Appendix 5.2 MON 810 Literature Review - Environment

MON 810 literature review (July 2016)

Appendix 5.2 - Environment

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Review of peer-reviewed publications

Area of the environmental risk assessment: Environmental Safety – Non-Target Organisms

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Holderbaum *et al.* Chronic responses of *Daphnia magna* under dietary exposure to leaves of a transgenic (event MON 810) Bt-maize hybrid and its conventional near-isoline (2015)	***Objective:*** To assess possible impacts of *Bacillus thuringiensis* (Bt) insect resistant maize on insect biodiversity present in different agro ecosystems. ***Experimental Design:*** Under field conditions, the incidence of *Spodoptera frugiperda*, the primary target pest of maize infesting the whorls and ears, and the insect community (non-target insect species, secondary pests and natural enemies) was evaluated in conventional and Bt maize expressing different proteins (Cry1Ab, Cry 1F, Cry1A105 + Cry2Ab2) in seven counties of Minas Gerais, Brazil. Samples were collected in November/December 2010 from crop areas of more than 350 ha with expected productivity of ca. 200 bags/ha. Conventional maize fields received three insecticide applications and Bt fields received none. To evaluate the cultivation effects of Bt maize on *S. frugiperda* abundance, variance analyses were performed on two factors, considering the effects of treatment (maize hybrid) and larvae size. Theses analysis were performed separately for data collected in the whorl/ear and grouped at the location, since the experimental design was incomplete (not all crop fields used the same Bt maize seed). Species richness was estimated using the Jacknife procedure. To estimate the diversity, the Shannon-Wiener index was used (EstimateS8.0 software). To evaluate the effect of Bt maize cultivation on estimated richness and diversity of secondary pests and natural enemies, variance analyses were performed on two factors, considering the effect of treatment and the type of organisms. As before, the analyses were performed separately for data collected in the whorl/ears/tassels and grouped at the location. Regression analyses were also performed to evaluate if the richness of natural enemies present in the fields was related to the richness of secondary pests and if the total area sown affected insect richness by location. To evaluate the insecticide spraying effect on estimated insect richness, Paired t-tests analyses were performed.	The authors concluded that: *"the results do not support the hypothesis that Bt protein affects insect biodiversity. The richness and diversity data of insects studied were dependent on the location and other factors, such as the use of insecticides"*.	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.

	Results: The abundance of *S. frugiperda* larvae of different sizes collected in whorl and ear samples was dependent on location. The overall pattern of estimated insect richness in the different maize plant parts was distinct among the seven crop field studies and the results did not support the hypothesis of Bt proteins having a negative effect on insect richness. The effects of Bt maize on the overall richness and diversity of insects appeared to be dependent on geographical location and crop management in the agro ecosystem. There is therefore an indication that other factors such as insecticides use may exert a stronger influence on this process.			

[1] The authors exhibit in this article adverse effects by MON 810 leaf tissue in comparison with near-isoline maize leaf tissue based on a comparison of several reproductive metrics in a 42-day chronic bioassay and recommend revisions of guidelines for risk assessment of genetically modified crops to include multiple endpoints over the full life cycle of model organisms, and that testing should be conducted using plant-produced proteins or plant material in place of the microbially-produced proteins. However, based on the demonstration of molecular and functional equivalence between plant-produced and microbially-produced Cry1Ab used in safety studies, a long history of safe use of Cry1Ab in animal feed, the well-understood mode of action of Cry proteins, and literature demonstrating that Cry1Ab is unlikely to cause adverse effects in non-target organisms, this conclusion is unsubstantiated.

Further, our review of this study questions the reliability and relevance of the study's results which need to be interpreted with caution. Specifically, the materials used in the study are not genetically identical and were not analyzed for compositional similarity; the study methods did not follow accepted guidelines; the endpoints collected within the accepted guideline study duration (survival, body size, reproduction) do not show any significant differences between treatment and control, and observations were collected after the accepted guideline study duration despite the assay control mortality exceeding 20%.

The study methods did not follow accepted testing guidelines. The duration of the tests was substantially longer (42 days vs. 21 days) than recommended (OECD, 2012). Control mortality must not exceed 20%; however, the control mortality exceeded 20% at day 21, long before the termination of the 42 day study. The authors noted that Daphnia fed maize-leaf diets displayed higher rates of mortality and reduced reproduction, indicating sub-optimal feed conditions, yet they drew conclusions based on these data.

Survival did not differ significantly between the maize treatments. Body size did not differ between maize treatments from day 9 to day 24. The authors conclude that body size differs between the treatments after day 27, which is far beyond the guideline duration and would be confounded by mortality. Incidence of reproduction did not differ between maize treatments. Age at first reproduction did not differ between maize treatments. Stage fecundity did not differ between maize treatments during the accepted guideline duration of the study of 21 days. Conclusions drawn beyond 21 days would be confounded by mortality within treatments. Cumulative fecundity did not differ significantly between maize treatments at any specific timepoint.

