

**Appendix 8.2: MON 810 Literature Review:  
Environment (June 2010)**

# MON 810 literature review (June 2010)

## Appendix 8.2 – Environment

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Porcar <i>et al.</i> , 2009)	<p><b>Objective:</b> To examine the effects of the <i>Bacillus thuringiensis</i> delta-Endotoxins Cyt1A, Cry1Ab, Cry3A, Cry4A/Cry4B, and Cry11, on the Pea aphid <i>Acyrtosiphon pisum</i>.</p> <p><b>Experimental design:</b> Full length and trypsin-digested Cyt1A, Cry4A/Cry4B, Cry1Ab and Cry3A, were incorporated into an aphid synthetic diet at different concentrations (32, 125, and 500 ug/mL). For each protein and concentration, 30 nymphs (10 nymphs/box and three repetitions) were bioassayed. Survival time was calculated from aphid deposition on the test diet (day 0). Mortality was surveyed daily, and body weights of survivors were noted at day 7.</p> <p><b>Results:</b> Four <i>B. thuringiensis</i> delta endotoxins, Cry3A, Cry4Aa, Cry11Aa, and Cyt1Aa, were found to exhibit low to moderate toxicity on the pea aphid, <i>A. pisum</i>, in terms both of mortality and growth rate. Cry1Ab was essentially nontoxic except at 500ug/mL.</p>	<p>The authors conclude that, although low, the susceptibility of aphids to <i>B. thuringiensis</i> could theoretically lead to the development of effective strategies for controlling these and other sucking insect pests with genetically modified crops expressing appropriate toxins, contingent on two factors; (i) toxins must be present in the plant phloem (route of exposure) (ii) more effective toxins need to be found.</p>	Environment	No adverse effects were detected in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Interactions between the GM plant with non-target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Kramarz <i>et al.</i> , 2009)	<p><b>Objective:</b> To investigate the influence of <i>Bt</i>-maize on survival and growth of the snail <i>Cantareus aspersus</i>. The effect of <i>Bt</i> soil on the hatchability of eggs from snails not previously exposed to <i>Bt</i> material was also assessed.</p> <p><b>Experimental design:</b> From the age of 4–88 weeks, snails were fed either powdered <i>Bt</i>-maize or non-<i>Bt</i>-maize and exposed to soil samples collected after harvesting either the <i>Bt</i>-maize (MON 810, variety MEB307) or non-<i>Bt</i>-maize (the near-isogenic variety Monumental). There were four treatments: non-<i>Bt</i> soil + non-<i>Bt</i>-maize (MM); <i>Bt</i> soil + <i>Bt</i>-maize (BB); non-<i>Bt</i> soil + <i>Bt</i>-maize (MB); <i>Bt</i> soil + non-<i>Bt</i> maize (BM and 10 replicates per treatment, each replicate consisting of one individual snail per test chamber. Maize material was harvested for silage in October 2002 and 2003 (two batches), freeze-dried before crushing in a mill and sieved through a 0.8 mm mesh, then fed to snails <i>ad libitum</i> three times a week (at the same time, uneaten food and feces were removed). Hatchability of eggs from snails not previously exposed to <i>Bt</i> material was also assessed. Three soil treatments were established: Foulum soil from non-<i>Bt</i> maize, from <i>Bt</i>-maize, and from a fallow soil without maize cultivation. Two series with three replicates of each treatment were performed. In each series, three clutches of snail eggs were used; 20 eggs from each clutch were assigned to one replicate of a given treatment.</p> <p><b>Results:</b> At the end of growth (47 weeks of exposure), snails exposed to <i>Bt</i>-toxin in food and soil (BB) had a growth coefficient (GC) 25% lower than unexposed snails (MM). After the first period of reproduction (68 weeks) a significant difference remained for body mass GC between the BB and MM treatments. Differences in body mass were not significant at the end of exposure (88 weeks). For snails not previously exposed to <i>Bt</i> material, hatchability of eggs was similar in the soils tested.</p>	<p>The authors conclude that, in growing snails, long-term exposure is needed to reveal an effect of <i>Bt</i> maize. The hazard analysis of <i>Bt</i>-maize, based on a worst-case scenario, i.e. snails having no food choice, should now be complemented by other simple measurements, e.g. food intake, to understand the underlying mechanisms involved<sup>1</sup>.</p>	Environment	No adverse effects were detected in this study.
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<sup>1</sup> This study does not constitute a hazard analysis as toxicity of *Bt* maize was not demonstrated. Indeed, differences in growth apparent at 47-68 weeks had disappeared by 88 weeks, and the authors could not rule out nutritional differences between varieties. Furthermore, the authors did not discuss the ecological relevance of their ‘worst case scenario’. It is unlikely that snails would be exposed to *Bt* (or non *Bt*) maize of the same nutritional quality continuously for 88 weeks under a no choice scenario. In addition, the authors did not include a more optimal control diet such as cabbage or lettuce against which to gage the nutritional quality of an all maize diet.

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Peterson <i>et al.</i> , 2009)	<p><b>Objective:</b> To identify the uptake of Cry1Ab <i>Bt</i>-endotoxin by non-target ground beetles (Coleoptera: Carabidae) in the field, in order to test the hypothesis that uptake of lepidopteran-specific <i>Bt</i>-endotoxin will occur at similar rates across single gene and stacked gene transgenic maize fields.</p> <p><b>Experimental design:</b> Four 50m x 50 m fields were planted with either YieldGard MON 810 (expressing Cry1Ab protein), MON 863 (expressing Cry3Bb1 protein), MON 810 x MON 863, and a non-transgenic isoline. Distances between fields ranged from 150 to 800 m and non-<i>Bt</i> crops, including soybean, alfalfa, and sweet pepper, surrounded the maize. Twenty wooden board refuge traps (25x46 cm, 2.5 cm thick) were aligned in transects between rows of maize (five refuge traps spaced 8 m apart in four rows 4 m apart) in each field. Adult ground beetles (Coleoptera: Carabidae) were collected weekly from each refuge trap in all fields between 4 June and 30 September 2007 and frozen prior to ELISA screening for presence of <i>Bt</i>-proteins. Corn samples were screened for presence of <i>Bt</i>-proteins by ELISA.</p> <p><b>Results:</b> Eleven species of carabid beetles were collected as adults from the field containing MON 810. Five species collected from the lepidopteran-specific field screened positive for Cry1Ab protein, and there was no evidence for uptake in the other six carabid species. The carabid species with the proportion of beetles screening positive for the Cry1Ab protein from the lepidopteran-specific field were: <i>Harpalus pensylvanicus</i> (39%, 25 of 64), <i>Stenolophus comma</i> (4%, 6 of 136), <i>Cratacanthus dubius</i> (50%, 1 of 2), <i>Clivina bipustulata</i> (50%, 1 of 2), and <i>Cyclotrachelus sodalists</i> (20%, 1 of 5). The highest proportion of Cry1Ab protein uptake was 4-6 weeks post anthesis. In the field containing MON 810 x MON 863, only one carabid species, <i>H. pensylvanicus</i> (5%, 4 of 75), screened positive for Cry1Ab protein, despite similar expression of this Cry1Ab in plant tissue harvested from both lines. In the coleopteran-specific (MON 863) field only <i>H. pensylvanicus</i> contained recognizable concentrations of the Cry1Ab protein. None of the carabid species screened positive for Cry1Ab protein in the non-transgenic isoline field. However, fewer <i>H. pensylvanicus</i> adults were collected from the isoline field compared to the three transgenic maize plots in this study.</p>	<p>Considerable variation was observed in the number of carabid species, and the percentage of individuals, screening positive for the presence of Cry1Ab protein among the maize plots in this study<sup>3</sup>. Incidence of detection of Cry1Ab protein in a few adult carabids collected from coleopteran-specific (MON 863) maize was not unexpected as the distance of 150 meters from maize plots expressing Cry1Ab protein was within reasonable distance for <i>H. pensylvanicus</i> movement. Based on the results of this study, the authors suggest further studies for the differential uptake of Cry1Ab by carabids, and suggest that risk-assessment of genetically modified crops should consider the exposure rates and routes of <i>Bt</i> protein in the field.</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Interactions between the GM plant with non-target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

<sup>3</sup> Discussion of differential uptake of Cry1Ab among multiple transgenic events, and differences in the percentage of individuals testing positive for the presence of Cry1Ab is of questionable value in a non-replicated study.

