Appendix 5.2. MON 810 literature review – Environment (July 2017)

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Andow and Zwalhen (2016) Ground beetle acquisition of CryAb from plant- and residue-based food webs	***Objective:*** To determine the direct and indirect exposure routes through which non-target organisms such as carabid beetles might acquire Cry1Ab toxin in fields of genetically modified (GM) *Bacillus thuringiensis* (Bt) maize (MON 810). ***Experimental:*** Four hypotheses were tested on abundant carabid species collected between July and September 2005: H1 - Beetles acquire Cry1Ab directly or indirectly from live *Bt* maize (crops from the current year 2005/'non-Bt/non-Bt/Bt'), H2 - Beetles acquire Cry1Ab directly or indirectly from one-year-old *Bt* maize residue (crops planted in the previous year 2004/'non-Bt/Bt/non-Bt), H3 - Beetles acquire Cry1Ab directly or indirectly from two-year-old *Bt* maize residue (Bt-maize crops planted in 2003/'Bt/non-Bt/non-Bt'), and H4 - Beetles can acquire Cry1Ab by directly consuming residue ('laboratory feeding trial'). The control treatment was achieved in non-Bt-crops for at least three consecutive seasons ('non-Bt/non-Bt/non-Bt'). Adult carabids were collected in barrier traps from 10 fields in Rosemound, Minnesota (USA). Samples of stalk and leaf tissues from live plant tissues and residue were analysed from three Bt maize varieties, i.e. DKC44-42 (DeKalb), K4688 (Kaltenberg) and P36N71 (Pioneer). In the 4-week laboratory feeding trial, *C. iowensis* (the most common specie trapped in maize fields of this region) were feed either with field –collected Bt maize residue or field –collected non-Bt maize residue. The positive control group was feed with ECB larvae that had been dipped in a solution of CryAb toxin. (Cry1Ab concentrations in beetles, maize residue, and live maize tissues were determined using an enzyme-linked immunosorbent assay (ELISA) and compared with control conditions. ***Results:*** The most frequent carabid species recorded were: *Cyclotrachelus iowensis, Poecilus lucublandus, Peocilus chalcites, Acarites quadriceps Chaudoir, Bembidion quadrimaculatum, and Elaphropus incurvus*. The amount of Cry1Ab per sample was related logarithmically with the size of the beetle. Samples from all species found in fields containing live Bt maize and samples of 5 out of 6 species found in fields containing one-year-old Bt residue tested	The authors concluded that *"significant differentiation among carabid species in their associations with live-plant and residue –based food webs in agricultural fields exist. For some species (C. iowensis, P. lucublandus and P. chalcites), both the live-plant and the residue-based food webs may have been important food sources, whereas for other species (B. quadrimaculatum, and E. incurvus), the live plant-based food webs might be predominant, or they (S. quadriceps) might feed on such a high trophic level that their prey does not contain detectable concentrations of Cry1Ab."* [1]	Environmental protection **Observed parameter** NTO	Direct or indirect transfer of Bt-endotoxins (Cry1Ab proteins) from living maize plant, residue or prey towards beetles **Feedback on initial environmental risk assessment** 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
	positive for Cry1Ab, suggesting that these species were participating or probably participating in live plant-based food webs and that they acquired Cry1Ab directly and/or indirectly from plant residue (H1 and 2). From fields with two-year-old Bt residue, Cry1Ab was not detected more frequently than from control field (H3). Finally, none of the *C. iowensis* adults that were offered either Bt or non-Bt maize residue contained any Cry1Ab. In contrast, Cry1Ab was detected in all of the beetles that were given ECB larvae dipped in a Cry1Ab solution (H4).			

[1] A key principle of ERA is risk-based testing (CLI, 2016; Wolt *et al.*, 2010), in which testing is limited to those scenarios under which there is plausible scientific rationale for an adverse environmental effect. The large body of literature in addition to the experience of safe cultivation of GM crops over the last 20 years supports a tiered based ERA for GM crops. The advantages of the tiered based ERA include repeatable, affordable, and commonly available bioassays using surrogate species that are predictive of risk to non-target organisms and related to clear protection goals. A biologically relevant effect observed at a lower tier of testing provides data to inform a hypothesis driven study at the next tier that includes additional variability found in natural systems. Based on experience, this tiered system has been accurately predictive of safety with currently available GM crops and is robust enough to be predictive of risk for novel products in the future.

Potential exposure to the Cry1Ab proteins for ground beetles could occur through direct feeding on plant tissue or indirectly through feeding on prey that contain the protein. Though a potential trophic transfer of these proteins exists, the initial expectation is that Cry1Ab activity is limited to within the Order Lepidoptera. Implications of the trophic transfer of this protein for ground beetles can be assessed by considering the specificity of the Cry1Ab protein and the non-exhaustive list of other invertebrates that have been studied without reports of direct effects of Bt proteins through the consumption of prey (i.e., secondary exposure), includes: ground beetles (Coleoptera: Carabidae) (Harwood *et al.*, 2006; Meissle *et al.*, 2005), lady beetles (Coleoptera: Coccinellidae) (Alvarez-Alfageme *et al.*, 2011; Li and Romeis, 2010), spiders (Araneae) (Meissle and Romeis, 2009), predatory mites (Acari: Phytoseiidae) (Obrist *et al.*, 2006), parasitic wasps (Hymenoptera) (Davidson *et al.*, 2006), brown lacewings (Neuroptera: Hemerobiidae) (Davidson *et al.*, 2006), and green lacewings (Neuroptera: Chrysopidae) (Rodrigo-Simon *et al.*, 2006).