Differences in ephippia production were emphasized in the discussion by the authors as a response indicative of stressful conditions. However, no details were provided on the timing of the ephippia production. It is not possible to evaluate the significance of any difference in ephippia production without knowing if the difference occurred within the 21 day accepted guidance duration, after day 21, or even if the ephippia production occurred at different times in the two treatments.

The discussion and conclusions drawn from the results extrapolate beyond the support of the results. The results presented in this study consistently show no differences between maize treatments for survival, growth, and reproduction during the first 21 days and within the accepted guideline study duration. Conclusions of adverse effects based on results and observations after day 21, and simultaneous with control mortality greater than 20% indicating poor testing conditions, should be interpreted with caution. Assertions that multiple endpoints should be included over the full life cycle of model organisms, and that testing should be conducted using plant-produced proteins or plant material in place of the microbially-produced proteins are not supported by the results from this study.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Resende *et al*. Does Bt maize cultivation affect the non-target insect community in the agro-ecosystem? (2016)	***Objective:*** To assess possible impacts of *Bacillus thuringiensis* (Bt) insect resistant maize on insect biodiversity present in different agro ecosystems. ***Experimental Design:*** Under field conditions, the incidence of *Spodoptera frugiperda (S. frugiperda)*, the primary target pest of maize infesting the whorls and ears, and the insect community (non-target insect species, secondary pests and natural enemies) was evaluated in conventional and Bt maize expressing different proteins (Cry1Ab, Cry 1F, Cry1A105 + Cry2Ab2) in seven counties of Minas Gerais, Brazil. Samples were collected in November/December 2010 from crop areas of more than 350 ha with expected productivity of ca. 200 bags/ha. Conventional maize fields received three insecticide applications and Bt fields received none. To evaluate the cultivation effects of Bt maize on *S. frugiperda* abundance, variance analyses were performed on two factors, considering the effects of treatment (maize hybrid) and larvae size. Theses analysis were performed separately for data collected in the whorl/ear and grouped at the location, since the experimental design was incomplete (not all crop fields used the same Bt maize seed). Species richness was estimated using the Jacknife procedure. To estimate the diversity, the Shannon-Wiener index was used (EstimateS8.0 software). To evaluate the effect of Bt maize cultivation on estimated richness and diversity of secondary pests and natural enemies, variance analyses were performed on two factors, considering the effect of treatment and the type of organisms. As before, the analyses were performed separately for data collected in the whorl/ears/tassels and grouped at the location. Regression analyses were also performed to evaluate if the richness of natural enemies present in the fields was related to the richness of secondary pests and if the total area sown affected insect richness by location. To evaluate the insecticide spraying effect on estimated insect richness, Paired t-tests analyses were performed. ***Results:*** The abundance of *S. frugiperda* larvae of different	The authors concluded that: *"the results do not support the hypothesis that Bt protein affects insect biodiversity. The richness and diversity data of insects studied were dependent on the location and other factors, such as the use of insecticides"*.	Environment	No adverse effects were determined in this study.
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Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
	sizes collected in whorl and ear samples was dependent on location. The overall pattern of estimated insect richness in the different maize plant parts was distinct among the seven crop field studies and the results did not support the hypothesis of Bt proteins having a negative effect on insect richness. The effects of Bt maize on the overall richness and diversity of insects appeared to be dependent on geographical location and crop management in the agro ecosystem. There is therefore an indication that other factors such as insecticides use may exert a stronger influence on this process.			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Shu *et al.* Multilevel assessment of Cry1Ab Bt-maize straw return affecting the earthworm *Eisenia fetida* (2015)	***Objective:*** To investigate the potential effects of *Bacillus thuringiensis* (Bt)-maize (5422Bt11 [event Bt11] and 5422CBCL [event MON 810]) straw return on *Eisenia fetida* using traditional (life-history traits, such as growth and reproduction), biochemical and molecular endpoints. ***Experimental Design:*** Soil was collected from the top layer of a conventional maize field at the Agricultural Experiment Station of South China Agriculture University, cleaned to remove plant debris and soil fauna, and air-dried. Two transgenic maize cultivars (5422Bt11 [event Bt11] and 5422CBCL [event MON 810]) and their near-isogenic cultivar (5422) were grown in a greenhouse. Three weeks after pollen shed, the straw, including leaves and stalks, was cut, freeze-dried, ground and sieved. The plant material was then stored at -20°C until use. In a series of experiments, adult *E. fetida* were bred in soil covered with powdered maize straw derived from either Bt11, 5422CCL or the non-Bt-maize for 3 generations. The growth of adult and juvenile *E. fetida* was determined by measuring the weight at Days 15, 30, 45 and 60 and calculating the relative growth rate (RGR). Every 30 days, juveniles and cocoons were counted. The guts of *E. fetida* were used to 1) extract proteins in order to measure the activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX); and 2) extract total RNA to measure by real-time PCR (RT-PCR) the expression of four genes involved in stress response, carcinogenesis and reproduction (heat shock protein 70 (*hsp70*), *sod*, translationally controlled tumour protein (*tcpp*) and annetocin (*ann*)). The concentrations of Cry1Ab protein in straw, soil, earthworm casts and guts were measured by ELISA. ***Results:*** 5422Bt11 straw return had no significant effect on the growth of adult earthworms after exposure of 90 days or more, while it showed a positive effect on the growth of juveniles and the reproduction of adults. Negative, no and positive effects on the growth and reproduction of adult earthworms treated with 5422CBCL straw were observed in the 1st, 2nd and 3rd generations, respectively. Negative and positive effects were seen on the growth	The authors concluded that: *"This study was the first to evaluate the generational effects of Bt-maize straw return on earthworms under laboratory conditions. The responses of enzymes activity and genes expression may contribute to better understand the different effects of Bt-maize straw return on earthworms from the 1st generation"*.	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.

