 10 days after hatching were not adversely affected (Shirai 2006).

**Coleoptera: Curculionidae (weevils) and Tenebrionidae (darkling beetles)** Bioassays did not reveal adverse effects on larval survival of the curculionids *Anthonomus eugenii*, *A. grandis* and *Sitophilus oryzae*, and the tenebrionid *Tribolium castaneum* after exposure to the Cry3Bb1 protein up to 200 ppm (Head et al. 2001). For *A. grandis*, the maximum concentration used was 50 ppm instead of 200 ppm.

**Hemiptera: Aphididae (aphids)** Lundgren and Wiedenmann (2005) reported that the aphids *Rhopalosiphum maidis* and *R. padi* weighed significantly less when reared on maize MON 863 than when reared on non-*Bt*-maize, but concluded that the reduction in aphid weight was attributed to variety differences between the *Bt*- and non-*Bt*-maize, rather than the Cry3Bb1 protein. That the effect was not due to the Cry3Bb1 protein is supported by the fact that aphids do not ingest detectable amounts of this protein when feeding on Cry3Bb1-expressing maize (Table 1).

**Lepidoptera: Crambidae, Danaidae and Noctuidae (butterflies and moths)** Sears and Mattila (2001) and Mattila et al. (2005) assessed the effects of maize MON 863 pollen (63.1 µg Cry3Bb1/g pollen) on the monarch butterfly *Danaus plexippus* (Lepidoptera: Danaidae). First instars were placed on milkweed leaves (*Asclepias syriaca*) dusted with doses of either *Bt*-maize pollen or non-*Bt*-maize pollen ranging from 50 to 3,200 pollen grains/cm<sup>2</sup>, or no pollen at all. Exposure to *Bt*-maize pollen for 4 days had no negative effects on larval mortality, development rate to third instars, growth (biomass) and leaf consumption relative to the controls. The European corn borer *Ostrinia nubilalis* (Lepidoptera: Crambidae) and the corn earworm *Helicoverpa zea* (Lepidoptera: Noctuidae) were shown not to be affected in mortality and weight by the Cry3Bb1 protein at a test concentration of 200 µg/ml after 7 days of exposure (Head et al. 2001).

**Higher-tier studies** In the USA, no negative impact of maize MON 863 was revealed on field densities of abundantly occurring species belonging to the families of Elateridae (Coleoptera), Aphididae (Hemiptera) and Gryllidae (Orthoptera) (Ahmad et al. 2005; Bhatti et al. 2005b) (Table S2 in Online Supporting Material). Bhatti et al. (2005b) reported decreased abundance of Chrysomelidae such as the corn flea beetle *Chaetocnema pulicaria* in *Bt*-maize. Foliar insecticide applications resulted in increased aphid populations probably due to reduced abundance of some spiders, coccinellids, the green lacewing and nabids (Al-Deeb and Wilde 2003; Bhatti et al. 2005a, b; McManus et al. 2005; Ahmad et al. 2006a). In the EU, no negative impact of maize MON 88017 was observed on the abundance of: chrysomelids such as *Phyllotreta vittula* (Coleoptera: Chrysomelidae); the hemipterans *Metopolophium dirhodum* and *R. padi* (Aphididae), *Zyginidia scutellaris* (Cicadellidae) and *Trigonotylus caelestialium* (Miridae); and the thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae) (Rauschen et al. 2008, 2009, 2010a, b; Svobodová et al. 2012a).

#### *Natural enemies (predators and parasitoids)*

**Lower-tier studies** Several lower-tier studies (Table S1 in Online Supporting Material) have been conducted to assess the toxicity of Cry3Bb1 proteins to several organisms that are valued for the pest regulation services they provide. Predators and parasitoids are likely to be exposed to plant-produced Cry proteins when feeding on phytophagous arthropods that contain Cry proteins or Cry-containing maize plant material. Several species of spiders, ground beetles, ladybirds, rove beetles, predatory bugs, and larvae of *Chrysoperla* spp. are known to be predators of herbivores (including pest insects) found in maize (Pilcher et al. 1997 and references therein; Ahmad et al. 2006a). Further, parasitic wasps attack a variety of herbivores occurring in maize ecosystems. In addition, many predators are facultative herbivores feeding on pollen, nectar and plant juices, while parasitoids primarily feed from (extra-)floral nectaries (Lundgren 2009; Romeis et al. 2009). Adults of the green lacewing *Chrysoperla carnea* (Neuroptera: Chrysopidae) are not predacious, but are prevalent pollen consumers in maize fields. Despite the broader feeding habits of some species, the species for which

**Table 1** Means and ranges in Cry3Bb1 protein levels in various plant parts of maize MON 88017 and MON 863, and aphids or spider mites fed maize MON 88017 and MON 863 plant material ( $\mu\text{g/g}$  fresh weight) (modified from EFSA 2011)

Plant parts	Maize MON 88017				Maize MON 863			
	2006 EU field trials	2002 USA field trials	<i>Rhopalosiphum padi</i>	<i>Metopolophium dirhodum</i>	<i>Sitobion avenae</i>	<i>Tetranychus urticae</i>	1999 USA field trials	<i>Rhopalosiphum maidis</i> – <i>Rhopalosiphum padi</i>
Leaf (OSL1; V2-4)	53 (37–67)	60 (22–84)	< LOD (0.001)–0.008	< LOD (0.002)	< LOD (0.002)	5.6–21.6	81 (65–93)	< LOD
Leaf (OSL2; V6-8)	59 (51–70)	59 (49–73)					NA	
Leaf (OSL3; V10-12)	49 (38–78)	55 (43–74)					NA	
Leaf (OSL4)	48 (25–70)	49 (39–61)					NA	
Root (OSR1; V2-4)	20 (12–34)	34 (21–44)					NA	
Root (OSR2; V6-8)	24 (13–37)	30 (22–48)					NA	
Root (OSR3; V10-12)	14 (10–18)	25 (18–44)					41 (25–56)	
Root (OSR4; pre-VT)	15 (12–21)	23 (14–30)					NA	
Grain	8 (5–13)	9 (6–13)					70 (49–86)	
Silk	16 (11–24)	31 (26–38)					10 (no range)	
Pollen	9 (5–12)	13 (10–17)					62 (30–93)	
Reference	Niemeyer and Silvanovich (2007); see also Nguyen and Jehle (2009a)	Bhakta et al. (2003)	Meissle and Romeis (2009b)	Meissle and Romeis (2009b)	Meissle and Romeis (2009b)	Meissle and Romeis (2009b), Li and Romeis (2010), Alvarez-Alfageme et al. (2011), Garcia et al. (2011)	Dudin et al. (2001)	Lundgren and Wiedenmann (2005)

LOD limit of detection; NA not assayed; OSL over season leaf; OSR over season root;  $V(n)$  nth vegetative growth stage; TV tasseling

their primary valued function is pest regulation are addressed in this section.