In summary, though Andow and Zwahlen report interesting and important results, the current body of knowledge does not indicate that Cry1Ab expressed in Bt maize negatively impacts predation ecosystem services provided by ground beetles. Evidence based on our understanding of the restricted activity spectra of Bt proteins and results from tri-trophic studies supports the conclusion that Bt maize is compatible with ground beetles and other generalist predators with no expected unacceptable negative ecological impact.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Di Grumo and Lovei (2016) Body size inequality in ground beetle (Coleoptera: Carabidae) assemblages as a potential method to monitor environmental impacts of transgenic crops	***Objective:*** To determine the body size distribution of ground beetle assemblages in order to monitor the environmental impact of genetically modified (GM) MON 810 maize, expressing the *Bacillus thuringiensis* insecticidal protein Cry1Ab. ***Experimental Design:*** The field trial was carried out in an experimental farm in Denmark from June to August 2014 within the frame of the AMIGA (Assessing and Monitoring the Impacts of Genetically modified plants on Agro-ecosystems) project. Specifically, a field of 0.5 ha was randomly divided into 10 plots of GM maize (MON 810) and 10 plots of its non-GM isogenic line. The ground beetles were sampled by pitfall traps. The cups were placed in the middle of each plot and covered with metal sheet to minimise the catch of undesired species. Traps were open from June to August, one week per month. All captured carabids were identified to species according to a published methodology. The geometric mean of the minimum-maximum values of the body size was calculated and the "decreasing body size hypothesis" was tested using the Lorenz curve. To quantify size inequality, the Gini coefficient was calculated. To compare the Gini and Lorenz asymmetry coefficients between GM and conventional maize crops, the normal distribution of data was tested by the Kolmogorov-Smirnov test and the Shapiro-Wilk normality test. ***Results:*** A total of 6339 carabids belonging to 38 species were captured and identified, with the dominant species being *Harpalus rufipes*. The analysis detected a significant shift in size inequality between months, indicating that a larger number of individuals of smaller sized species are present later in the season. However, no significant difference in inequality or mean body size was found between the assemblages in GM *versus* isogenic maize plots.	The authors concluded that: "*the evaluation of body size inequality was sensitive to subtle changes in the structure of the carabid assemblages, and this method had the potential to be used during monitoring of the unanticipated environmental effects of GM plants.*"	Environmental protection	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
Lee and Albajes (2016) Monitoring carabid indicators could reveal environmental impacts of genetically modified maize	***Objective:*** 1) To assess the abundance and frequency of carabid species present in maize agroecosystem across regions and years, providing baseline information; 2) to determine the most suitable carabid indicators for standardized monitoring based on distribution, statistical power, sample size and capacity for reflecting ecosystem diversity and functions; and 3) to determine the most suitable sampling sites and dates. ***Experimental:*** Carabids were sampled in three different maize cropping regions of northeast Spain to account for variability linked to landscape, cultural practices and agroclimatic conditions: Bujaraloz, Almacelles and La Seu. In Bujaraloz and Almacelles, applications of herbicides and the use of genetically modified (GM) maize expressing the insecticidal toxin Cry1Ab from *Bacillus thuringiensis* (Bt) (MON 810), are common practices. Ten conventionally managed maize fields (5 in 2011 and 5 in 2012) were sampled in each study region. 3 neighbouring habitats were also sampled when present. Each field and its neighbouring habitats were sampled three times: in July (V10-V14 vegetative stage), August (pollen-shed) and September (ripening of the grain). Carabids were pitfall-trapped using standard traps for a week. They were identified according to a well-established procedure. Species richness and Shannon's diversity index were calculated for each plot. Similarity of carabid assemblages between habitats was calculated using the estimated abundance-based Chao-Jaccard similarity index. Linear relationships for abundance between carabids species and groups compared with community measures were tested by Pearson's correlation coefficient. Frequency and dominance were calculated. For each habitat and sampling site, mean values of community measures and mean abundance of carabid species were calculated. Historic data from 2005 to 2012 were used to determine the dominant species of the carabid community. Prospective power analyses were carried out to determine sample sizes (number of paired sites of GM *versus* non GM crop) needed to detect a change in carabid populations, using a two tailed *t*-test. ***Results:*** 9193 carabids of 42 species were identified, aggregated into trophic groups and used for calculating community measures. The best indicator was *Pseudoophonus rufipes*, satisfying criteria of abundance, relevance, sensitivity, ease of sampling and sufficient statistical power. The best sampling location was the field margin where carabids were exposed to GM maize and are abundant enough to require smaller sample sizes to detect population changes. In addition, carabid abundance was shown to be the highest around pollen-shed.	The authors concluded that: "*the present study contributes toward the design of a post marketing monitoring plan for detecting impacts of GM maize and, additionally, it provides baseline data on carabids that are valuable for monitoring the effects of natural or anthropogenic changes on maize ecosystems*."	Environmental protection	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
Peterson *et al.* (2016) Spiders from multiple functional guilds are exposed to Bt-endotoxins in transgenic corn fields via prey and pollen consumption	***Objective:*** To evaluate the potential risk associated with the uptake by spiders of *Bacillus thuringiensis* (Bt)-proteins from genetically modified (GM) maize lines, and to identify the exposure pathways and the fate of the proteins in the field. ***Experimental:*** Spiders and their prey were collected weekly from maize fields located in Kentuchy, USA, not treated with insectides. The maize was modified to express the following proteins: Cry1Ab (MON 810), Cry3Bb1 (MON 863) or both (MON 810 x MON 863). A non-transgenic near isoline (4847) was also included. Spiders and prey were screened for Cry1Ab and Cry3Bb1 content using qualitative enzyme-linked immunosorbent assay (ELISA). Laboratory feeding trials were conducted in parallel to examine protein movement through multiple trophic levels. Maize leaf tissue and pollen collected at the VT/R1 stage (anthesis) from the 4 varieties was fed *ad libitum* for 1 h to two prey species: 'pinhead' crickets *Acheta domesticus* and springtails *Sinella curviseta.* One part of the insects was screened by ELISA and the remaining part was fed to spider predators for 1 h: *A. domesticus* were given to *Pardosa sp.* and *S. curviseta* to *Tennesseellum formica*. Afterwards, spiders were screened by ELISA. Additionally, direct consumption for 1 h of GM pollen by *Pardosa* sp., *T. formica* and *Cyclosa turbinata* (Araneidae, collected from non-transgenic corn fields) aimed to determine the direct transfer of the proteins. Exposed spiders were screened by ELISA. ***Results***: 1108 spiders belonging to 29 genera and 12 families were collected with the most common taxa belonging to the wandering sheet-tangle weavers, ground runners and orb-weavers. Spiders from those three functional guilds tested positive for Cry1Ab and Cry3Bb1 proteins, with the highest per cent positive (8.0 and 8.3%) during and after anthesis. Laboratory feeding trials showed that Bt proteins were detectable in the *Pardosa sp*. immature cricket-Bt maize pathway, but not in the *T. formica*-Collembola-Bt maize pathway. Collembola might be able to rapidly excrete the proteins and these could accumulate differently according to the species of spiders. Additionally, direct consumption of GM maize pollen by *Pardosa sp*., *T. formica*, and *C. turbinata* resulted in transfer of both proteins, suggesting that pollen also represents an exposure pathway.	The authors concluded that: "*Bt-endotoxins are taken up by diverse members of a spider community via pollen and prey consumption and should be factored into future risk assessment of Araneae in North America."* [2]	Environmental protection	Transfer of Bt-endotoxins (Cry proteins) through multiple trophic levels
			Observed parameter	**Feedback on initial environmental risk assessment**
			NTO	There are no changes to the conclusions of the safety of the initial risk assessment.

[2] A key principle of ERA is risk based testing (CLI, 2016; Wolt *et al.*, 2010), in which testing is limited to those scenarios under which there is plausible scientific rationale for an adverse environmental effect. The large body of literature in addition to the experience of safe cultivation of GM crops over the last 20 years supports a tiered based ERA for GM crops. The advantages of the tiered based ERA include repeatable, affordable, and commonly available bioassays using surrogate species that are predictive of risk to non-target organisms and related to clear protection goals. A biologically relevant effect observed at a lower tier of testing provides data to inform a hypothesis driven study at the next tier that includes additional variability found in natural systems. Based on experience, this tiered system has been accurately predictive of safety with currently available GM crops and is robust enough to be predictive of risk for novel products in the future.

Potential exposure to the Cry1Ab and Cry3Bb1 proteins for spiders could occur through direct feeding on plant tissue or indirectly through feeding on prey that contain the protein. Though a potential trophic transfer of these proteins exists, the initial expectation is that Cry1Ab and Cry3Bb1 activity is limited to within the Order Lepidoptera and Coleoptera respectively. Implications of the trophic transfer of these proteins for spiders can be assessed by considering the specificity of the Cry1Ab and Cry3Bb1 proteins and the non-exhaustive list of other invertebrates that have been studied without reports of direct effects of Bt proteins through the consumption of prey (i.e., secondary exposure), includes: ground beetles (Coleoptera: Carabidae) (Harwood *et al.*, 2006; Meissle *et al.*, 2005), lady beetles (Coleoptera: Coccinellidae) (Alvarez-Alfageme *et al.*, 2011; Li and Romeis, 2010), spiders (Araneae) (Meissle and Romeis, 2009), predatory mites (Acari: Phytoseiidae) (Obrist *et al.*, 2006), parasitic wasps (Hymenoptera) (Davidson *et al.*, 2006), brown lacewings (Neuroptera: Hemerobiidae) (Davidson *et al.*, 2006), and green lacewings (Neuroptera: Chrysopidae) (Rodrigo-Simon *et al.*, 2006).

In summary, though Peterson report interesting and important results, the current body of knowledge does not indicate that Cry1Ab and Cry3bb1 expressed in Bt maize negatively impacts predation ecosystem services provided by spiders. Evidence based on our understanding of the restricted activity spectra of Bt proteins and results from tri-trophic studies supports the conclusion that Bt maize is compatible with spider and other generalist predators with no expected unacceptable negative ecological impact.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Shu *et al.* (2017) Effects of Cry1Ab Bt maize straw return on bacterial community of earthworm *Eisenia fetida*	***Objective:*** 1) To assess the effects of genetically modified (GM) insect protected *Bacillus thuringiensis* (Bt) maize straw return on the bacterial community of earthworms. 2) To determine if the effects were direct (Cry1Ab protein) or unintended (soil nutrient status and bacterial community) results of Bt maize straw return. ***Experimental Design:*** Soil was collected from a conventional maize field (non-GM) in Guangzhou, China. In greenhouse, two GM Bt maize hybrids, event Bt11 and event MON 810, both expressing Cry1Ab protein, and a non-Bt parent line 5422 (no further details) were cultivated. After pollen was shed, the straw (including the leaves and the stalks) of the maize plants was collected, freeze-dried and ground. The effects of the Bt and non-Bt maize on bacterial community and soil nutrients were tested in a 90 d microcosm study. Twenty-four replicates per maize hybrid treatment, with 4 replicates per each maize variety treatment were sampled every 15 days. The organic matter, total N, total P, total K, available N, available P and available K in soil was determined. Gut contents of *Eisenia fetida* were collected and the Cry1Ab protein concentrations were measured with ELISA. DNA of the gut content of *E. fetida* was extracted and used for PCR amplification. Bacterial community in soil, earthworm gut contents and casts were investigated through T-RFLP analysis. Based on these analysis, partial 16S rRNA genes were amplified from the extracted genomic DNA by PCR and further used for PCR-DGGE. ***Results:*** Bt maize straw return had significant effects on soil nutrients, especially regarding available N. The differences were shown in soil bacterial community between Bt and non-Bt maize treatments on the 75th and 90th day, which was closely correlated with soil	The authors concluded that: *"These results suggested that Bt maize straw return caused changes in the living environment of the earthworm E. fetida, mainly including soil nutrient levels and bacterial community, which might ultimately affect the growth, metabolism and functions of earthworms. (…) Bt maize straw return caused changes in the bacterial community in E. fetida casts, which was possibly caused by the direct (Cry1Ab protein) and non-expected effects (N levels) of Bt maize straw.*[3]	Environmental protection	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.