	of juveniles produced from 1st- and 2nd-generation adults exposed to 5422CBCL straw, respectively. No significant differences in SOD activity were noted among the three maize treatments, while the GSH-PX activity of earthworms from Bt-maize treatments was significantly higher than that of controls on Day 90. While *tctp* and *sod* genes were down-regulated and *ann* expression was up-regulated in *E. fetida* from 5422Bt1 treatments, *tctp* and sod genes were up-regulated and *ann* and *hsp 70* genes were down-regulated in worms from 5422CBCL treatments. Cry1Ab released from 5422Bt1 and 5422CBCL straw degraded rapidly in the first 30 days and had a slow decline during the remaining time. Cry1Ab concentrations in the soil, casts and guts of earthworm significantly decreased over the course of the experiment.			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Arias-Martin *et al.* Effects of three-year cultivation of Cry1Ab-expressing Bt maize on soil microarthropod communities (2016)	***Objective:*** To evaluate possible effects of continuous cultivation of *Bacillus thuringiensis* (Bt) maize on the non-target soil microarthropods community in an experimental farm-scale field in Central Spain. ***Experimental Design:*** An experimental maize field was conducted with the DKC 6451 YG (event MON 810) and its near-isogenic line (DKC 6450), during the years 2009, 2010, 2011 and 2013 in the province of Madrid. The arrangement of treatments and plots was exactly the same across the year to assess potential cumulative effects. Maize was planted in April/May and harvested from late October/November, depending on the weather each year. Microarthropods were collected during three consecutive years (2009-2011) from soil cores. The presence of Cry1Ab protein in the soil of the experimental maize field was evaluated in 2013, when a higher accumulation might be expected. Cry1Ab rates were examined in soil samples collected from Bt and non-Bt plots from the experimental field, at different times (36, 78 and 99 days) after maize harvest. To obtain rhizosphere soil (RS), standing cut stalks were pulled out manually from the soil along with the roots. To obtain the organic matter (OM), the soil fraction discarded (size between 0.84 and 0.20 mm) was immersed in distilled water and recovered by flotation. Levels of Cry1Ab protein were measured by double-antibody sandwich enzyme-linked immunosorbent assays. A Generalized Estimating Equations model (GEE) was used to determine the effects of Bt maize cultivation on different population parameters of soil dwelling-microarthropods: abundance, species richness, species diversity and frequency of occurrence. ***Results:*** A total of 15,235 microarthropods belonging to different taxonomic groups were collected through the years. Considering the three years together, the majority of arthropods were mites (Acari, 73%) and springtails (Collembola, 15%), both maintaining similar rates throughout the study. Cry1Ab protein was detected in decaying soil OM from Bt maize plots up to three months after harvest, with values ranging between 0.10 and 0.18 ng Cry1Ab/mg OM, but it showed low insecticidal activity. Cry1Ab was detected	The authors concluded that: *"continuous cultivation of Bt maize does not negatively affect soil micro-arthropods, indicating that Bt maize could be compatible with this community"*.	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.