**Araneae (spiders)** Juvenile and adult *Phylloneta impressa*, formerly named as *Theridion impressum* (Araneae: Theridiidae), fed Cry3Bb1-containing prey and/or maize MON 88017 pollen were not adversely affected by the Cry3Bb1 protein after 8 weeks of exposure (Meissle and Romeis 2009b). Lacewings, WCR and spider mites, used as prey, contained mean concentrations of 0.74, 16.4 and 72.5 µg Cry3Bb1/g DW, respectively; field-collected pollen contained 27 µg Cry3Bb1/g DW; and greenhouse-collected pollen contained 8.3 µg Cry3Bb1/g DW. No differences in mortality, growth (biomass), adult weight, or fertility were observed between spiders provided with food containing or not containing the Cry3Bb1 protein. Quantification of the Cry3Bb1 protein in potential prey species collected in *Bt*-maize plots and prey spectrum analysis revealed that *P. impressa* ingests the Cry3Bb1 protein in the field. Exposure to the *Bt*-protein, however, was highly variable because some potential prey species, such as phloem-feeding herbivores and predators, contained little or no Cry3Bb1 protein, whereas tissue-feeding herbivores contained higher concentrations.

**Coleoptera: Carabidae (ground beetles)** Mullin et al. (2005) did not observe a significant effect on the survival time of field-collected adult carabid beetles of 12 different species from 8 genera (*Agonum*, *Amara*, *Chlaenius*, *Harpalus*, *Patrobus*, *Poecilus*, *Pterostichus* and *Scarites*) fed maize MON 863 pollen containing 30–90 µg Cry3Bb1/g fresh weight (FW). However, only for 7 out of the 12 species, the pollen feeding bioassay was replicated. Survival time ranged from 47 days for the predator *Pterostichus melanarius* to a year for the more omnivorous *Scarites quadriceps*. Similar observations for some of the carabid species tested by Mullin et al. (2005) were made by Ahmad et al. (2006a) and Duan et al. (2006b). No significant differences in mortality of adult *Harpalus caliginosus* and *H. pennsylvanicus*, reared on artificial diets with maize MON 863 or non-*Bt*-maize pollen were detected by Ahmad et al. (2006a). The Cry3Bb1 protein concentration in pollen was quantified to be 40.3 µg/g FW. In addition, no significant effects of *Bt*-maize pollen on fecundity and fertility of *H. caliginosus* were observed (Ahmad et al. 2006a). Duan et al. (2006b) showed that the Cry3Bb1 protein at a

concentration of 930 µg/g in an agar-based artificial diet had no adverse effect on the survival, development rate to adults and growth (biomass) of *Poecilus chalcites* larvae after 28 days of continuous exposure. The dose tested was about 10 times greater than the highest expected environmental concentration of the Cry3Bb1 protein in maize MON 88017 plant parts (Table 1). Priesnitz (2010) did not observe adverse effects on the survival of adult *Calathus fuscipes*, *C. ambiguus*, *Pseudophonus rufipes* and *P. melanarius* after 35 days of exposure to a 325 µg Cry3Bb1/ml artificial diet. Further research is, however, needed to confirm these preliminary data due to the low number of individuals tested per treatment and replications. ELISA analyses confirmed the uptake of the Cry3Bb1 protein by these carabids both under laboratory and field conditions (Priesnitz 2010).

**Coleoptera: Coccinellidae (ladybirds)** Several studies have evaluated the effect of the Cry3Bb1 protein on various ladybird species. Schmidt et al. (2009) reported a significantly increased mortality in first instars of *Adalia bipunctata* when continuously exposed to eggs of the Mediterranean flour moth *Ephestia kuehniella* (Lepidoptera: Pyralidae) that had been sprayed with 25 µg/ml of microbially produced Cry3Bb protein. However, no differences were observed in mortality of first and later instars at concentrations of 5 or 50 µg Cry3Bb/ml, and in development time and weight of newly emerged adults at all concentrations tested. The authors suggested that the increased mortality of first instars was caused directly by the Cry3Bb protein. However, this study was criticised by the scientific community (Meissle and Romeis 2008; ZKBS 2009, 2011; Rauschen 2010; Ricroch et al. 2010; Álvarez-Alfageme et al. 2011; Romeis et al. 2012; but see Hilbeck et al. 2012). Points of criticism included amongst others the observed high control mortality of first instars (up to 21.7 %), the limited exposure of larvae to the Cry3Bb protein as they puncture and subsequently suck out the egg contents, the fact that no adverse effects were observed at the highest Cry3Bb concentration, and the lack of sublethal effects for surviving individuals. Recent laboratory bioassays with *A. bipunctata* larvae exposed to the Cry3Bb1 protein, either continuously through spider mites (*T. urticae*) fed maize MON 88017 material (19.9 µg Cry3Bb1/g FW), or at intervals through purified protein at concentrations

approximately 10 times higher than measured in spider mites fed *Bt*-maize, revealed no effects on *A. bipunctata* mortality, development time and weight (Álvarez-Alfageme et al. 2011). Hilbeck et al. (2012) argued that the lethal effect was not observed by Álvarez-Alfageme et al. (2011) in the direct feeding study with purified protein due to the fact that the larvae were only provided with the test substance at intervals and were not exposed continuously as in the Schmidt et al. (2009) study. However, the two positive controls treatments included by Álvarez-Alfageme et al. (2011) revealed that the study was able to detect changes in development time and weight (Romeis et al. 2012). Further, in the spider mite bioassay of the latter study, no adverse effects on the same endpoints and mortality were observed.

Adults of *Coleomegilla maculata* were reared on diets containing equal amounts by weight of lyophilised eggs of the Mexican fruit fly *Anastrepha ludens* (Diptera: Tephritidae) and maize MON 863 pollen (37.4 µg Cry3Bb1/g FW). After 30 days of exposure, no negative effects were observed on the survival of *C. maculata* adults compared with those reared on non-*Bt*-maize pollen (Duan et al. 2001a, 2002). Using the same egg diet, *C. maculata* larvae were exposed to a diet containing 50 % maize MON 863 pollen (93 µg Cry3Bb1/g FW). The results showed that also the larvae were not affected in survival, nor in their development time to pupae and adults, and that the emerged adults did not differ in weight (Duan et al. 2001b, 2002). Further, no significant difference was detected in fecundity of females fed maize MON 863 pollen and non-*Bt*-maize pollen (Duan et al. 2002).

Other research groups also assessed the potential impact of maize MON 863 pollen on *C. maculata*. Lundgren and Wiedenmann (2002) compared amongst others larval survival, development time, and pupal weight of *C. maculata* reared to adult stage on diets containing pollen mixtures of 0, 25, 50, 75, or 100 % maize MON 863 pollen to control treatments. Further, amongst others adult traits, including pre-oviposition period and mobility were determined. No differences in any of the life-history parameters among *C. maculata* fed different mixtures of pollen were observed after 30 days of exposure. Similarly, Ahmad et al. (2006a) did not observe significant differences in *C. maculata* survival, development time, weight, elytra length and adult longevity between *Bt*- and non-*Bt*-maize pollen (40.3 µg Cry3Bb1/g FW) treatments.

Whether the predator *C. maculata* could be affected through the consumption of *R. maidis* and *R. padi* reared on maize MON 863 was examined by Lundgren and Wiedenmann (2005). No deleterious effects on survival to pupation, larval and pupal development time, adult weight, fertility and adult mobility were revealed for *C. maculata* larvae fed aphids reared on *Bt*- or non-*Bt*-maize plant material. Life-history parameters of *C. maculata* were similar between *Bt*- and non-*Bt*-treatments, despite a 33 % reduction in the weight of aphid prey reared on maize MON 863. Examination of the Cry3Bb1 protein content in aphids or *C. maculata* fed aphids using immunostrip tests showed that the Cry3Bb1 protein was not detectable (Table 1). Thus, this study does not allow a conclusion about the sensitivity of *C. maculata* to the Cry3Bb1 protein.