	available N, P and K rather than Cry1Ab protein. There was no difference in the bacterial community of earthworm gut contents between Bt and non-Bt maize treatments. The differences in the bacterial community of earthworm casts were found among the 3 maize varieties treatments, which were closely correlated with Cry1Ab protein and N levels. The differentiated bacterial species in earthworm casts mainly belonged to *Proteobacteria*, including *Brevundimonas*, *Caulobacter*, *Pseudomonas*, *Stenotrophomonas*, *Methylobacterium*, *Asticcacaulis* and *Achromobacter*, which were associated with the mineralization, metabolic process and degradation of plants residues.			

[3] We conclude that the results from this study do not impact the conclusion of the environmental risk assessment conducted on MON 810 and do not trigger adverse effect reporting for the following reasons:

(1) The authors state in their conclusion that bacterial community changes "possibly" could be caused by Cry1Ab protein. In the article, they state several times that the largest contributing factor on the bacterial community was the additional nutrients (OM, total N, P and K) from the addition of the maize straw.
(2) The study design is flawed because it did not include maize reference varieties for comparison. Since the differences observed were not consistent between the two Cry1Ab producing varieties, it is not possible to determine whether the differences observed were attributed to varietal differences unrelated to the Cry1Ab protein or event.
(3) The methodology used to determine the bacterial community is older technology compared to the NextGen sequencing used as standard in the industry. Their practice of selecting the brightest bands for sequencing may be hard to reproduce.
(4) The changes in the bacterial communities were not consistent between time points or when compared to different indices they used for evaluation.
(5) Several times the authors state that there were no statistically significant differences in the bacterial compositions of the gut contents associated with Bt and non-Bt maize straw across the whole experiment time. Therefore, there are no safety concerns on the microbial aspect.
(6) This article did not show adverse impacts to earthworms following exposure to maize straw containing Cry1Ab. Additionally there is an overwhelming weight of evidence in the published literature demonstrating a lack of hazard to beneficial non-target arthropods (including earthworms) following exposure at both field level, and several orders of magnitude above field level exposure. Therefore, this article should not pose any concern for the ERA of MON 810

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Mashiane *et al.* (2017) Metagenomic analyses of bacterial endophytes associated with the phyllosphere of a Bt maize cultivar and its isogenic parental line from South Africa	***Objective:*** To examine if genetic modification of maize with *Bacillus thuringiensis* (Bt) CRY proteins (MON 810) may predispose shifts in the bacterial endophytes community associated with maize shoots. ***Experimental Design:*** The diversity of bacterial endophytes associated with a Bt maize genotype and its isogenic non-transgenic parental line were investigated in South Africa. Plant parts, including healthy leaves, stem, tassels and seeds were collected from ten samples of each maize cultivar (MON 810 and non-Bt isoline) at two developmental stages: pre-flowering (50 d after emergence) and post flowering (90 d after emergence). PCR-DGGE and high throughput sequencing on the Illumina MiSeq sequencer were used to characterize bacterial 16S rRNA gene diversity in leaves, stems, seeds and tassels and for sequence processing, operational taxonomic units (OTUs) clustering and diversity analyses. ***Results:*** PCR-DGGE profile revealed similarity as well as differences between bacterial communities of shoots in both cultivars and at both developmental stages. A total of 1771 OTUs were obtained from the MiSeq and assigned into 14 phyla, 27 classes, 58 orders, 116 families and 247 genera. Differences in diversity measures of OTUs between the phyllospheres of both genotypes were not significant at all developmental stages. In all cultivars, OTU diversity reduced with plant development. OTUs belonging to the phyla *Proteobacteria* were dominant in all maize phyllospheres. The class *Gammaproteobacteria* was dominant in Bt maize while, *Alphaproteobacteria* and *Actinobacteria* were dominant in non-Bt maize phyllospheres. Differences in the abundance of some genera, including *Acidovorax*, *Burkerholderia*, *Brachybacterium*, *Enterobacter* and *Rhodococcus*, whose species are known beneficial endophytes were observed between cultivars. Hierarchical cluster analysis further suggests that the bacterial endophyte communities of both maize genotypes associate differently (are dissimilar).	The authors concluded that: *"bacterial endophytes community differed more across developmental stages than between maize genotypes. These differences were more pronounced between the diversity and abundance of particular species, rather than in the species richness of the maize bacterial community."*	Environmental protection	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.

Area of the environmental risk assessment: Environmental Safety – Agronomy

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Yinghua *et al.* (2017) Responses of the cutworm *Spodoptera litura (Lepidoptera: Noctuidae)* to two Bt corn hybrids expressing Cry1Ab	***Objective***: To examine the response of the secondary lepidopteran pest *Spodoptera litura* to two transgenic *Bacillus thuringiensis* (Bt) maize hybrids, 5422Bt1 (event Bt11) and 5422CBCL (event MON 810), expressing the insecticidal Cry1Ab protein. ***Experimental Design***: Two transgenic maize hybrids, 5422Bt1 and 5422CBCL, and their near-isoline cultivar (5422) were cultivated in a greenhouse. During the maturation stage, fresh maize leaves and kernels were used to feed the insects. *S. litura* larvae were reared for 7 days on the artificial diet under controlled conditions until the experiments were carried out. For the insect bioassay, 30 larvae, reaching the 3rd instar larval stage, were transferred to plastic bag and reared individually for 7 days with artificial diet, containing a final concentration of either 1, 2, 12, 24, 150 or 250 μ gg−1 of Cry1Ab protein. Each bioassay was performed in triplicate. Larvae were individually weighed daily and the larval survival rate was calculated. In the case of the insect bioassay with different tissues of Bt maize, *S. litura* 3rd instar larvae were reared individually in plastic boxes for 7 days. Diets of fresh leaves or kernels were introduced daily into the plastic boxes, with 30 replicates per diet for each maize variety. The survival rate and food utilization were recorded. The insect bioassay by bagging with Bt maize was carried in greenhouse. The 7th and 8th leaf of each plant were enclosed in a mesh bag and the 3rd instar larvae were placed in bags (5 larvae per bag); 20 bags were prepared for each maize variety. Every 3 days, the survival rate and the average weight of all larvae were recorded. When larvae developed into pre-pupae, the survival rate as well as the relative growth rate (RGR) were determined. The amount of Cry1Ab was determined in the protein extracts derived from diets, the gut contents, feces, midgut of larvae by performing the ELISA assay, while it was assessed in the protein extracts of maize tissues (leaves or kernel) by immunoblotting. RNA was extracted from midgut samples and used for quantitative PCR to determine the expression of S. *litura cadherin-like receptor* and *alkaline*	The authors concluded that: *"S. litura showed a significantly lower susceptibility to 5422CBCL in comparison with 5422Bt1."*	Environmental protection	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.