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Bohn <i>et al.</i> , 2010)	<p><b>Objective:</b> To perform a demographic analysis of previously published data (Bohn <i>et al.</i>, 2008) combined with unpublished data from a predation risk treatment to investigate whether <i>Bt</i>-maize might have a negative impact on a non-target model organism, <i>Daphnia magna</i>.</p> <p><b>Experimental design:</b> Ten individually housed <i>D. magna</i> neonates in 60 L of media were fed a ground grain suspension from a MON 810 hybrid or a local conventional variety (both identified as Dekalb 818 variety) over 42 days. The endpoints that were evaluated included survival, growth and reproduction. The experiment was replicated three times. Leslie projection matrixes were used to estimate the main demographic statistics for treatment. Cry1Ab was quantified in grain by ELISA.</p> <p><b>Results:</b> Higher mortality, a lower proportion of <i>Daphnia</i></p>	The observed effects were caused by a toxic effect rather than lower nutritional value of corn grain from MON 810 and consequently greater attention is needed to assess potential risks resulting from exposure of aquatic organisms to transgenic products. <sup>2</sup>	Environment	The authors of this paper reported a toxic effect of a diet containing MON 810 on <i>Daphnia magna</i> growth. However, given the poor experimental design, the physiological relevance of the findings is questionable; conclusions of adverse effects cannot be drawn from this study.

<sup>2</sup> Methodological deficiencies of the original Bøhn et al paper (2008), which forms the core of this current study, have been criticized by several authors (Bartsch et al (2010), Ricroch *et al* (2010)) who concluded that this is not a valid study to assess potential effects and risk to non-target aquatic organisms. In this study, *D. magna* were under extreme nutritional stress evidenced by measurements on growth, reproduction and days to first brood. Additionally, this study did not meet the validity criteria for a *D. magna* reproduction study as outlined in OECD 211. Significant deviations from OECD 211 guideline include:

- a) The validity criterion of greater than or equal to survival of 60 offspring per parent was not met. The mean number of young per female for the control treatment was approximately six, 10-fold lower than the acceptance criterion. This very low level of reproduction is even more significant because *D. magna* were allowed to brood until 42 days after the initiation of the study. The OECD 211 acceptance criterion is based on only a 21 day study.
- b) There is no indication that the test animals were derived from a healthy stock as is routinely required for these studies (showing no signs of stress as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloration).
- c) Dissolved oxygen measurements and pH measurements were not reported. So it is impossible to assess the water quality that this study was run under.

The authors report an earlier mean onset in reproduction for the MON 810 treatment (~15.5 days) versus the control treatment (18 days). Typically, the onset of reproduction for *D. magna* is about 8 days. This significant delay in the onset of reproduction for the control treatment is an additional sign of nutritional stress related to a planktivorous organism being fed corn grain. Another significant indicator of nutritional stress is the short measured body lengths of the *Daphnia* in this study. At the end of the study, lengths were approximately 2.5 mm. Typically, in a 21-day study *D. magna* are 4.5 mm in length. For *D. magna* there is strong correlation between length and body weight measurements, so it can be concluded that the masses of the *Daphnia* were also significantly lower than are typically measured in a study that follows an internationally approved test guideline. Based on the very significant flaws in this study, primarily related to extreme nutritional stress in both the control and test treatments, the results from this study cannot be used to assess the risk of MON 810 to aquatic organisms nor does it demonstrate risk to aquatic organisms.

	<p><i>magna</i> reaching reproductive status and reproduction reduction in the MON 810 treatment was observed.</p>		<p><b>Observed parameter</b></p>	<p><b>Feedback on initial environmental risk assessment</b></p>
			<p>Interactions between the GM plant with non-target organisms</p>	<p>There are no changes to the conclusions of safety of the initial risk assessment.</p>

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Perry <i>et al.</i> , 2010)	<p><b>Objective:</b> The objective of this paper was to develop a refined exposure assessment model to facilitate an evaluation of potential risk to non-target Lepidoptera resulting from exposure to pollen from MON 810 and other maize products.</p> <p><b>Experimental design:</b> An 11-parameter deterministic mathematical model was developed to evaluate the exposure for three lepidopteran species and their host plants, all of which occur widely throughout the EU. These were: the Peacock butterfly feeding on its nettle host-plant <i>Urtica dioica</i>; the Red Admiral butterfly also feeding on <i>U. dioica</i>; and the Diamond-backed moth and its host plant species within the family Brassicaceae. The moth was chosen as a ‘worst-case’ species because it has been shown to be very sensitive to the Cry1Ab protein expressed by MON 810. The parameter values chosen for the model were informed by field data where available from Germany, Italy, Spain, and Hungary and supplemented by the consensus estimates of experts.</p> <p><b>Results:</b> Estimated environmental impact to non-target lepidoptera in all regions was determined to be low. Neglecting density-dependent effects that might be important, a simplified analysis predicted that the expected population decline owing to maize MON 810 would not exceed 5 per cent over 10 years. Such a small decline would be difficult to detect in practice because of the natural fluctuations and trends in lepidopteran populations.</p>	The authors conclude that predicted environmental impact on the studied non-target lepidopteran larvae owing to exposure to potentially harmful amounts of pollen deposited on host plants in or near maize MON 810 fields was low and considered to pose no unacceptable effects to non-target lepidopteran populations.	Environment	No adverse effects were detected in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Interactions between the GM plant with non-target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Erasmus <i>et al.</i> , 2010)	<p><b>Objective:</b> to evaluate the impact of <i>Bt</i> maize events (MON 810, Bt11) on <i>Agrotis segetum</i> larval mass, development time, and survival, as well as fecundity of moths.</p> <p><b>Experimental design:</b></p> <p>(i) Two laboratory experiments involved first-instar larvae and fourth-instar larvae were laid out as a completely randomized design. Each experiment included four treatments: MON 810 maize, non <i>Bt</i> isolate for MON 810 maize, Bt11 maize, and non-<i>Bt</i> isolate for Bt11 maize. Seven to ten days old seedlings were placed in test tubes. One larva was placed per test tube that was plugged with cotton wool. Each maize hybrid was replicated 50 times for the experiment with first-instar larvae and 70 times for the experiment with fourth-instar larvae. Larval survival, larval development, and mean larval mass were recorded every 3-4 days until the onset of pupation. Percent pupation over time was also determined.</p> <p>(ii) The fecundity, fertility, and longevity of eighty mating pairs (20 per treatment) of moths derived from larvae fed on <i>Bt</i>- and non <i>Bt</i>-maize from the fourth-instar onward were also determined. The mating pairs were kept in a round plastic container with opening covered with gauze to serve as oviposition site. Water was provided by placing a sponge saturated with sugar water. Mortality of male and female moths was recorded at 24-h intervals, and eggs were collected until moths died. The number of eggs laid each night as well as the number of eggs that hatched per moth was recorded and expressed as a percentage.</p> <p><b>Results:</b> (i) The effects of the two <i>Bt</i> maize events on the different parameters measured in this study were not similar between the <i>Bt</i> events and their respective isolate hybrids. Compared with larvae that fed on conventional (non-<i>Bt</i>) maize, <i>Bt</i> maize did not affect survival of first-instar larvae. However, mean mass of larvae that fed on <i>Bt</i> maize (Bt11) was significantly lower. Feeding on <i>Bt</i> maize did not have a significant effect on development and survival of fourth-instar larvae or moth longevity. However, it delayed the larval development period to pupation. (ii) Fewer eggs were laid by moths fed as larvae on maize event Bt11 compared with MON 810.</p>	<p>The authors conclude that <i>Bt</i> maize events MON 810 and Bt11 expressing Cry1Ab toxin will have no effect as control method for <i>A. segetum</i>. Although some significant effects were observed on larval mass and fecundity of moth in some instances under laboratory conditions, it seems that the <i>Bt</i> maize events MON 810 and Bt11 will most likely not have any effect on this non-target pest under field conditions.</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Interactions between the GM plant with non-target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Jensen <i>et al.</i> , 2010)	<p><b>Objective:</b> to assess risk to non-target arthropods associated with transgenic corn debris entering streams.</p> <p><b>Experimental design:</b> (i) Corn tissue input into a stream in central Maryland was monitored with wire mesh cages placed every 20m within 1m of the stream and checked weekly from September 2006 to May 2007 and September 2007 to April 2008. Benthic surveys were also conducted for any identifiable corn tissue visible on the streambed.</p> <p>(ii) Plants from three hybrid families with different genetic backgrounds were grown at three locations as sources of senesced corn tissue for both the time-course bioassay and the non-target bioassay. Within each hybrid family three near isolines were used, all of which contained the Roundup Ready gene (NK603): (1) a non-<i>Bt</i> expressing near-isoline, (2) a near-isoline expressing the Cry1Ab gene (MON 810), and (3) a near-isoline stacked with both Cry1Ab and Cry3Bb1 genes (MON 810 x MON 863) for a total of nine near-isolines. To obtain a robust estimate of Cry1Ab protein activity in senesced leaves before environmental exposure, the growth response of the target European corn borer on corn leaves at time of harvest was measured with diet incorporation bioassays. To test the hypothesis that the Cry proteins remain biologically active in corn leaves after harvest in the environment, leaf tissue collected at 2-week intervals and tested using the same diet-incorporated feeding bioassay.</p> <p>(iii) Diet incorporation leaf tissue bioassays were also conducted on four non-target aquatic shredders: Larvae of two closely related caddisflies <i>Lepidostoma</i> spp. and <i>Pycnopsyche scabripennis</i>, a crane fly larva <i>Tipula abdominalis</i> and an isopod <i>Caecidotea communis</i>. All corn tissue was “conditioned” for 2 weeks before bioassay initiation in 20:80 filtered Clarksville site stream water to deionized water to ensure bacterial and fungal growth on the leaf tissue, which is an integral component of detritus for shredder feeding</p> <p><b>Results:</b> (i) Input of corn tissue debris occurred just after harvest, but peak input was delayed until February (2008) or March (2007).</p> <p>(ii) Bioassays using <i>Ostrinia nubilalis</i> (European corn borer) verified the bioactivity of the Cry1Ab proteins in senesced corn before environmental exposure. No bioactivity of Cry1Ab protein in senesced corn tissue was</p>	<p>The authors conclude that exposure of the senesced leaf tissue to environmental conditions in both terrestrial and aquatic environments eliminated any detectable bioactivity against the corn borer after 2 weeks and point out this is especially relevant because it is a shorter interval than the delay observed between harvest and peak input into a stream adjacent to a corn field. The lack of detectable bioactivity against <i>O. nubilalis</i> after 2 wk is also relevant to the non-target bioassay results because all of the corn tissue used in the non-target bioassays had been “conditioned” in stream water for 2 weeks before incorporation in the bioassay. Both of these factors (delayed input and conditioning time) suggest that any differences in the growth and survival of the non-target taxa are not likely caused by exposure to the Cry proteins. Furthermore the authors conclude that adverse effects to aquatic non-target shredders involve complex interactions arising from plant genetics and environment that cannot be ascribed to the presence of Cry1Ab proteins.</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Interactions between the GM plant with non-target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