Palmer and Krueger (1999a) showed that exposure of adult *Hippodamia convergens* beetles to 400 and 8,000 µg Cry3Bb1/ml artificial diet for 10 days resulted in no significant increase in mortality of ladybirds compared to the control group. The concentrations used were approximately 5 and 100 times the maximum protein concentration reported in maize MON 88017 plant parts. Further, it was demonstrated that survival of *H. convergens* adults was not adversely affected when offered a 50:50 honey: MON 863 pollen (55.9–72.5 µg Cry3Bb1/g FW) mixture for 15 days (Bryan et al. 2001).

Li and Romeis (2010) did not observe adverse effects on *Stethorus punctillum* larvae when fed spider mites (*T. urticae*) that were reared on maize MON 88017 or non-*Bt*-maize material. Larval survival to adults and development time, adult survival and weight did not differ for *S. punctillum* fed spider mites reared on *Bt*- or non-*Bt*-maize for over 2 months. Female beetles had a shorter pre-oviposition period, an increased fecundity, and a higher fertility in the *Bt*-treatment. The Cry3Bb1 protein content found in *T. urticae* reared on maize MON 88017 leaves and *S. punctillum* larvae fed spider mites that had consumed *Bt*-maize for 6 days was 55.9 and 7.5 %, respectively, of the mean concentration measured in *Bt*-maize leaves (37.6 µg/g FW). Adults that had consumed *Bt*-maize-fed mites for 56 days contained less than 1 µg Cry3Bb1/g FW.

*Coleoptera: Staphylinidae (rove beetles)* Prey-mediated effects of maize MON 88017 on the fitness



and predatory ability of *Atheta coriaria* were assessed using the spider mite *T. urticae* as prey (García et al. 2011). The Cry3Bb1 protein concentration through the trophic chain was shown to decrease significantly from *Bt*-maize (21.7 µg/g FW) over mites (5.6 µg/g FW) to *A. coriaria* adults (1.4 µg/g FW), but not from mites to *A. coriaria* larvae (4.1–4.6 µg/g FW). No significant differences were observed in larval survival to adults, larval and pupal development time, growth (head capsule width), elytra length and weight of newly emerged adults between rove beetles fed mites reared on maize MON 88017 or the corresponding non-*Bt*-maize line. Moreover, no negative effects on reproductive parameters (pre-oviposition period, fecundity and fertility) were reported in adults fed prey reared on *Bt*-maize after 30 days of exposure, and their survival was not affected after 60 days of exposure. In addition, the predatory ability of *A. coriaria* larvae and adults was not affected by exposure to the Cry3Bb1 protein.

**Hemiptera: Anthocoridae (true bugs)** The potential toxicity of the Cry3Bb1 protein has been evaluated on the minute pirate bug *Orius insidiosus*. Duan et al. (2008b) concluded that the Cry3Bb1 protein at a concentration of 930 µg/g of diet encapsulated in parafilm domes had no adverse effect on the survival and development rate to adults of *O. insidiosus* nymphs. They reported that 3–5 day old nymphs (probably late second instars) of *O. insidiosus* fed solely honeybee collected pollen (mixed to a 40:60 ratio with water/buffer or dissolved Cry3Bb1 protein) developed to the adult stage in about 5–7 days at 25 °C, with a survival rate close to 90 %. These rapid development and high survival rates on pollen (see also Duan et al. 2007) are, however, not in line with other studies on the developmental biology of this predatory bug (Isenhour and Yeorgan 1981; Kiman and Yeorgan 1985; Pilcher et al. 1997; Lundgren 2009; Tan et al. 2011).

**Neuroptera: Chrysopidae (net-winged insects)** Larvae of the green lacewing, *C. carnea*, fed eggs of *Sitotroga cerealella* (Lepidoptera: Gelechiidae) coated with the Cry3Bb1 protein at 400 and 8,000 µg/g diet for 10 days resulted in no significant increase in mortality of larvae compared to the control substance group (Palmer and Krueger 1999b). Due to the feeding mode of *C. carnea* larvae, which have piercing-sucking mouthparts, exposure to Cry proteins when feeding on the treated lepidopteran eggs is likely

to be low. Therefore, this protocol of exposure is no longer recommended by US EPA (Rose 2007). Li et al. (2008) conducted laboratory experiments to evaluate the impact of maize MON 88017 pollen on life-history parameters of adult *C. carnea*. Adults were fed pollen from *Bt*-maize (14.0–16.0 µg Cry3Bb1/g DW) or the corresponding non-*Bt*-maize line together with sucrose solution for 28 days. Survival, weight, pre-oviposition period, fecundity and fertility were not different between *Bt*- or non-*Bt*-maize pollen treatments. *Chrysoperla carnea* adults were also fed with artificial diet containing the purified Cry3Bb1 protein at about a 10 times higher concentration found in maize MON 88017 pollen. No differences in any of the above-mentioned life-history parameters were detected compared to the control diet. Li et al. (2010) further demonstrated that field-collected *C. carnea* females contained an average of approximately 5,000 maize pollen grains in their gut at the peak of pollen shed. Comparable numbers were found in females fed ad libitum maize pollen in the laboratory. When *Bt*-maize pollen passed through the gut of *C. carnea*, 79 % of the Cry3Bb1 protein (maize event MON 88017) was digested.

**Hymenoptera: Pteromalidae (parasitic wasps)** *Nasonia vitripennis* adults were exposed to 400 and 8,000 µg Cry3Bb1/ml in a honey diet, which is approximately equivalent to 5 and 100 times the maximum Cry3Bb1 protein content in maize MON 88017 plant parts (Palmer and Krueger 1999c). No significant differences in survival were observed at 400 ppm after 16 days of exposure. The LC<sub>50</sub> value was determined to be 8,000 ppm. In their lower-tier study, Sindermann et al. (2002b) did not observe significant differences in survival of *N. vitripennis* adults after 19 days of exposure to 1,860 µg Cry3Bb1/ml diet.

**Higher-tier studies** A series of higher-tier studies assessed the potential impact of Cry3Bb1-expressing maize on several natural enemies in the USA and EU (Table S2 in Online Supporting Material). Besides the untreated corresponding non-*Bt*-maize line, some studies included maize varieties treated with alternative pest control methods such as seed coating and/or soil or foliar insecticides as comparator. In the USA, no negative impact of maize MON 863 was revealed on field densities of abundantly occurring: Araneae; species belonging to the coleopteran families of

Carabidae, Cicindelidae, Coccinellidae (such as *C. maculata*, *Harmonia axyridis*, *H. convergens* and *Scymnus* spp.), and Staphylinidae; Syrphidae (Diptera); hemipterans, including the anthocorid *O. insidiosus*, and Nabidae; hymenopterans, the braconid *Macrocentrus cingulum*, and Formicidae; and the neuropteran *C. carnea* (Al-Deeb and Wilde 2003; Ahmad et al. 2005, 2006a; Bhatti et al. 2005a, b; McManus et al. 2005). Seed treatments and soil insecticides generally had no influence on natural enemies, except for some spiders and carabids. Foliar insecticide applications reduced the abundance of certain spiders, coccinellids, *C. carnea* and nabids (Bhatti et al. 2005a, b).