	phosphatase genes. The enzyme activity of protective enzymes (catalase and superoxide dismutase), detoxifying enzymes (true choline esterase and glutathione-S-transferase) and alkaline phosphatase was studied using the same samples as the ones used for the ELISA assay. ***Results***: Larvae displayed a similar performance when fed kernels, but not leaves of 5422Bt1, 5422CBCL and their near-isogenic non-Bt maize. Significantly higher Cry1Ab amounts were detected in larvae fed leaves than kernels of both Bt hybrids, with different molecular weights of protein band in plants (72 and 90 kDa for 5422Bt1 and 5422CBCL, respectively), gut contents (65 kDa) and feces (50 kDa). This indicated that larvae had lower ingestion, higher degradation and excretion of Cry1Ab when fed kernels not leaves of both Bt hybrids. Significantly higher levels of cadherin-like receptors and alkaline phosphatase transcripts were detected in larvae fed leaves than kernels of two Bt hybrids. Catalase, superoxide dismutase and glutathione-S-transferase activities in larvae fed 5422Bt1 leaves were significantly higher than that of 5422 treatments. Therefore, *S. litura* had low susceptibility to 5422Bt1 and 5422CBCL when larvae were fed kernels not leaves of Bt maize.			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Tefera *et al.* (2016) Resistance of Bt-maize (MON 810) against the stem borers *Busseola fusca* (Fuller) and *Chilo partellus* (Swinhoe) and its yield performance in Kenya	***Objective:*** To assess the performance of genetically modified insect resistant MON 810 maize against the stem borer *Busseola fusca* (Fuller) and against the spotted stem borer *Chilo partellus* (Swinhoe) in a biosafety greenhouse (BGH) and confined field trial (CFT) in Kenya. ***Experimental Design:*** The study comprised MON 810, non-Bt near isogenic and non-Bt commercial hybrids. First instar larvae were obtained from the Katumani Stem Borer Mass Rearing Laboratory. The CFT trial was repeated for three consecutive seasons (January 2013, August 2013 and May 2014) in Kiboko, Kenya. Plants were artificially infested twice with ten *C. partellus* neonates per plant, 14 and 24 days after planting. Foliar damage for stem borer was assessed using a 1-9 scale; where 1 = no visible damage and 9 = completely damaged. Foliar damage was assessed 24 and 39 days after planting (V2 and V4 stage, respectively). At harvest, the number of stem borer exit holes per plant was counted. Each tunnel length per stem was measured after splitting the stems of the infested plants and the cumulative tunnel length was expressed as percentage of total stem height. During the BGH, plants were sown in the biosafety level-two greenhouse in Nairobi. Each plant was artificially infested twice with ten *B. fusca* neonates per plant, 21 and 31days after planting. Leaf damage was assessed twice, 31 and 41 days after planting using the standard 1-9 scale. The insect succumbed to early mortality before making damage to stems and only leaf damage was subject to analysis. ***Results:*** In the CFT, the Bt hybrids significantly	The authors concluded that: "*the study demonstrated that MON 810 was effective in controlling B. fusca and C. partellus. It has great potential to reduce the risk of maize grain losses in Africa due to stem borers, and will enable the smallholder farmers to produce high-quality grain with increased yield, reduced insecticide inputs and improved food security*"	Environmental protection	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.

	differed from their non-Bt isolines for leaf damage, number of exit holes, percent tunnel length and grain yield. Bt hybrids had the least number of exit holes and percent tunnel length in all the seasons as compared to the non-Bt hybrids and commercial checks. When averaged over three seasons, Bt hybrids gave the highest grain yield (9.7 $t.ha^{-1}$), followed by non-Bt hybrids (6.9 $t.ha^{-1}$) and commercial checks (6 $t.ha^{-1}$). In BGH trials, Bt hybrids consistency suffered less leaf damage than their non-Bt isolines.			

Area of the environmental risk assessment: Environmental Safety – Protein/DNA fate in soil or in stream water

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Stenekamp *et al.* (2016) Effect of genetically modified Bt maize in an artificial diet on the survival of *Cydia pomonella* (Lepidoptera: Tortricidae)	***Objective:*** To evaluate the effect of diet containing genetically modified (GM) MON 810 maize expressing the *Bacillus thuringiensis* insecticidal protein Cry1Ab on *Cydia pomonella* (codling moth) larval mortality, development and dispersal. ***Experimental Design:*** The moth colony originated from larvae collected in a commercial apple orchard in Elgin, South Africa. The moths reared from these larvae were used to initiate a laboratory colony; wild larvae from apple orchards were introduced into the colony every second year to minimize inbreeding. MON 810 maize meal was a mixture of two cultivars (DKC 8012B and DKC 7815B). Bt-free, organic maize meal was used as negative control and in mixtures with Bt maize to obtain a range of 5 concentrations of Bt maize meal. The solidified diet was dispensed into sterile 25 mL plastic cups, before putting one first instar larva on the diet per container (50 larvae/group). Cry1Ab protein concentration in the diets was determined by ELISA. The insect bioassay was repeated twice on different dates. On Day 44, the following responses were recorded: 1) larval mortality, 2) larvae not reaching adulthood (delayed development), 3) larvae leaving the diet, and 4) total of the previous three responses. The concentrations causing 50 and 95% response for each case and the 95% fiducial limits were determined by probit analysis using PoloPC software. ***Results:*** The Bt maize meal diet adversely impacted the number of moths produced. Delayed larval development appeared to be the most important parameter affected based on the slopes of the probit regression analysis. The slope for delayed larval development was higher (although not significant at P = 0.05) than the slopes for the other 2 responses. In addition, the probit regression lines for delayed larval development more closely resembled the lines for total response than did those for larval mortality and larvae leaving the diet. The lower slope of the regression line for larvae leaving the diet, suggests that an increase in the Bt concentration in the diet resulted in a limited increase in the response.	The authors concluded that: "*since optimal rearing of codling moth is not feasible using meal from genetically modified maize with insecticidal properties, another nutritious meal lacking an insecticidal component must be substituted in the artificial diet.*"	Environmental protection	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
Griffiths *et al.* (2017) Occurrence, leaching, and degradation of Cry1Ab protein from transgenic maize detritus in agricultural streams	***Objective:*** To examine the occurrence, leaching and degradation of Cry1Ab protein from *Bacillu thuringiensis* (Bt) insect-protected MON 810 maize in streams. ***Experimental Design:*** First, it was investigated whether dissolved Cry1Ab protein concentrations in streams and tile drains (subsurface drainage pipes that underlay agricultural fields) varied spatially (across an agriculturally dominated region) and temporally (among 6 time periods encompassing 3 seasonally important time periods, i.e. the crop growing season, crop harvest, and snowmelt/spring flooding) in 11 low-order streams in north western Indiana, USA. In each stream, water samples were collected from 3 locations that spanned a distance of ca. 200 m. Samples were analysed for water velocity and discharge, Cry1Ab protein and nutrients concentrations. Second, recirculating, artificial streams were used to quantify the rate at which Cry1Ab protein leaches from maize leaves into the water column. The experiment took place over a 70 d period in order to quantify Cry1Ab leaching throughout the entire decomposition process. Finally, 3 laboratory microcosm experiments were conducted to determine the influence of microorganisms, light, and water collected from 3 different agricultural headwater streams (from Juday Creek and from 3 typical agricultural streams in Indiana) on Cry1Ab degradation in the water column. The concentration of Cry1Ab protein in maize leaf and husk detritus as well as in stream water was determined. Statistical analyses were performed using SYSTAT v.12. ***Results:*** The concentration of Cry1Ab protein in both stream and tile drain water was generally low and ranged from the detection limit of 3 ng/L up to 60 ng/L. There was no difference in Cry1Ab levels between stream water and tile drain water. There were also no differences in Cry1Ab concentrations in stream or tile drain water among seasons or between Bt maize and other fields. Maize detritus was found in the active channels of 82% of streams sampled at the end of harvest, in 73% at the start of snowmelt/spring floods, and in 18% at the end of snowmelt/spring floods. The percentage of streams containing	The authors concluded that: *"The common detection of Cry1Ab protein in streams sampled across an agricultural landscape, combined with laboratory studies showing rapid leaching and degradation, suggests that Cry1Ab may be pseudo-persistent at the watershed scale due to the multiple input pathways from the surrounding terrestrial environment."*	Environmental protection	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			Protein fate	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
	Cry1Ab-positive maize detritus varied over time. The mean concentration of Cry1Ab protein in maize detritus was higher directly after crop harvest. In the artificial stream experiment, the concentration of Cry1Ab in submerged Bt maize leaves decreased over time, resulting in the loss of 99% of Cry1Ab from Bt maize leaves on Day 70. Cry1Ab degraded over the experiment, more quickly in the presence of water-column microorganisms compared to the treatments without microorganisms. Light did not influence autotrophic uptake of the protein and there was also no influence of stream water source on Cry1Ab degradation.			