	detected after two weeks of exposure to terrestrial or aquatic environments.  (iii) Growth and survivorship of <i>Lepidostoma</i> spp. and <i>Pycnopsyche scabripennis</i> were not negatively impacted whereas <i>Tipula abdominalis</i> showed reduced growth rates and <i>Caecidotea communis</i> exhibited reduced growth and survivorship on the Cry1Ab near isolate but not on the stacked Cry1Ab and Cry3Bb1 near isolate.			
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Area of the environmental risk assessment: Environmental Safety – Non-Target Organisms

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Dorhout and Rice, 2010)	<p><b>Objectives:</b> To examine the influence of genetically-modified Bt corn (MON 810) on the survival of <i>Striacosta albicosta</i> (western bean cutworms) during intraguild competition with <i>Ostrinia nubilalis</i> (European corn borer) and <i>Helicoverpa zea</i> (corn earworm) larvae.</p> <p><b>Experimental design:</b> <i>S. albicosta</i>, <i>O. nubilalis</i> and/or <i>H. zea</i> larvae of various sizes were placed together either on artificial diet, corn silk (MON 810 or isoline) or on corn ears (MON 810 or isoline) in corn cages in the field. A completely randomized design was used with treatments replicated based on availability of western bean cutworm and corn earworm larvae. The experiment had three components and followed a 2 X 5 X 13 treatment design that was unbalanced and incomplete. The three components were competition arena, larval diet, and intraguild competition. For <i>S. albicosta</i>/<i>H. zea</i> interactions, 3-7 reps (one each from both species) were conducted. For 3-way interactions, 8-25 reps (one each from all three species) were conducted.</p> <p><b>Results:</b> <i>S. Albicosta</i> survival was significantly reduced (often to 0) in the presence of <i>H. zea</i> on non-Bt diet or silk. On Bt silk, <i>S. Albicosta</i> survival was significantly higher than controls in the presence of <i>O. nubilalis</i>. The addition of <i>O. nubilalis</i> did not affect the results. In field trials, there was no difference between <i>S. Albicosta</i> survival in the presence or absence of <i>H. zea</i> larvae.<sup>3</sup></p>	<p>These data suggest that MON 810 may confer a competitive advantage to <i>S. Albicosta</i> larvae during intraguild competition particularly from <i>H. zea</i>, and that <i>S. Albicosta</i> cutworms became equal competitors only when they are of equal or larger size. Therefore, if <i>H. zea</i> was restricting <i>S. Albicosta</i> movement or population increase prior to MON 810 introduction, the use of MON 810 may explain the increase in population and movement of <i>S. Albicosta</i> by reducing <i>H. Zea</i> predation of <i>S. albicosta</i>.</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Interactions between the GM plant with non-target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

<sup>3</sup> The study by Dorhout and Rice is inconclusive: An effect in the field with MON 810 was not seen. The only time an effect was observed was primarily when *S. Albicosta* and *H. zea* larvae are placed on the silk at the same time (a rare event in the natural field situation).