	for the first time in field collected collembolans, *Entomobrya* spp., demonstrating their exposure to the protein. The abundance of mites and collembolans and the frequency of occurrence of the main collembolan species did not rely on the type of maize except for *Parisotoma notabilis*, more abundant and frequent in Bt maize plots. However, significant differences among years were common in both groups. Higher values of species richness and diversity of collembolans were found in Cry1Ab-expressing Bt maize than in non-Bt plots.			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Zeng *et al*., The Cry1Ab protein has minor effects on the arbuscular mycorrhizal fungal communities after five seasons of continuous Bt maize cultivation.(2015)	***Objective:*** To assess the impact of five seasons of continuous *Bacillus thuringiensis* (Bt) maize cultivation on the colonisation and community structure of arbuscular mycorrhizal fungi (AMF) in maize roots, bulk soils and rhizospheric soils using the terminal restriction fragment length polymorphism (T-RFLP) analysis of 28S ribosomal DNA and sequencing methods. ***Experimental Design:*** Two Bt maize varieties (5422Bt1 (event Bt11) and 5422CBCL (event MON810)) and their conventional isoline (5422) were planted for five consecutive seasons following a randomised complete block design in a greenhouse at the South China Agricultural University, Guangzhou, China. Four replicates of ten individuals were planted for each maize variety. The first season of cultivation started in September 2009 and the subsequent seasons started in March, September 2010 and March, September 2011. In December 2011, the plants were gently dug up, and the soils attached to the roots were collected. The roots were rinsed with tap water and divided into two parts for the subsequent analyses (AMF colonisation, extraction of nucleic acids and analysis of the Cry1Ab protein). Concentrations of Cry1Ab protein in the soils and roots were determined by ELISA. ***Results:*** AMF colonisation was significantly higher in roots of the two Bt maize cultivars (5422Bt1 and 5422CBCL) than in the non-Bt isoline 5422 planted in the fifth season of continuous Bt maize cultivation. No significant differences were observed in the diversity of AMF community in the roots, rhizospheric soils and bulk soils from the Bt and non-Bt maize cultivars, as demonstrated by T-RFLP analysis. The AMF *Glomus* was dominant in most of the samples, as detected by DNA sequencing of the 16S rDNA fragment. A clustering analysis based on the DNA sequence data suggested that the sample types (i.e., the samples from the roots, bulk soils or rhizospheric soils) might have greater influence on the AMF community phytotypes than the maize cultivars.	The authors concluded that: *"this study suggests that the Cry1Ab protein has minor effects on the AMF communities after five seasons of continuous Bt maize cultivation"*.	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
Szénási Flea beetles (Coleoptera: Chrysomelidae, Alticinae) in Bt- (MON 810) and near isogenic maize stands: Species composition and activity densities in Hungarian fields. (2015)	***Objective:*** To examine potential effects of genetically modified *Bacillus thuringiensis* (Bt) maize expressing the Cry1Ab toxin on non-target flea beetles (Chrysomelidae, Alticinae). ***Experimental Design:*** The study was carried out in an isolated maize stand located near Budapest, Hungary, in 2002 and 2003. The following hybrids were used: DK 440 BTY (Bt maize, event MON 810) and its near-isogenic line (DK440). Each hybrid was planted in six repetitions in plots of 30 x 30 m. Sowing was in late April and maize was harvested in mid-October to early November, depending on the year. Flea beetle adults were collected with Pherocon AM yellow sticky traps. Sampling took place from late May to late September in 2002 and from early June to mid-September in 2003. The statistical analyses were performed by using the repeated measures analysis of variance (ANOVA), the Welch's test, the Greenhouse-Geisser test and the one-sample t test. ***Results:*** Overall, 51,348 flea beetle individuals from 26 species were collected. The dominant species were *Phyllotreta atra* (F.) and *Phyllotreta vittula* (Redtenbacher). Their abundance along with other (non-*P. atra* and non-*P. vittula*) flea beetle species showed no significant differences between Bt- and isogenic maize plots. Similarly, no difference was found between Bt maize and isogenic maize plots in the species richness of the flea beetle assemblages.	The authors concluded that: *"no adverse effects of Bt-maize (MON 810 event) on flea beetle assemblages or species, or on their abundance or species richness were observed"*.	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.

Area of the environmental risk assessment: Environmental Safety – Protein fate in stream water

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Böttger *et al.* Aquatic degradation of Cry1Ab protein and decompostition dynamics of transgenic corn leaves under controlled conditions (2015)	***Objective:*** To assess the effect of litter from genetically modified Bt maize plants expressing Cry1Ab protein on leaf litter decomposition rates in the aquatic environment, under controlled temperature and dissolved oxygen conditions. ***Experimental Design:*** Leaves of transgenic maize expressing Cry1Ab protein (PAN 6Q-321B) and of its isoline (PAN 6Q-121) were used in the experiments. Green and senescent leaves were harvested in parallel from the same plants after the fourth month of growth. Leaf disks (2 cm diameter) of green and senescent plant material in natural proportions of 70:30 (green/senescent) were used. These were placed into 10L polyethylene vessels which were filled with 5L of stream water from the Federal Environment Agency of Germany in Berlin-Marienfelde. The experiment was conducted in a climate chamber at 18°C. Aeration was performed by air diffusers and an aeration pump at a rate of 0.5 L/min to stabilize dissolved oxygen concentrations in water. A 12h/12h light-dark cycle was set to simulate natural light conditions. Four replicates of Bt maize litter and isoline litter were used. The duration of the experiment was 21 days. ***Results:*** No significant differences in the aquatic degradation of maize leaves were observed between the leaves of transgenic maize expressing Cry1Ab and its isoline. The loss of total mass in dry weight [43% (isoline) and 45% (Bt maize)], the lignin content increased [137.5% (isoline) and 115.7% (Bt maize)], and the phenol loss decreased [53.6% (isoline) and 62.2% (Bt maize)] were measured during the experiment. At the end of the experiment, Cry1Ab protein was still detectable at 6% of the initial concentration. A slightly but significant lower cellulose content was found for the Cry1Ab treatment compared to the isoline litter at the end of the experiment. The significant higher total protein (25%) and nitrogen (25%) content in Bt maize, most likely due to the additionally expression of the transgenic protein, may increase the microbial cellulose degradation and decrease microbial lignin degradation. A relevant year by year input of protein and therefore nitrogen rich Bt maize litter into aquatic environments may affect the balanced nutrient turnover in aquatic ecosystems.	The authors concluded that: *"Cry1Ab protein had no effect on litter mass loss and lignin and phenol contents… but it affected the nitrogen and total protein content of leaf litter during decomposition process and therefore may affect the carbon turnover and nutrient spiralling in freshwater ecosystems in the long-term. Furthermore, a slightly decreased cellulose content of leaves during aquatic decomposition was found".*	Environment	The described fate of Cry1Ab is consistent with previous study results showing rapid degradation of the protein in freshwater systems[1]. The compositional differences observed are marginal and their predicted consequences are not supported by the reported data[2].
			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.