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Buuk *et al.* (2016) Is there any change in susceptibility of European corn borer (*Ostrinia nubilalis*) to Cry1Ab protein?	***Objective:*** To detect, in a timely manner, shifts relative to baseline susceptibility of the maize pest *Ostinia nubilalis* (European corn borer, ECB) to the *Bacillus thuringiensis* (Bt) protein Cry1Ab expressed by insect resistant MON 810 maize that could result in inadequate protection against this target species. This program was intended to enable early detection of potential resistance development and allow implementation of additional risk mitigation measures, if necessary. ***Experimental Design:*** From 2005 to 2014, 140 samples of ECB were collected from 15 areas in the most important European maize growing regions where the adoption of MON 810 was expected to be greater than 20% (Czech Republic, France, Germany, Italy, Hungary, Slovakia, Poland, Portugal, Romania and Spain). For each region, sampling sites separated by at least 50 km. In naturally infested fields, ECB were mainly collected as larvae by dissecting maize stalks but also as adults by using light traps or collecting egg masses on leaves. Field-collected larvae were cultured in the laboratory and exposed to increasing concentrations of Cry1Ab protein. A laboratory reference non-diapausing ECB strain (G04) from Niedernberg, Germany, highly susceptible to Cry1Ab, was tested as a positive control. Two batches of Cry1Ab protein were used: batch 1 (2 mg Cry1Ab/ml) from 2005 to 2011 and batch 2 (1.64 mg Cry1Ab/ml) since 2012. For batch 1, eight concentrations were tested (0.5-80 ng Cry1Ab/cm²), for batch 2 nine concentrations (0.2-28.22 ng Cry1Ab/cm²) and a control (bicarbonate buffer) was also included in the study. The bioassays were performed in 128 well trays. In each cell, 1 ml of artificial diet was dispensed to which 100 µl of protein solution was applied to the surface. Egg masses from	The authors concluded that: *"The results indicate that the variation in ECB populations in their susceptibility to Cry1Ab reflects their susceptibility to this Bt protein. There was no evidence of a decrease in the susceptibility to Cry1Ab in populations over the period 2005-2014."*	Environmental protection	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			IRM	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
	each location sampled were incubated and neonate larvae, within 12 h of hatching, were transferred to the cells (one per cell), which were covered with a lid. All assays were conducted at 25°C, 70% RH and a photoperiod of 0:24h (L:D). After 7 days of exposure, larval mortality and developmental stage were recorded. Statistical analyses were conducted with SYSTAT 10.0, except for the concentration-response analysis for which PoloPlus 1.0 was used. ***Results:*** Susceptibility of ECB collected in different geographic regions and exposed to purified Cry1Ab toxin differed only slightly. Variation in Cry1Ab susceptibility moulting inhibition concentrations (MIC50) of field samples was up to 13.1-fold. A smaller variability was found for ECB pooled according to geographic and climatic conditions (up to 6.6-fold). It was planned that all *O. nubilalis* larvae from field collections that survived the bioassay at the highest dose should be transferred to plastic boxes in groups of approximately 50 larvae, provided with newly detached MON 810 maize leaves, and fed *ad libitum* to record any survivors. As for the seasons reported, no surviving larvae were found after 10 days and thus confirmatory experiments were not conducted.			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Yao *et al.* (2017) Comparisons of transcriptional profiles of gut genes between Cry1Ab-resistant and susceptible strains of *Ostrinia nubilalis* revealed genes possibly related to the adaptation of resistant larvae to transgenic Cry1Ab corn	***Objective:*** Compare gut gene transcriptional responses using microarrays, between Cry1Ab-resistant and susceptible strains of *Ostrinia nubilalis* when larvae were fed transgenic Cry1Ab maize leaves. ***Experimental Design:*** A Bt-resistant strain (R-strain, Sky) and its susceptible isoline strain (S-strain, Meads) of *O. nubilalis* were provided by Blair Siegfried at the University of Lincoln-Nebraska, in which R-strain had been selected from S-strain by feeding them an artificial diet containing Cry1Ab protoxin (0.3 µg/mL) for 137 generations. The transgenic Cry1Ab maize (Pioneer 34P88 with MON 810) and its isoline (Pioneer 34P86) were planted in the greenhouse and grown until the six-leaf stage at Kansas State University, USA. To assess the resistance level, LC_{50} values of Cry1Ab protoxin were determined in both R- and S-strains. Each bioassay was conducted with more than 360 third-instar larvae that were fed artificial diet, after 24 h starvation, containing various concentrations of Cry1Ab protoxin (0 – 234 µg/mL, each concentration replicated three times, with ± 16 larvae per replicate). Mortality was recorded after seven days. To evaluate the resistance level of R-strain to transgenic maize expressing Cry1Ab toxin, LT_{50} values were determined by feeding early third-instar larvae of both R- and S-strains, after 24 h starvation, with transgenic maize leaves at 26 °C and 16L:8D photoperiod (each strain replicated three times, with ± 50 larvae per replicate). Fresh leaves were replaced daily and the survival was recorded daily for one week. For microarray analysis,	The authors concluded that: *"The study suggests enhanced adaptation of Cry1Ab-resistant larvae on transgenic Cry1Ab maize as revealed by lower number and lower ratios of differentially expressed genes in R-strain than in S-strain of O. nubilalis. Further studies will be necessary to validate or clarify functional roles of differentially expressed genes involved in Cry1Ab toxicity and resistance in O. nubilalis."*	Environmental protection	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			Gene expression	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
	forty early third-instar larvae of each strain (S- or R-) were starved for 24 h and then individually transferred to 20 rearing cups containing either transgenic maize leaves or non-transgenic isoline maize leaves. After feeding for 6 h, guts of these larvae were dissected and five guts were pooled as one sample. Total four replicates of each treatment were obtained for total RNA extraction. Total RNA for each sample was extracted after which the quantity and quality of the RNA were determined. Two Agilent custom microarray slides (each containing 8 microarrays) for a total of 16 microarrays were used to compare gut transcriptional responses in the larvae of R- and S-strains after fed transgenic and non-transgenic maize leaves. Each microarray was tested with 12,972 probes representing 2895 unique larval gut genes of *O. nubilalis*. The expression of each gene for each strain was expressed as the ratio between the transgenic and the non-transgenic maize leave-fed groups. These ratios were then tested statistically at a p value <0.05 (one-way ANOVA) with expression ratio ≥ 2.0 as significant. Gene ontologies for those significantly differential expressed genes were further analyzed by using Blast2go at level 2. The expression ratio of each candidate gene with significantly differential expressions was validated further by reverse transcription quantitative PCR (RT-qPCR). The specific primers for 20 selected genes and the reference gene ribosome protein L18 (*RPL18*) were designed using Beacon 7 Designer™ (Primer Biosoft, Palo Alto, CA, USA). RT-qPCR was performed with 2-step amplification protocol (40			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
	cycles of 95 °C for 30 s, 56 °C for 30 s). Expression levels of the selected gut genes were normalized to the reference gene *RPL18*. Using the normalized gene expression, the expression ratio was calculated between the transgenic and the non-transgenic maize leave-fed groups. ***Results:*** Respectively 398 and 264 gut genes were differentially expressed in S- and R-strain after being fed transgenic maize leaves. The expression ratios of down-regulated genes were much higher in S-strain than in R-strain. Respectively, 17 and 9 gut genes were either significantly up- or down-regulated in the S and R-strain, including serine proteases and aminopeptidases. These genes may be associated with Cry1Ab toxicity by degradation, binding and cellular defense.			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Castañera *et al.* (2016) Sixteen years of Bt maize in the EU hotspot: Why has resistance not evolved?	***Objective:*** 1) To investigate whether *Sesamia nonagroides* developed resistance to maize hybrids expressing the *Bacillus thuringiensis* Cry1Ab toxin (Bt maize) in the northeast region of Spain after 16 years of use, and 2) to determine possible reasons of the resistance management success using evolutionary models to consider factors expected to accelerate or delay resistance in the F1 generation. ***Experimental Design:*** Cry1Ab protein (81% purity) isolated from a bacterial culture was used for all the bioassays. Last instar pre-diapausing larvae of *S. nonagrioides* from the final generation of the season were collected from the maize fields of northeast, central and southwest regions of Spain (2–4 sampling sites from each geographical area, between 100–200 larvae from each site) and the susceptibility to Cry1Ab was tested on the F1 progeny. In addition, a laboratory colony from before Bt maize cultivation was used as control. Bioassays were performed in plastic trays containing 128 wells, where about 0.5 ml of rearing diet were placed and let solidify. Serial dilutions (7 to 10 different concentrations) of Cry1Ab toxin were tested. Three replicates of 16-32 larvae at each concentration and 32-64 larvae for the control were performed. Larval mortality was recorded after 7 days. Lethal concentrations (LC50 and LC90) values were estimated by probit analysis using POLO-PC software. Resistance Ratios (RR) were calculated with respect to the LC50 or MIC50 (molt inhibition concentration) values obtained for the susceptible laboratory strain each year to normalize the data. The resistance evolution model was used to determine the causes of lack of resistance to Cry1Ab. It is a single-locus, two-allele, deterministic, three-	The authors concluded that: *"Low initial adoption rates and the EU policy decision to replace Event 176 with MON 810 Bt maize were key to delaying resistance evolution. Model results suggest that if refuge compliance continues at the present 90%, Bt maize might be used sustainably in northeast Spain for at least 20 more years before resistance might occur. However, obtaining good estimates of the present R allele frequency and level of local assortative mating are crucial to reduce uncertainty about the future success of resistance management."*	Environmental protection	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			IRM	There are no changes to the conclusions of the safety of the initial risk assessment.

