Appendix 5.2 MON 810 Literature Review - Environment

MON 810 literature review (July 2015)

Appendix 5.2 - Environment

Table of contents

Review of peer-reviewed publications

Area of the environmental risk assessment: Environmental Safety – Non Target Organisms (NTO)

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Meissle *et al.*, 2014)	***Objective***: To determine: 1) the suitability of maize pollen as a food source for larvae of *Chrysoperla carnea*, 2) the effect of pollen from different maize varieties on the development of *C. carnea* larvae, and 3) differences in total protein content and pollen diameter among maize cultivars and cultivation batches, and their effect on lacewing performance. ***Experimental Design:*** The following nine maize cultivars were used: genetically modified (GM) MON 810 maize expressing the *Bacillus thuringiensis* (Bt) Cry1Ab insecticidal protein (DKC 3421YG), the GM MON 88017 expressing the Bt Cry3Bb1 insect control protein (DKC5143Bt), the GM Compa CB expressing the Bt Cry1Ab insecticidal protein (Bt176), the corresponding three non-transformed cultivars, and 2 conventional cultivars (Radiance and the Swiss Iandrace Rheintaler). Maize plants were grown individually in 12 L plastic pots in the glass house. Pollen was collected daily, passed through a mesh and dried. For each maize cultivar, pollen from several plants and days was pooled and stored at -80°C. In Experiment 1, eggs collected from a *C. carnea* colony were separated in Petri dishes 1 day before hatching. The larvae were provided *ad libitum* with either pollen of one of the nine maize cultivars or eggs of *Ephestia kuehniella* (control treatment). The experiment ended when larvae either reached the pupal stage or died. In Experiment 2, larvae were assigned to one of the following ‘‘life-stage’’ treatments: (a) maize pollen in the first instar, *E. kuehniella* eggs in the second and third instar, (b) maize pollen in the second instar, eggs in the first and third instar, (c) maize pollen in the third instar, eggs in the first and second instar. As a control treatment, larvae were fed eggs in all instars. For Experiments 1 and 2, 15-25 replicates were set up for each treatment. Each experiment was repeated twice. Protein content of pollen and grain size were measured with the Bradford method and steromicroscope, respectively. ***Results:*** Maize pollen was utilized by *C. carnea* larvae. Complete development was not possible when larvae were provided with pollen only, with 25% of neonates reaching the third instar. When larvae were provided with pollen in one instar and eggs in the other two instars, 58–87% of the larvae reached the pupal stage. The protein content and diameter of the different pollens greatly varied and the differences were inconsistent and depended on the batch used. Lacewing performance was not affected by maize cultivar.	The authors concluded that "*For the environmental risk assessment of GM plants, in planta studies must consider the variability among conventional cultivars, individual plants, batches, and environmental conditions when evaluating the ecological significance of differences observed between GM and near-isolines*."	Environment	No adverse effects were determined in this study
			Observed parameter	**Feedback on initial environmental risk assessment**
			NTO	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Grabowski *et al*., 2014)	***Objective:*** To determine whether *Adalia bipunctata* L. (Coleoptera: Coccinelidae) larvae accumulate and/or degrade Cry1Ab protein in relation to different exposure time to Bt (*Bacillus thuringiensis*)-loaded prey. ***Experimental Design:*** DKC 3421 (event MON 810) and the corresponding near-isogenic hybrid DKC 3420 maize plants were grown individually and kept in a greenhouse. The spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) was used as herbivore and reared on Bt maize or isogenic plants (from 4-5 leaf stage to anthesis stage) for several generations. The bioassay was conducted in two subsequent runs of 10 replicates each, resulting in a total of 60 *A. bipunctata* larvae per treatment. The predatory ladybird beetle larvae were placed individually in Petri dishes (55mmØ×33mm Height) and were fed by *T. urticae* reared on different plants.The exposure treatments used in a trial consisted of 24, 48 and 72 h feeding periods, prey-Bt or non-Bt maize, respectively. All experiments were conducted in a climate chamber. The level of Cry1Ab protein in maize leaves, herbivores and predatory larvae was determined by a sandwich ELISA adapted for quantitative purposes, using a kit for Cry1Ab/Cry1Ac. ***Results:*** The concentration of Cry1Ab protein through the trophic chain decreased from Bt maize plant (60.49 µg/g fresh weight (FW)) to spider mite (0.12 µg/g FW). The third trophic level, predatory larvae of *A. bipunctata*, showed different mean concentration of Bt protein between feeding periods. In a first repetition, no significant difference was noticed in the level of Cry1Ab protein in *A. bipunctata* larvae between the 24 h (0.019 µg/g FW) and 48 h (0.011 µg/g FW) feeding treatments. However, a significant difference in Bt content (0.128 µg/g FW) was obtained during 72 h feeding. In the second repetition, all three treatment times differed from each other in the level of Cry1Ab protein in predatory larvae. The 72 h treatment (0.133 µg/g FW) was significantly greater than both the 24 h (0.026 µg/g FW) and the 48 h (0.085 µg/g^{-1} FW) ones.	The authors concluded that: *"the analysis in both bioassays showed a predominantly similar pattern of Cry1Ab degradation through the tritrophic system (Bt maize plants – herbivorous prey – predatory ladybird beetle larvae).* *Since we do not know all potential Bt protein sources in coccinellids via multi-trophic food webs in agro-ecosystems, an accurate environmental risk assessment of genetically modified plants could not be made.* *One of the solutions is to combine laboratory bioassays and field surveys as a quantitative approach for such predatory arthropods."* [1]	Environment	This article shows that Cry1Ab protein could be transferred to predatory ladybird beetle larvae through a tritrophic chain. The transfer pattern is consistent with other previous publications. No adverse effects could be concluded by the detected Cry1Ab level only.
			Observed parameter	**Feedback on initial environmental risk assessment**
			NTO	There are no changes to the conclusions of the safety of the initial risk assessment.

[1] The potential exposure for coccinellid beetles to Bt protein produced in GM crops can be characterized based on the expression level of the protein and potential exposure pathways in agro-ecosystems. The majority of peer-reviewed studies where ladybird beetles were directly fed purified proteins or exposed to proteins in tri-trophic assays using non-sensitive herbivores as prey, revealed no adverse effects of Cry1Ab to *Adalia bipunctata* (Alvarez-Alfageme et al., 2011; Porcar et al., 2010), *Coleomegilla maculata* (Lundgren *et al*., 2004), *Stethorus punctillum* (Álvarez-Alfageme *et al*., 2008), *Cryptolaemus montrouzieri* (Porcar *et al*., 2010), and *Propylea japonica* (Bai *et al*., 2005; Bai *et al*., 2006; Zhang *et al*., 2004; Zhang *et al*., 2014). Conflicting results were reported for Cry1Ab effects on *Cheilomenes sexmaculatus* (Dhillon and Sharma, 2009), *Coleomegilla maculata* (Moser *et al*., 2008) and *Adalia bipunctata* (Schmidt *et al*., 2009). These studies appear to have suffered from flawed methodologies (Rauschen, 2010; Romeis *et al*., 2012; Romeis *et al*., 2013) and, in the case of *Adalia bipunctata*, could not be confirmed in subsequent studies (Alvarez-Alfageme *et al*., 2011; Porcar *et al*., 2010).