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
<p>(Cancino-Rodezno <i>et al.</i>, 2010)</p>	<p><b>Objective:</b> To further elucidate the response of insects (Lepidoptera: <i>Manduca sexta</i>; Diptera: <i>Aedes aegypti</i>) to intoxication of <i>Bt</i> toxins by measuring the activation of mitogen-activated protein kinase p38 (MAPKp38) after <i>Bt</i> Cry protein intoxication (including non-toxic <i>Bt</i> Cry protein mutants), and to knock out MAPK p38 using MAPK p38 dsRNA and see the response to <i>Bt</i> Cry protein intoxication</p> <p><b>Experimental design:</b> Cry1Ab (lep-active) and Cry11Aa (mosquito-active) produced from single gene clones. <i>M. sexta</i> diet overlay bioassays with Cry1Ab (and mutants) conducted with 24 larvae. Mortality assessed after 7 days and LC50 values determined using Probit Analysis. <i>A. aegypti</i> bioassays with Cry11Aa (and mutants) conducted in 100 ml water. Ten 4<sup>th</sup> instar larvae were added. Mortality recorded after 24 hours and LC50 values conducted as above. Western blot and RT-PCR analysis was conducted on excised insect midguts after <i>Bt</i> intoxication to check for MAPK p38 expression. RNAi bioassays using dsMAPK p38 dsRNA (made from both <i>M. sexta</i> and <i>A. aegypti</i>) were conducted on 100 <i>M. sexta</i> neonates (a single dose of 1 µl drop containing 5µg Ms-p38 dsRNA) or 200 <i>A. aegypti</i> neonates (200 µg Ms-p38 dsRNA) until third or fourth instar, respectively, in which guts were extracted for RT-PCR and Western Blots.</p> <p><b>Results:</b> <i>M. sexta</i> was susceptible to Cry1Ab, but not Cry1Ab mutants. Gut extracts from <i>M. sexta</i> show that MAPK p38 expression increased in the presence of Cry1Ab but not mutants. Gene silencing of <i>M. sexta</i> MAPK p38 in the presence of Cry1Ab resulted in an increase in toxicity of 8 fold. <i>A. aegypti</i> was susceptible to Cry11Aa, but not Cry11Aa mutants. Gut extracts from <i>A. aegypti</i> show that MAPK p38 expression increased in the presence of Cry11Aa but not mutants. Gene silencing of <i>A. aegypti</i> MAPK p38 in the presence of Cry11Aa resulted in an increase in toxicity of 10 fold.</p>	<p>MAPK p38 is activated at the posttranslational level after Cry toxin intoxication in Lepidoptera and Diptera. Gene silencing of MAPK p38 resulted in increased susceptibility to <i>Bt</i> Cry toxins suggesting that the MAPK p38 pathway is involved in insect defense against <i>Bt</i> Cry toxins.</p>	Environment	No adverse effects were detected in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Interactions between the GM plant with non-target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Prasifka <i>et al.</i> , 2010)	<p><b>Objective:</b> To examine how resistance to <i>Bacillus thuringiensis</i> Cry1Ab toxin (in diet and <i>in planta</i>) influences movement and survival of <i>Ostrinia nubilalis</i> (European corn borer) as it relates to <i>Bt</i> resistance risk.</p> <p><b>Experimental design:</b> The Cry1Ab-resistant <i>O. nubilalis</i> colony (R-Kandi; relatively high levels of resistance (<math>\approx 1000</math>-fold) enabling it to be useful for short-term tests) used was capable of survival on MON 810 maize. A parental susceptible population was used as negative control. For tracking, larvae were placed on untreated and <i>Bt</i> treated diets [<i>Bt</i> maize leaf powder was incorporated into the diet (no final Cry1Ab concentration provided)] for 12 hours with 30 larvae per treatment. A generalized linear model (PROC GLM) was used to test for effects of insect line, diet, and insect line X diet interactions. Four aspects of larval movement were assessed. Comparisons among treatments were made with least-squares estimates and t-tests. Relationships between pairs of video-tracking variables were examined using Pearson's correlation coefficients. <i>In planta</i>, 3-4 <i>O. nubilalis</i> egg masses were placed on a <i>Bt</i> plant, with a non-<i>Bt</i> plant on each side to assess possible movement. Plants were dissected after 48-72 hours to count dead and living insects. PROC GLM was used to test for the effect of line (resistant or susceptible) on each of the three proportions (n=15-78): (i) survival for dispersing larvae, (ii) survival after 48-72 hr, and (iii) movement to adjacent plant.</p> <p><b>Results:</b> On <i>Bt</i> treated diet, susceptible and hybrid larvae demonstrated increased larval movement compared to Cry1Ab-resistant <i>O. nubilalis</i> (but Cry1Ab-resistant <i>O. nubilalis</i> had higher larval meander values). On Cry1Ab maize, movement off of <i>Bt</i> maize and survival were greater for the Cry1Ab-resistant <i>O. nubilalis</i>.</p>	<p>The results suggest that simplified Petri dish tests may not be predictive of larval movement among non-<i>Bt</i> and insect-resistant <i>Bt</i> maize plants. The results showed lower estimates of susceptible larvae dispersing to non-<i>Bt</i> crops than estimated by other researchers, which would suggest that evolution of resistance to <i>Bt</i> maize could tend to be faster than previously estimated<sup>4</sup>.</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Interactions between the GM plant and target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

<sup>4</sup> In the discussion the authors state that resistant *O. nubilalis* could move from resistant to non-*Bt* plants, implying *Bt* resistance concerns. This happens only under a refuge in a bag (RIB) scenario, and currently there are no RIB proposals using a single *Bt* protein (Cry1Ab) to control *O. nubilalis*.

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Crespo <i>et al.</i> , 2009)	<p><b>Objective:</b> To use a field-derived resistant strain of <i>Ostrinia nubilalis</i> (European corn borer) to define the nature of resistance to Cry1Ab toxin by examining the inheritance and on-plant survival of susceptible and resistant insects and their F1 progeny.</p> <p><b>Experimental design:</b> A field derived Cry1Ab-resistant <i>O. nubilalis</i> colony underwent further <i>in vitro</i> and <i>Bt</i> maize leaf disk selection in the laboratory resulting in a strain (at generation 21) capable of tolerating a 20-fold excess of a Cry1Ab diagnostic dose (1000 ng/cm<sup>-2</sup>, trypsin-activated Cry1Ab). Additionally, two different susceptible strains were also used for the on-plant bioassays and inheritance experiments. Bioassays were conducted using CellCap<sup>®</sup> Cry1Ab protoxin initially and after 21 generations a trypsin-resistant Cry1Ab toxin. Dominance of resistance, sex linkage and maternal effects were determined using progeny from F1 reciprocal mass crosses. For all crosses, 100 pupae of each strain/sex were pooled. Between 512 and 1640 larvae were used to determine responses to Cry1Ab. Pollen (48 larvae), silk (32 larvae) and on-plant survival (10 plants/treatment with 10 neonates/plant) was conducted using Bt11 (N4242YG), MON 810 (Pioneer 38G17Bt) and Event 176 (Mycogen 2657 Bt) and their isolines (pollen and silk bioassays) and MON 810 (RX 634) and isolate (RX634) for on-plant bioassays. The data obtained in survival bioassays with backcross progeny were used to determine the number of loci by three different approaches including Lande's method, the direct test for monogenic inheritance and indirect tests of models with one, two, five and ten loci. Larval weights were tested for normality using the Shapiro–Wilk test. Weight values were square root transformed. Data were analyzed using a two-way ANOVA with <i>O. nubilalis</i> type and <i>Bt</i> events as main factors. Treatment means were separated using least-squares means tests at a 5% significance level. The survival data were analyzed as binomial with 95% confidence intervals for binomial proportions calculated using the modified Wald method. The traits (and combinations) used in the calculation of effective dominance on plants were larval survival and percentage weight gain. Survival on Cry1Ab maize was estimated by adjusting for mortality observed on non-expressing plants. Percentage weight gain was calculated relative to the larval weight of each type on isolate plants. For each <i>O. nubilalis</i> type, the frequency distribution of instars found on MON 810 plants was compared against the frequency distribution of instars found on isolate plants using a chi-square test.</p> <p><b>Results:</b> The <i>O. nubilalis</i> resistant strain exhibited &gt;800-fold resistance to Cry1Ab. Resistance was primarily autosomal and controlled by more than one locus or multiple alleles at one locus. The degree of dominance <i>D</i> calculated on the basis of LC50 values was -0.45, indicating that resistance was incompletely recessive. No survivors were found on vegetative-stage <i>Bt</i> corn, although both resistant larvae and their F1 progeny were able to survive on reproductive corn 15 days after infestation.</p>	<p>The authors conclude that a field derived <i>O. nubilalis</i> strain can exhibit high levels of resistance<sup>5</sup> to Cry1Ab but cannot survive on transgenic corn vegetative tissue. Also, the Cry1Ab resistance is primarily autosomal, incompletely recessive and polygenic. Finally, the dominance of the resistance is dependant on the plant stage.</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Interactions between the GM plant and target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

<sup>5</sup> Bioassays were conducted only for 15 days and therefore survival does not reflect survivorship to adult stage. Because development was delayed even on reproductive tissue, incomplete recessiveness in a field situation is questionable

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Lopez <i>et al.</i> , 2010)	<p><b>Objective:</b> to examine the potential impact of <i>Nosema pyrausta</i> infections on survival and developmental rate of <i>Bt</i>-resistant and susceptible populations of <i>Ostrinia nubilalis</i> (European corn borer). These results will help determine if <i>N. pyrausta</i> infections will have an affect on <i>O. nubilalis</i> mating between potential resistant insects emerging from a <i>Bt</i> maize field and susceptible insects emerging from the non-<i>Bt</i> structured refuge.</p> <p><b>Experimental design:</b> Infected (I) and non-infected (NI) susceptible (S) and Cry1Ab-resistant (<i>Bt</i>-R) <i>O. nubilalis</i> were placed on control diet (CD) or Cry1Ab diet (Cry) and examined daily for pupation, adult emergence or death. The six treatments (n = 100) were: (i) S CD (NI), (ii) S CD (I), (iii) <i>Bt</i>-R CD (NI), (iv) <i>Bt</i>-R Cry (NI), (v) <i>Bt</i>-R CD (I), and (vi) <i>Bt</i>-R Cry (I). Data were log<sub>10</sub>-transformed. Survival estimates and developmental data were analyzed by using a mixed-model ANOVA.</p> <p><b>Results:</b> Mortality and developmental time for susceptible <i>O. nubilalis</i> were significantly increased when infected with <i>N. pyrausta</i> compared to untreated control. Mortality and developmental time for Cry1Ab-resistant <i>O. nubilalis</i> were significantly increased when fed on Cry1Ab toxin (and similar to susceptible <i>O. nubilalis</i> infected with <i>N. pyrausta</i>) compared to the Cry1Ab-resistant untreated control. Mortality and developmental time for Cry1Ab-resistant <i>O. nubilalis</i> were significantly increased (and mortality was significantly increased when compared to Cry1Ab-resistant <i>O. nubilalis</i> feeding on Cry1Ab toxin) when infected with <i>N. pyrausta</i> compared to Cry1Ab-resistant untreated control. When challenged with both Cry1Ab toxin and <i>N. pyrausta</i>, 96% of Cry1Ab resistant <i>O. nubilalis</i> died (significantly greater than any other treatment)<sup>6</sup>.</p>	<p>The authors conclude that greater larval delays of resistant <i>O. nubilalis</i> feeding on <i>Bt</i> maize could lead to temporal isolation from adults emerging from refuge maize. The resulting assortative mating could hasten the evolution of resistance. However, developmental delays caused by infection with <i>N. pyrausta</i> may increase the likelihood of mating between resistant and infected susceptible adults emerging from refuge maize, producing infected offspring that are also more susceptible to <i>Bt</i> maize.</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Interactions between the GM plant and target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