	patch, discrete generation model with stochastic weather that determines the diapause rate and survival of the third generation. The three habitat patches were MON810 Bt maize, Event 176 Bt maize and non-Bt maize. Parameters, such as population density and allele frequencies through adult emergence, pre-mating movement, mating, post-mating movement, reproduction, selection, density-dependent larval survival, and adult emergence, were evaluated. The best parameter values (BPV) were used to simulate the most likely evolutionary trajectory in northeast Spain, and a range of parameters were used to investigate possible alternative trajectories. The impact of refuges and cross-resistance on resistance timing was also assessed. ***Results:*** No shift in susceptibility to Cry1Ab in northeast Spanish field populations of *S. nonagroides* was observed after 16 years of cultivation of Bt maize. The factors that could delay resistance evolution dominated those that could accelerate. The low initial adoption of Bt maize in northeast Spain and the decision to replace Event 176 with Mon 810 maize were key to delaying resistance evolution. Model results suggest that if two conditions hold, i.e. an initial R allele frequency not higher than 0.0015 and a mating that has been and continues to be locally random, the Cry1Ab Bt maize is likely to be used for at least 20 more years in northeast Spain.			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Dos Santos *et al.* (2016) Development of *Helicoverpa* spp. in Bt maize expressing different proteins	***Objective:*** To evaluate the biological response of *Helicoverpa zea* and *Helicoverpa armigera* (Lepidoptera: Noctuidae) in genetically modified (GM) insect resistant maize hybrids expressing different *Bacillus thuringiensis* (Bt) proteins. ***Experimental Design:*** The experiment was conducted in Sete Lagoas, Brazil in 2013/2014. The Cry1F (30F35HX), Cry1Ab (30F35YG – MON 810), Cry1A.105+Cry2Ab2 (DKB390 VT PRO – MON 89034), Vip3A (Impacto Viptera) proteins and their isogenic conventional counterparts (30F35, Impacto and DKB 390) were evaluated. The bioassay was conducted in the laboratory with F1 and F2 generation of *H. zea* and F2 and F3 generation of *H. armigera*. Larvae were fed on spikelets of Bt and conventional maize, which were changed every two days. The variables evaluated were: survival 48 h after hatching, larval survival, larval and pupal weight, larval development period and pre-imaginal period. ***Results:*** There was a significant interaction between Bt maize events and *Helicoverpa* species for all evaluated biological variables. The survival percentage of both *Helicoverpa* species was similar between Cry1Ab and conventional DKB 390 maize. However *H. armigera* larvae showed higher survival 48 h after hatching on maize expressing Cry1F and Vip3A proteins, when compared to *H. zea*. A similar observation was made with the isogenic conventional 30F35 and Impacto. Larval survival was close to zero percent for all evaluated Bt hybrids. Lethal period was greater for *H. armigera* than for *H. zea*, but in maize expressing Cry1Ab protein, it was four times lower. There was significant interaction in relation to the larval development period, the pre-imaginal period and the larval and pupal weight, where only the larvae fed on conventional maize completed the developmental period. Highest larval weight values were found for *H. zea* on Impacto, followed by 30F35, which were respectively about 40 and 22% higher than the *H. armigera* weights in the same events.	The authors concluded that: *"in isogenic conventional counterparts, the adaptation index of H. armigera was higher than that of H. zea, which indicates greater ease of adaptation to the environment of that species"*.	Environmental protection	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			IRM	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
Erasmus *et al.* (2016) Movement and survival of *Busseola fusca* (Lepidoptera: Noctuidae) larvae within maize plantings with different ratios of non-Bt and Bt seed	***Objective:*** To determine to what extent migrating *Busseola fusca* larvae of different ages are effectively controlled by genetically modified (GM) *Bacillus thuringiensis* (Bt) insect resistant plants using the seed mixture strategy and if there is potential for using this strategy to manage resistance evolution. ***Experimental Design:*** The study included two Bt maize lines (Cry1Ab and Cry1A.105 + Cry2Ab2)[1] and their near-isogenic non-Bt isolines. The larvae used to inoculate plants in the laboratory and field trials were F1 generation of diapause larvae collected from maize in the Venters-drop area, South-Africa. This population was previously shown to survive on Bt maize. In the laboratory, two experiments were conducted with maize whorl and ear tissue. The migration of larvae of different ages (3, 9 and 21 days old) was simulated between Bt and non-Bt maize. The field trial was conducted over two growing seasons (2011/2012 and 2012/2013) at Potchefstroom. Five treatments with different ratios of Bt and non-Bt seeds (5, 10, 15 and 20% non-Bt) were used. At planting, the position of the various seeds was recorded within each plot. A single non-Bt plant was inoculated with 50 neonates *B. fusca* larvae 4 weeks after seedling emergence. The levels of natural infestation in both experiments were determined 6 weeks after seedling emergence. Monitoring of stem borer damage commenced 3 days after inoculation and the incidence of damaged plants was recorded thereafter at weekly intervals for 9 weeks. ***Results:*** In the laboratory trials, larval survival after the initial 7 day feeding period was high (52-92%) in all movement scenarios. Many 21 day old larvae survived the 7 day period in nearly all treatment scenarios, although survival was significantly lower for larvae that were reared	The authors concluded that: "*larval movement continued for 5 weeks and resulted in a significant incidence of Bt and non-Bt damaged plants, indicating that the movement behaviour of B. fusca is of such a nature that seed mixtures as an IRM strategy may not be effective to delay resistance evolution*"	Environmental protection **Observed parameter** IRM	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] The publication does not give details on the events used.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
	on Cry1Ab Bt maize for the whole period or for those that were transferred to the stacked-trait after 21 days. Further, larval mass was very low after the 7 day feeding period in all treatments involving exposure to the stacked-trait maize tissue and none of the larvae survived longer than 9 days. In the field trials, natural infestation during both seasons was low, with 5 and 3% of plants showing symptoms of larval feeding damage 8 weeks after crop emergence. The mean percentage larvae recovered per plant in the reference plot decreased over 4 weeks to 26 to 18% respectively in the two seasons. The first prepupae were observed in the samples taken on day 35 (5 weeks) after inoculation and larval numbers were not recorded further. The mass of a 21 day old larva 7 days after being transferred from non-Bt to stacked-trait maize was approximately the same as that of a 14-16 day old larva that fed on non-Bt maize under field conditions, while the mass of a 21 day old larva transferred from Cry1Ab maize to stacked-trait maize was the same as that of a 14 day old larva on non-Bt maize. The overall mean incidence of damaged plants was in the same range across the two seasons. The incidence of damaged plants increased over the first 6 weeks after inoculation, after which it levelled off. There was a high rate of *B. fusca* movement in non-Bt plots until pupation commenced. In general, the incidence of damaged plants in the Cry1Ab seed mixture treatments was similar to that of the control treatment for the season 1, but the incidence of damage was lower than in the non-Bt control in season 2. The overall incidence of damaged plants per plot in the stacked-trait treatments was lower and tanged between 4.4 and 12.3% in the first season, and between 2.4 and 5.4% in the second season. In both seasons the stacked-trait plots with 5% non-Bt seed mixtures suffered the lowest incidence of damaged plants, which in most cases differed significantly from the other treatments with stacked-trait.			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Kotey *et al.* (2017) Monitoring resistance to Bt maize in field populations of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) from smallholder farms in the Eastern Cape Province of South Africa	***Objective:*** To evaluate the resistance of populations of stem borer (*Busseola fusca*) collected from different genetically modified (GM) insect resistant *Bacillus thuringiensis* (Bt) maize cultivating areas. ***Experimental Design:*** Third to fourth instar *B. fusca* larvae were collected from fields in 2 Bt maize cultivating areas and a non-Bt maize cultivating area of the Eastern Cape region, South Africa (2016). In the laboratory, rearing colonies and subsequent neonate larvae from each population were used to infest two varieties of Bt maize plants (DKC8012B, event MON 810, Cry1Ab protein; DKC8012BGEN, event MON 89034, Cry2Ab2 + Cry1A.105 proteins) and one variety of non-Bt maize (DKC8010, iso-hybrid of the two Bt maize varieties). Leaf sheaths of field collected maize plants (4 week old) of each of the maize varieties were fed to neonate larvae of each population. The number and mass of the surviving larvae were determined at 7, 10, 14, 17 and 21 days after inoculation. Data on field incidence, larval survival and mass of *B. fusca* were subjected to analysis of variance (ANOVA). ***Results:*** All larvae maintained on MON 89034 maize died within 7 days of infestation. Survival of all *B. fusca* populations maintained on MON 810 maize declined rapidly during the first 7 days and was significantly lower than larval survival on non-Bt maize. Similarly, the mass of surviving larvae of all populations on MON 810 from the first two weeks to the 21st day was significantly lower than the mass of larvae surviving on non-Bt maize.	The authors concluded that: *"These results indicate that field-collected populations screened in this study are still susceptible to Bt maize."*	Environmental protection	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			IRM	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
Niu *et al.* (2016) Performance of Cry1A.105-selected fall armyworm (Lepidoptera: Noctuidae) on transgenic maize plants containing single or pyramided Bt genes	***Objective:*** To evaluate the survival and plant injury of two strains of *Spodoptera frugiperda*, which are resistant to the transgenic *Bacillus thuringiensis* (Bt) Cry1A.105 protein, a Cry1A.105-susceptible strain and two F1 heterozygous populations, on eight commercial or experimental Bt maize hybrids/lines containing single or pyramided Bt genes. ***Experimental Design:*** Five *S. frugiperda* populations were used in the study: 2 Cry1A.105-resistant strains (FL32 and FL67), 1 Cry1A.105-susceptible strain (SS) and 2 F1 heterozygous populations (FL32-RS and FL67-RS). 12 maize products were used: 4 non-Bt maize, 5 single-Bt maize (1 experimental line expressing Cry1A.105 protein, 2 experimental lines expressing Cry2Ab2 protein, Pioneer (TC 1507 event, expressing Cry1F protein), DKC 69-70 (MON 810 event, expressing Cry1Ab protein) and 3 pyramided Bt maize hybrids/lines (DKC 64-04 (MON 89034 event, expressing Cry1A.105 and Cry2Ab2 proteins), DKC 62-08 (MON 89034 x TC1507 x MON 88017 x DAS-59112-7, expressing Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, Cry34Ab1 and Cry35Ab1 proteins) and N78N-3111 (Bt11 x MIR162 x MIR604, expressing Cry1Ab, Vip3A and Cry3A proteins)). Two independent leaf tissues bioassays were performed, using leaves from maize plants at V5-V8 stages. 2-3 pieces of leaves (about 3-4 cm in length) were placed in each well of 32-well trays. 4 neonates of each population were placed on the surface of the leave tissue in each well. Larval mortality was recorded on the 7^{th} day after release of the neonates. In each trial, there were four replications for each combination of maize product and insect population, and each replication contained 32 neonates in eight wells. Two independent whole-plant tests were carried out in the greenhouse to evaluate the performance of all 5 insect populations on 11 of the 12 maize products (one experimental line was not included due to limited seed supply). In each trial, 4 neonates of an insect population were placed into the whorl of a plant at the V5-V9 stage. Treatments were replicated 4 times in a randomized complete block design with 1 pot (2 plants) per	The authors concluded that:*"Data generated from this study will be useful in developing resistance management strategies for the sustainable use of Bt maize technology"*.	Environmental protection **Observed parameter** IRM	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.