[1] (Li *et al.*, 2013; Prihoda and Coats, 2008; Strain and Lydy, 2015)

[2] The results of the publication show no consistent or significant differences between test and control for nitrogen and protein content, cellulose degradation or lignin accumulation throughout the course of the experiment, which discredits the conclusion that "*a relevant year by year input of protein and therefore nitrogen rich Bt maize litter into aquatic environments may affect the balanced nutrient turnover in aquatic ecosystems*". Further, as also highlighted by the authors, no reference samples were tested to determine the natural variability in the components tested. Finally, to support the conclusion provided, changes in nutrient content within the water phase should be measured.

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
Valldor *et al.* Fate of the insecticidal Cry1Ab protein of GM crops in two agricultural soils as revealed by ^{14}C-tracer studies (2015)	***Objective:*** To characterize the fate of Cry1Ab protein in two agricultural soils with contrasting amounts of clay but high similarities in other parameters, including pH. To trace ^{14}C-labelled Cry1Ab protein in the background of natural soil proteins and indigenous microbial activities and to quantify its mineralization, adsorption and incorporation into soil microbial biomass. ***Experimental Design:*** Two soils, originating from maize fields of the "Oderbruch" region in Brandenburg, Germany, with no previous cultivation of Bt maize or other genetically modified crops, were selected for this study. The soils were collected in November, six weeks after harvesting, and immediately transported to the laboratory, where they were sieved and stored. The ^{14}C-labelled Cry1Ab protein was produced by an *Escherichia coli* stain, genetically modified to synthetize the Cry1Ab protein identical to the protein from Bt-maize MON 810. ^{14}C-labelled Cry1Ab protein was applied to soil microcosms at two concentrations (14 and 50 µg/g soil) to quantify the mineralization of Cry1Ab, its incorporation into the soil microbial biomass and its persistence in two soils which strongly differed in their texture but not in silt or pH. ELISA was used to quantify Cry1Ab and its potential immunoreactive breakdown products in aqueous soil extracts. ***Results:*** In both soils, $^{14}CO_2$ production was initially very high and then declined during the total monitoring period of up to 135 days. A total of 6 to 23% of the ^{14}C activity was incorporated after 29 to 37 days into the soil microbial biomass, indicating that Cry1Ab protein was utilized by microorganisms as a growth substrate. Adsorption in the clay-rich soil was the most important factor limiting microbial degradation; as indicated by higher degradation rates in the more sandy soil, extremely low concentrations of immunoreactive Cry1Ab molecules in the soils' aqueous extracts and a higher amount of ^{14}C activity bound to the soil with more clay.	The authors concluded that: *"Ecological risk assessment of Bt-crops should consider that the very low concentrations of extractable Cry1Ab do not reflect the actual elimination of the protein from the soils but that desorbed proteins mineralize quickly due to efficient microbial degradation"*.	Environment	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.

Area of the environmental risk assessment: Environmental Safety – Gene flow

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Taverniers *et al.* Influence of plant developmental stage on DNA yield and extractability in MON 810 maize (2015)	***Objective:*** To investigate the influence of sampling stage on genetically modified (GM) DNA quantification from different samples of *Bacillus thuringiensis* (Bt) insect resistant maize, taken at various periods during the growth season. To follow the evolution of different plant parts during the growth season and to evaluate differences in DNA extractability from different types of maize tissues. ***Experimental Design:*** Seeds of MON 810 maize were sown on April 2010 at ILVO (Institute of Agricultural and fisheries Research) test fields situated in Wetteren, Belgium. Between the end of pollination and harvest, plant samples were taken on four occasions (with kernels at R2, R3, R4 and R5 stages). Per individual plant, the weights of the whole plant, the cob and the vegetative part of the plant were determined. Subsequently, three kernels were removed from the cob (top, middle and bottom) and their weights determined. The mean mass of fresh material for each vegetative part and cob was determined and the standard deviations were calculated over ten samples. These averages were used to calculate the (fresh) vegetative/cob weight ratio. The total DNA concentration per lyophilized plant part was determined. The DNA concentrations were averaged by sampling stage and a vegetative/cob DNA ratio was calculated. Weight and DNA concentration ratios between endosperm and embryo within a single kernel were also determined. The overall GM percentage of one kernel was calculated by the formula provided by Zhang *et al.* (2008)[1] where *F* represents the DNA ratio endosperm/overall kernel. The GM percentage for the corn cob maize (CCM) was determined by multiplying the GM percentage for grain maize by the DNA content of the total amount of kernels on the cob and subsequently dividing it by the DNA content in one cob. The DNA content of all kernels on one cob was defined by	The authors concluded that: *"when examining the percentage GM contamination level in a non-GM maize field neighbouring a GM maize field, decreasing rates of GM cross-pollination percentage might be expected with increasing distance from the neighbour GM field. Based on empirical testing, different models could be composed to follow the decrease of GM percentage from the border to the center of the field"*.	Environment	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			DNA fate	There are no changes to the conclusions of the safety of the initial risk assessment.

