

**Appendix 5.2. MON 810 Literature Review – Environment**

# MON 810 literature review (July 2011)

## 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	Conclusions	Protection goal	Adverse effects
(Álvarez-Alfageme <i>et al.</i> , 2010)	<p><b>Objective:</b> (1) To evaluate whether the coccinellid <i>Adalia bipunctata</i> (Coleoptera: Coccinellidae) larvae are adversely affected by Cry1Ab or Cry3Bb1 at exposure levels (approx. 10 times higher) above those to which the predator larvae would be exposed to in <i>Bt</i> maize fields. (2) To evaluate the effect of Cry1Ab or Cry3Bb1 proteins on <i>A. bipunctata</i> larvae under more realistic routes of exposure through tritrophic studies with the spider mite <i>Tetranychus urticae</i> Koch (Acari: Tetranychidae) fed maize MON 810 and MON 88017. (3) To assess the ability of <i>A. bipunctata</i> larvae to consume whole <i>E. kuehniella</i> eggs and ingest test compounds deposited on the outside of the egg shell, to determine whether the exposure method reported by Schmidt <i>et al.</i> (2009) was suitable for laboratory toxicity studies.</p> <p><b>Experimental Design:</b> (1) purified Cry1Ab and Cry3Bb1 protein feeding studies were conducted to evaluate larval and pupal development of <i>A. bipunctata</i> and weight of emerging adults. Thirty-four to 41 neonate larvae were tested per diet treatment, in which each larva received doses of a 2 M sucrose solution that was prepared with deionized water (untreated control), or a sucrose solution containing 45 µg/ml Cry1Ab, 200 µg/ml Cry3Bb1, 10,000 µg/ml GNA, or 300 µg/ml potassium arsenate on the first day of each instar. Snowdrop lectin (GNA), and potassium arsenate treatments were included as positive controls. (2) Maize varieties, DKc3421Bt (MON 810) and DKc5143Bt (MON 88017), and their corresponding near isolines, were used for tritrophic studies. Four treatment groups of <i>A. bipunctata</i> larvae (1<sup>st</sup> through 3<sup>rd</sup> instar; L1-L3) were fed ad libitum with <i>T. urticae</i> reared separately on each of the maize varieties. ELISAs were used to measure the Cry proteins through the trophic chain. (3) <i>E. kuehniella</i> eggs were provided as the sole food source to 8 larvae, feeding behaviour was observed, and every moth egg that was eaten by the <i>A. bipunctata</i> larvae were tracked.</p> <p><b>Results:</b> (1) No effect on any of the life-history parameters were detected when purified Cry1Ab or Cry3Bb1 were dissolved in sucrose solution (at an approx. 10X high dose higher than measured in <i>Bt</i> maize fed spider mites.) and fed to <i>A. bipunctata</i> larvae. In contrast, the positive controls (GNA and potassium arsenate) caused adverse effects to <i>A. bipunctata</i> larvae on all life-history parameters measured. (2) There were no significant differences for <i>A. bipunctata</i> larval mortality, weight, or development time with ingestion of <i>T. urticae</i> fed MON 810, MON 88017, or the corresponding non transgenic near isolines. ELISA showed a drastic reduction (&gt; 90%) of the Cry proteins through the trophic chain from plant leaf tissue to <i>A. bipunctata</i> larvae. (3) First and second instars of <i>A. bipunctata</i>, when preying on <i>E. kuehniella</i> eggs, both sucked out their contents until they were depleted. “No larva was observed consuming whole eggs or even parts of the egg shell.”</p>	<p>Ingestion of the Cry1Ab protein by <i>A. bipunctata</i> did not affect larval mortality, weight, or development time.</p> <p>The authors overall conclusion is that their results “show that the harmful effects of the two Cry proteins reported in Schmidt <i>et al.</i> (2009) were likely false positives, i.e., artifacts of poor study likely due to factors other than direct toxicity of the two Cry proteins”.</p>	Environment	No effect on any of the measured life-history parameters were detected
			Observed parameter	Feedback on initial environmental risk assessment
			Interaction between the GM plant and NTO	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Balog <i>et al.</i> , 2010)	<p><b>Objective:</b> to investigate the mechanism of abundance, species richness, diversity, and similarity of rove beetles (Coleoptera: Staphylinidae) in MON 810 and near-isogenic maize.</p> <p><b>Experimental Design:</b> The study was conducted over 3 years in Hungary. MON 810 and its near isogenic control were planted in 30m x 30m plots with a final population of 50,000 plants/ha. Plots were arranged alternatively with 6 replications. Plots were separated by a 3m alley.</p> <p>Two pitfall traps were placed in the center of the each plot 10 m from each other to collect rove beetles (Coleoptera: Staphylinidae). Sampling occurred weekly from late May (2002, 2003) or July (2001) until harvest. Staphylinids were identified to species level and enumerated. The abundance of the most abundant prey aphid species, <i>Rhopalosiphum padi</i>, was assessed weekly by collecting the aphids from 10 randomly selected maize plants.</p> <p>Staphylinid species were classified into 3 guilds: parasitoids; predatory guilds that eat aphids (aphidophagous); predatory guilds that don't eat aphids (non-aphidophagous). Fisher alpha diversity index was calculated as a measure of biodiversity for each treatment. Metric ordination, principal components analysis, and the Horn index were used to determine the similarities of staphylinid communities.</p> <p><b>Results:</b> No differences in the activity-density pattern of staphylinids were found between MON 810 and the near isogenic control. No statistically significant differences in diversity were detected between MON 810 and the near isogenic control. Significant differences in relative abundance were detected MON 810 and the near isogenic control. However, the direction of the significant difference was not consistent. No significant differences were detected between MON 810 and the near isogenic control for non-aphidophagous predators and parasitoids. MON 810 demonstrated significantly lower abundances for aphidophagous predators in two of three years. Abundance of <i>R. padi</i> fluctuated between treatments and years and was numerically higher in the isogenic treatment in the second half of the growing season. The abundance of aphidophagous predatory guilds demonstrated a linear correlation with total annual number of <i>R. padi</i> across years but not within year.</p>	<p>The authors conclude that Staphylinid community structure (activity–density pattern) was not influenced by the presence of the <i>Cry1Ab</i> protein in MON 810 maize. Also after grouping staphylinids into guilds the authors did not find significant differences for non-aphidophagous predators and parasitoids, whereas “there were significantly and marginally significantly higher abundances for predators with aphids in their diet in isogenic maize stands in 2002 and 2003 respectively”. The go on explaining that “the abundance of the prey <i>R. padi</i> (L.) showed a high fluctuation between stands and years and was numerically higher only in isogenic stands in the second half of the maize-growing season”. The abundance of predatory guilds including aphids in their diet did not correlate with the total annual number of <i>R. padi</i> in the same year.<sup>1</sup></p>	Environment	<p>Although few differences in relative abundance were found in this experiment, the direction of these was not consistent. In addition, the differences found in the abundance of aphidophagous predators could have been a result of the tendency of aphid infestations to be patchy, thus skewing beetle distributions within a field. The authors overall conclusions is that “The Cry1Ab protein expressed by the MON 810 maize hybrid did not influence the overall community structure”. Thus, no adverse effects were determined in this study.</p>
			Observed parameter	Feedback on initial environmental risk assessment
			Interaction between GM plant and NTO	There are no changes to the conclusions of the initial risk assessment

<sup>1</sup> Differences detected in the abundance of aphidophagous predators could have been a result of the tendency of aphid infestations to be patchy, thus skewing beetle distributions within a field.

Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Garcia <i>et al.</i> , 2010)	<p><b>Objective:</b> To evaluate the prey-mediated effects of <i>Bacillus thuringiensis</i> (<i>Bt</i>) Cry1Ab on the rove beetle <i>Atheta coriaria</i> (Coleoptera: Staphylinidae) using the two-spotted spider mite <i>Tetranychus urticae</i> (Acari: Tetranychidae) as prey in a tritrophic bioassay.</p> <p><b>Experimental Design:</b> For feeding trails, <i>Bt</i> maize (cv. DKC 6575 YG, event MON 810) expressing Cry1Ab and a non-<i>Bt</i> maize (Tiétar) were used at the five-leaf stage to feed <i>T. urticae</i>. Newly emerged larvae and adults of <i>A. coriaria</i> were added to plastic test arenas containing maize MON 810 or non-<i>Bt</i> maize infested with <i>T. urticae</i>, and allowed to feed <i>ad libitum</i> on the <i>T. urticae</i>. A selection of <i>A. coriaria</i> first instar larvae (L<sub>1</sub>) were removed after 2 days, second instars (L<sub>2</sub>) after 4-5 days, and third instars (L<sub>3</sub>) after 6-8 days. The adults were removed after 4 days and initial and final weights recorded. All samples, including leaf samples from maize MON 810 and non-<i>Bt</i> maize were frozen at -20°C and Cry1Ab protein levels were determined by ELISA. Three control treatments were assayed consisting of <i>A. coriaria</i> larvae and adults fed on maize MON 810 and non-<i>Bt</i> maize without <i>T. urticae</i>. A separate bioassay was performed to determine the detection time of Cry1Ab protein. <i>A. coriaria</i> were fed on maize MON 810 and non-<i>Bt</i> maize infested with <i>T. urticae</i> for 4 days, adults were then transferred to new plastic containers; 10 adults were removed at different time intervals (0, 4, 8, 12, 24, and 48 h), weighed, and frozen at -20°C. This was replicated four times and levels of Cry1Ab protein were determined by ELISA. To evaluate the effect of <i>Bt</i> maize on survivorship, fecundity, and egg fertility, neonate <i>A. coriaria</i> were exposed to three treatments: (1) maize MON 810 infested with <i>T. urticae</i> (2) non-<i>Bt</i> maize infested with <i>T. urticae</i>, or (3) rearing food. Once larvae emerged from the eggs they were allowed to feed <i>ad libitum</i>. This experiment was repeated 3 times with 30 larvae per treatment. Proteolytic activities of <i>A. coriaria</i> abdomens exposed to <i>T. urticae</i> raised on maize MON 810 and non-<i>Bt</i> maize infested with <i>T. urticae</i> were characterized using specific substrates and inhibitors (40 adults per treatment).</p> <p>Data that did not meet the assumptions of normality was analyzed using non-parametric. Other data were analyzed using one-way ANOVA followed by multiple comparisons tests and analysis of the covariance.</p> <p><b>Results:</b> Cry1Ab protein detected in <i>T. urticae</i> and <i>A. coriaria</i> adults and larvae, decreased rapidly through the trophic chain. Cry1Ab protein levels (per gram of fresh weight) were 4-fold lower in <i>T. urticae</i> (1.28 µg) than MON 810 plants (5.49 µg), and 6-fold lower in <i>A. coriaria</i> adults (0.21 µg) than <i>T. urticae</i>. The mean concentration of Cry1Ab protein in <i>A. coriaria</i> larvae was similarly lower at: 0.16µg for L<sub>1</sub>, 0.42µg for L<sub>2</sub>, and 0.72 µg for L<sub>3</sub> per gram of fresh weight.</p>	<p>This study shows that <i>A. coriaria</i> fed on <i>T. urticae</i> raised on maize MON 810, are exposed to the Cry1Ab protein, but the protein degrades rapidly through the trophic chain. Analysis of multiple endpoints determined that the exposure to Cry1Ab protein had no adverse effects on <i>A. coriaria</i> larvae or adults. Additionally, the data suggests the quality of the prey (<i>T. urticae</i>) was not affected when reared on maize MON 810.</p>	Environment	No adverse effects were determined in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Interactions between the GM plant and NTOs	There are no changes to the conclusions of the safety of the initial risk assessment.

	<p>Adults from the three control treatments (with no <i>T. urticae</i>) showed significant reduction in weights. Cry1Ab protein in <i>A. coriaria</i> decayed at an exponential rate, and was not detectable in <i>A. coriaria</i> adults 24 h after exposure to maize MON 810-fed mites. No differences were found in any of the parameters analyzed for <i>A. coriaria</i> (duration of immature stages, sex ratio, survivorship, fecundity and egg fertility) between <i>A. coriaria</i> reared on maize MON 810-fed <i>T. urticae</i>, non-<i>Bt</i> maize-fed <i>T. urticae</i>, or rearing food. Proteolytic activities of <i>A. coriaria</i> adults fed with mites raised on maize MON 810 did not show differences with those reared on non-<i>Bt</i> fed-prey, indicating that the nutritional quality of the prey was not affected by exposure to the Cry1Ab protein.</p>			
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Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Knecht and Nentwig, 2010)	<p><b>Objective:</b> To evaluate the effects of maize tissue expressing Cry3Bb1 (MON 88017), and Cry1Ab pure protein, on <i>Drosophila melanogaster</i> (fruit fly) and <i>Megaselia scalaris</i> (humpbacked/scuttle fly) representing two saprophagous families of Diptera (Drosophilidae and Phoridae), important decomposers in soil known to consume leave litter in maize fields).</p> <p><b>Experimental Design:</b> Fly larvae were fed maize leaf treatments incorporated into instant <i>Drosophila</i> diet. Treatments included: leaves of MON 88017 expressing Cry3Bb1 protein (DKC5143Bt), leaves of an isolate of the MON 88017 maize (DKC5143), leaves of a non-<i>Bt</i> isolate of Syngenta Bt11 maize (N4640), and N4640 leaves incorporated with Cry1Ab pure protein. The diets contained a ratio of 1:2 (instant diet: maize leaves). Maize plants were grown at optimal conditions in fertilized soil in a climate chambers maize tissue. Following pollen shed, only desiccated leaves were harvested for inclusion in the study. The test was maintained for 4 generations. Each treatment included 35 replicates per treatment at each generation for development time data, and 35 replicate adult pairs per treatment each generation for the counts of female offspring.</p> <p>Protein analysis was conducted by ELISA to determine the Cry protein levels in the senescent maize leaves, the milled leaves, the prepared diets containing milled leaves or the pure Cry protein, and in larvae and adult insects. mixed into and</p> <p>Statistical analysis included one-way ANOVA to analyze mean number of offspring per pair and mean developmental time of larvae. Comparisons were conducted with a Tukey's test. The authors used a Generalized linear model for testing log-transformed data for the interaction between generation and treatment for number of offspring and mean developmental time of larvae.</p> <p><b>Results:</b> Effects of <i>Bt</i> maize on <i>Drosophila melanogaster</i>: "The interaction between treatment and generation was significant. The lowest number of offspring was measured in the second generation followed by the first and the third. On average, the fourth generation had the highest number of offspring."</p> <p>The number of offspring did not differ between transgenic and isolate diets in the 1<sup>st</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> generation. Significantly more progeny were produced in the second generation with the diets Cry protein containing maize leave diets Cry3Bb1 (MON 88017) and the Cry1Ab pure protein diet incorporated diet -- compared to the isolate maize leave diets. In the 1<sup>st</sup>, 2<sup>nd</sup>, and generations, the development times did not differ between larvae fed the MON 88017 maize leaves, the Cry1Ab protein incorporated diet, and the corresponding isolate diets. Larvae fed with the MON 88017 diet had a significantly prolonged developmental time at the third and the fourth generation. The authors report, "More generally, developmental times had very low variability, and significant</p>	The authors conclude that "the performance of saprophagous dipteran larvae is not affected by Cry proteins".	Environment	No adverse effects were determined in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Interactions between the GM plant and NTOs	There are no changes to the conclusions of the safety of the initial risk assessment.