	replication. Maize leaf injury ratings were determined using the Davis scale on the 14th day after larva inoculation. Statistical analysis was performed by using two-way analysis of variance. ***Results:*** Cry1A.105 and Cry1F maize killed 92.2-100% susceptible larvae in both test methods (leaf tissues in the laboratory and whole plants in the greenhouse), while resistant larvae survived well on these two maize products. Performance of the two F1 populations on Cry1A.105 and Cry1F maize varied between the two test methods. In leaf tissue bioassay, Cry1Ab maize was marginally effective against the susceptible population. In contrast, few live larvae and little leaf injury from any of the five populations were observed on Cry2Ab2 and the three pyramided Bt maize products. The results of this study showed evidence of cross resistance of the Cry1A.105-resistant *S. frugiperda* to Cry1F and Cry1Ab maize, but not to the Bt maize products containing Cry2Ab2 or Vip3A.			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Omoto *et al*. (2016) Field-evolved resistance to Cry1Ab maize by *Spodoptera frugiperda* in Brazil	***Objective:*** To evaluate the susceptibility of fall armyworm (FAW), *Spodoptera frugiperda*, to Cry1Ab toxin and the performance of maize hybrids expressing the Cry1Ab from *Bacillus thuringiensis* (Bt) (event MON 810). ***Experimental Design:*** FAW populations were sampled for resistance monitoring during each maize-growing season from 2010 to 2015 in multiple regions of Brazil. Each crop season consisted of two maize-growing seasons, designated as first (spring/summer) and second crop (autumn/winter). Approximately 1000 FAW larvae were collected on non-Bt maize at each sampling location and reared on artificial diet. For both the diet-incorporated and the diet overlay assays, 7–8 concentrations of Cry1Ab in artificial diet were used. An FAW laboratory strain, obtained from EMBRAPA Maize in 1996, was used as a susceptible reference (SUS). Functional mortality (i.e. larvae did not moult to second instar) was assessed after 7 days. A total of 66 FAW field populations were tested from 2009 to 2015. Leaves were removed from the whorl region of MON 810 and non-Bt maize plants cultivated in a greenhouse at the V4, V6 and V8 phenological stages. Leaf discs were placed in 12-well plates and one FAW neonate larva was placed on each leaf disc. Plates were sealed with plastic film and placed in a climatic chamber. The experimental design was completely randomised, with ten replicates per treatment; each replicate consisted of 12 neonate larvae, for a total of 120 neonate larvae tested for each phenological stage. Functional mortality, weight and instars of survivors were assessed after 5 days. MON 810 and non-Bt maize were sown in a greenhouse at a density of 5 seeds per linear metre following a completely randomised experimental design. At phenological stage V6, each plant was infested with 100 FAW neonates. After 7 days, foliar damage was evaluated using the Davis scale and the number of living caterpillars in the whorl was determined. MON810 and non-Bt maize were sown in the greenhouse into 12	The authors concluded that: "T*he decrease in susceptibility to Cry1Ab was expected, but the specific contributions to this resistance by MON 810 maize cannot be distinguished from cross-resistance to Cry1Ab caused by exposure to Cry1F maize. Technologies combining multiple novel insecticidal traits with no cross-resistance to the current Cry1 proteins and high activity against the same target pests should be pursued in Brazil and similar environments.*"	Environmental protection	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			IRM	There are no changes to the conclusions of the safety of the initial risk assessment.