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Truter *et al.*, 2014)	***Objective:*** *1)* To describe the biodiversity of arthropods occurring in maize in South-Africa, *2)* to compare the diversity and abundance of arthropods and the functional groups on *Bacillus thuringiensis* (Bt) maize and non-Bt maize. ***Experimental Design:*** Collections of arthropods were carried out during the 2008-2009 and 2009-2010 growing seasons in Bt maize and non-Bt maize fields in the Northern Cape and Limpopo provinces in South-Africa. Three maize fields were sampled for each of the two localities once during each season. All arthropods were collected and kept such that abundance and diversity could be calculated on a per-plant basis. Arthropods were classified to morphospecies level and grouped into functional groups (detritivores, herbivores, predators and parasitoids) to provide information on the potential exposure of species to Bt protein produced by Bt maize. Using the Shannon (H^1) and Margalef (d) indices, the total number of species and the total number of individuals for each site were compared over seasons and between maize varieties (Bt vs. non-Bt) for determining total arthropod diversity and different functional guilds. ***Results:*** A total of 8,771 arthropod individuals, comprising 288 morphospecies, were collected from the 480 plants sampled during this study. These 288 morphospecies were representative of 20 arthropod orders. Only 28.8% of these species occurred at both localities. The species accumulation curve for these 480 plants had not reached an asymptote, suggesting that the number of species will further increase as more maize plants are sampled. Arthropod diversity indicated no statistical differences between Bt and non-Bt maize for the indices and the number of species or individuals at any of the sites.	The authors concluded that: *"from this short-term study, abundance and diversity of arthropods were not significantly affected by Bt maize. This study provided a start in the study of biodiversity of arthropods on maize in South-Africa and generated a basic checklist of these species".*	Environment	No adverse effects were determined in this study
			Observed parameter	**Feedback on initial environmental risk assessment**
			NTO	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	**Summary of research and results**	**Conclusion[1]**	**Protection Goal**	**Adverse effects**
(Campos and Hernandez, 2015)	***Objective:*** To evaluate differences in dung beetle community composition and structure in forest fragments next to conventional vs. Bt (*Bacillus thuringiensis*) transgenic maize crops, and to reveal possible impacts caused by these environmental changes in organisms via trophic cascade interactions. ***Experimental Design:*** The study was conducted in Campos Novos (Brazil) in February 2011. Twenty sample areas were established in the forest fragments, 10 areas surrounded by a matrix of transgenic maize crops and 10 areas surrounded by conventional maize crops. The genetically modified crops expressed Cry1Ab proteins (DKB240YG, AS1555YG) and Herculex® Technology (30F53H Pioneer). Sampling of coprophagous and necrophagous dung beetles was performed at a distance of 10 m from the edge of the fragment when possible and the total sampling effort consisted of 200 traps (across the 20 forest fragments sampled). A total of 1,502 beetles belonging to 33 species were collected. 805 and 697 dung beetles from 27 species were collected in the fragments adjacent to conventional and Bt-maize, respectively. ***Results:*** Principal component analysis (PCA) showed that vegetation complexity varied little, regardless of the type of crop adjacent to the fragments, but dung beetle community composition was affected by fragment size, environmental complexity and distance between fragments. However, it did not explain the differences related to the two crop types, i.e., the functional group of dwellers was significantly over-represented in the fragments surrounded by transgenic maize. Hence, the dweller species *Eurysternus francinae* and *Eurysternus parallelus* were more frequent and abundant in fragments located near the transgenic maize, while the tunneler species *Onthophagus tristis*, *Uroxys terminalis*, *Ontherus sulcator* and the roller species *Canthon lividus seminitens* were more frequent and abundant in fragments surrounded by conventional maize. Further, for some species that were found in both fragment types, some had strong differences in abundance.	The authors concluded that: *"The observed impact of transgenic crops on functional group dynamics within dung beetle communities could potentially lead to impaired capacity for feces removal, seed dispersal, edaphic aeration, and incorporation of organic matter in the soil in these areas, as such ecosystem services are not performed by the dominant functional group (i.e. dwellers)"*.	Environment	The observed variation cannot be objectively attributed to the matrix of conventional or transgenic maize crops adjacent to the forest fragment collection sites[1].
			Observed parameter	**Feedback on initial environmental risk assessment**
			NTO	There are no changes to the conclusions of the safety of the initial risk assessment.

[1] This study provides a survey of Scarabaeinae species in Atlantic forest fragments of Campos Novos, in Santa Catarina, Brazil. There are several reasons why the observed variation cannot be objectively attributed to the matrix of conventional or transgenic maize crops adjacent to the forest fragment collection sites.

- The Campos Novos region is a patchwork of anthropic environments of various agricultural and land uses among the fragmented forests, which results in highly dynamic conditions between and within collection areas that also includes geophysical variation and elevation contours with slopes and valleys. These factors affect soil structure and moisture levels, critical to defining the distribution of populations in the Scarabaeinae (Halffter and Matthews, 1966). The measurements taken in the forest fragments cannot fully characterize the multitude of variables affecting the communities of Scarabaeinae species in the collections areas.
- The history of the land use (e.g. crop management regime, rangeland pastures, flooding or fires) in and around the farmland near the collection sites should also be taken into account. Measurements in the study to determine the distance from a forest fragment to the closest neighboring forest fragment, cannot characterize the integral environmental patchiness and edge affects along the varied environmental patches. Diaz *et al.* (2010)found that edge effects at tropical forests and pasture and anthropogenic landscape modifications, have affects on the dung and carrion beetles communities.
- The rate of movement and dispersal ability for the dung beetle species (Roslin and Koivunen, 2001) would help to understand the fragmented environments' affects of on the abundance, distrtibution, and the patchy or eveness, of populations of Scarabaeinae species.
- There are no baseline data from undisturbed Atlantic forests from which comparisons could be made to understand changes in the species richness, abundance or biomass of dung beetle communities with the various land uses around remnant fragments of the Atlantic forests.
- This study lacks replication. A collection period within a single month of a single year is not sufficient to characterize the composition and structure of dung beetle communities in the forest fragments in the agriculture region Campos Novos.

The authors provided no empirical evidence to substantiate their conclusion. In the case of insecticidal traits in maize, the assessment of the potential hazard to a non-target beneficial insect population (e.g. dung beetle) is typically carried out within the context of an ecological risk assessment whereby knowledge of the insecticidal activity spectrum of the toxin is combined with data on the environmentally relevant levels and routes of exposure. Additionally, information is not provided with regard to the growth stages of the various matrices of maize fields, crop management regime, and pesticide or nutrient application in either transgenic or non-transgenic maize fields. Therefore it is impossible to place the results of this study into any context in relationship to the maize crop, regardless of the presence of the transgenic maize varieties.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Habustova *et al.*, 2015)	***Objective:*** To examine the safety of genetically modified *Bacillus thuringiensis* (Bt) insect resistant MON 810 maize on a range of species that could be considered as bioindicators for the post-market environmental monitoring (PMEM) defined in Directive 2011/18/EC. To examine variations in the abundance of ground-dwelling, rove beetles and spiders and species richness associated with maize type (Bt and non-Bt) and year of sampling. ***Experimental Design:*** The field trial was performed in the vicinity of České Budějovice (Czech Republic) in 2003-2005. The weather conditions differed from year to year. Bt maize cultivar YieldGard® (MON 810) and its parental non-transgenic cultivar Monumental were planted in the same plots in all three years. The expression of Cry1Ab protein in the Bt maize was verified with ELISA and confirmed by the observation of European corn borer mortality. The ground-dwelling insects were collected once before maize sowing, four times during the growing season and once after the harvest. Five pitfall traps were placed on each plot and exposed for about 14 days. ***Results:*** The impact of maize type (Bt versus non-Bt) on the overall abundance and species richness was examined separately for each year to minimize interference with variable weather conditions. The abundance and species richness of all studied groups greatly varied over the season and between the seasons but without statistically significant differences between the Bt and non-Bt plots. A single spider species (*Oedothorax apicatus*) and three ground beetle spices (*Poecilus cupreus*, *Pterostichus melanarius* and *Bembidion quadrimaculatus*) dominated in the catches every year, whereas a set of 1-4 most abundant rove beetle species changed every year. The magnitude of seasonal variations and the extent of inter-annual differences in both abundance and species richness indicate great impact of weather conditions, field management (e.g., the term of sowing), type of crops grown in adjacent fields and the initial composition and size of arthropod communities in the monitored field.	The authors concluded that: "*All our results demonstrate that the examined arthropod communities are not affected by the Bt maize." In this three year field study that compared maize plots of Bt YieldGard® (MON 810) with the non-transgenic parental cultivar, there were no significant differences between the Bt and non-Bt maize treatments for the abundance and species richness of "assemblages of the plant-dwelling arthropods (Habuštová et al., 2014) and ground-dwelling arthropods (present study).*" The authors suggested that: *"The total counts of ground beetles, rove beetles and spiders collected once or twice per season are proposed to serve as bioindicators on the post-market environmental monitoring (PMEM)"*.	Environment	No adverse effects were determined in this study. This article further supports previous laboratory and field studies demonstrating no adverse effect of Cry1Ab protein, expressed in MON810 maize, on ground dwelling arthropods including ground beetles, rove beetles and spiders. The authors offer suggestions for the potential use of counts of ground beetles, rove beetles and spiders, as bioindicators for agronomic post-market environmental monitoring (PMEM)[1].
			Observed parameter	**Feedback on initial environmental risk assessment**
			NTO	There are no changes to the conclusions of the safety of the initial risk assessment.

[1] The authors further conclude: “Seasonal changes of species abundances in the Bt and non-Bt plots exhibited virtually identical profiles. All our results demonstrate that the examined arthropod communities are not affected by the Bt maize. This conclusion is consistent with the results of other studies on the effect of maize expressing Cry1Ab on the ground dwelling arthropods (Comas *et al*., 2014; Farinós *et al*., 2008; Leslie *et al*., 2007; Rose and Dively, 2007). In Central Europe, no significant differences in the abundance of ground beetles were found in Poland (Twardowski *et al*., 2012) of rove beetles in Hungary (Balog *et al*., 2010; Twardowski *et al*., 2014) and of spiders in the Czech Republic (Rezac *et al*., 2006) and Germany (Toschki *et al*., 2007)."