In the EU, no negative impact of maize MON 88017 was observed on the abundance of: Araneae such as *Pardosa agrestis*, *Pachygnatha degeeri* and *Oedothorax apicatus*; coleopteran species belonging to the families of Carabidae such as *C. fuscipes*, *C. ambiguus*, *P. rufipes*, *P. melanarius* and *Poecilus cupreus*, Coccinellidae such as *A. bipunctata*, *Coccinella quinquepunctata*, *C. septempunctata*, *H. axyridis*, *Propylea quatuordecimpunctata* and *Psyllobora vigintiduopunctata*, and Staphylinidae such as *Aloconota sulcifrons*, *Amischa analis*, *Anotylus rugosus*, *Mycetoporus eppelsheimianus* and *Tachyporus hypnorum*; the dipteran *Episyrphus balteatus*; the hemipterans *Orius* spp.; and the neuropterans *C. carnea* and *Osmylus fulvicephalus* (Priesnitz 2010; Rauschen et al. 2010a; Rosca and Cagan 2012a, b; Svobodová et al. 2012a, b). Soil insecticides had generally no influence on the abundance of natural enemies, except on some Araneae and carabids (Svobodová et al. 2012a, b).

Meta-analyses of independent higher-tier studies carried out across different continents showed that the mean abundance of non-target Araneae, Coleoptera and Hemiptera was not significantly different in maize MON 863 fields from that in the corresponding non-*Bt*-maize line treated either with or without pyrethroid insecticides (Marvier et al. 2007). Overall, minimal to undetectable changes in natural enemies due to cultivation of *Diabrotica*-resistant *Bt*-maize events were reported (Marvier et al. 2007; Wolfenbarger et al. 2008; Naranjo 2009).

### Pollinators

Honeybees can be exposed to plant-produced Cry proteins, as they collect, store and consume maize

pollen, mainly when alternative pollen sources are scarce. In most cases, however, the proportion of maize pollen as a total of all pollen collected and fed to larvae during summer will be low. It is therefore unlikely that maize pollen would regularly comprise more than 50 % of the honeybee diet.

Larvae of honeybee *Apis mellifera* (Hymenoptera: Apidae) exposed to 1,790 and 2,550 µg Cry3Bb1/ml inoculated in brood cells (which is more than 100 times the maximum Cry3Bb1 protein content found in maize MON 88017 pollen) were not affected after up to 18 days of exposure in terms of survival (Maggi 1999a, 2002) (Table S1 in Online Supporting Material). No effects on survival of honeybee larvae and pupal weight were observed when larvae were fed syrup supplemented with Cry3B protein at levels up to 2,000 times than that found in *Bt*-maize pollen (Arpaia 1996). The source of the Cry3B protein and therefore the degree of similarity of the Cry3B protein to the maize MON 88017 Cry3Bb1 protein is not specified in the study. Hendriksma et al. (2012) reported that the survival of honeybee larvae and the weight of prepupae remained unaffected, even after exposure to the highest test concentration of 0.3 µg Cry3Bb1/µl. Lower-tier studies with adult honeybees exposed for 11 days to an aqueous feeding solution containing 360 µg Cry3Bb1/ml in a 30 % sucrose:deionised water solution (corresponding to 20 times the maximum Cry3Bb1 protein content found in maize MON 88017 pollen) did not indicate statistically significant differences in mortality between the control and test substance groups (Maggi 1999b).

Richards (2011a, b) assessed the effect of maize MON 88017 pollen on honeybee larvae and adults. No effects on survival and larval development time were observed after up to 17 days of exposure to maize MON 88017 pollen: larvae were exposed to approximately 2 mg of pollen that was distributed to each brood cell, whilst adults were fed ad libitum a honey diet containing 4 g pollen. Since no data on the concentration and bioactivity of the Cry3Bb1 protein in thawed pollen were provided, COGEM (2011) questioned the usability of the honeybee studies. Experience, however, shows that the activity of Cry proteins is preserved after storage at -80 °C for at least 30 months (Nguyen and Jehle 2009b; Meissle and Romeis 2009a). Ideally, bioactivity should be confirmed using sensitive insect bioassays or ELISA analyses (Romeis et al. 2011).

Feeding honeybee larvae with a diet containing 2 mg pollen from maize event MON 89034 × MON 88017 that expresses the Cry1A.105, Cry2Ab2 and Cry3Bb1 proteins had no adverse effects on larval survival and the prepupal weight after 120 h of exposure until the prepupal stage (Hendriksma et al. 2011).

Based on a meta-analysis of lower-tier studies assessing direct effects on honeybee survival of Cry proteins from currently commercialised Lepidoptera- and *Diabrotica*-resistant *Bt*-crops, Duan et al. (2008a) concluded that the Cry3Bb1 protein does not negatively affect the survival of either honeybee larvae or adults in laboratory settings.

### Decomposers

Decomposers can be exposed to plant-produced Cry proteins remaining in plant residues, dead arthropod bodies, or faeces. Knecht and Nentwig (2010) quantified the effects of maize MON 88017 on the saprophages *Drosophila melanogaster* (Diptera: Drosophilidae) and *Megaselia scalaris* (Diptera: Phoridae) over 4 and 3 generations, respectively (Table S1 in Online Supporting Material). Freshly hatched larvae were reared on a diet containing decaying maize MON 88017 leaves. The few significant differences in development time to the adult stage and fertility found between transgenic and non-transgenic treatments were not consistent over the generations and did not indicate negative effects of the Cry3Bb1 protein. Quantitative ELISA analyses detected low Cry3Bb1 protein concentrations in larvae feeding on *Bt*-maize, and did not detect the Cry3Bb1 protein in adults, indicating that predators of the larvae are exposed to low Cry protein levels, whereas predators of adult flies are not.

Higher-tier studies did not indicate adverse effects of maize MON 863 on the abundance of various species belonging to: Chilopoda; Lathridiidae and Nitidulidae (Coleoptera); and Japygidae (Diplura) in the USA (Bhatti et al. 2005a). In the case of maize MON 88017, no adverse effects were observed on the abundance of: the coleopteran species *Cortinicara gibbosa*; various dipteran species; and the mecopteran *Panorpa communis* in the EU (Hönemann et al. 2008; Rauschen et al. 2010a; Svobodová et al. 2012a, b) (Table S2 in Online Supporting Material).