	L plastic pots (two seeds per pot). Completely expanded leaves were removed from the whorl region of the plants when they reached V6 and cut into sections. Leaves were placed into sterilised glass tubes and one FAW neonate larva was placed into each glass tube. Leaves were changed every 48 h over the larval development period. The experimental design was randomised with 20 replicates, each one consisting of ten tubes. For each treatment, the following biological parameters were evaluated: length and survival of egg, larval and pupal stages; total cycle (egg to adult); larval weight at 10 days after infestation; pupal weight; sex ratio; length of pre-oviposition, oviposition and post-oviposition periods; adult longevity, fecundity and fertility. Adult longevity, fecundity and fertility were determined from 15 couples, raised on either MON 810 or non-Bt leaf tissue. The number of eggs and the mortality of adults were assessed daily. To determine the embryonic period and survival, 50 eggs were obtained from each couple. The eggs were observed daily, and the number of hatched larvae was counted. The data from the bioassays and whole plant assays were subjected to analysis of variance to determine statistical significance. ***Results:*** Baseline susceptibility results indicated that FAW larvae sampled on maize-producing areas were moderately susceptible to Bt maize. The results also indicated moderate susceptibility of FAW to Cry1Ab protein in leaf-disc and whole plant assays. The surviving FAW larvae on MON 810 (22.4%) had a 5.5 day increase in life cycle time and a 24% reduction in population growth rate. Resistance monitoring (2010–2015) showed a significant reduction in Cry1Ab susceptibility of FAW over time. Additionally, a significant reduction in the field efficacy of MON 810 maize against FAW was observed in different regions from crop season 2009 to 2013.			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Sousa *et al.* (2016) Life-history traits of *Spodoptera e*xposed to low-dose Bt maize	***Objective:*** To determine whether exposure to *Bacillus thuringiensis* (Bt) toxins in low-and moderate-dose transgenic crops induces sub-lethal effects and increases the rate of Bt resistance evolution. ***Experimental Design:*** *Spodoptera frugiperda* late instar larvae were collected from Bt-maize expressing Cry1Ab protein (event MON 810) and from non Bt-maize fields in 2010 at five locations in Brazil. The progeny was reared on leaves of either Bt-maize 30F35Y, (event MON 810) or non-Bt isoline maize 30F35. Specifically, 48 batches of five neonates were assigned to either control or Cry1Ab foliage. First instar survival rates were recorded after allowing the larvae to feed for 48 h. To record life-history traits up to the adult stage, 72 survivors from the original cohort of 240 larvae were followed throughout larval development: larval survival was recorded every two days, as were larval weight at 14 days, pupal weight 24 h after pupation and development time from neonate to pupa. The potential for population growth was analysed by following 10 male-female pairs: 1) the number of eggs laid down by each female was recorded until the end of oviposition period; 2) the number of neonates hatched from each egg mass was recorded daily; 3) the age and proportion of the cohort surviving to adult, as well as the sex ratio and the number of females produced by each parental female were determined; and 4) the intrinsic rate of population increase or daily rate of female offspring production per parental female was determined. Linear statistical modelling, two-way analysis of variance and three-way analysis of variance were used for statistical analysis. ***Results:*** Larval survival on Cry1Ab maize leaves varied from 20 to 80%. Larvae reared on Cry1Ab maize had a delay of seven days in development time in relation to control larvae, and such delay was shorter in offspring of armyworms collected from Cry1Ab maize. Population growth rates were 50-70% lower for insects continuously exposed to Cry1Ab maize relative to controls, showing the population-level effect of Cry1Ab, which varied	The authors concluded that: *"The field-derived populations of S. frugiperda with increased fitness on Cry1Ab maize provide the opportunity to investigate the genetics/molecular basis of Cry1Ab resistance and update information to refine resistance management strategies for lepidopteran pest species for which it is difficult to obtain high-dose Bt transgenic events"*.	Environmental protection	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
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	among the populations and prior exposure to Cry1Ab maize in the field. In three out of five populations, armyworms derived from Bt maize reared on Cry1Ab maize showed higher larval weight, faster larval development and better reproductive performance than the armyworms derived from non-Bt maize, and one of these populations showed better performance on both Cry1Ab and control diets, indicating no fitness cost of the resistance trait. Altogether, these results indicate that offspring of armyworms that developed on field-grown, single-gene Cry1Ab maize had reduced performance on Cry1Ab maize foliage in two populations studied, but, in the other three populations, these offspring had better overall performance on the Bt maize foliage than that of the armyworms from non-Bt maize fields, possibly because of Cry1Ab resistance alleles in these populations.			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Waquil *et al.* (2016) Fitness index and lethal time of fall armyworm on Bt corn	***Objective:*** To evaluate the fitness index and lethal time of fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) population on *Bacillus thuringiensis* (Bt) maize expressing the Cry1Ab, Cry1F, Cry1A.105/Cry2Ab2 and Vip3Aa20 proteins. ***Experimental Design:*** Three bioassays were conducted with larvae collected at the municipalities of Inhaúma (from Bt Cry1Ab maize in 2008/2009 and 2010/2011) and Sinop (from Bt Cry1A.105/Cry2Ab2 maize in 2014), in the state of Minas Gerais and Mato Grosso respectively, Brazil. *S. frugiperda* corresponding to the control group were collected in non-Bt maize (DKB 390 and Impacto). Larvae from these populations were fed with leaves of non-Bt and Bt maize (P30F35Hx for Cry1F, P30F35 YG for Cry1Ab, DKB Bt VT PRO for Cry1A.105/Cry2Ab2 and Impacto Bt Vip for Vip3Aa20). The following variables were evaluated: larval survival, pupae mass and larval development period. The fitness indexes and lethal time of these populations were then calculated. For the lethal time analysis, mortality data of a population collected from Cry1F Bt maize in 2010/2011 from the municipality of Piumhi, in the state of Minas Gerais, were used. ***Results:*** The Cry1Ab Bt maize showed a limited but durable efficiency. The Cry1A.105/Cry2Ab2 and Vip3Aa20 Bt maize reduced *S. frugiperda* performance to less than 5 and 0% respectively, regardless of the origin of the colony. The population from Sinop showed high larval performance in Cry1F Bt maize, showing its resistance to this protein and a certain level of fitness cost.	The authors concluded that: *"the lethal time can be a variable that indicates evolution of resistance, since it is greater for populations with greater fitness"*.	Environmental protection	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			IRM	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
Yang *et al.* (2016) Performance of Cry1Ab-susceptible and heterozygous resistant populations of sugarcane borer in sequential feedings on non-Bt and Bt maize plant tissue	***Objective:*** To compare the performance of Cry1Ab-susceptible and Cry1Ab-heterozygous resistant populations of *Diatraea saccharalis* in sequential feedings on non-*Bacillus thuringiensis* (Bt) and Bt maize (MON 810) plant tissue. ***Experimental Design:*** Two populations of *D. saccharalis* were tested: a Cry1Ab-susceptible strain (SS) (established from larvae collected from non-Bt plants in northeast Louisiana in 2009) and a heterozygous resistant population (RS). SS is susceptible to purified Cry1Aa, Cry1Ab, Cry1Ac, Cry1A.105 and Cry2Ab2 proteins as well as to Bt maize plants expressing Cry1Ab, Cry1A.105 and Cry2Ab2. RS was developed by crossing SS with a Cry1Ab-resistant strain that was established using an F2 screen in 2004. A Bt maize hybrid, DKC69-70 (MON 810) and a closely related non-Bt maize hybrid, DKC62-95, were used and planted in a greenhouse. Expression/non-expression of the Cry1Ab protein for the Bt and non-Bt maize hybrids was confirmed using ELISA. Survival, development and progeny production of SS and RS in sequential feedings on non-Bt and Bt plant tissue were evaluated. For each insect population, there were nine feeding sequences (Feed-1 to Feed-9). In Feed-1, which served as a negative control, newly hatched larvae (<24 h old) were placed on non-Bt maize plant tissue where they fed until pupation. In Feed-2, which served as a positive control, larvae were fed on Bt plants for their entire larval stage. Feed-3 to 7 were designed to simulate the sequential feeding behaviour of maize borer larvae moving from non-Bt to Bt plants in seed mix plantings, whereas Feed-8 and 9 were conducted to mimic larvae moving from Bt to non-Bt plants. For each bioassay, there were four replications, each containing 24 larvae. For Feed-1 to 4, 8, and 9, three neonates (<24 h old, for Feed-1 and 2) or three larvae of 1, 2, 3, or 6 d old (for Feed-8, 9, 3, and 4, respectively), collected from the insect rearing trays, were assayed in the wells containing maize leaf tissue. Insect survival and pupation for each bioassay were checked on the 3rd day after the assay was started and every 3 d thereafter.	The authors concluded that: *"our results suggest that the larval dispersal behaviour of maize borers could result in survival and successful reproduction of RS populations in seed mix plantings of non-Bt and Bt maize plants even with high dose expression"*.	Environmental protection	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			IRM	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
	Results: SS and RS feeding on non-Bt plants for their entire larval stages survived well and > 60% of the adult pairs produced viable eggs, with an average of 269 progeny per female, whereas none of the two populations on Bt maize plants survived to the pupal stage. SS larvae could not develop to adults if the larvae fed on non-Bt plants for ≤ 15 d and then moved to Bt plants. In contrast, 4.2–29.2% of RS larvae that fed on non-Bt plants for ≥ 9 d and then moved to Bt plants developed to adults, and 63.6% of pairs of these adults produced viable eggs, with an average of 185 progeny per female. For SS larvae that fed on Bt plants for 1 or 2 d and then moved to non-Bt plants, few larvae developed to adults with varied emergence times, whereas 28.1 and 13.5% RS larvae feeding on Bt plants for 1 and 2 d, respectively, successfully developed to adults; 43.8% of pairs of these adults produced viable eggs, with an average of 220 progeny per female. For the case of the single Bt gene maize plants (MON 810), the results suggest that RS insects may have advantages in survival and reproduction over SS if RS larvae hatch and feed on Bt plants during the first 1 or 2 d and then move to non-Bt plants. This advantage is less for RS larvae that hatch and feed on non-Bt plants first and then move to Bt plants, unless the larval movement occurs in the later stages (e.g., fourth or fifth instars).			

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