<sup>6</sup> There were no published citations provided for the highly Cry1Ab-resistant *O. nubilalis* colony capable of surviving on Bt maize. All research was conducted in the lab and therefore relevance in the field is unknown. Additionally, conclusions by the authors are ambivalent in that they cannot say if their results would increase resistance risk or reduce resistance risk. However, as *N. pyrausta* infection rates are probably population dependant, this would suggest that susceptible insects would be more likely to become infected with *N. pyrausta* than the relative rare Cry1Ab-resistant *O. nubilalis*. Therefore the scenario where susceptible adults (delayed due to *N. pyrausta* infection) mate with Bt-resistant *O. nubilalis* (delayed due to development on Bt maize) seems the more likely scenario.

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Goldstein <i>et al.</i> , 2010)	<p><b>Objective:</b> Determine the differences in <i>Ostrinia nubilalis</i> (European corn borer) neonate dispersal behaviour when exposed to <i>Bt</i> corn vs non-<i>Bt</i> corn under controlled environmental conditions; especially quantifying rates of plant abandonment and silking as well as neonate behaviour and movement in a group of plants representing typical row spacing in the field.</p> <p><b>Experimental design:</b> Pioneer 34K78 (MON 810 <i>Bt</i> corn), and Pioneer 34K77 (non-<i>Bt</i> corn) were used and grown in a greenhouse. <u>Plant Abandonment study:</u> Conducted in growth chambers with continuous air flow. Compared one <i>Bt</i> plant vs 1 non-<i>Bt</i> plant; (six leaf stage). Replicated 9 times. One <i>O. nubilalis</i> egg mass (ca. 30 eggs) placed on fifth leaf of both plants. After 24 hrs, plants checked for presence of larvae: Neonates on leaf or in whorl considered as staying on plant, those silking off, on sticky trap, or in water pan were considered as abandoning. <u>Dispersal among group of plants study:</u> Grouped 21 plants from each variety on benches in greenhouse. One center plant surrounded by plants in two circles 76 and 152 cm away. Three treatments and two replicats: (i) All non-<i>Bt</i> corn, (ii) center <i>Bt</i>-corn plant and the rest non-<i>Bt</i> corn, (iii) Center <i>Bt</i> corn, 76 cm way <i>Bt</i> corn and the 152 cm circle non-<i>Bt</i> corn. Only center plant infested with single <i>O. nubilalis</i> egg mass (ca. 30 eggs). Larvae counted on all plants after 24 hr. <u>Silking behavior study:</u> <i>Bt</i> and non-<i>Bt</i> plant placed on table in room with controlled airflow. <i>O. nubilalis</i> neonates pre-exposed to either <i>Bt</i> or non-<i>Bt</i> corn for 1 hr. Randomly, 1 larvae from one pre-exposure treatment placed on <i>Bt</i>, larvae from the other pre-exposure treatment placed on non-<i>Bt</i>. Neonates transferred to center of the top of the leaf at six-leaf corn. Neonates observed for 10 minutes for silking. Replicated 50 times.</p> <p><b>Results:</b> Significantly more neonates abandoned the plant from <i>Bt</i> than from non-<i>Bt</i> corn. Neonates are unable to detect <i>Bt</i> in corn within 10 minutes, but can detect it within the first hour. Neonates that were given the <i>Bt</i> pre-exposure and then transferred to <i>Bt</i> plants silked significantly sooner than neonates transferred to non-<i>Bt</i> plants<sup>7</sup>.</p>	<p>The authors conclude that <i>O. nubilalis</i> neonates hatching on <i>Bt</i> plants are more likely to move to another plant than when hatching on non-<i>Bt</i> plants. Thus, the high dose strategy for IRM could be less effective if larvae are able to get to a non-<i>Bt</i> plant, especially with the mixed plant refuge<sup>8</sup>.</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Interactions between the GM plant and target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

<sup>7</sup> None of these studies were conducted in the field. All plants were grown in greenhouses and *Bt* plants were not checked for expression levels.

<sup>8</sup> In the discussion the authors state that resistant *O. nubilalis* could move from resistant to non-*Bt* plants, implying *Bt* resistance concerns. This happens only under a refuge in a bag (RIB) scenario, and currently there are no RIB proposals using a single *Bt* protein (Cry1Ab) to control *O. nubilalis*.