Though the authors offer suggestions for the use of these arthropods as bioindicators for PMEM, it is important to point out that this suggestion is made in relation to the existence Directive 2001/18/EC and improving PMEM techniques and is not a call for more monitoring based upon the identification of adverse effects of MON 810 on the examined arthropod communities. As demonstrated in this and the other studies mentioned above, the cultivation of Bt maize did not adversely affect the examined arthropod species/communities. Therefore the suggestion to continue PNEM for MON 810 is not supported by the weight of evidence of this and the above-mentioned studies.

Publication	Summary of research and results	Conclusion[1]	Protection Goal	Adverse effects
(Leite *et al*., 2014)	***Objective:*** To evaluate possible prey-mediated effects of *Spodoptera frugiperda* larvae fed with Bt (*Bacillus thuringiensis*) maize (Cry 1Ab) on the biology and behaviour of the predatory stinkbug *Podisus nigrispinus*. ***Experimental Design:*** MON 810 (Cry1Ab) and its near isogenic non-Bt maize hybrid were used. A colony of *P. nigrispinus* was started with adults collected in the field and kept for more than 4 years in the laboratory. Fall armyworm (*Spodoptera frugiperda)* neonates (~2,000) were fed Cry1Ab maize or non-Bt maize leaves (~500) until the 4^{th} instar. Two groups of 50 *P. nigrispinus* nymphs were fed *S. frugiperda* larvae (fed with Bt or non-Bt maize). No plant material was offered to the predator. A bioassay assessed the duration, the total and the stage of each instar as well as the weight of the 5^{th} instar nymphs. A randomized experiment was run with 3 and 7 day-old *S. frugiperda* larvae, reared on Bt or non-Bt maize leaves and 2^{nd} and 4^{th} instar of *P. nigrispinus* nymphs and adults. *S. frugiperda* larvae were confined in Petri dishes and a predator was released in the centre. The duration from release of the predator to the first capture of a larva was timed. A bioassay was also installed in a greenhouse. Plants at the V6 stage were manually infested with five *S. frugiperda* neonates. Five days after larva infestation, half the plants were further infested with five *P. nigrispinus* 2^{nd} instar nymphs. *S. frugiperda* injuries on the plants were graded using a scale of 0 to 5 on Days 7, 15 and 21 after larva infestation. On Day 21 after larva infestation, the number of pupae removed from the pot soil was also evaluated. ***Results:*** Predator showed 43.7% delay in nymphal development time and 15% biomass reduction in the 5^{th} instar, probably due to the low nutritional quality of prey exposed to Bt maize. Survival curves were similar in predators fed with *S. frugiperda* larvae exposed and not exposed to Bt maize. The predator search time was slightly influenced by the development delay of *S. frugiperda* fed with Bt maize. In the greenhouse assay, *P. nigrispinus* was important in controlling *S. frugiperda* density in Bt maize expressing the Cry1Ab protein.	The authors concluded that: *"Bt maize may cause indirect effects on P. nigrispinus and suggest that nutritional prey-quality factors other than the Bt protein determine the observed negative effects. However, the semi-field greenhouse assays demonstrate that indirect negative effects of Bt maize on the predator's performance and search behaviour are not substantiated, and that plant damage is lowest if Bt maize is used concurrently with biological control by P. nigrispinus for managing S. frugiperda"*.	Environment	No adverse effects were determined in this study
			Observed parameter	**Feedback on initial environmental risk assessment**
			NTO	There are no changes to the conclusions of the safety of the initial risk assessment.

[1] The diet consumed by the prey can affect the growth of predatory stinkbugs (Strohmeyer *et al*., 1998), and the potential for indirect effects on insect predators due to the prey-quality of insects that fed on a *Bt* crop, to which the prey is susceptible, are well documented ((Lawo *et al*., 2010; Romeis *et al*., 2006) However, the similarity between *Bt* and non-*Bt* crops, in the abundance and activity of beneficial insect predators and parasitoids has been confirmed by field studies (Romeis *et al*., 2006).

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Erasmus and Van den Berg, 2014)	***Objective:*** To determine the effects of *Bacillus thuringiensis* (Bt) maize expressing the Cry1Ab protein on non-target Coleoptera and Lepidoptera pests. ***Experimental Design:*** Laboratory and greenhouse experiments were conducted with F_1 field-collected larvae from ears of non-Bt maize in the Potchefstroom area of the North West Province, South-Africa. Larval survival and mass gain on Bt- and non-Bt maize leaves and ears were evaluated. The maize was DKC 78-15B (expressing Cry1Ab protein) with the corresponding non-Bt iso-hybrid CRN 3505 and NK Mayor B (event Bt11) with its iso-hybrid Brasco. Two laboratory experiments were conducted to compare beetle mass, mortality and fertility when feeding on 2-4 weeks old stems of Bt and non-Bt maize seedlings. Effects of Bt-maize on various life-history parameters of *S. angulatus* larvae were determined. Survival of second-instar larvae and fourth-instar larvae on maize seedlings of different hybrids was evaluated in test tubes. Test tubes were filled with autoclaved soil in a sufficient quantity to cover stem cuttings completely. Filed-collected beetles were used to determine the effects of Bt-maize on fecundity and fertility. ***Results:*** In both the greenhouse and laboratory studies with *H. armigera*, no larvae survived to the pupal stage on either Bt hybrid over the first four days compared to the non-Bt maize. Further, larval survival was significantly lower on DKC 78-15B compared to RN 3505 as well as on NK Mayor B compared to Brasco. Mass of larvae fed on Bt-maize ears was significantly lower than that of larvae fed on non-Bt ears. Larval survival decreased slowly over time and differed significantly between Bt and non-Bt ears. Bt-maize had no effect on *H. arator* mortality, mass, fertility or fecundity. Similar results were observed for *S. angulatus* with no effects on survival of second or fourth instars, larval mass, fecundity and fertility.	The authors concluded that: *"Cry1Ab protein did not have effects on the coleopteran species evaluated in this study. Cry1Ab-producing maize may protect the crop against H. armigera feeding damage. This effectiveness may, however, contribute to resistance development by H. armigera".*	Environment	No adverse effects were reported, nor can any potential adverse effects be implied by the data
			Observed parameter	**Feedback on initial environmental risk assessment**
			NTO	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Zeng *et al*., 2014)	***Objective:*** To investigate the effects of cultivation and return of *Bacillus thuringiensis* (Bt) maize on arbuscular mycorrhizal fungi (AMF) in soils and roots. ***Experimental Design:*** Two Bt maize varieties, 5422Bt1 (event Bt11) and 5422CBCL (event MON 810) and their conventional (non-Bt) isoline 5422 were planted with four replications for five consecutive seasons from 2009 to 2011. Plants from each variety were sampled and Cry1Ab protein content was determined (9451 and 7455.37 ng/g for 5422Bt1 and 5422CBCL, respectively). At each sampling time, three individual plants from each straw treatment were sampled. Plants were gently dug up and the soils attached to roots were collected. The roots were analysed for AMF colonization, nucleic acids and Cry1Ab protein content. AMF communities were investigated through T-RFLP analysis. The quality of T-RFLP data was first visually inspected by Gene Scanner Software V1.0 and then transferred to T-Rex. Curtis distance measure and 10,000 permutations were used to assess the similarity of the fungal communities in root and soil samples. AMF sequences were identified using the BLASTn program provided by NCBI online. ***Results:*** The concentration of Cry1Ab protein in roots and soils of subsequently planted conventional maize (SCM) ranged from 0.04 to 0.70 ng/g in all samples. No significant difference was observed in AMF colonisation in roots of SCM grown in soil with previously cultivated Bt or non-Bt maize. AMF community diversity assessed by T-RFLP showed no consistent difference related to the richness of soils and roots of SCM, and neither did the diversity of AMF communities in SCM grown with different straw varieties. DNA sequencing showed that typical AMF communities included *Glomus*, *Paraglomus*, *Diversispora*, *Acaulospora* and *Rhizophagus*, of which *Glomus* was the most abundant. No significant effects related to the presence of Bt maize straw were found by general linear analysis. However, plant growth stage had a greater influence on AMF diversity than Bt traits.	The authors concluded that: *"5422Bt (event Bt11) and 5422CBCL (MON 810), which express Cry1Ab protein, had only minor effects on the diversity of the AMF community in soils and roots of subsequently planted conventional maize in soils where Bt maize had been grown for consecutive seasons. Plant growth stage had greater influence on AMF diversity than Bt traits. "*	Environment	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			NTO	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Cotta *et al.*, 2014)	***Objective:*** To assess the abundance and structure of nitrogen-transforming (ammonia-oxidizing) *Archaea* (AOA) and bacteria (AOB) as well as nitrogen-fixing bacteria driven by genetic modification of their maize host plants. ***Experimental Design:*** Two different soils from Brazil, one from the Cerrado region (a dark red acid dystrophic lacyosol with a clayey texture) and another from Várzea areas (a low-humus, eutrophic gley soil with a clayey texture and subneutral pH) were used. In these soils, two maize hybrids, both expressing *Bacillus thuringiensis* protein were planted: MON 810 and TC1507. The near isogenic parental lines of each served as a control. Plants were cultivated during "safra" (in August during the rainy season) and "safrinha" (in February during the dry season). Sampling expeditions were performed at three time points: during the transition from the vegetative to the reproductive growth stage (30 days), at flowering (60 days) and during grain filling and maturity (90 days)[1]. The soil adhering to the roots was pooled, homogenized and considered as a sample of the rhizosphere soil. The samples were used for determination of rhizosphere pH and ammonium / nitrate concentrations, extraction of DNA from rhizosphere samples, quantification of AOA, AOB and diazotrophic communities by qPCR, and PCR-denaturing gradient gel electrophoresis (DGGE) analysis of the AOA, AOB and diazotrophic communities. ***Results:*** All maize types tested (non-Bt protein-expressing maize and Bt-protein-expressing hybrids MON 810 and TC1507) revealed similar growth rates during the safra and safrinha cropping seasons and in both soil types (Cernado and Várzea). Significant changes in the abundances (revealed by quantitative PCR) of ammonia-oxidizing bacterial and archaeal communities occurred as a result of the maize host being genetically modified[2]. In contrast, the structures of the total communities (determined by PCR-denaturing gradient gel electrophoresis) were mainly driven by factors such as soil type and season and not by plant genotype.	The authors concluded that: *"community abundance, in particular AOA, should be explored as candidate bioindicators to further explore the disturbances or potential impacts caused by GM plants"*.	Environment	The authors of this study report observed transient changes in AOA community abundance in the presence of GM plants. However, the study and observations are limited in scope[1, 2] and thus such conclusions cannot be drawn from this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			NTO	There are no changes to the conclusions of the safety of the initial risk assessment.