### Non-target soil arthropods

Non-target arthropods occurring in the soil ecosystem can be exposed to plant-produced Cry proteins introduced into the soil via physical damage to plant tissues, via decomposition of shed root cells during plant growth, via decomposing plant residues remaining in fields after harvest, which might be incorporated into the soil during tillage operations, and possibly via root exudates (reviewed by Icoz et al. 2008). Lower-tier studies have been conducted with springtails (Collembola), some of which are important in soil-related processes such as the breakdown and recycling of crop residues (Table S3 in Online Supporting Material). Cry3Bb1 proteins are degraded rapidly in decaying plant residues (Prihoda and Coats 2008b; Miethling-Graff et al. 2010; Zurbrügg et al. 2010) and occur—if detected—at extreme low concentrations in soil (<1.0 ng Cry3Bb1/g soil) without accumulation during subsequent years of *Bt*-maize cultivation (Ahmad et al. 2005; Icoz and Stotzky 2007; Icoz et al. 2008; Miethling-Graff et al. 2010). Teixeira (1999) reported that the survival of juvenile (10-day-old) *Folsomia candida* (Collembola: Isotomidae) fed leaf material of maize MON 859 in a yeast diet ( $\leq 872.5$  µg Cry3Bb1/g diet), and their offspring production were not adversely affected as compared to organisms exposed to non-*Bt*-maize leaf tissue after 28 days of exposure.

No significant differences were reported on field densities of springtails and soil mites (Acari) in a 9 months leaf litter-bag field study conducted in Switzerland with maize MON 88017 as compared to the non-*Bt*-treatment (Hönemann et al. 2008). Similar results were obtained in field trials conducted with maize MON 863 in the USA for springtails and soil mites (Al-Deeb et al. 2003; Ahmad et al. 2005; Carter et al. 2004; Bitzer et al. 2005).

### Non-target aquatic arthropods

Exposure of non-target arthropods to plant-produced Cry proteins in aquatic ecosystems is very low (reviewed by Carstens et al. 2011): in surveyed water streams in Indiana (USA), the mean concentration of the Cry1Ab protein was  $14 \pm 5$  ng/l with a maximum concentration of 32 ng/l (Tank et al. 2010). In decomposing maize MON 863 residues (i.e., leaf,

stalk and root), a half-life of less than 3 days was found for the Cry3Bb1 protein (Prihoda and Coats 2008a). Based on exposure estimates, Carstens et al. (2011) identified shredders (Cummins et al. 1989) as the functional group most likely to be exposed to Cry proteins in aquatic environments (see also Rosi-Marshall et al. 2007). Lower-tier studies with non-target aquatic arthropods have been conducted with 6 species belonging to 4 orders (Table S4 in Online Supporting Material).

*Cladocera: Daphniidae (daphnids)* No adverse effects on survival of *Daphnia magna* larvae were observed after 2 days of exposure up to 120 mg maize MON 858 pollen/l water, containing 18.8 µg Cry3Bb1 µg/g FW (Drottar and Krueger 1999). Despite the observational evidence that daphnids can ingest pollen (US EPA 2010), there is no clear evidence that these filter feeders are capable of digesting pollen grains. Therefore, exposure to the Cry3Bb1 protein, including through diffusion into an aquatic environment (see supplementary data 3 reported by Li et al. 2010), remains unclear.

*Diptera: Chironomidae and Tipulidae (flies)* Prihoda and Coats (2008a) observed a decrease in survival but no effect on growth (biomass) of the larvae of *Chironomus dilutus* when exposed to maize MON 863 root extracts at concentrations  $\geq 30$  ng Cry3Bb1/ml for 10 days. As noted by the authors, the study results are inconclusive, as it remains unclear if the observed effects were due to the presence of the Cry3Bb1 protein or other compounds in the root extracts, given that no control treatments of non-*Bt*-maize root extracts were included. Jensen et al. (2010) reported no effect on growth (biomass) of *Tipula (Nipoptipula) abdominalis* larvae fed senesced leaf tissues from the Cry1Ab- and Cry3Bb1-expressing maize event MON 810 × MON 863 ad libitum for 30 days.

*Isopoda (isopods)* When exposed to senesced leaf tissues from maize MON 810 × MON 863 ad libitum for 30 days, no effects on survival, growth (length) and weight of *Caecidotia communis* larvae were observed (Jensen et al. 2010).

*Trichoptera: Lepidostomatidae and Limnephilidae (caddisflies)* No adverse effects on growth (head capsule width) and weight of larvae of *Lepidostoma* spp. and *Pycnopsyche scabripennis* were reported

when fed senesced leaf tissues from Cry3Bb1-expressing maize (maize event MON 810 × MON 863) ad libitum for 30 days (Jensen et al. 2010). In addition, no effect on survival was reported for *P. scabripennis*.

#### Non-arthropod NTOs

Several studies investigated the potential of maize MON 88017 to have direct or indirect adverse effects on NTOs that are not arthropods (Table S5 in Online Supporting Material) such as Annelida, Mollusca, Nematoda, mammals & birds, and soil microorganisms.

*Annelida (enchytraeid worms and earthworms)* Annelida play an important role in decomposing plant litter, soil structure and nutrient cycling processes (Didden 1993; Curry and Schmidt 2006). A lower-tier study in which the earthworm *Eisenia fetida* (Haplotaxida: Lumbricidae) was exposed to 166.6 mg Cry3Bb1/kg dry soil did not indicate adverse impacts on survival and weight after 14 days of exposure (Sindermann et al. 2002a). However, as the Cry3Bb1 protein concentration was not monitored throughout the test period, it remains unclear for which period of time the earthworms were exposed to biologically active Cry3Bb1 protein. Exposure to maize MON 863 roots or maize MON 863 biomass at 2 % (w/w) of soil had no effect on survival and weight of the earthworm *Lumbricus terrestris* (Haplotaxida: Lumbricidae) up to 45 days of exposure (Ahmad et al. 2006b). Feeding the *Enchytraeus albidus* (Haplotaxida: Enchytraeidae) with diets containing leaf material from maize MON 88017 did not affect the adult survival after 21 days of exposure, nor fertility (Hönemann and Nentwig 2009). A 9 months leaf litter bag field study conducted in Switzerland with maize MON 88017 revealed no difference in decomposer communities (including lumbricids and enchytraeids) when compared to non-*Bt*-maize (Hönemann et al. 2008). In a higher-tier field study conducted in the USA, Zeilinger et al. (2010) did not observe significant differences in weight of juvenile and adult earthworms (*Aporrectodea caliginosa*, *A. trapezoides*, *A. tuberculata* and *L. terrestris*; Haplotaxida: Lumbricidae) between non-*Bt*-maize and *Bt*-maize (event MON 863) varieties during 4 years of cultivation.

*Mollusca (molluscs)* Slugs can be abundant and play an important role in the food web of maize ecosystems

as prey of spiders, carabids, birds and hedgehogs (Symondson et al. 2006). The Cry3Bb1 protein was detected in the gut and faeces of *Arion lusitanicus* (Pulmonata: Arionidae) and *Deroceras reticulatum* (Pulmonata: Agriolimacidae), after the slugs had been fed maize MON 88017 leaves for 3 days. After 3 days of feeding, the Cry3Bb1 protein concentration in gut and faeces was shown to be 6 % of that measured in *Bt*-maize leaves. Upon exposure, no differences in growth (biomass) and leaf consumption were observed compared to slugs fed non-*Bt*-maize (Zurbrügg and Nentwig 2009). In a follow-up study, no significant effects of maize MON 88017 were detected on longevity, weight change and oviposition of the slug *A. vulgaris* (Pulmonata: Arionidae) over an exposure period of 16 weeks (Hönemann and Nentwig 2010).