[1] Zhang D, Corlet A and Fouilloux S, 2008. Impact of genetic structures on haploid genome-based quantification of genetically modified DNA: theoretical considerations, experimental data in MON 810 maize kernels (*Zea may*s L.) and some practical applications. Transgenic Research, 1-10.

	multiplying the average DNA content of one kernel by the average total amount of kernels on one cob. Finally, the GM percentage for silage maize was calculated by multiplying the GM percentage for CCM by the DNA content of the cob and dividing it by the DNA content of the overall plant. The DNA content of the plant was defined by the summation of the DNA content of all different plant parts (vegetative parts plus cob). ***Results:*** The evolution of weights, absolute DNA yields, DNA densities and ratios of endosperm and embryo relative to total maize kernel were studied. Sampling at the four stages during the growth showed an influence on relative GM quantification based on haploid genome equivalents, due to the specific maize seed composition and differences in DNA extractability from different seed tissues. During plant growth, plant parts with potential GM genes (embryo in kernel and cob on total plant) increased in importance at the weight and DNA concentration levels, while the endosperm dropped in relative importance. Expected percentage GM maize values were calculated for a whole field harvest of grain maize.			

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
García *et al.* Inheritance, fitness costs, incomplete resistance and feeding preferences in a laboratory-selected MON 810-resistant strain of the true armyworm *Mythimna unipuncta*, (2015)	***Objective:*** To analyse the inheritance of resistance, the fitness costs associated and the existence of incomplete resistance in the moth *Mythimna unipuncta*. To investigate the feeding preferences of resistant and susceptible *M. unipuncta* larvae. To discuss the potential of this secondary pest to develop resistance in nature and the implications for resistance monitoring. ***Experimental Design:*** All experiments were performed with a resistant (MR) and a control (MC) strain of *M. unipuncta* derived from the same field population (collected in conventional maize in Monzón – Spain in 2009, maintained in laboratory without exposure to *Bacillus thuringiensis* (Bt) maize or Cry toxins for six generations). Two hybrids were used: Bt maize DKC6451YG (event MON 810) and its near isogenic conventional line DKC6450. Plants were grown in a controlled greenhouse at 25 ± 5°C and > 60% RH with a 16:8h (L:D) photoperiod. The Cry1Ab toxin used was obtained from *Escherichia coli* cultures. Susceptibility to Cry1Ab of neonate larvae (< 24h) was assessed using a leaf-disc bioassay. For each concentration of Cry1Ab, 6 replicates of 12 larvae were used. After 7 days, larval mortality was recorded. The concentrations needed to cause 50% mortality were obtained by probit analysis, which automatically corrects for control mortality. Dominance of resistance (D_X) was calculated as described by Bourguet *et al.*, $D_x = (X_{RS} – X_{SS})/(X_{RR} – X_{SS})$, where X_{SS}, X_{RS} and X_{RR} are the quantitative values calculated for a trait (x) for susceptible homozygotes, heterozygotes and resistant homozygotes respectively. To calculate the dominance level of Cry1Ab resistance, the trait assessed was logLC50, estimated by using mortality data from LM7 days (mortality when fed on maize discs during a 7 day period) and LMlarval cycle (mortality when fed on maize leaf pieces throughout the larval cycle). For direct testing of monogenic inheritance, the observed mortalities in the backcrosses were compared to each concentration tested with the expected mortalities when assuming a monogenic model.	The authors concluded that: *"both resistant and heterozygous larvae of M. unipuncta survive the Cry1Ab toxin expressed in Bt maize, with a weak fitness cost for the homozygous larvae, indicating the potential risk of field-evolved resistance and its relevance to resistance monitoring"*.	Environment	No field-relevant adverse effects expected. This is a secondary pest, with no observed change in field performance, and no outbreaks of *M. unipuncta*, since the introduction of MON 810 maize in 1998. Resistant insects were developed in the lab from a susceptible population, not in the field with plants expressing Cry1Ab; low field relevance. As shown in Table 1, even susceptible *M. unipuncta* has relatively low susceptibility to Cry1Ab, meaning that selection pressure by feeding on MON 810 maize also should be low. At time of experiments, only 10X resistance to Cry1Ab suggesting that various factors (probably many of which are environmental such as refuge) is contributing to this absence of observed change in field performance after so many years of MON 810 use. This is particularly