[1] No preplanting analysis of the plots was performed to establish whether there was a pre-existing bias or variability in the soil with respect to AOA/AOB community abundance. Furthermore, samples were limited to very small soil masses (0.5g) from pooled rhizopheric soil so it is unclear what variability in AOA/AOB would have been observed throughout a particular plot.

[2] Changes in AOA/AOB community abundance were typically half a log or less (the approximate equivalent of just 2 PCR cycles) and only observed at a single and earliest time point (30 days). Futhermore, plant genotype had no effect on diazotroph abundance nor overall community structures for AOA/AOB/diazotrophs. Although the authors propose these communities play minimally redundant roles in the nitrogen cycle, no real-time functional measurements were examined to determine whether the transient changes in AOA/AOB abundance could be detected at a functional level. Endpoint measurements of ammonium or nitrate did not correlate with changes in AOA/AOB abundance.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(da Silva *et al.*, 2014)	***Objective:*** To examine the effects of genetically modified (GM) insect-resistant maize (MON 810 and TC1507) on their endophytic microbial communities (bacteria, archaea and fungi) in comparison with the microbial community found in near-isogenic maize. ***Experimental Design:*** The study was conducted in the state of Minas Gerais, in Brazil. Two *Bacillus thuringiensis* (Bt)-maize hybrids were used: i) Guardian (MON 810 genotype 30F35Y), ii) Herculex (TC1507 genotype 30F35H). The near-isogenic parental line of both GM cultivars was used as control. Sampling was performed during grain filling and maturity (90 days). Roots were submitted to total microbial community DNA extraction. The structure of the endophytic communities and their composition were evaluated by denaturing gradient gel electrophoresis (DGGE) and by the construction of clone libraries. A nested PCR approach was used to amplify the bacterial and archaeal 16S rRNA gene sequences and the fungal internal transcribed spacer (ITS) region from surface sterilized roots. Fragments of PCR-amplified bacterial 16S rRNA gene were obtained from each maize genotype. After transformation of *Escherichia coli* JM109 competent cells, clones were picked and the presence of insects of the correct size was verified by PCR using the primers M13F and M13R. ***Results:*** The three maize genotypes did not show any apparent difference in growth conditions or productivity. No signs of disease or nutrient scarcity were noted. DGGE analysis and the clone libraries of the bacterial community showed that genotype TC1507 slightly differed from the other two genotypes, although high similar (≥ 89%) was found among the two transgenic genotypes and their near-isogenic maize. However, TC1507 showed a higher diversity within its endophytic bacterial community when compared to the other genotypes. Although some bacterial genera, such as *Burkholderia*, *Achromobacter* and *Stenotrophomonas,*were found in all genotypes, some were unique to TC1507. Moreover, OTUs associated with *Enterobacter* predominated only in TC1507 clone libraries.	The authors concluded that: *"the endophytic bacterial community of the maize genotype TC1507 differed from the communities of both MON 810 and near-isogenic genotypes. The differences observed among the maize genotypes studied may be associated with insertion of the gene coding for the PAT protein present only in the transgenic genotype TC1507. The precise impact of transgene expression on endophytic community shifts is something that needs to be further investigated"*.	Environment	The authors of this study report differences in endophytic bacterial community structure in TIC1507 which is not a Monsanto product. No significant differences observed between MON810[1] and controls.
			Observed parameter	**Feedback on initial environmental risk assessment**
			NTO	There are no changes to the conclusions of the safety of the initial risk assessment.

[1] In the discussion, while trying to explain differences between experimental groups, the authors incorrectly claim that MON 810 expresses Cry1F. However, the impact is negligible given the focus of the explanation is on the *pat* gene which is not a Monsanto product.

<table>
<tr><th>Publication</th><th>Summary of research and results</th><th>Conclusion</th><th>Protection Goal</th><th>Adverse effects</th></tr>
<tr><td rowspan="3">(Hurej et al., 2014)</td><td rowspan="3">Objective: To determine the quantitative changes in the proportion of fungi-infected aphids and possible differences in the species composition of entomopathogenic fungi on conventional and Bacillus thuringiensis (Bt) insect resistant maize.
Experimental Design: The experiments were performed in Budziszów, Poland from 2008 to 2010. Three cultivars of maize were cultivated: 1) genetically modified, MON 810 (DKC 3421 Yield Gard®), 2) an isogenic non-Bt crop (DKC 3420) and 3) a conventional crop (Bosman). The fungi-infected aphids were counted in situ on 18 plants per plot (72 per treatment) every two weeks from the beginning to the end of the maize growing season. If maize plants were found infested by aphids, the aphid colonies were visually examined to find individuals with external symptoms of fungal infection. Additionally, twice a growing season the fundi-infected aphids were also collected for fundi identification.
Results: In the three years of the study, in total 10,461 aphids infected by entomopathogenic fungi were recorded on all the observed maize cultivars. Almost the same number of fungi-infected insects occurred on both Bt maize (4,059) and the isogenic non-Bt cultivar (3,866), whereas the incidence of infected aphids was somewhat lower on the conventional reference “Bosman” (2,536). The number of fungi-infected aphids and their time of occurrence were similar on the three compared cultivars. Four species of entomopathogenic fungi were the most abundant and infected aphids on all the maize cultivars: Pandora neoaphidis, Conidiobolus thromboides, Entomophthora planchoniana and Neozygites fresenni. P. neoaphidis was the most numerous species in July 2008 and 2009, while E. planchoniana dominated in October 2009 and July 2010.</td><td rowspan="3">The authors concluded that: “in the three years of the study no influence of the Bt maize on the number, quantity changes of fungi-infected aphids or on the spectrum of fungal species was found”.</td><td>Environment</td><td>Feedback on initial environmental risk assessment</td></tr>
<tr><td>Observed parameter</td><td>There are no changes to the conclusions of the safety of the initial risk assessment.</td></tr>
<tr><td>NTO</td><td>No adverse effects were determined in this study</td></tr>
</table>