**Nematoda (nematodes)** Nematodes are considered key indicators for soil-related processes due to their near omnipresence and large diversity (Sochova et al. 2006). *Caenorhabditis elegans* exposed to aqueous Cry3Bb1-containing solutions showed a dose-dependent inhibitory response in terms of growth (body length) and offspring production, with EC<sub>50</sub> values of 22.3 mg/l and 7.9 mg/l, respectively (Höss et al. 2011). However, when exposed to rhizosphere soil obtained from plots cropped with maize MON 88017, containing up to 1 ng Cry3Bb1/g soil, no adverse effects were observed. Higher-tier studies conducted with Cry3Bb1-expressing maize in the USA (event MON 863; Al-Deeb et al. 2003) and EU (event MON 88017; Höss et al. 2011) did not indicate significant differences in the abundance and diversity of field nematodes in soil planted with *Bt*- and non-*Bt*-maize.

**Mammals & birds** No toxic effects of the Cry3Bb1 protein were observed in toxicity studies with northern bobwhite quail *Colinus virginianus* (Galliformes: Phasianidae) chicks fed a diet containing 10 % ground grains from Cry3Bb1-expressing maize events for 5 days (Gallagher et al. 1999), and with rat *Rattus norvegicus* (Rodentia: Muridae) fed a diet containing maize MON 88017 or MON 863 grains (approximately 11 and 33 % (w/w)) during 90 days (MON 88017: WIL-50283 2005; WIL-50284 2005; MON 863: Hammond et al. 2006; EFSA 2007).

**Soil microorganisms** Meissle et al. (2009) studied the interaction of the entomopathogenic fungus

*Metarhizium anisopliae*, used as a microbial pest control agent, with maize MON 88017 and WCR in a lower-tier study. There was no difference in infection rates of WCR larvae fed MON 88017 and control maize, indicating that maize MON 88017 does not interfere with the biological control provided by *M. anisopliae*. No deleterious effects on soil microbial biomass, activity or community structure were observed in higher-tier studies with maize MON 863 in the USA (Devare et al. 2004, 2007; Icoz et al. 2008; Lawhorn et al. 2009; Xue et al. 2011). Miethling-Graff et al. (2010) did not detect any significant differences between the rhizosphere bacterial community structure of maize MON 88017 compared with non-*Bt*-maize varieties in a 3-year field study in Germany. Zurbrügg et al. (2010) found that leaf litter from maize MON 88017 is readily degraded and that degradation did not differ from the corresponding non-*Bt*-maize line, but varied among conventional maize varieties commonly planted in Switzerland.

## Discussion

NTOs occurring in and around Cry3Bb1-expressing *Bt*-maize fields (including aquatic environments) can be exposed to Cry3Bb1 proteins when consuming plant material (including pollen and crop residues), through plant exudates, or when feeding on prey/host organisms, which have previously been feeding on *Bt*-maize (Romeis et al. 2009). Many studies on arthropod and non-arthropod species have therefore been conducted to assess the potential impact of Cry3Bb1-expressing maize events on NTOs. However, not all the data reported in the scientific literature and compiled in this review paper are equally informative to the ERA of maize event MON 88017; their relevance depends upon: (1) the representativeness of the species tested for a valued group of NTOs, including their likely exposure to maize MON 88017 under field conditions; (2) the experimental design of the study; (3) the type of study conducted; and (4) the Cry3Bb1 protein variant considered.

(1) To assess potential harm to NTOs, studies need to be conducted with representative species of maize ecosystems and their relevant life stages that are likely to be exposed to the Cry3Bb1 protein under field conditions. Species that have been tested represent different habitats (terrestrial, soil and aquatic),

**Table 2** Overview of non-target species tested in lower- and higher-tier studies investigating potential adverse effects of purified Cry3Bb1 protein or Cry3Bb1-containing food of vegetal or animal origin

Non-target organisms	Functional group <sup>a</sup>	Type of study	Order	Family (species)	Online Supporting Material			
Terrestrial (plant- and ground-dwelling) arthropods	Herbivore	Lower-tier	Acanthoptera	Tetranychidae ( <i>Tetranychus urticae</i> )	Table S1			
			Coleoptera	Chrysomelidae ( <i>Callosobruchus maculatus</i> , <i>Galernella vittaticollis</i> , <i>Gastrophysa viridula</i> , <i>Leptinotarsa decemlineata</i> , <i>Oulema melanopus</i> , <i>Phaedon cochleariae</i> ); Coccinellidae ( <i>Epilachna vigintioctopunctata</i> ); Curculionidae ( <i>Anthonomus eugenii</i> , <i>Anthonomus grandis</i> , <i>Stiphilus oryzae</i> ); Tenebrionidae ( <i>Tribolium castaneum</i> )				
			Hemiptera	Aphidae ( <i>Rhopalosiphum maidis</i> , <i>Rhopalosiphum padi</i> )				
			Lepidoptera	Grambidae ( <i>Ostrinia nubilalis</i> ); Danaidae ( <i>Danaus plexippus</i> ); Noctuidae ( <i>Helicoverpa zea</i> )				
			Coleoptera	Chrysomelidae (various species such as <i>Chaetocnema pulicaria</i> , <i>Oulema lichenis</i> , <i>Phyllotreta vittula</i> ); Elateridae (various species)				
			Hemiptera	Aphididae (various species such as <i>Metopolophium dirhodum</i> , <i>Rhopalosiphum maidis</i> , <i>Rhopalosiphum padi</i> ); Cicadellidae ( <i>Zyginidia scutellaris</i> ); Miridae ( <i>Trigonotylus caelestium</i> )				
			Orthoptera	Gryllidae (various species)				
			Thysanoptera	Thripidae (various species such as <i>Frankliniella occidentalis</i> )				
			Natural enemy	Lower-tier		Aranee	Theridiidae ( <i>Phylloneta impressa</i> formerly named as <i>Theridion impressum</i> )	Table S1
						Coleoptera	Carabidae ( <i>Agonum cupripenne</i> , <i>Agonum placidum</i> , <i>Anara pennsylvanicus</i> , <i>Catalus ambiguus</i> , <i>Calathus fuscipes</i> , <i>Chlaenius tricolor</i> , <i>Harpalus affinis</i> , <i>Harpalus caliginosus</i> , <i>Harpalus pennsylvanicus</i> , <i>Patrobus longicornis</i> , <i>Poecilus chalcites</i> , <i>Poecilus lucublandus</i> , <i>Pseudophonus rufipes</i> , <i>Pterostichus melanarius</i> , <i>Scarites quadriceps</i> ); Coccinellidae ( <i>Adalia bipunctata</i> , <i>Coleomegilla maculata</i> , <i>Hippodamia convergens</i> , <i>Stethorus punctillum</i> ); Staphylinidae ( <i>Atheta coriaria</i> )	
			Higher-tier	Higher-tier		Hemiptera	Anthocoridae ( <i>Oritus insidiosus</i> )	Table S2
						Hymenoptera	Peromalidae ( <i>Nasonia vitripennis</i> )	
						Neuroptera	Chrysopidae ( <i>Chrysoperla carnea</i> )	
						Aranee	Linyphiidae (various species such as <i>Oedothorax apicatus</i> ); Lycosidae (various species such as <i>Paradosa agrestis</i> ); Tetragnathidae (various species such as <i>Pachygnatha degeeri</i> )	
						Coleoptera	Carabidae (various species such as <i>Amara aene</i> , <i>Amara similata</i> , <i>Bembidion lampros</i> , <i>Bembidion quadrimaculatum</i> , <i>Calathus ambiguus</i> , <i>Calathus fuscipes</i> , <i>Calathus melanoccephalus</i> , <i>Harpalus aeneus</i> , <i>Harpalus distinguendus</i> , <i>Platynus dorsalis</i> , <i>Poecilus cupreus</i> , <i>Pseudophonus rufipes</i> , <i>Pterostichus melanarius</i> , <i>Trechus quadristriatus</i> ); Cicindelidae (various species); Coccinellidae (various species such as <i>Adalia bipunctata</i> , <i>Coccinella quinquepunctata</i> , <i>Coccinella septempunctata</i> , <i>Coleomegilla maculata</i> , <i>Harmonia axyridis</i> , <i>Hippodamia convergens</i> , <i>Propylea quatuordecimpunctata</i> , <i>Psylliobora vigintiduopunctata</i> , <i>Scymnus</i> spp.); Staphylinidae (various species such as <i>Aloconota sulcifrons</i> , <i>Amischa analis</i> , <i>Anotylus rugosus</i> , <i>Mycetoporus eppelsheimianus</i> , <i>Tachyporus hypnorum</i> )	
						Diptera	Syrphidae (various species such as <i>Episyrphus balteatus</i> )	
						Hemiptera	Anthocoridae (various species such as <i>Oritus insidiosus</i> ); Nabidae (various species)	
Hymenoptera	Braconidae (various species such as <i>Macrocentrus cingulum</i> ); Formicidae (various species)							
Neuroptera	Chrysopidae ( <i>Chrysoperla carnea</i> ); Osmylidae ( <i>Osmylus fitchicephalus</i> )							