	<p>differences were only about 1 day.</p> <p>Protein Analysis: Cry3Bb1 protein decreased quickly in senescent leaves over time and through processing to prepare the experiment diet medium. After 1 week and 2 weeks, the Cry3Bb1 protein concentrations were approximate 60% and 4%, respectively from the initial diet medium concentrations that averaged 21.3 µg/g diet. The authors “estimated a value of 13.3 µg/g as the maximum environmental concentration that the soil-dwelling organisms would encounter in nature”. “Similarly, in the diet containing a negative control (isoline) senescent maize leaves with added pure Cry1Ab protein, about 50% of the protein was degraded after 1 week and only 10% remained after 2 weeks. At the start of the feeding experiments the diet medium containing the incorporated pure protein contained an average of 20.8 µg Cry1Ab /g of diet. No Cry proteins were detected in the negative control isoline milled senescent diet medium. Larvae of <i>D. melanogaster</i> and <i>M. scalaris</i> reared on diets contain Cry proteins contained low but detectable amounts of the Cry protein, averaging, respectively, 0.06±0.02 and 0.05±0.02 µg/g dry weight for Cry1Ab, and 0.07±0.03 and 0.13±0.06 µg/g dry weight for Cry3Bb1. Cry1Ab and Cry3Bb1 was not detected in any adult flies.</p>			
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Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Porcar <i>et al.</i> , 2010)	<p><b>Objective:</b> To determine the effects of dietary exposure to <i>Bacillus thuringiensis</i> (<i>Bt</i>) Cry1Ab protein on three species of predatory Coleoptera.</p> <p><b>Experimental Design:</b> Three coleopteran species were chosen based on their use as biocontrol agents: <i>Adalia bipunctata</i> (two-spotted ladybird; Coccinellidae) feeds on aphids, <i>Atheta coriaria</i> (a rove beetle; Staphylinidae) feeds on fungus gnats, and <i>Cryptolaemus montrouzieri</i> (mealybug destroyer; Coccinellidae) feeds on mealybugs. Cry1Ab-producing <i>Escherichia coli</i> recombinant strain was “cultured and inclusion bodies extracted and solubilized”. The protein was analyzed using SDS-PAGE and protein sizes were determined by comparing with broad range protein markers. The bioactivity of the Cry1Ab protein was confirmed using a sensitive species bioassay with <i>Ostrinia nubilalis</i>. Cry1Ab was incorporated into an artificial diet for dietary exposure to the three coleopteran species. Six different treatments were used: water, solubilization buffer, trypsin-treated buffer, solubilized Cry1Ab (50 µg/ml), trypsin-treated Cry1Ab (50 µg/ml), and a positive control (5% Boric acid or 0.5% ZZ Copper (Zelnova). Insects were maintained in small Petri dishes and allowed to feed <i>ad libitum</i> on the artificial diet treatments. The diets were renewed every 3 days. For <i>A. bipunctata</i>, a single first or second-instar larva was placed in the dish for dietary exposures, with 30 insects per treatment. For <i>A. coriaria</i> and <i>C. montrouzieri</i>, four to five adult insects were included in each dish with five dishes per treatment for <i>A. coriaria</i>, and two dishes per treatment for <i>C. montrouzieri</i>. These bioassays were then repeated four times with <i>A. bipunctata</i>, and three times with <i>A. coriaria</i> and <i>C. montrouzieri</i>. The total number of insects assayed was: 720 <i>A. bipunctata</i>, 640 <i>A. coriaria</i>, and 240 <i>C. montrouzieri</i>. The bioassays with <i>A. bipunctata</i> larvae were conducted for 6 days. The adult bioassays with <i>A. coriaria</i> and <i>C. montrouzieri</i> were extended to 15 days.</p> <p><b>Results:</b> The Cry1Ab protein was confirmed to be highly active against the susceptible target <i>O. nubilalis</i>. There were no significant differences in the percent mortality of <i>A. bipunctata</i> larvae among control and test treatments (18 to 24% at five days). On day six, the mortality in the control treatments, including water, were &gt;30% and considered too high to continue the bioassays. In the bioassays with <i>A. coriaria</i> or <i>C. montrouzieri</i> adults, there were no treatment effects after 15 days with mortality ranging from 16 to 20% for <i>A. coriaria</i>, and a maximum of 7% for <i>C. montrouzieri</i>. In contrast, the positive control treatments resulted in approximately 100% mortality in all bioassays with the three coleopteran species.</p>	The authors conclude that their results suggest that Cry1Ab protein is innocuous to the three important predatory Coleoptera ( <i>A. bipunctata</i> , <i>A. coriaria</i> , and <i>C. montrouzieri</i> ) under the conditions tested. The concentration tested (50 µg/ml) is about fivefold higher than the concentration of Cry1Ab in transgenic maize.	Environment	No adverse effects were determined in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Interactions between the GM plant and NTOs	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Rauschen <i>et al.</i> , 2010)	<p><b>Objective:</b> To determine the effects of two <i>Bt</i>-maize varieties (MON 810 and MON 88017) on occurrence and field densities of Coleoptera (Coccinellidae and Chrysomelidae).</p> <p><b>Experimental Design:</b> Two field studies were carried on in Germany. One of them lasted from 2001 to 2003 and consisted on 3 maize treatments: MON 810, is the near-isogenic maize line without insecticide treatment and the isogenic maize treated with a synthetic pyrethroid insecticide. The other field study lasted from 2005 to 2007 and consisted on 4 maize treatments: MON 88017, its non-<i>Bt</i> near isoline and two conventional maize lines.</p> <p>The field-release experiment with MON 88017: The plots were 0.13 ha big and included eight replications per treatment in a systematically randomized plot design. Several collection methods were used: Cob samples, Sweep net samples (three times each year), Visual assessments (4 plants in each plot assessed 7 times during the growing season) and Panicle samples (25 flowers per plot were sampled during the peak of anthesis).</p> <p>For comparisons of field abundances a test of equivalence (by simulation study and confidence interval approaches) were used to define the null-hypothesis as the state of either unacceptable increase or unacceptable decrease in the test treatments to the conventional treatments. For the simulation study the case is considered that abundance in the genetically engineered (GM) test plots does not differ from that in the convention plots.</p> <p><b>Results:</b> A total of 6529 beetles from 9 families, and 29 genera or species, were caught over the 6 seasons at the two field sites. The most diverse families represented groups were Coccinellidae and Chrysomelidae. Only a single species of Lathridiidae, collected in Cob samples was however, the most numerous species in the study. Staphylinids were indentified in 2005, and not investigated further due to very low abundances. One genus of Carabidae was observed in this study</p> <p>Overall abundance was low for all organisms observed and where analyses of equivalence were possible, abundance of coccinellids and chrysomelids were equivalent in MON 88017 and non-GM maize hybrids.</p>	<p>The authors conclude that Coccinellidae and Chrysomelidae were comparatively abundant and diverse, but still low in numbers.</p>	Environment	No adverse effects were determined in this study.
		<p>The authors also conclude that Chrysomelidae do not meet all of the characteristics for relevant non-target organisms, as these are mostly pests within the agricultural landscape. Therefore, their value as indicator species in field tests is limited.</p>	<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
		<p>The authors discuss that given the large natural variability in ladybird densities in the field, Environmental Risk Assessment questions need to be addressed in low-tier laboratory tests. Non-target Chrysomelidae can be addressed in a similar fashion as non-target Lepidoptera in Cry1Ab expressing <i>Bt</i>-maize.</p>	Interactions between the GM plant and NTOs	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Lumbierres <i>et al.</i> , 2011)	<p><b>Objective:</b> The study aimed to compare three transgenic <i>Bt</i> maize varieties, including two from Event MON 810 and one from Event Bt176, and their corresponding near-isogenic varieties, and the possible effects these events have on aphid parasitism and the aphid-parasitoid complex composition.</p> <p><b>Experimental design:</b> The experiment consisted of a complete randomized block design with four replicates conducted during two years. Each block contained six maize varieties: three <i>Bt</i> varieties (two from Event MON 810 (DKC6575 (Monsanto) and PR33P67 (Pioneer), and one from Event Bt176 (Syngenta)), and their respective near-isogenic varieties. In the first year, aphids and parasitoids were sampled on two dates, and in the second year samples were collected on three dates. For each date, 20 plants per plot were selected randomly for inspection where aphid density was evaluated and several aphid colonies were collected. At least 100 aphids were enclosed in plastic cages in the laboratory, and inspected every two days for up to 8 weeks for emerged aphid parasitoids or hyperparasitoids. Total parasitism rate was calculated as final number of mummified aphids in relation to number of aphids initially caged, effective parasitism rate was the number of parasitoids emerged in relation to the number of aphids caged, and hyperparasitism rate was the number of hyperparasitoids emerging in relation to the final number of mummies. The experimental design was a split-split-plot-like model (years as main plots, variety as subplot, and sampling date as sub-subplot). As aphid abundance was expected to have an effect on the parasitism rate, aphid abundance was considered as a covariable and an ANCOVA analysis of the parasitism rate was performed. To compare mean aphid abundance and parasitism or hyperparasitism rates between pairs of <i>Bt</i> varieties and their isogenics we used the least significant differences (LSD) test.</p> <p><b>Results:</b> Significant differences in aphid abundance among varieties were observed in the first year only. However, in both years there were no significant differences in aphid abundance among MON 810 maize and their near-isogenic varieties. In the first and second years respectively, total parasitism ranged from 1.0% to 18.6% and from 0.0% to 63.8%, and effective parasitism ranged from 0.49% to 9.70% and from 0.0% to 51.7%. When aphid abundance is added as a covariable, no significant effect is found on parasitism rate for both years. <i>Lysiphlebus testaceipes</i> (Cresson), <i>Lipolexis gracilis</i> Förster (Hymenoptera, Braconidae, Aphidiinae), and <i>Aphelinus</i> sp. (Hymenoptera, Aphelinidae) were the most common parasitoids. In both years of the study, the percentage of mummies that developed in hyperparasitoids ranged from 0% to 70%.</p>	The authors conclude “the results show that the <i>Bt</i> maize lines studied do not affect the aphid– parasitoid complex and parasitoid relative occurrence. Observed differences in aphid abundance and <i>L. testaceipes</i> or <i>L. gracilis</i> parasitism rates are probably due to varietal traits rather than to the genetic modification. [These] results add to the mounting evidence that <i>Bt</i> maize has no negative impact on the non-target maize biocenosis”.	Environment	No adverse effects were determined in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Interactions between the GM plant and NTOs	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Wolt and Peterson, 2010)	<p><b>Objective:</b> To assess potential risk to sensitive aquatic species from maize-expressed Cry1Ab protein (MON 810) using a conservative worst-case scenario screening level approach.</p> <p><b>Experimental Design:</b> “a worst-case scenario was developed for temperate zone maize production on the basis of a high-end exposure estimate to estimate the risk level where risk is based on a combined probability of the high-end exposure and susceptibility to the stressor.” For the high-end exposure estimate data for the spatial and temporal distribution of Cry1A(b) produced in a standing crop of <i>Bt</i> maize the authors used published data which represent the season-long pattern of Cry1A(b) expression, distribution, and dry matter partition for MON 810 maize. Estimated environmental concentrations (EEC) of Cry1Ab occurring in receiving waters due to the presence of residual plant materials in fields where GE maize is grown used the US EPA GENEEC and FIRST tier 1 models for ecological risk assessment of pesticides. Aquatic EECs for Cry1A(b) were determined for three standard default scenarios: the GENEEC pond; a shallow semi-aquatic wetland; and the FIRST index reservoir. “These aquatic EECs are considered to represent Cry1A(b) to which aquatic micro-and macro-invertebrates maybe exposed.” Species sensitivity distributions were used to develop a probabilistic profile of acute effects for arthropod exposure to Cry1A(b) using acute LD50 values for susceptible lepidopteran larvae ingesting the purified protein as surrogate data describing toxicity for a putative susceptible aquatic arthropod.</p> <p><b>Results:</b> The high-end environmental load of Cry1A(b) protein present in the field immediately post-harvest used to develop the tier 1 EEC was 50 g/ha. For the farm pond (GENEEC) and reservoir (FIRST) scenarios, the peak EEC, occurring immediately following harvest were 1.3 and 1.2 mg/L, respectively, since under the worse-case assumption of near instantaneous loading of Cry1A(b) to surface water there was little impact of physical and chemical processes to dissipate the protein. The distribution of terrestrial lepidopteran larvae sensitivity to acute Cry1A(b) exposure ranged over four orders of magnitude.” “As a first estimate of sensitivity of a putatively susceptible aquatic arthropod to Cry1A(b), the multi-species distribution indicates 96% of species will be less acutely sensitive than the EEC for the standard pond and reservoir scenarios, and 90% of species will be less sensitive than the EEC for the semi-aquatic wetland.”</p>	The authors conclude, “The high-end risk expressed as the combined probability of short-term exposure and acute effects to a sensitive species indicated no concern in 99% of cases with limited opportunity for chronic effects due to the rapid decline of Cry1A(b) from the environment”.	Environment	No adverse effects were determined in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Interactions between the GM plant and NTOs	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Zeilinger <i>et al.</i> , 2010)	<p><b>Objective:</b> To investigate potential effects on earthworm populations in soil cultivated with <i>Bt</i> maize in a large multiple-year field study.</p> <p><b>Experimental design:</b> Six maize varieties (two Cry1Ab <i>Bt</i> maize (Bt11 and MON 810), one Cry3Bb1 <i>Bt</i> maize (MON 863), and three conventional control varieties) were planted in US fields for four growing seasons in 40m×40m plots (0.16-ha) in a complete randomized block design with four replicates. Plots (there was a total of 24) were separated by a 20 m non-<i>Bt</i> maize buffer. The maize was planted using standard agricultural practices. Earthworm populations were surveyed and biomass of each earthworm specimen was estimated as ash-free dry mass (afdm), using the body length of each worm in an allometric growth equation.</p> <p>Using the data collected in 2005, a prospective power analysis was performed in order to have an adequate sample size to detect a significant difference in earthworm populations due to maize variety. Based on this analysis, the number of samples per plot in 2006 was doubled to sixteen, and the collection of samples was performed only inside the maize rows during (in 2005 also mid way between the rows samples were taken).</p> <p><b>Results:</b> A total of 1276 earthworms were collected over the sampling dates. Four earthworm species were identified: <i>Aporrectodea caliginosa</i>, <i>Aporrectodea trapezoides</i>, <i>Aporrectodea tuberculata</i> (collectively, the <i>A.caliginosa</i> species complex), and <i>Lumbricus terrestris</i>. No significant differences were found in the biomass of juveniles and adults for any of the four species between <i>Bt</i> and non-<i>Bt</i> maize varieties. Although five contrasts for the interactions of the maize variety by location and maize variety by year effects were statistically significant, no biologically meaningful patterns were discernible from them.</p>	<p>The authors found “no significant differences in the biomass of juveniles and adults for all four species between <i>Bt</i> and non <i>Bt</i> maize varieties. From this and previous studies, we conclude that the effects of Cry1Ab <i>Bt</i> maize on the <i>A. caliginosa</i> species complex and <i>L. terrestris</i> are small”.</p>	Environment	No adverse effects were determined in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Interactions between the GM plant and NTOs	There are no changes to the conclusions of the 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	Conclusions	Protection goal	Adverse effects
(Emmerling <i>et al.</i> , 2011)	<p><b>Objective:</b> To assess the fate of Cry1Ab protein in <i>Bt</i>-maize litter in the presence of earthworms (<i>Lumbricus terrestris L.</i>) and study its fragmentation in the earthworm gut.</p> <p><b>Experimental design:</b> Four soil microcosms (n=4), each containing three earthworms containing <i>Bt</i>-maize litter (MON 810) were incubated at 15°C and 65% humidity for 2 weeks with and without earthworms. The experimental soil, a sandy loamy Fluvisol, was collected from the Ap-horizon (0-30 cm depth) of an arable field near Trier (Germany) and sieved less than 2 mm. Each microcosm was filled with 1000 g moist soil which was wetted previously to a water content of 20% by weight. Samples of <i>Bt</i>-maize litter, earthworm tissue, earthworm gut compartments (foregut, midgut, hindgut), and earthworm casts were analyzed for Cry1Ab protein using an Agdia ELISA. The molecular weight (MW) of degraded Cry1Ab-protein fragments was determined by Western Blot analysis.</p> <p>Statistical comparison of <i>Bt</i> maize, gut and cast material, and gut compartments (foregut, midgut, hindgut) were done by a non-parametric KruskalWallis <i>H</i>-test and subsequently pairwise MannWhitney-U-tests</p> <p><b>Results:</b> There was a significantly greater degradation of Cry1Ab protein residues in <i>Bt</i>-maize litter containing earthworms (initial 8.8 µg/g down to 0.77 µg/g after 2 weeks) versus litter without earthworms (8.8 µg/g down to 2.3 µg/g). There was additional degradation into smaller protein fragments in the earthworm gut, from initial Cry1Ab MW of 65 kD down to fragments of 31 kD, 23 kD and 17 kD, as measured by Western blot. In the hind gut and cast samples containing non-detectable fragments in the Western blot (i.e., &lt; 17 kD), the ELISA still produced a detectable response, suggesting that the Agdia ELISA could detect fragments of the Cry1Ab protein that were smaller than 17 kD but still containing an immunoreactive epitope. A 99% decrease in Cry1Ab concentration occurred between the soil litter and the earthworm foregut, suggesting that most protein digestion occurs in the foregut. No Cry1Ab fragments were found in the earthworm hindgut or casts by Western blot analysis. Only the foregut and midgut had detectable protein fragments.</p>	The paper concludes that degradation of the Cry1Ab protein in <i>Bt</i> -maize litter is accelerated by the presence of earthworms.	Environment	No adverse effects were determined in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Interactions between the GM plant and NTOs	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Lehman <i>et al.</i> , 2010)	<p><b>Objective:</b> To investigate the relative decomposability of maize (<i>Zea mays</i> L.) residues from insect (<i>Bt</i>)-protected hybrids (carrying the <i>cry3Bb1</i> and the <i>cry1Ab</i> genes) and conventional hybrids cultivated under insect pressure.</p> <p><b>Experimental Design:</b> Field studies were conducted in the US. For the first decomposition study, 6 replicate plots arranged in a completely randomized design were cultivated under rainfed conditions during 2006. Chopped residues (stalks and leaves) were used from a corn rootworm-protected (Cry3Bb1) hybrid and its non-<i>Bt</i> near isoline that were grown under corn rootworms pressure (CRW, <i>Diabrotica</i> spp.). The number of larva and adult CRW were determined by stereoscopic observation of samples from duplicate soil cores collected from each plot at weekly intervals. Aboveground biomass, residue macromolecular composition and stalk physical strength were also measured. ANOVA (1-way analysis) was used to analyze the data. For the second study, also held in 2006, 3 European corn borer (ECB, <i>Ostrinia nubilalis</i> Hübner)-resistant (Cry1Ab) hybrids, their non-<i>Bt</i> near isolines, 2 Cry3Bb1-protected isolines, and 3 additional conventional hybrids were cultivated in replicated plots under rain fed, no-till conditions with fertilizer application and with elevated ECB pressure. Residue (intact stalk sections) was used for the comparisons. Aboveground biomass was harvested, and in ~6 stalks/plot the number of ECB entrance/exit holes were counted and recorded. Half of these stalks were split longitudinally and the length of each tunnel was recorded. The other half of the stalks was used for the decomposition study.</p> <p><b>Results:</b> Corn rootworm larval counts documented greater CRW pressure on the non-protected hybrids. Measurements of the percentage of stalks with an ECB tunnel demonstrated that the <i>cry1Ab</i> protected hybrids were minimally affected by the ECB infestation compared to non-protected hybrids. In both studies, insect-resistant residues decomposed at rates similar to their non-protected near isolines. No evidence was found that insect-protected hybrids produced more above-ground biomass or had distinct residue composition. While some measures of mechanical stalk strength indicated that stalks from non-protected hybrids were not as stiff as those from protected hybrids (probably due to ECB-damage), these physical differences did not translate into differences in residue decomposition</p>	The authors conclude that while individual hybrids may vary in their production of biomass, residue composition or residue decomposability, these characteristics do not systematically vary with the presence of the <i>Bt</i> gene conferring insect resistance, even under conditions of insect pressure.	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

Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Sander <i>et al.</i> , 2010)	<p><b>Objective:</b> To study the mechanism of Cry1Ab binding to polar surfaces and to determine if Cry1Ab undergoes conformational changes during the adsorption process.</p> <p><b>Experimental Design:</b> Cry1Ab adsorption was measured at pH 5 to 8 and at constant ionic strength (50 mM), using QCM-D sensors and OWLS waveguides coated with negatively-charged SiO<sub>2</sub> or positively-charged poly-L-lysine (PLL) as models for polar soil minerals. Both techniques allow direct quantification of the kinetics and reversibility of protein adsorption. Conformational stability of Cry1Ab was studied by adsorbing Cry1Ab to an apolar gold surface at pH 6.</p> <p><b>Results:</b> Electrostatic interactions govern Cry1Ab adsorption. Increasing pH decreases electrostatic attraction in the Cry1Ab-SiO<sub>2</sub> system, while increasing attraction in the Cry1Ab-PLL system. Cry1Ab adsorption is reversible: adsorbed and solution-phase Cry1Ab are in dynamic equilibrium, Cry1Ab-SiO<sub>2</sub> interactions are relatively weak at pH&gt;5, and there are no extensive, irreversible conformational changes of Cry1Ab in the adsorption/desorption process. During adsorption Cry1Ab is oriented with positively charged patches toward the SiO<sub>2</sub> surface, and with negatively charged patches toward the PLL surface. This patch-controlled electrostatic attraction (PCEA) is consistent with the non-uniform surface charge distribution discussed in a companion paper (Madliger <i>et al.</i>, 2010).</p>	<p>The authors conclude that the “electrostatic forces control the adsorption of monomeric Cry1Ab to charged polar surfaces”.</p> <p>The environmental implication is that “adsorbed Cry1Ab proteins will desorb and, hence, be mobilized upon decreasing the solution protein concentration or increasing the solution pH”.</p> <p>The authors also indicate that study “provides a plausible explanation for earlier findings that Cry1Ab protein can retain its insecticidal activity in soil”.</p>	Environment	The paper does not report any new E-fate findings. The biological relevance of the findings is not addressed and conclusions of adverse effects cannot be drawn from this study. <sup>2</sup>
			Observed parameter	Feedback on initial environmental risk assessment
			Effects on biochemical processes	There are no changes to the conclusions of the safety of the initial risk assessment.