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Čerevková and Cagan, 2015)	***Objective:*** To determine the effect of the cultivation of *Bacillus thuringiensis* (Bt) maize, expressing the transgenic Cry1Ab insecticidal protein, on soil nematodes communities, and to analyze the impact of fertilisation and moisture on the community structure of soil nematodes. ***Experimental Design:*** The study was carried out at Borovce in western Slovakia in 2012 and 2013. The following hybrids were used: DKC442YG (Bt maize, event MON 810) and its near-isogenic line DK440 (FAO 350) in 2012, DKC3872YG (Bt maize, event MON 810) and its near-isogenic line DKC3871 (FAO 270) in 2013. Each hybrid was sown in 10 repetitions in plots of 10 m x 10 m. Fertilizers used were: Eurofertil Plus NP 35®, granulated urea and Polidap®. For each hybrid, 10 bulked samples were collected during the maize flowering and used to isolate nematodes according to a well-established procedure. All isolated nematodes were identified at species level and the juveniles were identified at genus level. Nematode species were assigned to different trophic groups on the basis of the feeding habits. The nematode communities were assessed by calculating the maturity index (MI), the plant parasitic index (PPI), the PPI:MI ratio, the ratio of bacterial feeders to fungal feeders, the enrichment index (EI) and the structure index (SI). The composite footprint, the enrichment footprint, the graphical scheme of cp triangles and the graphical scheme of the soil food web were calculated using the web-based software NINJA. ***Results:*** A total of 39 species belonging to 35 genera were identified, with the dominant taxa in both Bt maize and the near-isogenic lines being *Acrobeloides nanus*, *Cephalobus persegnis*, *Aphelenchoides composticola*, *Aphelenchus avenae*, *Eudorylaimus carteri* and *Filenchus vulgaris*. The calculation of MI, PPI, EI and SI did not confirm any influence of year or hybrid type on the soil nematode community. The proportional representation of cp-1, cp-2 and cp-3-5 groups of nematode fauna indicated conditions of low stability and high stress. Faunal profiles showed an environment with a high C:N ratio and high levels of fungal feeders. The calculation of the metabolic footprint of nematodes in the soil food web indicated a difference between the Bt maize and the near-isogenic line in 2012; this difference was not observed in 2013. The occurrence of nematodes, their abundance, proportion of feeding types and ecological indices did not depend on the type of maize. The application of fertilizers at certain periods did not influence the nematode community, while the significant higher abundance of nematodes was correlated with the soil moisture.	The authors concluded that *'soil nematode communities are not influenced by the cultivation of Bt maize hybrids. Fertilizers treatment may affect nematode densities, but the minor differences in their application that are typical in agronomic practice do not significantly influence soil nematode populations. Soil moisture is an important factor influencing soil nematode community. Attack of the European corn borer larvae may cause some changes in the nematode soil communities. Different maize hybrids may influence nematode communities in a specific year, but there are many other, more important factors that are more influential'.*	Environment **Observed parameter** NTO	No adverse effects were determined in this study **Feedback on initial environmental risk assessment** There are no changes to the conclusions of the safety of the initial risk assessment.

Area of the environmental risk assessment: Environmental Safety – Non Target Organisms (NTO) / Insect Resistance Management (IRM)

Publication	**Summary of research and results**	**Conclusion**	**Protection Goal**	**Adverse effects**
(Giron-Perez *et al.*, 2014)	***Objective:*** To assess the variability in Cry1Ab protein susceptibility among different populations of the sugarcane borer (*Diatraea saccharalis*) and to select for resistance in a mixed population in the laboratory. ***Experimental Design:*** Sugarcane borer egg masses and larvae obtained from five locations in Brazil were placed in glass jars and kept in the laboratory under controlled conditions. Offspring from the next generation were used for the bioassays. Cry1Ab protein was prepared from a recombinant strain of *Escherichia coli* (ECE53). Forty eight to sixty four larvae were tested in trays at protein concentrations of 2 – 128 ng/cm^2. The trays were maintained under controlled conditions for 7 days, after which severe growth inhibition / mortality was determined. The weight of larvae surviving exposure was also recorded. The LC50 and EC50 values obtained were used to estimate resistance ratios and 95% confidence intervals. Selection for Cry1Ab resistance was performed using surviving neonates fed on MON 810 maize leaves at the V3 and V5 stages. Larvae from each population that survived exposure to transgenic maize were collected upon moulting to the 2nd instar. The surviving pupae from each population were counted, sexed and pooled into a single "mixed" population. Eggs obtained from this population were used for the Cry1ab resistance selection process. Neonates were fed for 2 days with maize leaves. The procedure was continued for three more consecutive generations. Survival and growth inhibition results as well as heritability data were subjected to regression analysis. ***Results:*** LC50 values ranged from 0.32 to 10.32 ng/cm², which corresponds to a 32-fold variation in the resistance ratios at the LC50 (RR50). There was substantial variation in Cry1Ab susceptibility amongst populations and relatively low variation in intra-population heterogeneity. Similar results were obtained on larval growth, with little variability in intra-population heterogeneity and resistance ratios reaching 12-fold. The mixed population in its F1 generation showed resistance ratios of 10 and 30-fold based on mortality and larval growth inhibition, respectively. The mixed population that was subjected to selection showed 55-fold increase of Cry1ab resistance in the fourth generation. Larval survival for the Cry1Ab-selected strain reached levels similar to the non-selected larvae maintained on the non-transgenic isoline after four generations of selection. The weight gain of surviving larvae also differed between the Cry1Ab-selected and non-selected insects, without exhibiting significant variation along successive generations of Cry1Ab selection. Offspring-parent regression for survivorship data indicated a significant gain of survivorship on Cry1Ab maize upon selection for resistance, while no-significant offspring-parent regression coefficient was observed for insects maintained on non-transgenic maize as expected.	The authors concluded that: *"these findings spark concerns regarding the future use of transgenic maize and sugarcane expressing Cry1Ab toxins in Brazil because the evolution of sugarcane borer resistance to this toxin could take place upon their large-scale field use, which would require resistance management tactics to maximize the sustainable use of these transgenic crops. The mixing of the gathered populations allowed for sufficient variability for quick (four-generation) laboratory selection for Cry1Ab resistance in larvae of the sugarcane borer, although based on these results, we cannot predict whether resistance will develop in the field because the conditions of exposure and intensity of selection will differ under field settings"*.	Environment	The field relevance of this study is questionable because of the conduction of only a three-day bioassay together with the use of maize leaves instead of stalks for feeding.
			Observed parameter	**Feedback on initial environmental risk assessment**
			NTO	There are no changes to the conclusions of the safety of the initial risk assessment

Area of the environmental risk assessment: Environmental Safety – Insect Resistance Management (IRM)

<table>
<tr><th>Publication</th><th>Summary of research and results</th><th>Conclusion[1]</th><th>Protection Goal</th><th>Adverse effects</th></tr>
<tr><td rowspan="3">(Cruz and Eizaguirre, 2015)</td><td rowspan="3">Objective: To determine whether gravid females of Sesamia nonagrioides could discriminate between genetically modified insect protected Bacillus thuringiensis (Bt) maize plants versus their respective near isogenic counterparts, in a dual-choice olfactometer assays. To consider the role of vision in the host location process of gravid females.
Experimental Design: S. nonagrioides larvae were collected from maize fields in the Lleida area – Spain. Larvae were fed on a semi-artificial diet at 25°C and a photoperiod of 16:8 (L:D) h, and the culture was renewed every three or four generations with larvae or pupae from the field. Bt maize DKC 6667 and its near isogenic counterpart DKC 6666; transgenic MV (not commercialized yet) and its near isogenic counterpart M37W, were sowed in regular potting soil kept in a greenhouse. Between 15 and 20 days after planting, seedlings with four to five leaves were used for the experiments. A Y-tube olfactometer was used to test the olfactory and visual responses of 24h-old adult mated females of S. nonagrioides to the odours emitted by the different maize plant varieties. After 3h, the number of females that reached each of the two varieties of the experiment was recorded. Each experiment was repeated until at least 40 insects responded. Only the females that performed the oviposition behaviour and laid eggs were taken into account for the statistical analysis. The same experiment was performed by eliminating the role of vision. The plants were hidden behind a black screen perforated with holes to allow the insects to perceive the plant volatiles without the vision cues.
Results: Olfactory cues were the predominant ones when gravid females looked for a suitable host to lay eggs, and no synergistic effects were observed when both visual and olfactory cues were present. When the plant was visible, the females preferred the odours emitted by M37W to its multivitamin transgenic counterpart and when they only could detect the volatiles they also preferred the M37W variety to the Bt corn variety. However, they did not discriminate between DKC 6666 and M37W, DKC 6666 and DKC 6667, DKC 6666 and MV, or MV and DKC 6667.</td><td rowspan="3">The authors concluded that: “the possibility that gravid females of S. nonagrioides are less attracted to maize with an increased level of vitamins could have important unintended consequences for insect resistance management. The stacked genetically modified plant could be less attractive to gravid females than the refuge plants, which would increase the value of refuge plants for managing resistance”.</td><td>Environment</td><td>No adverse effects were determined in this study</td></tr>
<tr><td>Observed parameter</td><td>Feedback on initial environmental risk assessment</td></tr>
<tr><td>IRM</td><td>There are no changes to the conclusions of the safety of the initial risk assessment.</td></tr>
</table>

[1] Note that this result above would be positive for IRM. Further, as maize with increased vitamin levels has not yet been commercialized, it is premature to determine what impact the commercial event (especially in different genetic backgrounds) will have on preference. Finally, these were lab studies and would need to be validated in the field.