**Table 2** continued

Non-target organisms	Functional group <sup>a</sup>	Type of study	Order	Family (species)	Online Supporting Material
Soil arthropods	Pollinator	Lower-tier	Hymenoptera	Apidae ( <i>Apis mellifera</i> )	Table S1
	Decomposer	Lower-tier	Diptera	Drosophilidae ( <i>Drosophila melanogaster</i> ); Phoridae ( <i>Megaselia scalaris</i> )	Table S1
		Higher-tier	Chilopoda	(various species)	Table S2
			Coleoptera	Lathrididae (various species such as <i>Corticara gibbosa</i> ); Nitidulidae (various species)	
			Diptera	Japygidae (various species)	
			Diptera	(various species)	
			Mecoptera	Panorpidae (various species such as <i>Panorpa communis</i> )	
		Lower-tier	Collembola	Isotomidae ( <i>Folsomia candida</i> )	Table S3
		Higher-tier	Acari	(various species); Acaridae (various species such as <i>Rhizoglyphus robini</i> )	
			Collembola	(various species)	
		Cladocera	Daphnidae ( <i>Daphnia magna</i> )	Table S4	
Aquatic arthropods		Lower-tier	Chironomidae ( <i>Chironomus dilutus</i> ); Tipulidae ( <i>Tipula (Nippotipula) abdominalis</i> )		
			( <i>Caecidotea communis</i> )		
			Lepidostomatidae ( <i>Lepidostoma</i> spp.); Limnephilidae ( <i>Pyenopsyche scabripennis</i> )		
			Enchytraeidae ( <i>Enchytraeus albidus</i> ); Lumbricidae ( <i>Eisenia fetida</i> , <i>Lumbricus terrestris</i> )		
			Agriolimacidae ( <i>Deroceras reticulatum</i> ); Arionidae ( <i>Arion lusitanicus</i> , <i>Arion vulgaris</i> )		
			Rhabditidae ( <i>Caenorhabditis elegans</i> )		
			Enchytraeidae (various species); Lumbricidae (various species such as <i>Aporrectodea caliginosa</i> , <i>Aporrectodea trapezoides</i> , <i>Aporrectodea tuberculata</i> , <i>Lumbricus terrestris</i> )		
			(various species)		
			Nematoda (phylum)		

<sup>a</sup> Functional group is the primary functional group for which a species is valued

taxonomic groups, and valued ecosystem services (Table 2). Given the target specificity of Cry3Bb1 to chrysomelid beetles, many studies have focused on non-target species belonging to the order of Coleoptera. Beetle species belonging to the families of Carabidae (ground beetles), Coccinellidae (ladybirds) and Staphylinidae (rove beetles) are representatives of functional groups important for pest regulation and are abundant and widely distributed in arable ecosystems including maize, and were therefore selected for study purposes. Species not belonging to the order of Coleoptera were selected as representatives of potentially exposed taxonomic groups and functional groups that provide valued ecosystem services (natural enemies such as spiders, predatory true bugs, lacewings and parasitoids; plant bugs, plant- and leafhoppers and molluscs as herbivores; honeybees as pollinators; springtails, earthworms and soil microorganisms as decomposers; daphnids and caddisflies as representatives of aquatic arthropods), or because they are of conservation concern (e.g., the monarch butterfly, birds). The only non-coleopteran species tested in lower-tier studies that is not related to maize ecosystems, but which may still serve as a surrogate for hymenopteran parasitoids, is *N. vitripennis*. However, it has been suggested to use more appropriate species that are abundant in maize, or at least in cropping systems, like certain ichneumonids, braconids, mymarids, or scelionids (Rose 2007; Albajes et al. 2012). The remaining species that were studied and considered in this review paper were selected to meet regulatory data requirements (i.e., mammals), to determine the target specificity of Cry3Bb1 proteins within the order of Coleoptera (i.e., Curculionidae, Tenebrionidae, *E. vigintioctopunctata*), or to quantify the between-species variability in sensitivity to Cry3Bb1 proteins (Chrysomelidae). Of the Chrysomelidae tested in lower-tier studies, only *G. vittaticollis* and *O. melanopus* have been observed in maize fields.

(2) It is needless to say that a study is not relevant for an ERA or any assessment if it does not adhere to good experimental design criteria, including good laboratory and field practices (Candolfi et al. 2000; Perry et al. 2009; WHO 2009; Romeis et al. 2011). Any study should be reproducible and be carried out in such a way that it minimises the probability of erroneous or inconclusive results. A lower-tier study with the purified Cry3Bb1 protein that has been

questioned for its reproducibility is the study on *O. insidiosus* carried out according to the design described in Duan et al. (2008b) as the development rates did not fall within previously reported ranges. Also the lower-tier study with larvae of *C. carnea* by Palmer and Krueger (1999b) and the Schmidt et al. (2009) lower-tier study with larvae of *A. bipunctata* do not provide relevant data on the direct toxic effect of the Cry3Bb1 protein since the exposure to the test substance was very low or questionable (Rose 2007; Romeis et al. 2012). Another study questioned for its design was that by Sindermann et al. (2002a) on *E. fetida*. Since the Cry3Bb1 protein concentration was not monitored throughout the test period, doubts were raised on the robustness of the study. Further, the usability of the honeybee studies done by Richards (2011a, b) was questioned as the bioactivity of the Cry3Bb1 protein in thawed pollen was not proven. In the case of *D. magna* fed *Bt*-maize pollen (Drottar and Krueger 1999), exposure to the Cry3Bb1 protein remains unclear, as it is not known to what extent daphnids are able to digest maize pollen grains. Likewise, exposure of predatory larvae to Cry3Bb1 proteins via aphids reared on Cry3Bb1-expressing maize (Lundgren and Wiedenmann 2005) is not likely, as phloem sap-sucking species such as aphids, do not ingest Cry3Bb1 proteins when feeding on Cry3Bb1-expressing maize (Romeis and Meissle 2011). For several lower-tier studies (i.e., Mullin et al. 2005; Prihoda and Coats 2008a; Priesnitz 2010), the conclusions drawn are less reliable, due to the low number of individuals tested, lack of replication, or inappropriate controls. Likewise, poorly replicated higher-tier studies will have little probability to detect real effects of a given size (as emphasised by Duan et al. 2006a; Perry et al. 2009; Albajes et al. 2012).