<sup>2</sup> This paper studies the adsorption of Cry1Ab to SiO<sub>2</sub> surfaces as a surrogate for adsorption to soil minerals that contain SiO<sub>2</sub> (e.g., clay or quartz). It does not report any new E-fate findings, but suggests a molecular mechanism to account for the slow soil dissipation of Cry1Ab previously reported in the literature. The molecular mechanism was further tested and validated in the companion paper (Madliger *et al.*, 2010).

Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
<p>(Yanni <i>et al.</i>, 2011b)</p>	<p><b>Objective:</b> To evaluate difference in <i>Bt</i> vs. non-<i>Bt</i> maize for biomass, maize residue chemical composition and residue decomposition rates.</p> <p><b>Experimental design:</b> Nine <i>Bt</i> maize hybrids and their near-isolines were evaluated in a field study over a two year period (2008 and 2009) in Quebec, Canada. The study included MON 810 maize hybrids. The study was done in a complete randomized design with three replicates. The plants were harvested when most were at the R5 or black layer stage. Grain and stover yields were measured and the dried and ground leaves, stems and roots were analyzed for fiber content, carbon, and nitrogen.</p> <p>The field decomposition study used one <i>Bt</i>/non-<i>Bt</i> hybrid pair (Pioneer 38W22 and 38W21) with six replicates and seven sampling dates (each month up to 6 months and 1 year after planting). Eighty-four litter bags were buried at a depth of 5 cm in no-till plots. Mass loss and C/N ratio were measured.</p> <p><b>Results:</b> There were no differences in grain yield, stover yield, grain moisture, or lignin concentration among near isolines. Grain and stover yield did not differ between <i>Bt</i> and non-<i>Bt</i> hybrids. The authors note that they did not expect a yield effect due to the <i>Bt</i> gene since there wasn't any European corn borer (ECB) stress. However, the hybrid type had a significant effect on grain yield and lignin concentration in leaves and roots in the first year and stover yield and lignin concentration of stems and roots in the second year. The C and N concentrations, and C/N ratio were not affected by hybrid type. However, the <i>Bt</i> gene had a significant (<math>P &lt; 0.05</math>) effect on the N concentration of stems and roots in 2009.</p> <p>Stem material in the decomposition study had comparable chemical composition. After one year, the <i>Bt</i> stems lost 56% of their mass vs. 43% for non-<i>Bt</i> stems and had a significantly lower C/N ratio. The non-<i>Bt</i> stems had a greater mass loss after five months, but no difference was noted after one year.</p>	<p>The authors conclude that the <i>Bt</i> gene does not affect the agronomic performance or the chemical composition of maize in fields without herbivory and that <i>Bt</i> maize residue may decompose more rapidly than non-<i>Bt</i> maize residue.</p>	<p>Environment</p>	<p>No adverse effects were detected in this study</p>
			<p><b>Observed parameter</b></p>	<p><b>Feedback on initial environmental risk assessment</b></p>
			<p>Effect on biogeochemical processes</p>	<p>There are no changes to the conclusions of safety of the initial risk assessment</p>

Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Yanni <i>et al.</i> , 2011c)	<p><b>Objective:</b> To investigate the effect of European corn borer injury and the <i>Bt</i> gene on lignin, carbon and nitrogen in corn tissues and on decomposition of corn residues.</p> <p><b>Experimental design:</b> A greenhouse study was conducted over two growing seasons in 2008 and 2009. Treatments were arranged in a factorial experiment with two levels of ECB injury (ECB and No-ECB) and two genetic lines (Bt and NBt). Four Bt corn hybrids produced by Bt11 and MON 810 and their non-Bt (NBt) near-isolines were used. The study was a completely randomized design with four replicate pots for each hybrid. ECB hybrids were manually infested twice with ECB eggs. Corn was harvested after 128 days of emergence in 2008 and 126 days of emergence in 2009. Plants were separated into leaves, stems and roots. Samples were dried, ground through a 1-mm screen before analysis. Stems from ECB and no-ECB infestation of two Bt and two NBt hybrids from 2008 were placed in litterbags to determine decomposition. The study was a completely randomized design with five replicate bags for each of five sampling dates for each hybrid. Bags were buried at 5 cm depth in a field in May 2009 and collected monthly for five months.</p> <p>ANOVA were used to test the effect of hybrid, genetic modification (Bt, NBt), ECB injury and the interaction of Bt and ECB on lignin content, N concentration and C:N ratio in stem and leaf tissue, and the effects of Bt and ECB on decomposition rate. Monthly decomposition rate constants were calculated by fitting the data into the single exponential model</p> <p><b>Results:</b> ECB injury had no consistent effect on lignin concentration in leaves and stems of the Bt and NBt hybrids across the two years of the study. Neither was there a consistent effect of the <i>Bt</i> gene on lignin concentration. ECB injury did consistently lower (<math>p &lt; 0.05</math>) the C:N ratio in stems of the Bt and NBt hybrids whereas the <i>Bt</i> gene had only a small effect on N concentration. ECB injury affected the composition of lignin derived phenols, however ECB infested and non-infested stems lost the same amount of mass after 5 months in buried field litterbags.</p>	The authors concluded that the effect of ECB injury corn tissue chemistry was subtle and included changes in levels of lignin-derived phenolics. There was no consistent effect of the Bt gene on lignin content of corn stems and leaves.	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

Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Yanni <i>et al.</i> , 2011a)	<p><b>Objective:</b> To evaluate the effects of maize plant components, the presence of the <i>Bt</i> gene, and elevated-lignin inputs on decomposition.</p> <p><b>Experimental Design:</b> This experiment used a complete factorial design with roots, stems, and leaves from MON 810 maize and isolines enriched with <sup>13</sup>C and <sup>15</sup>N added to soil, with and without indulin lignin (commercial lignin source), for a total of 12 treatments. Maize plants that were grown in a greenhouse in sandy loam soil and enriched with <sup>13</sup>C-CO<sub>2</sub> and <sup>15</sup>N-KNO<sub>3</sub> were harvested at the V9 - 10 growth stage and separated into leaves, stems, and roots. Incubation jars were prepared so that there were four replicates for each of ten sampling dates (1, 2, 4, 8, 12, 16, 20, 24, 30, and 36 weeks). Each replicate consisted of 50 g of air-dried soil mixed with 0.5 g of dried and ground maize tissue. Jars were capped with an air-tight lid, incubated in the dark at 20°C, and the lids were removed to aerate the jar for 15 min weekly. The initial isotope enrichment levels (<sup>13</sup>C and <sup>15</sup>N), organic C, total N, and lignin content of the incubation soil, maize residues and indulin lignin were measured. The CO<sub>2</sub> concentration of the incubation jar headspace was measured until week 20, when a constant rate of decomposition was reached. On the designated weeks soil subsamples from each replicate were analyzed for mineral N (NH<sub>4</sub>-N + NO<sub>3</sub>-N), <sup>13</sup>C, and <sup>15</sup>N. At 1 and 36 weeks, CuO oxidation analysis was used to determine the acid to aldehyde ratio (Ad/Al) of samples as an indicator of biodegradation and lignin alterations.</p> <p><b>Results:</b> Initial measurements indicated that roots tended to have a greater lignin content, a smaller C content, and greater lignin:N ratio than leaves or stems. The C:N and lignin:N ratios tended to be lower in <i>Bt</i> tissues than in non-<i>Bt</i> (<i>NBt</i>) tissues.</p> <p>Slight differences were noted between treatments receiving <i>Bt</i> and <i>NBt</i> tissue in decomposition rate (CO<sub>2</sub> production) of stem tissue, N mineralized from root tissue<sup>3</sup>, Ad/Al ratios of all tissues, and residue-N in leaf and stem tissue. However, no differences were detected between treatments that received <i>Bt</i> or <i>NBt</i> tissue in the decomposition rate of leaf and root tissue, N mineralized from leaf and stem tissue, residue-N of root tissue, or residue-C in all tissues.</p>	<p>Some differences in measurements from <i>Bt</i> and <i>NBt</i> maize treatments were detected, but the authors conclude “that lignin and N contents, and consequently the lignin:N ratio, of maize tissue control its decomposition.”</p> <p>Furthermore, the authors conclude that “the results strongly suggest that <i>Bt</i> maize does not differ from <i>NBt</i> maize in terms of decomposition and should have no effect on the soil C dynamics in <i>Bt</i> maize agroecosystems.”</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>3</sup> The authors state that no definite conclusions can be drawn from this result as it may be due to soil contamination in root samples and that the Cry1Ab protein is not produced in the roots and should not have affected the composition of the root tissue.

**Area of the environmental risk assessment: Environmental Safety – Protein/DNA Fate in Stream Water.**

Publication	Summary of research and results	Conclusion	Protection goal	Adverse effects
(Tank <i>et al.</i> , 2010)	<p><b>Objective:</b> to determine the extent of maize detritus and presence of Cry1Ab protein in maize detritus and dissolved in stream water using a synoptic survey of the stream network in Indiana.</p> <p><b>Experimental Design:</b> 217 stream sites were sampled across an intensively farmed region of northwestern Indiana 6 months after maize harvest. The study addressed three questions: “What is the spatial distribution of maize detritus in streams across an agricultural landscape, and is Cry1Ab protein detectable in that material?” “Is Cry1Ab protein detectable in stream water, and what is the spatial distribution of this dissolved protein across the landscape?” “Given the connectedness of stream networks, are there any longitudinal patterns in Cry1Ab in maize detritus and dissolved in the water column?”</p> <p><b>Results:</b> 86% of stream sites contained maize leaves, cobs, husks, and/or stalks in the active stream channel. Cry1Ab protein was detected in stream-channel maize at 13% of sites and in the water column at 23% of sites. 82% of stream sites were adjacent to maize fields, and Geographical Information Systems analyses indicated that 100% of sites containing Cry1Ab positive detritus in the active stream channel had maize planted within 500 m during the previous crop year.</p>	The authors conclude, “This study demonstrates that maize detritus can be dispersed throughout a stream network and that compounds associated with <i>Bt</i> maize, such as Cry1Ab proteins, may be a more common occurrence in watersheds draining maize-growing regions than previously recognized. In addition, tile drains, which drain row-crop agriculture in much of the midwestern United States, are a likely source of dissolved Cry1Ab to streams. Cry1Ab proteins are distributed beyond field boundaries and persist after initial crop harvest”	Environment	The authors of this paper reported that <i>Bt</i> maize detritus can be dispersed throughout the stream. However, the authors recognize that the study does not address the question of whether the concentrations of Cry1Ab protein reported in this study have any effects on non-target organisms. The biological relevance of the findings is not addressed and conclusions of adverse effects cannot be drawn from this study. <sup>4</sup>
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Protein persistence	There are no changes to the conclusions of the safety of the initial risk assessment.

<sup>4</sup> In decades of peer-reviewed, scientific studies, plant-incorporated protectants have been found to be safe and do not pose significant threat or damage to non-target organisms or the environment.

- Numerous studies have shown *Bt* crops to have negligible effects on non-target organisms and the environment (e.g. Naranjo (2009)).

Cry proteins are known to readily degrade in soil and aquatic systems, thereby significantly reducing the possibility of persistence or long-term exposure to high concentrations either in soil or water (e.g. (Accinelli *et al.*, 2008; Clark *et al.*, 2005; Head *et al.*, 2002; Herman *et al.*, 2001; Icoz and Stotzky, 2007; Marchetti *et al.*, 2007; Prihoda and Coats, 2008a; Prihoda and Coats, 2008b).

This paper lacks transparency and does not include enough data to accurately judge the validity of the methods used to generate concentration results and draw conclusions. The analytical methods cannot be reproduced or interpreted based on the information provided in the paper; therefore we look forward to a full data/methods review.

- Without using a sensitive insect bioassay to corroborate the Enzyme-Linked ImmunoSorbent Assay (ELISA) detection of Cry1Ab in maize tissue or water in the study conducted by Tank *et al.* (2010), it is impossible to know how much of the detected Cry1Ab protein is bioactive, and how much is simply degraded protein fragments still containing the immunophoric sequence.
  - Using a sensitive test organism (European maize borer), Jensen *et al.* (2010) found no bioactivity of Cry1Ab proteins in senesced maize tissue after only two weeks of exposure to the elements in a terrestrial or aquatic environment.
  - Not enough information is presented to be able to judge whether immunoassay method validation or “optimization” occurred, or to allow repetition of the study. This is a critical point since ELISA methods are known to be sensitive to matrix effects that could result in false positive readings, thus calling into question low levels detected in stream water.
  - It is unclear if appropriate control site(s) (reference streams) were used to test for possible matrix interference of the stream water with the ELISA.
- In addition, this study cannot be reproduced based on the information provided regarding field collection methods.
  - It is unclear how sites were selected for sampling, and no details are provided on detritus sampling methods.

The concentrations of Cry1Ab in water reported in this study, if reliable, are so low (parts per trillion) that the biological significance of the study is called into question. When placed in the context of an environmental risk assessment, the concentrations of Cry1Ab detected by Tank *et al.* (2010), pose negligible risk to aquatic organisms.