	For the life history traits assay, forty neonate larvae were randomly collected from each reproductive cage and reared individually, where they were fed *ad libitum* on either non-Bt or Bt maize leaf pieces. All vials were checked daily to assess development time, survival, larval (L2) and pupal (24-48 h after pupation) weight, emergence of adults and sex ratio. Newly emerged virgin adults were paired within groups representing the number of reproductive pairs. Eggs were collected and counted every day. The preoviposition period of copulated females, the total fecundity, fertility and adult longevity were estimated. The feeding preference bioassays were conducted with fifth-instar larvae (< 24h) starved for 3h and individually placed in each petri dish at 23 ± 0.2°C and 80 ± 5% RH with a 16:8h (L:D) photoperiod in a growth chamber. Results: The resistance in the MR strain is autosomal and inherited as a partially dominant trait. A lack of fitness costs in the stain for essential life history traits, reproductive potential and most of the population growth parameters analysed were found. The only exception being an increment in the mean generation time. Larvae of the MR strain reared on Bt maize took longer to develop, presented a high adult cumulative emergence time and had lower growth rate than those reared on non-Bt maize, suggesting the existence of incomplete resistance. Feeding preference assays reveal a low discrimination between Bt and conventional maize.			true if resistance is inherited as a partially dominant trait as the authors describe. Also, with only 10X resistance. to Cry1Ab, low-no fitness costs would be expected.
			Observed parameter	**Feedback on initial environmental risk assessment**
			IRM	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)/ Impact of Management Practices

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Crespo (Survival of corn earworm (Lepidoptera: Noctuidae) on Bt maize and cross-pollinated refuge ears from seed blends, (2015)	***Objective:*** 1) To investigate whether a blend refuge with transgenic maize, containing the events 1507, MON 810 and MIR 162 (1507xMON 810xMIR 162) - which express the *Bacillus thuringiensis* (Bt) insecticidal proteins Cry1F, Cry1Ab and Vip3Aa, respectively - causes changes in survival and development of the ear-feeding pest *Helicoperva zea* and 2) to estimate the relative survival of *H. zea* on Bt events. ***Experimental Design:*** Three trials with natural insect infestations of *H. zea* were performed in the Southern United States in 2012. The trials consisted of 8 treatments with 6 replicates per planting, arranged in a randomized block design. Planting patterns consisted of pure stands of refuge, MON 810, 1507, MIR 162 or 1507xMON 810xMIR 162. In addition, 1507xMON 810xMIR 162 was planted as a seed blend, using 5, 10 and 20% refuge seed blending rates. Since mature larvae only leave one exit hole per ear, larval densities was estimated by counting ears with exit holes. In addition, given that not all *H. zea* larvae leave holes in ears, a subsample of ears within the pure stand treatments were bagged to estimate the total number of pupating *H. zea* per treatment. Ears were not bagged in blended refuge entries to allow larval movement among plants. After silking, ca. 30 plants were randomly marked in each plot and the ears were covered mesh bags. When larvae started to pupate on refuge plants, the numbers of larvae and pupae were determined for each ear. Two trials with artificial insect infestations were also performed in 2012. These included 9 treatments with 6 replicates, arranged in a randomized block design. Planting patterns consisted of pure stands of refuge, MON 810, 1507, MIR162 and 1507xMON 810xMIR 162, or four different seed blend clusters made of 1507xMON 810xMIR 162 plants in combination with refuge plants. The 4 central rows of each six-row plot were artificially infested. To evaluate larval survival, the ears of the five-plant clusters were stripped and checked for the presence of larvae when most larvae had reached 4th and 5th instars. Larval samples were staged based on the width of their	The authors concluded that: *"These results can be used in computer simulation models to evaluate the feasibility of seed blends as a refuge deployment strategy with the pyramid 1507xMON 810xMIR 162. Because the reduction in survival of H. zea due to blending was variable, a sensitivity analysis that includes all possible scenarios of reduction in survival should be considered".*	Environment	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.

	head capsules. Statistical analysis was conducted by performing pairwise comparison. ***Results:*** Very few larvae were recovered from pure stands of MIR 162, 1507xMON 810xMIR 162, or blended 1507xMON810xMIR162. This indicates that both MIR162 and 1507xMON 810xMIR 162 were highly efficacious in reducing the number of larvae at all locations. The results showed variation of the production of *H. zea* on refuge plants from seed blends as compared to pure stand refuge plants. The relative survival of *H. zea* on the events 1507, MON 810, MIR 162, and 1507xMON 810xMIR 162 was similar in the three locations tested.			