Area of the environmental risk assessment: Environmental Safety – Insect Resistance Management (IRM)/ Impact of Management Practices

<table>
<tr><th>Publication</th><th>Summary of research and results</th><th>Conclusion</th><th>Protection Goal</th><th>Adverse effects</th></tr>
<tr><td rowspan="3">(Reisig et al., 2015)</td><td rowspan="3">Objective: To compare plant injury from five species of Lepidoptera between: 1) single transgenic maize expressing the insecticidal protein derived from Bacillus thuringiensis (Bt) and pyramided Bt traits; and 2) a blended and a structured refuge.
Experimental Design: A two-year study (2010-2011) evaluated seven treatments using small plots in separate tests across 12 states in the southern part of the United States. Treatments were as follows: 1) Cry1F x Cry1Ab pyramid of TC1507 (expressing the Bt cry1F insecticidal protein) x MON 810 (expressing the Bt Cry1Ab insecticidal protein) x NK603 (resistant to glyphosate herbicide); 2) Cry1F x Cry1Ab pyramid (same as Treatment 1) plus a blended refuge non-Bt hybrid with only NK603; 3) Cry1Ab x Vip3Aa20 pyramid of Bt11 (expressing Cry1Ab insecticidal protein) x MIR162 (expressing the Vip3Aa20 insecticidal protein) x GA21 (resistant to glufosinate herbicide); 4) Cry1F with events TC1507 x NK603; 5) and 6) two treatments of non-Bt structured refuge, containing herbicide tolerance only (NK603); and 7) Cry1Ab, containing events MON 810 x NK603. A randomized block design was used with four replications. Planting time was within the optimal range of dates in 2010 and up to three weeks later than the optimal dates at some locations in 2011 because of weather conditions. Natural infestations of at least one of the target pests (Spodoptera frugiperda, Helicoverpa zea, Diatraea grandiosella, Diatraea saccharalis and Elasmopalpus lignosellus) took place in each experiment except for S. frugiperda in KY and D. saccharalis in LA: in both cases, artificial infestation was carried out. Leaf and ear rating were collected on 20 consecutive plants within the treatment row. Leaf injury, kernel damage and stalk tunneling were evaluated according to standard procedures. The data were analyzed for statistical significance using Turkey's honestly significant difference test.
Results: The lepidopteran pests did not differentially injure non-Bt plants blended with Cry1F x Cry1Ab plants compared to non-Bt plants in a structured refuge with a seed treatment. One exception was E. lignosellus which injured or killed 42% more plants in a blended seed mixture, compared to a structured refuge of non-Bt plants. Although neighbouring non-Bt plants within the pyramided Cry1F x Cry1Ab plus non-Bt blended refuge treatment were more injured by pests than the plants producing two Bt proteins, a pure stand of pyramided Cry1F x Cry1Ab effectively managed overall plant injury caused by S. frugiperda and H. zea to the leaf, kernel injury caused by H. zea on the ear, and injury caused by tunneling of D. grandiosella. The pyramided Cry1Ab x Vip3Aa20 structured refuge was the most effective treatment tested against all insect pests. Pyramided dual-gene Bt treatments were more effective in protecting the plants from fall</td><td rowspan="3">The authors concluded that '... hybrids with pyramided Bt traits were more effective for managing S. frugiperda and H. zea. Both single-Bt trait and pyramided-Bt trait hybrids were effective against D. grandiosella, D. saccharalis, and E. lignosellus. Cry1Ab x Vip3Aa was either the most effective pyramid or among the most effective for all insect pests. The efficacy of plants containing Cry1F x Cry1Ab was not influenced by a blended refuge scenario, compared to plots mimicking a pure stand for major southern US pests. Non-Bt plants within the blended refuge did not differ significantly in injury than non-Bt plants (except with E. lignosellus) in plots mimicking a structured refuge.......These data suggest that it is likely that the period for development of resistance to these traits would remain static, compared to the traditional structured refuge in the southern United States'.</td><td>Environment</td><td>No adverse effects were determined in this study</td></tr>
<tr><td>Observed parameter</td><td>Feedback on initial environment al risk assessment</td></tr>
<tr><td>IRM</td><td>There are no changes to the conclusions of the safety of the initial risk assessment.</td></tr>
</table>