- Concentrations of Cry1Ab in water reported in this study are 1000 times less than levels shown to kill the most sensitive species (e.g. Wolt (2003)).
- A recent publication by Wolt and Peterson (2010) estimated risk to aquatic organisms based upon worst case scenario estimates of Cry1Ab protein in water and maize tissue.
  - Wolt and Peterson (2010) used a worst case estimate for Cry1Ab protein concentrations in water at 1.3 µg/L (pond) and 7.2 µg/L (wetland). Tank *et al.* (2010) report levels approximately 100 times lower at 32 ng/L (0.032 µg/L).
  - Wolt and Peterson (2010) list a worst assumption exposure in maize tissue of 6 ng/mg. Tank *et al.* (2010) found means of 0.095 ng/mg and 0.2 ng/mg, with large SD, (>10x difference).
  - Wolt and Peterson (2010) conclude that their worst case assumptions are well below any levels likely to cause hazard to non-target organisms, and therefore risk to aquatic ecosystems is negligible.
- These findings are confirmatory of earlier field studies (Swan *et al.*, 2009), that likewise found no adverse effects of *Bt* proteins to aquatic invertebrate communities. These studies represent the most realistic examination of effects from *Bt* maize on the aquatic invertebrate community available.
- Tank *et al.* (2010) in a separate peer-reviewed paper, recently reported that invertebrate communities in streams next to *Bt* maize fields are unaffected by exposure to *Bt* maize (Chambers *et al.*, 2010).

The presence of *Bt* proteins in maize detritus in and around maize fields (including streams) is expected given the broad adoption by US farmers of *Bt* maize. Thus, the presence of *Bt* proteins in water, while not convincingly demonstrated, is also expected. In addition to inputs from maize detritus, these proteins could enter the water column following spray applications of *Bacillus thuringiensis* strains as microbial pesticides, which are used by organic growers and in forests to manage gypsy moth populations.

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The authors conclude that Cry1Ab protein is widely distributed and persistent in streams in the Maize Belt, and suggest that tile drainage systems are a likely source of Cry1Ab to streams.

- Drawing conclusions on tile drainage as major source of Cry1Ab concentrations in Midwestern streams based on only two actual samples (only one of which contained measureable concentrations of Cry1Ab) is not valid and is contrary to published studies (e.g. Wolt and Pederson (2010)).
- The authors chose to cite only a portion of the scientific literature available on persistence of Cry proteins in soil and water. Cry proteins are known to readily degrade in soil and aquatic systems, thereby significantly reducing the possibility of persistence or long-term exposure to high concentrations in either soil or water (e.g. (Accinelli *et al.*, 2008; Clark *et al.*, 2005; Head *et al.*, 2002; Herman *et al.*, 2001; Icoz and Stotzky, 2007; Marchetti *et al.*, 2007; Prihoda and Coats, 2008a; Prihoda and Coats, 2008b).

The authors also conclude their paper with the suggestion that further research is needed to determine the effects of Cry1Ab insecticidal proteins on non-target ecosystems; however, the author's own research in a separate peer-reviewed paper (Chambers *et al.*, 2010) found that the invertebrate communities in streams next to *Bt* maize fields are unaffected by exposure to *Bt* maize.

### Summary

The results of the Tank *et al.* (2010) study are neither new nor surprising. It is not clear how much of the detected Cry1Ab protein is biologically active, but even if the protein is biologically active, concentrations of protein detected by Tank *et al.* (2010) are below levels of concern for non-target aquatic organisms. This conclusion is supported by realistic field exposure studies examining the effects from *Bt* maize on the aquatic invertebrate community which showed no adverse impact (Chambers *et al.*, 2010; Swan *et al.*, 2009).

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Andow <i>et al.</i> , 2010)	<p><b>Objective:</b> To determine the in-field refuge planting patterns Cry1Ab (MON 810) and Cry3Bb1 (MON 863)-expressing maize in Minnesota in 2005</p> <p><b>Experimental Design:</b> All maize fields in southwestern Minnesota were sampled late June, 2005 for the presence of <i>Bt</i> (taking leaf tissue samples from plants in two different rows) and September, 2005 for in-field refuges. Compliance on in-field refuges was measured by evaluating planting patterns of <i>Bt</i> and non-<i>Bt</i> maize seed throughout the field. The size (number of rows) of the planter and the area of the field was determined, then the number of times the planter boxes would have to be refilled to plant the entire field was calculated. At each sample location, every row of the planter was tested for the presence or absence of Cry1Ab and Cry3Bb1. In each row, leaf tissue (0.9 cm disk) was taken from two plants. Strip refuges were divided into compliant (refuge at least 4 rows wide) or non compliant fields (refuge with less than 4 rows wide). The percentage of refuge was calculated by averaging all planter patterns in a field.</p> <p><b>Results:</b> Although most in-field refuges contained at least the required overall 20% Cry(-) seed, only 5% of the MON 810 fields and 2% of the MON 863 fields were in compliance with USEPA requirements because the Cry(-) seed was not planted in enough contiguous rows.</p>	<p>The authors concluded that out that most growers in Minnesota “had planted their fields with either finely mixed refuges or with strips that were too narrow” and that” there was a high diversity in planting patterns, and the occurrence of Cry seed was in random rows”.</p> <p>Despite lack of compliance, the authors point out that “resistance failures have not been documented for either <i>O. nubilalis</i> or <i>D. virgifera virgifera</i>, so better education programs will need to be undertaken to encourage growers to plant in-field refuges properly</p>	Environment	In 2005 in Minnesota, there was substantial lack of compliance with refuge implementation. However, no resistance failure has been documented and therefore no adverse effects were determined in this study. <sup>5</sup>
			Observed parameter	Feedback on initial environmental risk assessment
			Insect resistance management	There are no changes to the conclusions of the safety of the initial risk assessment.

<sup>5</sup> The strip row option for in-field refuges is an option for *Bt* maize refugia in the EU. The percent of fields using this option is expected to be relatively low, therefore any increase in overall resistance risk due to the planting of <4 contiguous rows of non-*Bt* maize (assuming that the overall field contained at least 20% non-*Bt*) would be negligible (as evidenced in the US where this study was conducted in 2005, yet there still is no report of Cry1Ab resistance in *O. nubilalis*).