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Xu *et al.* Transcriptome differences between Cry1Ab resistant and susceptible strains of Asian corn borer, (2015)	***Objective:*** To compare the transcriptional profile of an Asian corn borer (ACB) strain resistant to Cry1Ab toxin (ACB-AbR) with a susceptible strain (ACB-BtS) using high throughput RNA sequencing (RNA-seq) and bioinformatics tools. ***Experimental Design:*** The corn borers were originally collected from a summer maize field of central China. The strain, with no contact to pesticides, was considered to be susceptible (ACB-BtS). The corn borers were exposed to trypsin-activated Cry1Ab toxin (94% pure protein) throughout larval development for 135 generations, in order to obtain a resistant ACB-AbR strain. Two biological replicates of the transcriptome of the ACB-BtS and ACB-AbR strains were sequenced using Solexa/Illumina RNA-Seq technology. Transcriptome *de novo* assembly was carried out through the short reads assembling program Trinity. The assembled unigenes were used for BLAST searches and annotation against 6 public databases: NCBI non-redundant (NR), NCBI non-redundant nucleotide (NT), Swiss-prot protein, Kyoto Encyclopedia of Genes and Genomes (KEGG), Cluster of Orthologous Groups of proteins (COG), and Gene Ontology (GO) databases. Since the Fragments Per Kilobase Of Exon Per Million Fragments Mapped (FPKM) method is able to eliminate the influence of different gene length and sequencing level on the calculation of gene expression, the calculated gene expression was directly used for comparing the difference of gene expression between samples. The genes differentially expressed between ACB-BtS and ACB-AbR were analyzed using NOISeq. The transcriptome results were verified by quantitative RT-PCR. ***Results:*** Based on the NOISeq method, 3,793 unigenes were considered to be differentially expressed between ACB-BtS and ACB-AbR. Cry1Ab resistance appeared to be associated with change in the transcription level of enzymes involved in growth regulation, detoxification and metabolic/catabolic process. Among previously described Bt toxin receptors, the differentially	The authors concluded that: *"To our knowledge, this is the first comparative transcriptome study to discover candidate genes involved in ACB Bt resistance. This study identified differentially expressed unigenes related to general Bt resistance in ACB. The assembled, annotated transcriptomes provide a valuable genomic resource for further understanding the molecular basis of ACB Bt resistance mechanisms*."	Environment	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.

	expressed unigenes associated with aminopeptidase N and chymotrypsin/trypsin were up-regulated in ACB-AbR whereas other putative Cry receptors, cadherin-like protein, alkaline phosphatase, glycolipid, actin, V-type proton ATPase vatalytic, heat shock protein, were down-regulated. Finally, GPI-anchor biosynthesis was found to be involved in the significantly enriched pathway and all genes mapped to the pathway were substantially down-regulated in ACB-AbR.			

Area of the environmental risk assessment: Environmental Safety - Agronomy

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
Rocha Gene expression profile and fumonisin production by *Fusarium verticillioides* inoculated in Bt and non-Bt maize, (2016)	***Objective:*** To verify the levels of fumonisins (FUM) produced by *Fusarium verticillioides* on *Bacillus thuringiensis* (Bt) and non-Bt maize, post-harvest, in different periods of incubation under controlled conditions. To compare *FUM* gene expression between Bt and non-Bt hybrids and to study the association between fumonisin production and *FUM* gene expression by *F. verticillioides* for each of the hybrids. ***Experimental Design:*** Samples of maize cultivar Bt 2B710 Hx and 30F35 YG (MON 810) and their isogenic non-Bt 2B710 and 30F35 were provided by the Agronomic Institute of Campinas, Brazil. The seeds were sown in November 2010 and the plants harvested in March 2011. All samples were sterilized by gamma radiation to eliminate the natural mycoflora. The values of fumonisin from the grains were considered to be a basal FB1 or FB2 contamination and were used to calculate the final fumonisin concentration produced by *F. verticillioides* after each period of incubation. Samples were then inoculated with *F. verticillioides* and analysed under controlled conditions of temperature and relative humidity for FB1 and FB2 production and *FUM1*, *FUM3*, *FUM6*, *FUM7*, *FUM8*, *FUM13*, *FUM14*, *FUM15* and *FUM19* expression. 2B710 Hx and 30F35 YG kernel samples were virtually intact when compared to the non-Bt hybrids that came from the field. After each incubation period (10, 20 and 30 days), 10 samples of each hybrid were analysed. ***Results:*** Statistical analysis showed that FB1 production was significantly lower in 30F35 YG and 2B710 Hx than in the 30F35 and 2B710 hybrids ($P < 0.05$). However, there was no statistical difference on FB2 production. The kernel injuries observed in the non-Bt samples possibly facilitated *F. verticillioides* penetration and promoted FB1 production under controlled conditions. *FUM* genes were expressed by *F. verticillioides* in all of the samples. However, there was indication of lower expression of a few *FUM*	The authors concluded that: *"wounded grains possibly facilitated F. verticillioides penetration, leading to higher fumonisin production in non-Bt hybrids during the period of incubation due to the increase of fungal biomass. Management of insects through the use of Bt maize can greatly reduce insect damage, thus indirectly controlling fungal and mycotoxin contamination in the field and the accumulation of mycotoxin during storage. The gene expression analyses demonstrated that further studies should be conducted to determine an acceptable qualitative genetic marker for identifying fumonisin production in early stages of infection by F. verticillioides in maize"*.	Environment	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.

	genes in the Bt hybrids and a weak association between FB1 production and the relative expression of some of the *FUM* genes in 30F35 YG.			

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