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
<p>(Arenas <i>et al.</i>, 2010)</p>	<p><b>Objective:</b> To determine whether the glycosylphosphatidylinositol (GLP)-anchored alkaline phosphatase (ALP) could act as a functional receptor of Cry1Ab toxin in <i>Manduca sexta</i>.</p> <p><b>Experimental design:</b> <i>M. sexta</i> brush border micro-vesicles (BBMV) were prepared from midgut tissue from each larval instar by the magnesium precipitation method. (i) Expression of aminopeptidase N (APN) and ALP in different larval instars was determined by ligand blot analysis. (ii) To determine the role of <i>M. sexta</i> ALP in Cry1Ab toxicity, the binding of Cry1Ab oligomer to pure ALP preparations was analyzed and compared to the binding to pure APN samples. APN was purified from fourth instar larvae to avoid ALP contamination, and ALP was purified from third instar larvae purified fractions containing only APN or ALP activity were examined by SDS-PAGE and silver staining. (iii) To analyse the interaction of oligomeric or monomeric Cry1Ab structures with both receptors, APN or ALP Cry1Ab oligomers were purified by size exclusion chromatography from Cry1Ab protoxin samples activated with <i>M. sexta</i> midgut juice in the presence of a cadherin fragment that contains cadherin repeats (CR) 7–12, whereas monomeric Cry1Ab was prepared by activating Cry1Ab protoxin with trypsin. (iv) Cry1Ab mutants were produced by site-directed mutagenesis Two mutants were located in loop 2 of domain II, and the third mutant was located in the <math>\beta</math>-16 of domain III.</p> <p><b>Results:</b> (i) APN and ALP are present during all instar stages, although at different levels. APN was detectable at low levels in early instars, and higher ALP enzymatic activity than APN during the first instars, whereas APN activity increased after the third instar, showing higher APN activities than ALP in the fifth instar. (ii) APN appears as a single band at 120 kDa, whereas ALP appeared as two bands of 65 and 60 kDa. A Cry1Ab toxin ligand blot showed that both the 120-kDa APN and the 65-kDa ALP bound Cry1Ab. These also indicate that the 65- and 120-kDa bands previously identified by Cry1Ab binding in BBMV samples correspond to ALP and APN proteins, respectively. (iii) Oligomeric Cry1Ab exhibits at least 200-fold higher affinity to both APN and ALP proteins in comparison with the Cry1Ab monomer. (iv) Toxicity assays showed that the three mutants were not toxic to <i>M. sexta</i>. These studies showed that the monomeric structure of the mutant, located in domain III, was severely affected in binding to ALP protein. In contrast, it is clear in this figure that this mutant retained significant binding to APN. The monomeric structures of the domain II loop 2 mutants were not affected in their binding interaction with both APN and ALP. The binding of oligomeric structures of the Cry1Ab mutants to both APN and ALP revealed that domain II loop 2 mutations are affected on binding to both receptors, whereas the domain III mutant had just a marginal effect in its interaction with both receptors.</p>	<p>The authors suggest that APN and ALP fulfil two roles. First APN and ALP are initial receptors promoting the localization of toxin monomers in the midgut microvilli before interaction with cadherin. Then APN and ALP function as secondary receptors mediating oligomer insertion into the membrane. However, the expression pattern of these receptors and the phenotype of L511A mutant suggest that ALP may have a predominant role in toxin action because Cry toxins are highly effective against the neonate larvae that is the target for pest control programs.</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Interactions between the GM plant and target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Xu <i>et al.</i> , 2010)	<p><b>Objective:</b> To report results from selection for Cry1Ab resistance in <i>Ostrinia furnacalis</i> (Asian corn borer) and the different levels of resistance to other <i>Bt</i> toxins.</p> <p><b>Experimental design:</b> <i>O. furnacalis</i> was selected on Cry1Ab toxin for 34 generations before any characterization was initiated. Selection and characterization continued up to generation 71. Tissue (leaves and silk) bioassays on MON 810 were initiated after 34 generations. Susceptible and resistant larvae were assayed with 96 larvae replicated 3 times. Field evaluation using MON 810 and Bt11 of susceptible and resistant <i>O. furnacalis</i> was initiated after 53 generations of selection. Each ear was infested with two egg masses. Number of surviving larvae was recorded after 18 days. Each treatment consisted of 20 plants with 4-7 replications. Cross resistance bioassays were conducted with Cry1Ac, Cry1Ah, Cry1F and CryIIe protoxin after 50 generations of selection with 288 insects tested per treatment. For all bioassays, mortality included dead insects plus insects with less than 0.1mg in weight.</p> <p><b>Results:</b> <i>O. furnacalis</i> could be selected for resistance to Cry1Ab toxin after 35 generations. However, resistance dropped after 51 and 71 generations, presumably because Cry1Ab protoxin was used for resistance determination instead of Cry1Ab toxin. No Cry1Ab-resistant <i>O. furnacalis</i> could survive on MON 810 whorl leaves for 3 days even with 107-fold level of resistance. However, Cry1Ab-resistant <i>O. furnacalis</i> could survive (22% survival vs 67% survival on non-<i>Bt</i> silk) on MON 810 silk for 7 days. A slight number of Cry1Ab-resistant <i>O. furnacalis</i> (5%,6%) could survive on MON 810 and Bt11, respectively when reared on corn ears for 18 days in the field. However, only 7% and 11%.of larvae survived even on the non MON 810 and non Bt-11 plants respectively when infested on corn silk for 18 days in the field. However, only 7% and 10%.of larvae survived even on the iso-negative controls for MON 810 and Bt11, respectively. Cry1Ab-resistant <i>O. furnacalis</i> was highly cross resistant to Cry1Ah, Cry1Ac, slightly cross resistant to Cry1F, and not cross resistant to CryIIe<sup>9</sup>.</p>	<p>The authors were successful in creating a highly Cry1Ab toxin-resistant <i>O. furnacalis</i> colony, but the first determination of resistance was after 35 generations. These insects could survive when fed MON 810 silk, but not whorl leaves. Cry1Ab toxin-resistant <i>O. furnacalis</i> was not cross resistant to CryIIe, with only low cross resistance to Cry1F, indicating that the availability of multiple toxins could improve IRM strategies</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Interactions between the GM plant and target organisms	There are no changes to the conclusions of safety of the initial risk assessment.

<sup>9</sup> In this study, toxin was used for selection, but resistance ratios were determined using protoxin for 2 of the 3 dates. Furthermore, all cross resistance bioassays were conducted with protoxin instead of toxin. In field studies, there was between 90 and 93% mortality in the untreated controls.

Area of the environmental risk assessment: Environmental Safety - Protein / DNA Fate in Soil

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Icoz <i>et al.</i> , 2009)	<p><b>Objective:</b> To determine whether the Cry1Ab protein released in root exudates and from decaying plant residues of <i>Bt</i> maize<sup>10</sup> was taken up by plants from soil in the field on which <i>Bt</i> maize had previously grown and by carrot, as a representative plant, in sterile hydroponic culture to which purified Cry1Ab protein had been added.</p> <p><b>Experimental design:</b> A number of crops, mostly vegetables (basil, carrot, kale, lettuce, okra, parsnips, radish, snap beans, beet, spinach and soybeans) were planted in fields where <i>Bt</i>-maize was grown in a previous year, as well as in fields where no GM-maize was grown for at least 3 years (since 2002). Field experiments were performed during two growing seasons (2005 and 2006). Field design was a randomized complete block in 4 blocks; 1m x 1m plots; 15 varieties in a total of 60 plots. Plant tissue and soil samples were collected and analyzed by Western blot from 2 commercial sources; quantitative immunoassays from 3 commercial sources; and insect bioassay using <i>Manduca sexta L.</i> In addition, hydroponic uptake from a Cry1Ab-spiked solution was investigated using carrot as an uptake model. Each of four replicate field-grown plants was evaluated at least three times by Western blot and at least twice by ELISA, resulting in at least 12 Western blots and eight ELISAs plant per variety. At least five randomly selected carrot plants in hydroponic cultures were evaluated by Western blot and by ELISA. The results of Western blots were expressed as the presence (+) or absence (-) of the Cry1Ab protein. The results of ELISA (ng/g of fresh plant tissue) were expressed as the means ± the standard errors of the means.</p> <p><b>Results:</b> Some plant species grown in soils where <i>Bt</i>-maize had been cultivated took up small amounts of the Cry1Ab protein (typically 0.02 to 0.09 ng/g, with a few samples ranging up to 0.53 ng/g). However, results were inconsistent between detection methods and sampling times. In many cases, results were not detectable in plants collected from <i>Bt</i>-maize fields, but were detectable in plants collected from non <i>Bt</i>-fields. In 2005, ELISA assays showed 0.05 ng/g (basil), 0.02 ng/g (okra) and 0.34 ng/g (snap bean), no ELISA detection in the other 8 crops, some insect mortality (12% to 28%) in all crops, except beets and spinach, and no Cry1Ab detection in any of the soils analyzed. In 2006 there was no detection using Agdia ELISA (except snap beans, 0.04 ng/g), some detection (0.02 to 0.53 ng/g) using the Abraxis</p>	<p>The authors conclude that “Because of the different results obtained in this study with different commercial Western blot and ELISA kits, it is not clear whether the presence of the Cry1Ab protein in the tissues of some plants under field condition and in carrot in sterile hydroponic culture was the result of the uptake of the protein by the plants or of the accuracy and sensitivity of the different commercial kits used. More detailed studies with additional techniques are obviously needed to confirm the uptake of Cry proteins from soil by plants subsequently planted after a <i>Bt</i> crop.”</p>	Environment	The authors imply an uptake of Cry1Ab protein by crop planted in a field where MON 810 was previously cultivated. However, these results are dubious given the lack of biological replication. Therefore, conclusions of adverse effects cannot be drawn from this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Effect on biogeochemical processes	There are no changes to the conclusions of safety of the initial risk assessment.

<sup>10</sup> It is not possible to determine from the text if MON 810 or/and another Bt maize was planted

	<p>ELISA, positive detection of Cry1Ab in soil using ImmunoStrips, and virtually no insect mortality (0 to 6%) in crops collected from <i>Bt</i>-maize fields. However, almost every vegetable tissue collected in 2006 from non-<i>Bt</i> fields showed detection by ELISA (0.02-0.09 ng/g), presence in soil by ImmunoStrip, and insect mortality (12.5% max)<sup>11</sup>. Uptake by carrot from a sterile hydroponic mixture spiked with 100 microgram Cry1Ab showed a maximum concentration of 0.08 ng/g (leaves and stems) and 0.6 ng/g (root), but no detection when Cry1Ab was not added to the hydroponic medium.</p>			
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<sup>11</sup> The field results were inconsistent with a previous paper by the same authors in which no uptake was detected for non-GM corn, carrot, radish and turnip grown in Bt-soil (greenhouse) or in hydroponic medium (Saxena and Stotzky, 2002). It is possible that there was some minor uptake into some crops, but it is also likely that many of the findings reflect false-positive detections due to interference by co-extracted materials at the sub-ng/g level. The authors acknowledge this possibility, as quoted in the conclusions, and suggest further study is needed.