(3) Amongst risk assessors, it is generally accepted that detecting an adverse effect is more likely in a lower-tier study compared to a higher-tier study, as the former uses more controlled conditions with less variables (Garcia-Alonso et al. 2006; Rose 2007; Romeis et al. 2008). Laboratory studies, considered to be the most controlled studies, are often conducted at the highest possible concentration of the test substance that can be delivered with the test system, typically at amounts that exceed the concentration present in the *Bt*-plant by a factor 10–100. In case no adverse effect can be observed under such worst-case exposure conditions, there may be no need to conduct any



further testing because of minimal probability of adverse effects in the fields where NTOs are exposed to much lower concentrations of the active protein. Testing in a more controlled setting such as under laboratory and greenhouse conditions is therefore overall considered to be conservative in predicting effects of *Bt*-crops and to resolve questions more thoroughly than do higher-tier studies (Duan et al. 2010).

With the exception of the study of Höss et al. (2011) on *C. elegans*, all undisputed lower-tier studies concluded that Cry3Bb1 proteins have no adverse effects to the NTOs under consideration even at worst-case exposure conditions. Hence, according to the tiered approach, further testing under more realistic conditions of exposure at a higher-tier would add little information on the risks of Cry3Bb1 proteins to NTOs, except for *C. elegans*. Indeed, higher-tier study results for non-target terrestrial and soil organisms confirmed the conclusions from lower-tier studies with the same species that their abundance would not be affected. The inhibitory responses at high Cry3Bb1 protein doses were no longer observed for *C. elegans* when exposed to rhizosphere soil, or for field nematodes under cultivation conditions (Höss et al. 2011).

(4) Several Cry3Bb1 protein variants, derived from the *Bt*-maize events/lines MON 853, MON 858, MON 859, MON 863 and MON 88017, have been used as test substances in NTO studies. However, only those variants that have been shown to be equivalent to the MON 88017 Cry3Bb1 protein variant can provide information on the safety of maize MON 88017. This was the case for the MON 853 and MON 863 Cry3Bb1 protein variants (see Box 1). For the remaining Cry3Bb1 proteins, no such information could be found and these are therefore not taken into consideration in the overall conclusion of this review paper.

## Conclusion

The available data show that the expression of the Cry3Bb1 protein in maize MON 88017 (and maize MON 853 and MON 863) has no toxic effect on non-target terrestrial (plant- and ground-dwelling), soil and aquatic arthropod NTOs and non-arthropod NTOs (Annelida, molluscs, nematodes, mammals & birds, and soil microorganisms) outside the coleopteran family of Chrysomelidae, and that the insecticidal

activity of the Cry3Bb1 protein is limited to arthropod species of the Chrysomelidae family (leaf beetles).

Chrysomelid beetle species, however, are only at risk if they are exposed to harmful concentrations of the plant-produced Cry3Bb1 protein under realistic conditions of exposure. Leaf beetles are regularly found in maize fields and can be exposed to Cry3Bb1 proteins due to their herbivorous feeding habits, as shown for the genera *Chaetocnema*, *Longitarsus*, *Oulema* and *Phyllotreta* in the EU (Kiss et al. 2002, 2004; Knecht et al. 2010; Rauschen et al. 2010a; Meissle et al. 2012) and the species *C. pulicaria* in the USA (Bhatti et al. 2005b). Some leaf beetles frequently feed on maize and are considered pests; a potential reduction of their abundance is therefore not considered an environmental concern in several jurisdictions (US EPA 2010; Rauschen et al. 2010a; EFSA 2011). Non-target Chrysomelidae are low in abundance in maize fields due to their preference for other habitats or host plants, but larvae may come into contact with the Cry3Bb1 protein when ingesting maize MON 88017 pollen deposited on their host plants. The risk to larvae is, however, minimal due to the low likelihood of encountering harmful amounts of pollen on their host plants in and around maize MON 88017 fields (US EPA 2010; EFSA 2011). Given the limited exposure, the identified hazard does not translate into risk. Non-target adult leaf beetles, which may occasionally feed on maize MON 88017 plants, are not expected to be affected due to the low activity of the Cry3Bb1 protein on adult non-target chrysomelid species, as has been shown for *O. melanopus* (Meissle et al. 2012). Low activity of the Cry3Bb1 protein on WCR adults was also reported (Nowatzki et al. 2006; Meissle et al. 2009, 2011a). Further, the only protected chrysomelid species (*Macrolea pubipennis*) considered at risk across the EU does not occur in maize fields (Dewar 2010), and no endangered and threatened chrysomelid beetle species are listed for the USA (US EPA 2002a, 2010).

Besides the assessment of potential adverse effects on NTOs due to the intended genetic modification (i.e., the expression of the Cry protein), those arising from potential unintended changes in the GM plant, which go beyond the primary objectives of the genetic modification, are usually also considered in an ERA. The molecular characterisation of the DNA insert and flanking regions of maize MON 88017 did not indicate unintended changes due to the insertion (EFSA

2009, 2011). Moreover, no biologically relevant differences in the composition of key analytes, or agronomic and phenotypic characteristics were identified between maize MON 88017 and the corresponding non-*Bt*-maize (near-isogenic) line (McCann et al. 2007; EFSA 2009, 2011; Poerschmann et al. 2009). Some compositional plant properties such as lignin contents among conventional maize varieties differed more than between maize MON 88017 and its near-isogenic line, with maize MON 88017 falling within the variation found in conventional maize varieties (see also Lehman et al. 2008a, b, 2010 for other Cry3Bb1-expressing maize events). As outlined above, none of the NTO studies conducted with maize MON 88017 or plant material derived from maize MON 88017 revealed altered interactions between the plant and natural enemies, pollinators, herbivores and decomposers.

In conclusion, there are no indications of adverse effects on NTOs due to the expression of the Cry3Bb1 protein in maize MON 88017 or due to unintended changes in maize MON 88017. The available data show that maize MON 88017 poses negligible risks to NTOs and the ecosystem services they provide. Further, corn rootworm management in maize MON 88017 may differ from that currently practiced in conventional maize cropping systems, given that less or no treatments with soil or foliar broad-spectrum insecticides are needed. It is this reduced environmental load of insecticides that may ultimately lead to fewer adverse side-effects on non-target arthropods in maize ecosystems. However, an integrated pest management approach reliant on multiple tactics is required (Gassmann et al. 2011) to ensure effective long-term corn rootworm management and sustainable use of *Diabrotica*-resistant *Bt*-maize events such as maize MON 88017.

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