Area of the environmental risk assessment: Environmental Safety – Agronomy

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Bowers *et al*., 2014)	***Objective:*** To determine 1) the effect of Western bean cutworm (WBC) and European corn borer (ECB) infestation on both *Fusarium* infection of maize and fumonisin contamination; and 2) the ability of various maize hybrids (MON 810 and TC1507), expressing the *Bacillus thuringiensis* (*Bt*) insecticidal proteins, to control and mitigate this issue. ***Experimental Design:*** Field trials wee conducted from 2007 to 2010. The following six commercial maize hybrids were used: 2 MON810 hybrids producing Cry1Ab insecticidal protein (DKC57-79 and DKC61-69), 2 TC1507 hybrids producing Cry1F insecticidal protein (33D14 and 34A20) and 2 near-isogenic non-*Bt* hybrids (DKC61-72 and 34A14). The hybrids were grown in plots (6 x 6.1 m) consisting of four rows. Only the middle two rows were used for insect infestation and grain harvest. The experiment included 5 replications of each combination of maize hybrid and insect infestation. ECB infestation was performed as follows: in 2007, 50 neonates were applied at the base and 50 on the silks of the primary ear; in 2008, 50 neonatal larvae were applied in two consecutive days, the first day on the silks of the primary ear and the second day on the silks of the second-highest ear; in 2009 and 2010, approximately 100 neonatal larvae were applied on the primary ear, 50 at the base and 50 on the silks. WBC infestation methods differed each year: in 2008, three second or third-instar larvae were applied on the silks of each primary ear on 10 ears per plot; in 2009 and 2010, egg masses on maize leaf tissue were attached at the base of the primary ear of 20 plants per plot, on 10 consecutive ears in each of the treatment rows; in 2009 the maize leaves were first fixed to a section of plastic screen and then attached at the base of the ear, while the screen was not used in 2010. 10 ears per plot were harvested; ears were inspected for the presence of insect injury and then dried. To determine the presence of fumonisin contamination, maize extract samples were analyzed by 1) ELISA for the presence of Fumonisin B_1 in 2007 and total fumonisin in 2008 and 2009, and 2) HPLC for the presence of total fumonisin in all 4 years. ***Results:*** *Bt* hybrids showed less insect injury, *Fusarium* ear rot, and fumonisin contamination as compared to non-*Bt* hybrids. WBC infestation increased fumonisin content as compared to natural infestation in non-Bt and hybrids expressing Cry1Ab protein in five of eight possible comparisons, while it did not have any effect in Cry1F hybrids. Results of the ELISA and HPLC methods were highly correlated, with the ELISA estimates being higher.	The authors concluded that *'WBC is capable of increasing fumonisin levels in maize. Under WBC infestation, Cry1F mitigated this risk more consistently than Cry1Ab or non-Bt hybrids. Transgenically expressed Bt proteins active against multiple lepidopteran pests can provide broad, consistent reductions in the risk of fumonisin contamination'.*	Environment	No adverse effects were determined in this study
			Observed parameter	**Feedback on initial environmental risk assessment**
			Plant health	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Bowen *et al*., 2014)	***Objective:*** To evaluate insect damage, yield improvement and aflatoxin levels as affected by Bt (*Bacillus thuringiensis*) maize compared to near isogenic non-Bt maize. ***Experimental Design:*** YieldGard Corn Borer (expressing Cry1Ab), Herculex I (expressing Cry1F), Genuity VT Triple PRO (expressing Cry1A.105 and Cry2Ab2), Agrisure Viptera 3111 (expressing Vip3Aa20 and Cry1Ab), Genuity SmartStax (expressing Cry1A.105, Cry2Ab2 and Cry1F) and the near isogenic non-Bt maize were planted at eight sites across Alabama over three years (from 2010 to 2012). Experiments were planted in the first year of maize following rotation. To determine lepidopteran feeding damage, 10 ears were arbitrarily collected from each plot at dough stage or dent to clack layer. Silk feeding damage with or without corresponding feeding on the kernels was often observed: this was rated from 0 to 2 where 0 = no cut silks, 1 = <50% of cut silks and 2 = >50% of cut silks due to feeding damage. Ten grams of ground maize from each sample was assayed for aflatoxin using the Veratox test. ***Results:*** Conditions differed from site to site within each year and maize was irrigated at some locations. When examined over all sites and years, hybrids with any of the included Bt traits had lower insect damage and higher yields. Although not significant, hybrids with a Bt trait had higher yields in 2010 (3.6%) than non-Bt maize. However, insect damage was not consistently correlated to yield. Bt traits expressing multiple proteins provided greater protection from maize earworm feeding than traits for single proteins. Hybrids with a Bt trait had 21.5, 35.9 and 42.9% lower ear damage than those without Bt in 2010, 2011 and 2012, respectively. In 2010, 2011 and 2012, Bt traits had 15, 34 and 31% lower silk damage, respectively, than hybrids without a Bt trait. Yields and aflatoxin levels were highly variable among sites although irrigated sites had higher yields than non-irrigated sites. Aflatoxin concentrations averaged 72, 109 and 21 ppb over all cultivars and sites in the three years.	The authors concluded that: *"Bt maize hybrids can provide yield advantages in many situations, but did not impact aflatoxin concentrations under the conditions in this study… Bt hybrids expressing multiple proteins provide greater protection from ear damage by lepidopteran pests than those with single Bt traits"*.	Environment	No adverse effects were determined in this study
			Observed parameter	**Feedback on initial environmental risk assessment**
			Plant health	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusion[2]	Protection Goal	Adverse effects
(Gulli *et al*., 2015)	***Objective:*** To assess the effect of water deficit on physiological parameters, the global transcriptional pattern and *Cry1Ab* gene expression in the genetically modified (GM) maize variety DKC6575, compared to the near-isogenic non-GM Tietar. ***Experimental Design:*** Both field and growth chamber experiments were conducted. For the field experiment, seeds of Tietar and DKC6575 were grown in Parma (Italy), with a typical continental climate on a coarse-loamy, mixed, calcareous soil. Each plot was fully irrigated until plants reached the vegetative V6 stage, then they were irrigated differently to apply drought stress: i) irrigated at 100% field capacity; ii) irrigated at 25% field capacity; iii) not irrigated. For the growth chamber experiment, fourteen plants of each hybrid were grown until the V8 stage, at 28/25°C (day/night), 50% relative humidity, photoperiod 14h , watered daily to field capacity. At the V8 stage, plants were randomly divided into different experimental sets: i) watered at 100% during the whole experiment, ii) not irrigated for 6 days.[1] Net photosynthesis, leaf transpiration, stomatal conductance and sub-stomatal CO_2 concentration were measured with a portable infrared gas analyser. The ratio between sub-stomatal and external CO_2 concentration as well as water use efficiency were calculated. Chlorophyll a (Chl a) fluorescence was measured using the Fluorescence Monitoring System. Sequences of *Zea mays* encoding the actin binding protein (*Zmabp3*), the 18S small subunit of ribosomal RNA (*Zm18SRNA*), dehydrin (*Zmdn1*) and the transgene (*Cry1Ab*) were selected from GenBank. Specific primers and probes for qRT-PCR were designed using "Primer Express 2". Samples of leaves were collected from plants at the V and V8 stages and at the silking R1stage, all at the same hour of the day, immediately frozen in liquid nitrogen and stored at -80°C. ***Results:*** Main photosynthetic parameters were significantly affected by drought stress in both GM and non-GM varieties to a similar extent. Although DKC6575 had a greater sensitivity in the early phase of stress response as compared with Tietar, after six days of stress they behaved similarly, and both varieties recovered from stress damage. Profiling gene expression in water deficit regimes and in a generalised drought stress condition showed an up-regulation of many stress-responsive genes, but a greater number of differentially expressed genes was observed in Tietar, with genes belonging to transcription factor families, genes encoding heat stock proteins, late embryogenesis abundant proteins and detoxification enzymes. Since induction of these genes have been indicated from the literature as typical of stress response to drought. DKC6575 was also analysed for the expression of the transgene Cry1Ab in water deficit conditions. In all the experiments, the *Cry1ab* transcript was not influenced by drought stress, but was expressed at a constant level.	The authors concluded that: *"i) although the main photosynthetic parameters were affected by drought to a similar extent in both the GM and non-GM varieties, under controlled environmental conditions DKC6575 was demonstrated to have a greater sensitivity to stress in the early phase with respect to Tietar;* *ii) a whole genome transcriptomic analysis demonstrated that the water deficit regimes determined the up-and down- regulation of many genes, but with an up-regulation of stress-responsive genes to a greater extent in Tietar, suggesting more efficient drought responses in this genotype than in DKC6575;* *iii) the expression of the transgene Cry1Ab was not influenced by the water regime, being expressed at a constant level, suggesting that any eventual greater sensitivity to drought stress in the GM variety did not concern the level of transgene expression, which was stable through conditions".*	Environment **Observed parameter** Plant gene expression	No adverse effects were determined in this study **Feedback on initial environmental risk assessment** There are no changes to the conclusions of the safety of the initial risk assessment.

[1] At the V8 stage, the growth chamber experiment only considered seven plant replications per treatment for measuring several parameters such as photosynthesis. A small replication number leads to a low power of the study.

[2] It cannot be ruled out that the differential drought response observed by the authors is caused by the difference in genetic background between DKC6575 and Tietar. This has been studied by Venkatesh *et al.* (2015), who demonstrated that differences between GM crops and their non-GM comparators can be attributable to minor genomic differences in near-isogenic lines.

References

Alvarez-Alfageme F, Bigler F and Romeis J, 2011. Laboratory toxicity studies demonstrate no adverse effects of Cry1Ab and Cry3Bb1 to larvae of *Adalia bipunctata* (Coleoptera: *Coccinellidae*): The importance of study design. Transgenic Research, 20, 467-479.

Álvarez-Alfageme F, Ferry N, Castañera P, Ortego F and Gatehouse A, 2008. Prey mediated effects of *Bt* maize on fitness and digestive physiology of the red spider mite predator S*tethorus punctillum Weise* (Coleoptera: *Coccinellidae*). Transgenic Research, epub ahead of print,

Bai YY, Jiang MX and Cheng JA, 2005. Effects of transgenic cry1Ab rice pollen on Þtness of *Propylea japonica* (Thunberg). J. Pest Sci., 78, 123-128.

Bai YY, Jiang MX, Cheng JA and Wang D, 2006. Effects of Cry1Ab toxin on *Propylea japonica* (Thunberg) (Coleoptera: *Coccinellidae*) through its prey, *Nilaparvata lugens Sta*° l (Homoptera: *Delphacidae*), feeding on transgenic *Bt* rice. Environmental Entomology, 35, 1130-1136.

Balog A, Kiss J, Szekeres D, Szenasi A and Marko V, 2010. Rove beetle (Coleoptera: *Staphylinidae*) communities in transgenic *Bt* (MON 810) and near isogenic maize. Crop Protection, 29, 567-571.

Bowen KL, Flanders KL, Hagan AK and Ortiz B, 2014. Insect damage, aflatoxin content, and yield of *Bt* corn in Alabama. Journal of Economic Entomology, 107, 1818-1827.

Bowers E, Hellmich R and Munkvold G, 2014. Comparison of fumonisin contamination using HPLC and ELISA methods in *Bt* and near-isogenic maize hybrids infested with european corn borer or western bean cutworm. Journal of Agricultural and Food Chemistry, 62, 6463-6472.

Campos RC and Hernandez MIM, 2015. Changes in the dynamics of functional groups in communities of dung beetles in Atlantic forest fragments adjacent to transgenic maize crops. Ecological Indicators, 49, 216-227.

Čerevková A and Cagan L, 2015. Effect of transgenic insect-resistant maize to the community structure of soil nematodes in two field trials. Helminthologia, 52, 41-49.

Comas C, Lumbierres B, Pons X and Albajes R, 2014. No effects of *Bacillus thuringiensis* maize on nontarget organisms in the field in southern Europe: a meta-analysis of 26 arthropod taxa. Transgenic Research, 23, 135-143.

Cotta SR, Franco Dias AC, Marriel IE, Andreote FD, Seldin L and van Elsas JD, 2014. Different effects of transgenic maize and non-transgenic maize on nitrogen-transforming *Archaea* and *Bacteria* in tropical soils. Applied and Environmental Microbiology, 80, 6437-6445.

Cruz D and Eizaguirre M, 2015. Do *Sesamia nonagrioides* (Lepidoptera; *Noctuidae*) gravid females discriminate between *Bt* or multivitamin corn varieties? role of olfactory and visual cues. Journal of Insect Science, 15, 5.

da Silva DAF, Cotta SR, Vollu RE, Jurelevicius DD, Marques JM, Marriel IE and Seldin L, 2014. Endophytic microbial community in two transgenic maize genotypes and in their near-isogenic non-transgenic maize genotype. Bmc Microbiology, 14, 9.

Dhillon M and Sharma H, 2009. Effects of *Bacillus thuringiensis* -endotoxins Cry1Ab and Cry1Ac on the coccinellid beetle, *Cheilomenes sexmaculatus* (Coleoptera, Coccinellidae) under direct and indirect exposure conditions. Biocontrol Science and Technology, 19, 407-420.