Area of the environmental risk assessment: Environmental Safety - Protein / DNA Fate in Soil

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
<p>(Daudu <i>et al.</i>, 2009)</p>	<p><b>Objective:</b> To investigate the decomposition of residues of <i>Bt</i>-maize and its near-isogenic non-<i>Bt</i> maize, changes of their C and N contents, the activity of the Cry1Ab protein in <i>Bt</i>-maize residues incubated in the field in litterbags and to investigate the effects of lignin content on decomposition.</p> <p><b>Experimental design:</b> A split plot in a randomized complete block design was used. <i>Bt</i>-maize (DKC 78-15B) or near-isogenic maize (CRN 3505) were the main plot treatments, replicated three times. Residues of <i>Bt</i>-maize (MON 810) leaves or stems and near-isogenic leaves or stems were buried in litterbags in the sub-plots two weeks after planting at 10 cm below soil surface. Three litterbags from each subplot were taken at 2, 4, 6, 8, 12, and 16 weeks and analyzed for C, N, Cry1Ab protein, ash-free dry matter (AFDM) and nutrient values. Values of AFDM, C, and N (as percentage of initial amounts) were subjected to ANOVA to determine the effects of (i) the growing maize (<i>Bt</i> or non-<i>Bt</i>-maize), (ii) residues (<i>Bt</i> or non-<i>Bt</i>-maize), (iii) residue type (stem or leaf), and (iv) incubation time. Least significant differences at <math>p = 0.05</math> were used to separate treatment means. Contents of C, N, and AFDM and carbon to nitrogen (C/N) ratios of the different residues were regressed against incubation time. Initial residue characteristics and decomposition rate constants were subjected to correlation analysis.</p> <p><b>Results:</b> Residues of <i>Bt</i>-maize (leaves and stems) had relatively higher contents of P, lignin, cellulose, and total polyphenols than corresponding residues of the near-isogenic line. Neither transgenic nor non-transgenic maize affected the decomposition patterns of the maize residues in the litterbags. Contents of AFDM, C, and N in the leaf and stem residues decreased significantly over time. Decomposition of leaf residues was significantly faster than that of stems, but there were no differences between <i>Bt</i>-maize and its near-isogenic line. The <i>Bt</i>-maize leaf residues had higher initial levels of the Cry1Ab protein (120 ng/g) than stems (75 ng/g). The concentrations of Cry1Ab protein in <i>Bt</i>-maize leaf residues declined in litterbags to &lt;0.02% of initial levels under both cropping systems within 14 days after placement. Furthermore, no measurable concentrations of the protein were observed in soils immediately below the litterbags throughout the study.</p>	<p>The authors conclude that “The Cry1Ab protein did not accumulate as it degraded rapidly under field conditions. The growing of <i>Bt</i>-maize in South Africa and incorporation into the soil may not result in undesirable ecological consequences in soils.” Confirmation studies with different events and other transgenic crops was recommended.</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Effect on biogeochemical processes	There are no changes to the conclusions of safety of the initial risk assessment.

Area of the environmental risk assessment: Environmental Safety - Protein / DNA Fate in Soil

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Swan <i>et al.</i> , 2009)	<p><b>Objective:</b> To investigate the effect of the transgenic nature of senesced maize (<i>Zea mays L.</i>), tissue on breakdown rates, invertebrate abundance and invertebrate community composition in nine streams draining agricultural fields over 2 years (2004–2006).</p> <p><b>Experimental design:</b> Field studies were conducted over two years in nine streams using four hybrids of <i>Bt</i> maize, each with its single (MON 810), stacked (MON 810 x MON 863), and non-<i>Bt</i> conventional near-isoline. In 2004, maize tissue (one hybrid) degradation was assessed gravimetrically using n=4 litterbags x 3 isolines x 6 sites sampled at 7, 14, 21, 28, 36 and 42 days (432 litter bags). In 2005-2006, n=3 litterbags x 3 isolines x 3 hybrids x 4 sites x 4 time points over ~120-day period (432 litter bags) were assessed. Invertebrate abundance was determined by removing organisms from litter bags obtained from streams, combusting, and measuring ash-free dry mass. Total invertebrates were counted, and all trichopteran and other shredder taxa identified to the lowest possible taxonomic level. Litter decay rates were assessed using a multi-factorial indicator variables regression analysis. Partial redundancy analysis was used to assess potential effects on invertebrate community composition. Potential effects of environmental (abiotic) factors on litter breakdown were assessed using principle components analysis.</p> <p><b>Results:</b> In 2004, two instances were identified whereby <i>Bt</i> leaf litter degraded slower (67-68% of control) than corresponding non-<i>Bt</i> near-isolines. In 2005-2006, no differences in litter decay were observed between <i>Bt</i> and non-<i>Bt</i> near-isolines. Multivariate analysis of invertebrate communities found no differences associated with <i>Bt</i> treatment. Principle components analysis identified important abiotic factors (e.g., temperature, dissolved oxygen) as variables influencing litter breakdown, but no interaction was found between the abiotic factors and <i>Bt</i> treatment.</p>	<p>The authors conclude “that maize tissue breakdown is unlikely to be altered by the presence of the <i>Bt</i> trait, but more so by hybrid-germplasm and site-specific factors such as nutrients. Management of agricultural streams will need to consider multiple sources of stress at larger scales, such as nutrient loading and temperature, which probably overwhelm the potential for consumer mediation of ecosystem processes in these ecosystems.”</p>	Environment	No adverse effects were detected in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Effect on biogeochemical processes	There are no changes to the conclusions of safety of the initial risk assessment.

Area of the environmental risk assessment: Environmental Safety - Protein / DNA Fate in Soil

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Zurbrugg <i>et al.</i> , 2010)	<p><b>Objective:</b> Evaluate the decomposition and structural plant components of leaves from <i>Bt</i> and conventional maize hybrids.</p> <p><b>Experimental design:</b> Nine maize hybrids evaluated, including three <i>Bt</i> hybrids (two containing Cry1Ab (Bt11, MON 810) and one Cry3Bb1 (MON 88017)), their near-isolines, and three conventional hybrids. Plants were grown in a climate chamber and leaves were obtained when senescent after 12 weeks. Leaf material was placed in polyethylene mesh litterbags (nine bags per hybrid) and buried at a depth of 5 cm in ten maize fields. Litterbags were collected from each field for each hybrid once per month from Nov 2005 to Jun 2006 (with some exceptions due to limited test materials). Decomposition was measured by loss of dry wt. C:N ratio, cellulose, hemicellulose, and lignin were determined by chemical analyses. Cry1Ab and Cry3Bb1 were determined by ELISA and by insect bioassay (mortality). Differences in decomposition and <i>Bt</i> protein were determined by using a linear mixed effect model. Differences in plant components were determined using one-way ANOVA followed by Tukey HSD multiple comparison post hoc test.</p> <p><b>Results:</b> No differences were detected in decomposition between <i>Bt</i> hybrids and their near isolines, while decomposition did vary significantly among maize hybrids. Differences were observed between C:N ratios of leaves collected directly from plants of <i>Bt</i> hybrids and their near isolines (two lower and one higher), but differences were also observed among conventional hybrids. After five months in soil, none of the <i>Bt</i> hybrids differed in C:N ratio from their near isolines. Levels of cellulose, hemicellulose, and lignin in leaves from <i>Bt</i> hybrids did not differ from near isolines collected directly from plants or after five months in soil. Greater than 99% of the Cry1Ab and Cry3Bb1 proteins in leaves was degraded after nine months in soil, and activity of the Cry proteins determined by insect bioassay was not different from the corresponding conventional near isolines after four months in soil.</p>	<p>The authors conclude that “the decomposition dynamics of transgenic hybrids were similar to the non-transgenic near-isolines, but varied among conventional hybrids, demonstrating that <i>Bt</i>-transgenic maize hybrids lie within the variation found in conventional agroecosystems. The present study gives no indication of deleterious effects of <i>Bt</i> maize on the activity of the decomposing community.”</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Effect on biogeochemical processes	There are no changes to the conclusions of safety of the initial risk assessment.