Diaz A, Eduardo G and Favila ME, 2010. The effect of the landscape matrix on the distribution of dung and carrion beetles in a fragmented tropical rain forest. Journal of Insect Science, 10, 1-17.

Erasmus A and Van den Berg J, 2014. Effect of *Bt*-maize expressing Cry1Ab toxin on non-target *Coleoptera* and *Lepidoptera* pests of maize in South Africa. African Entomology, 22, 167-179.

Farinós GP, de la Poza M, Hernandez-Crespo P, Ortego F and Castanera P, 2008. Diversity and seasonal phenology of aboveground arthropods in conventional and transgenic maize crops in Central Spain. Biological Control, 44, 362-371.

Giron-Perez K, Oliveira AL, Teixeira AF, Guedes RNC and Pereira EJG, 2014. Susceptibility of Brazilian populations of *Diatraea saccharalis* to Cry1Ab and response to selection for resistance. Crop Protection, 62, 124-128.

Grabowski M, Lipska A, Zmijewska E, Kozak M and Dabrowski ZT, 2014. Transfer of the Cry1Ab toxin in tritrophic bioassays involving transgenic maize MON 810, the herbivore tetranychusurticaeKoch and the predatory ladybird beetle Adaliabipunctata L. (Coleoptera: *Coccinelidae*). Egyptian Journal of Biological Pest Control, 24, 11-16.

Gulli M, Salvatori E, Fusaro L and Pallacani C, 2015. Comparison of drought stress response and gene expression between a GM maize variety and a near-isogenic non-GM variety. PLOs, 1-21.

Habustova OS, Svobodova Z, Spitzer L, Dolezal P, Hussein HM and Sehnal F, 2015. Communities of ground-dwelling arthropods in conventional and transgenic maize: background data for the post-market environmental monitoring. Journal of Applied Entomology, 139, 31-45.

Halffter G and Matthews EG, 1966. The natural history of dung beetles of the subfamily *Scarabaeinae* (Coleoptera, *Scarabaeidae*). Folia Entomol. Mex., 12 (14), 1-312.

Hurej M, Mietkiewski R and Twardowski JP, 2014. The effect of Cry1AB insecticidal protein on the incidence of entomopathogenic fungi infecting aphids on *Bt* maize. Zemdirbyste-Agriculture, 101, 279-284.

Lawo NC, Wäckers FL and Romeis J, 2010. Characterizing indirect prey-quality mediated effects of a *Bt* crop on predatory larvae of the green lacewing, *Chrysoperla carnea*. Journal of Insect Physiology, 56, 1702–1710.

Leite AN, Mendes MS, Dos Santos AC and Pereira EJ, 2014. Does Cry1 Ab maize interfere in the biology and behavioural traits of *Podisus nigrispinus*? Bulletin of Insectology, 67, 265-271.

Leslie TW, Hoheisel GA, Biddinger DJ, Rohr JR and Fleischer SJ, 2007. Transgenes sustain epigeal insect biodiversity in diversified vegetable farm systems. Environmental Entomology, 36, 234-244.

Lundgren JG, Razzak AA and Wiedenmann RN, 2004. Population responses and food consumption by predators *Coleomegilla maculata* and *Harmonia axyridis* (Coleoptera : Coccinellidae) during anthesis in an Illinois cornfield. Environmental Entomology, 33, 958-963.

Meissle M, Zund J, Waldburger M and Romeis J, 2014. Development of *Chrysoperla carnea* (Stephens) (Neuroptera: *Chrysopidae*) on pollen from *Bt*-transgenic and conventional maize. Scientific Reports, 4, 9.

Moser SE, Harwood JD and Obrycki JJ, 2008. Larval feeding on *Bt* hybrid and non-*Bt* corn seedlings by *Harmonia axyridis* (Coleoptera : Coccinellidae) and *Coleomegilla maculata* (Coleoptera : Coccinellidae). Environmental Entomology, 37, 525-533.

Porcar M, Garcia-Robles I, Dominguez-Escriba L and Latorre A, 2010. Effects of *Bacillus thuringiensis* Cry1Ab and Cry3Aa endotoxins on predatory Coleoptera tested through artificial diet-incorporation bioassays. Bulletin of Entomological Research, 100, 297-302.

Rauschen S, 2010. A case of "pseudo science"? A study claiming effects of the Cry1Ab protein on larvae of the two-spotted ladybird is reminiscent of the case of the green lacewing. Transgenic Research, 19, 13-16.

Reisig DD, Akin DS, All JN, Bessin RT, Brewer MJ, Buntin DG, Catchot AL, Cook D, Flanders KL, Huang FN, Johnson DW, Leonard BR, McLeod PJ, Porter RP, Reay-Jones FPF, Tindall KV, Stewart SD, Troxclair NN, Youngman RR and Rice ME, 2015. Lepidoptera (*Crambidae, Noctuidae*, and *Pyralidae*) injury to corn containing single and pyramided *Bt* traits, and blended or block refuge, in the Southern United States. Journal of Economic Entomology, 108, 157-165.

Rezac M, Pekar S and Kocourek F, 2006. Effect of *Bt*-maize on epigeic spiders (*Araneae*) and harvestmen (*Opiliones*). Plant Protection Science, 42, 1-8.

Romeis J, Alvarez-Alfageme F and Bigler F, 2012. Putative effects of Cry1Ab to larvae of *Adalia bipunctata* - reply to Hilbeck *et al.*(2012). Environmental Sciences Europe, 24, 18.

Romeis J, McLean MA and Shelton AM, 2013. When bad science makes good headlines: *Bt* maize and regulatory bans. Nature Biotechnology, 31, 386-387.

Romeis J, Meissle M and Bigler F, 2006. Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. Nature Biotechnology, 24, 63-71.

Rose R and Dively GP, 2007. Effects of insecticide-treated and lepidopteran-active *Bt* transgenic sweet corn on the abundance and diversity of arthropods. Environmental Entomology, 36, 1254-1268.

Roslin T and Koivunen A, 2001. Distribution and abundance of dung beeltes in fragemented landscapes. Oecologia, 217, 69-77.

Schmidt J, Braun C, Whitehouse L and Hilbeck A, 2009. Effects of activated *Bt* transgene products (Cry1Ab, Cry3Bb) on immature stages of the ladybird *Adalia bipunctata* in laboratory ecotoxicity testing. Archives of Environmental Contamination and Toxicology, 56, 221-228.

Strohmeyer HH, Stamp NE, Jarzomski C and Bowers MD, 1998. Prey species and prey diet affect growth of invertebrate predators. Ecological Entomology, 23, 68-79.

Toschki A, Hothorn LA and Ross-Nickoll M, 2007. Effects of cultivation of genetically modified *Bt* maize on epigeic arthropods (A*raneae; Carabidae*). Environmental Entomology, 36, 967-981.

Truter J, Van Hamburg H and Van Den Berg J, 2014. Comparative diversity of arthropods on *Bt* maize and non-*Bt* maize in two different cropping systems in South Africa. Environmental Entomology, 43, 197-208.

Twardowski J, Beres P, Hurej M and Klukowski Z, 2014. A quantitative assessment of the unintended effects of *Bt*-maize (MON 810) on rove beetle (Col., *Staphylinidae*) assemblages. Polish Journal of Environmental Studies, 23, 215-220.

Twardowski JP, Beres PK, Hurej M, Klukowski Z, Dazbrowski ZT, Sowa S and Warzecha R, 2012. The quantitative changes of ground beetles (Coleoptera: *Carabidae*) in *Bt* and conventional maize crop in southern Poland. J. Plant Prot. Res. , 52, 404-409.

Venkatesh TV, Cook K, Liu B, Perez T and Willse A, 2015. Compositional differences between near-isogenic GM and conventional maize hybrids are associated with backcrossing practices in conventional breeding. Plant Biotechnology Journal, 1-11.

Zeng HL, Tan FX, Zhang YY, Feng YJ, Shu YH and Wang JW, 2014. Effects of cultivation and return of *Bacillus thuringiensis* (*Bt*) maize on the diversity of the arbuscular mycorrhizal community in soils and roots of subsequently cultivated conventional maize. Soil Biology & Biochemistry, 75, 254-263.

Zhang GF, Wan FH, Guo JY and Hou ML, 2004. Expression of *Bt* toxin in transgenic *Bt* cotton and its transmission through pests *Helicoverpa armigera* and *Aphis gossypii* to natural enemy *Propylaea japonica* in cotton plots. Acta Entomologica Sinica, 47, 334-341.

Zhang X, Li Y, Romeis J, Yin X and Wu K, 2014. Use of a Pollen-Based Diet to Expose the Ladybird Beetle Propylea japonica to Insecticidal Proteins. PLOs, 9 (1), e85395.