Area of the environmental risk assessment: Environmental Safety – Protein / DNA Fate in Soil

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
<p>(Badea <i>et al.</i>, 2010)</p>	<p><b>Objective:</b> Investigate if there is a correlation between soil-type and Cry1Ab expression of MON 810 (root, leaf and seed) planted in three different soils, and monitor the time-dependent degradation of Cry1Ab in the same 3 soils following the soil-incorporation of the MON 810 plants.</p> <p><b>Experimental design:</b> Conventional (DKC5783; non-GM) and MON 810 corn (DKC5784YG) were grown in a greenhouse, in soils collected from 3 corn-producing regions of Romania. For each soil, 8 pots x 18 Kg soil/pot, 4 non-GM replicates and 4 MON 810 replicates (2 plants/pot). For correlation of expression vs. soil type, tissue samples of root and leaves were collected at the 10-leaf stage and at senescence, and seed samples at maturity. Soil samples (rhizosphere) were also collected from 2 plants at each time of tissue collection. To monitor time dependence of soil degradation, large pieces of senescent plants were incorporated into soil, simulating post-harvest farming practice. Soil samples were collected prior to plant incorporation and at 3, 6, 9, 12 and 15 weeks after plant incorporation from each of 4 replicate soil pots per soil type. Soil specimens from each of the 4 replicates were bulked into a single analytical sample per time-point per soil. Analysis of <i>Bt</i> protein was conducted in two homogenized samples of each soil (MON 810) and one control (non-GM) from each sample-collection time. Analysis was done using a commercial Cry1Ab/Cry1Ac ELISA kit. Soil specimens (1 g) were vortexed with 10X PBST buffer and centrifuged. Supernatants were analyzed by ELISA. Extraction efficacy and detection limits were determined by adding known amounts of Cry1Ab protein to control soil samples.</p> <p><b>Results:</b> Soils ranged from pH 5.6 to 8; organic matter from 1.8 to 3.5% and clay 19.7 to 39.3%. For expression studies, no significant differences were observed in plants grown in the 3 soils: Mean leaf expression ranged 8.78 to 9.56 µg/gfw; senescent leaf 0.11 to 0.25 µg/gfw; root 1.67 to 2.20 µg/gfw; senescent root 0.73 to 0.92 µg/gfw and seed 0.44 to 0.50 µg/gfw. No Cry1Ab residues were found in the rhizospheric soil samples collected at time of tissue-sample collection. For monitoring the degradation of Cry1Ab in soil after MON 810 plant incorporation, the reported LOD was 0.01 ng Cry1Ab/g soil. Maximum detected Cry1Ab in soil was 0.6 ng/g, at 6 weeks after soil-incorporation of MON 810. The initial increase in Cry1Ab soil concentration was detected in all soils until about 6-9 weeks, followed by degradation to the 0.1 to 0.2 ng/g Cry1Ab range at the 12-15 week sampling intervals.</p>	<p>Data from this study indicates that expression of Cry1Ab protein in a variety of tissues is not dependent on the soil type on which the MON 810 plants are grown. The lack of Cry1Ab protein residue in rhizospheric soil samples during the growing season, and its detection in bulk soil after soil-incorporation of plant residues indicates that plant tissues are the main source of the Cry1Ab present in soils after harvest. After MON 810 plant incorporation into soil, residues of Cry1Ab increase in soil until peaking at 6-9 weeks. Cry1Ab protein in soil then declines slowly toward the 12-15 week interval (3-4 months after soil-incorporation). The authors conclude that "...the Cry1Ab protein does not persist or accumulate in different soil types after incorporation of <i>Bt</i>-corn plants".</p>	Environment	No adverse effects were detected in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Effect on biogeochemical processes	There are no changes to the conclusions of safety of the initial risk assessment.

Area of the environmental risk assessment: Environmental Safety – Protein / DNA Fate in Soil

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Raubuch <i>et al.</i> , 2010)	<p><b>Objective:</b> This study evaluated the effects of straw from two transgenic <i>Bt</i>-maize varieties and two near-isogenic varieties on microbial growth and maintenance in soil during early decomposition.</p> <p><b>Experimental design:</b> A laboratory experiment was performed consisting of 5 treatments replicated 5 times in which maize tissue was dried, ground and mixed with soil. The treatments were: a non-amended control, 40 mg Nobilis (non-<i>Bt</i>)/g moist soil, 40 mg Novelis (MON 810)/g soil, 40 mg Prelude (non-<i>Bt</i>)/g soil, and 40 mg Valmont (<i>Bt</i>176)/g soil. The soil-tissue mixtures were adjusted to 50% water holding capacity and incubated at 15°C in the dark. Soil respiration was measured after 1, 2, 4, 7, 9, 11, 17, and 21 days. Another series of five replicates were prepared and destructively sampled for detection of ATP and microbial biomass C at day 2 and 21. ATP and microbial biomass carbon were measured as biomass indices and CO<sub>2</sub> production rate as an indicator of activity. Specific respiration rate (CO<sub>2</sub>-C/ATP and CO<sub>2</sub>-C/microbial biomass C), adenylate energy charge (AEC), and energy loss rates were calculated as indices of maintenance requirements and substrate use efficiency.</p> <p><b>Results:</b> Microbial activity as measured by CO<sub>2</sub> production rate was significantly higher for <i>Bt</i> maize varieties compared to non-<i>Bt</i> varieties on days 2 and 4, but was significantly lower on day 21. Microbial biomass carbon from <i>Bt</i> varieties was also significantly lower than in non-<i>Bt</i> varieties on day 21. ATP content indicated a growth phase up to day 2 followed by a stationary phase. An exception to this was the non-<i>Bt</i> variety Prelude which took approximately 9 days to reach the same ATP content as the other maize varieties. Transient differences were observed in measurements of microbial maintenance requirements and substrate use efficiency. Specific respiration rate as measured by CO<sub>2</sub>-C/ATP was significantly higher for <i>Bt</i> compared to non-<i>Bt</i> varieties at day 2 but not at day 21. There was no difference between <i>Bt</i> and non-<i>Bt</i> varieties for AEC at day 2 or 21. Energy production as measured by CO<sub>2</sub> was significantly higher for <i>Bt</i> varieties from days 0-2 and most of this energy was stored in microbial biomass. From days 3-21 each <i>Bt</i> variety was no longer significantly different from its respective control. A similar pattern occurred for energy loss rate. At day 2 the loss rate was significantly higher for the <i>Bt</i> maize varieties, but on day 21 the <i>Bt</i> varieties were no different from their controls.</p>	<p>The authors conclude that microbial use of the two <i>Bt</i>-maize straw varieties was less efficient than the two corresponding near-isogenic non-<i>Bt</i> varieties<sup>12</sup>. However, the authors do acknowledge that the reasons for the lower use efficiency of <i>Bt</i>-maize straw cannot be fully explained by the present experiment.</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Effect on biogeochemical processes	There are no changes to the conclusions of safety of the initial risk assessment.

<sup>12</sup> In contrast to the authors' reported results, results from several published studies indicate no or negligible impact from the use of *Bt* crops on the soil microflora (Baumgarte and Tebbe, 2005; Cortet *et al.*, 2006; Devare *et al.*, 2007).

**Area of the environmental risk assessment: Environmental Safety (Import) – Spillage and consequences of thereof**

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Park <i>et al.</i> , 2010)	<p><b>Objectives:</b> To investigate whether imported GM maize is released into Korean environment during the transportation of grain in the Republic of Korea.</p> <p><b>Experimental design:</b> Monitoring was conducted in two major grain receiving ports, 15 feed manufacturing plants, and 14 livestock barns in five provinces of the Republic of Korea from July to September 2007. Gel-based PCR was used for the detection of transgenes. For GM maize detection, both 35S promoter and <i>Nos</i> terminator targets were assayed. Event-specific primers were used for identification.</p> <p><b>Results:</b> Based on the event-specific PCR analysis, three maize events (NK603, MON 810, and TC1507) were identified. Spillage of GM seeds was identified in ports, feed manufacturing plants and livestock barns.</p>	<p>Though several GM maize plants were found around the port and feed manufacturing plants, most of these facilities were located inside the industrial park and were far from cultivated fields, likely rendering the impact of these GM maize on the natural environments negligible. However, most of the livestock barns were close to cultivated areas. Moreover, maize plants were cultivated for food or feed near some livestock barns. GM grain imports and transportation may facilitate gene flow from GM maize to non-GM maize plants. Therefore, continuous monitoring is necessary to detect the occurrence of GM maize, and appropriate action should be taken to prevent genetic admixture in our environment.</p>	Environment	No adverse effects were detected in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Persistence and invasiveness	There are no changes to the conclusions of safety of the initial risk assessment.

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