Publication	Summary of research and results	Conclusions	Protection goal	Adverse effects
(Alcantara <i>et al.</i> , 2011)	<p><b>Objective:</b> Quantify baseline susceptibility of Philippine <i>Ostrinia furnacalis</i> (Asian corn borer-ABC) populations to Cry1Ab, develop a diagnostic Cry1Ab concentration for monitoring <i>O. furnacalis</i> resistance to Cry1Ab-expressing maize, and use this diagnostic concentration to monitor changes in susceptibility of field populations of <i>O. furnacalis</i> to Cry1Ab.</p> <p><b>Experimental design:</b> Baseline susceptibility was conducted between 2002-2004. 25-50 <i>O. furnacalis</i> eggs masses from maize leaves were collected primarily from major maize-planting regions. Egg masses from Laguna province were used as the reference population. Neonates were placed on artificial diet containing at least six concentrations of Cry1Ab. All assays had three replications of 30 larvae per dose. Each assay was repeated twice. A series of linear mixed model analyses were used to evaluate whether results varied across years and provinces. All baseline bioassay data were reanalyzed by combining all data sets to increase sample size to develop the diagnostic concentration. Three diagnostic concentrations were chosen and evaluated by conducting bioassays with <i>O. furnacalis</i> populations in 2007 from similar sites where baseline susceptibility study insects were collected; 200 insects per population. The designated diagnostic concentration was used in 2009 for monitoring susceptibility to insects where <i>Bt</i> maize was frequently planted for 2-3 cropping seasons. Confirmatory tests were conducted on all populations with less than 99% mortality; survivors were pooled and insects were reared on non-<i>Bt</i> treated diet to adult stage and mass mated. F1 progeny were tested on <i>Bt</i> maize leaves using three replications of 50 neonates per assays and repeating assay three time.</p> <p><b>Results:</b> There was only about a 6-fold level of variation in susceptibility between all field populations assayed with Cry1Ab, all populations were highly susceptible to Cry1Ab. The upper limit of the estimated LC99 from these bioassay results was used as the diagnostic concentration (104 ng/cm<sup>2</sup>). When this diagnostic concentration was used to monitor the susceptibility of field populations in 2009 after 3 years of <i>Bt</i> maize cultivation, there was some enhanced survival of neonates, but all F1 progeny died when placed on <i>Bt</i> maize.</p>	The authors conclude that “Monitoring of field populations during 2009 in areas where <i>Bt</i> corn had been grown for 3 years found some enhanced survival of neonates at the diagnostic concentration but progeny of the diagnostic concentration survivors did not survive on <i>Bt</i> corn, indicating that ACB populations in the Philippines remain susceptible to Cry1Ab-containing <i>Bt</i> corn hybrids”.	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Insect Resistance Management	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
(Crespo <i>et al.</i> , 2010)	<p><b>Objective:</b> To determine the fitness costs of a field-derived population of Cry1Ab-resistant <i>Ostrinia nubilalis</i> to help with evaluating resistance management practices.</p> <p><b>Experimental Design:</b> Cry1Ab was purified from two sources. Five <i>O. nubilalis</i> strains or crosses were used for fitness comparisons; a resistant strain, reselected resistant strain, control strain, and two F1 progenies using reciprocal crosses. Developmental time, pupal weight, fecundity and number of offspring produced per strain were determined by rearing neonates (128/strain) on artificial diet. 45 adults/sex were randomly mated. Number of egg masses were recorded, weighed, and neonates produced per female recorded. Larvae were transferred to rearing diet to estimate neonate-to-adult survivorship and number of female adult offspring produced. Population growth parameters estimated using standard ecological equations. Spermatophore volume estimated by measuring spermatophore and calculating volume of a prolate ellipsoid. Mating frequency estimated by counting number of spermatophores. Pupal weight, developmental time, and spermatophore volume analyzed by ANOVA. Means separated using Fisher's protected LSD. Frequency of mating analyzed using chi square.</p> <p><b>Results:</b> There were significant fitness costs in Cry1Ab-resistant <i>O. nubilalis</i> in pupal weight, developmental time, number of egg masses/female, weight of egg masses/females offspring produced, and proportion of unmated females. There was essentially no difference in fitness costs between susceptible and F1's suggesting all fitness cost parameters were either recessive or incompletely recessive.</p>	The authors conclude "Selection for resistance to Cry1Ab significantly reduced the fitness of <i>O. nubilalis</i> .	Environment	No adverse effects were determined in this study.
		Resistant insects exhibited reduced pupal weight and increased developmental time compared with susceptible and F1 larvae derived from reciprocal crosses of resistant and susceptible parents. In addition, it was observed that resistant insects exhibited a higher proportion of unsuccessful matings and lower fertility than the susceptible strain. Despite the differences observed in resistant insects, our results did not indicate strong evidence of fitness costs in the F1 progeny".	<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Insect Resistance Management	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
(Desneux <i>et al.</i> , 2010)	<p><b>Objective:</b> to assess in the laboratory whether <i>Cotesia marginiventris</i> (Cresson) (Hymenoptera: Braconidae), is equally attracted to host-related volatiles derived from <i>Bt</i> and conventional maize plants.</p> <p><b>Experimental Design:</b> Four experiments were conducted to test the attraction of the moth parasitoid <i>C. marginiventris</i> to fall army worm (FAW) (<i>Spodoptera frugiperda</i> (Smith), Lepidoptera) and maize plant interactions. <i>Bt</i> maize seedlings were hybrid Pioneer 35N05 expressing a Cry1Ab protein (MON 810). Conventional plants were near-isogenic hybrid Pioneer 34A55. The frequency and time of visit of parasitoid females to test odors were observed in a four-arm olfactometer. The plants or frass were then placed individually in closed glass bells or flasks to serve as odor sources. Exp.1 consisted of FAW injured plants, representing complete plant–host systems, (maize seedling, FAW larvae, &amp; FAW frass). Exp. 2 used artificially injured plants to which FAW regurgitate was applied to exclude potential effects of FAW feeding differences on MON 810 or conventional maize seedlings. For Exp. 3 FAW were allowed to feed on MON 810 or conventional maize plants; frass was collected and placed in glass flasks as separate odors sources. The numbers of visits and the time spent in each olfactometer field and the effects of treatment within the experiment were analyzed via Friedman’s two-way ANOVA, and means were separated using Student–Newman–Keuls tests. Exp. 4, consisting of 4assays, each set with 3 clean air fields and 1 test odor, compared parasitoid response to frass from FAW fed: conventional maize, MON 810 maize, conventional maize treated with tetracycline, or MON 810 maize treated with tetracycline. Three independent statistical analyses were conducted. First, parasitoid attraction to frass odor versus clean air. Second, the influences of <i>Bt</i> maize and tetracycline on parasitoid attraction to frass were assessed by comparing the frequencies of parasitoid females selecting the frass odor in the assay with conventional maize without tetracycline against the frequencies of females selecting the frass odor in each of the three remaining sets of assays using permuted Fisher’s exact tests. Third, a Kruskal–Wallis test was used to compare the times spent in the frass odor fields among the four sets of assays.</p> <p><b>Results:</b> Parasitoid females: (Exp.1) Visited more frequently and spent more time in host-injured MON 810 or conventional seedling air fields than in clean air fields; visited host-injured conventional seedlings more frequently than host-injured <i>Bt</i> seedlings, but the time spent between these air fields did not differ. (Exp.2) Visited more frequently and spent more time in artificially-injured MON 810 or conventional seedling air fields than in clean air fields; frequency and time of visits between artificially-injured MON 810 and conventional seedlings more were not significantly different. (Exp.3) more frequently visited</p>	<p>The authors conclude, “The results of our experiments showed that <i>C. marginiventris</i> females responded positively to host-associated and host-induced odors derived from both conventional and <i>Bt</i> maize seedlings when searching for FAW hosts.” And that the “results suggested that while the responses of foraging parasitoids to odors that are useful at medium to long ranges, such as those omitted by host injured plants, may not differ between conventional and <i>Bt</i> maize plants, their responses to odors useful at close-range, such as those emitted by frass, may be weakened by a host’s ingestion of <i>Bt</i> maize tissue.”</p>	No adverse effects were determined in this study.	No adverse effects were determined in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Interactions between the GM plant and NTOs	There are no changes to the conclusions of the safety of the initial risk assessment.

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	<p>and spent more time air field of frass derived from conventional maize, followed by frass derived from MON 810 maize, and finally to fields of clean air. (Exp.4) selected with a greater frequency than random, the odor field of frass involving hosts fed conventional maize without tetracycline, but not in the remaining assays; spent more time in the frass odor field with in hosts fed conventional maize without tetracycline compared to the other remaining assays.</p>			
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Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Engels <i>et al.</i> , 2010)	<p><b>Objective:</b> To investigate <i>Bt</i> resistance alleles in European corn borer populations using F-2 screening</p> <p><b>Experimental Design:</b> 784, 455, 80, and 26 isofemale lines were F-2 screened in France, Germany, Italy, and Slovakia between 1999-2005, respectively. Each country performed the F-2 screen slightly different, especially using various stages of the F0 for starting material. Insects were tested for resistance using either MON 810-expressing maize or diet containing Cry1Ab. Results from each F-2 screen were analyzed for the expected resistance allele frequency with its 95% credibility interval using Bayesian analysis and probability of a false negative (using a custom program compiled in C++)</p> <p><b>Results:</b> Many isofemale lines were lost due to various factors, especially due to females being unmated. No resistant F-2 larvae were found in any of the 1,345 isofemale lines evaluated. Allele frequency ranged from <math>9 \times 10^{-3}</math> – <math>3 \times 10^{-4}</math>. The cost for conducting the F-2 screen varied from US\$1,300/screened line (Slovakia) to US\$300 (France)</p>	<p>The authors conclude that “Making the assumption that European corn borer populations in these countries belong to the same genetic entity, the frequency of alleles conferring resistance to the Cry1Ab produced by the MON 810 maize in western and central Europe was <math>1.0 \times 10^{-4}</math>, with a 95% confidence interval of <math>3.0 \times 10^{-4}</math>”. Also “F-2 screen is probably the best option for resistance monitoring in European regions where maize fields are infested by bivoltine or multivoltine populations of <i>O. nubilalis</i>, this question remains open in other regions where maize fields host univoltine populations of this pest”. However, the authors recognize this method is costly.</p>	Environment	No adverse effects were determined in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Insect Resistance Management	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
(Feng <i>et al.</i> , 2010)	<p><b>Objective:</b> To compare the induced response in undamaged parts of MON 810 maize resulting from <i>Ostrinia furnacalis</i> (Asian corn borer) damage. To explore how the Cry protein may interact with herbivore damage with respect to systemic defense responses in maize.</p> <p><b>Experimental Design:</b> MON 810 maize (5422CBCL) expressing Cry1Ab, and a conventional non-<i>Bt</i> maize (5422) were compared in this experiment.</p> <p>The maize was grown individually in growth chamber at 79% relative humidity, and 22 -28°C, 12 h/12h (dark/light) photoperiod. Two treatments were applied to maize plants with two fully expanded leaves in triplicate. For the herbivore damage (HD) treatment, 3 third-instar <i>O. furnacalis</i> were placed onto the first leaf of the maize. The larvae and the leaf were then sealed within a bag. For the control maize, the first leaf was sealed with a bag but without <i>O. furnacalis</i> infestation. After 6 h, the second leaf and root of each plant were collected separately and were used to determine the contents of Cry protein (ELISA), DIMBOA (HPLC), total phenolics, and some defense related genes (RT-PCR)</p> <p>Analysis of group t-Test was carried out, and significant differences between treatments were tested at the 0.05 level.</p> <p><b>Results:</b> There was no difference in the Cry1Ab content in the second leaf after the <i>O. furnacalis</i> treatment of MON 810 compared to the control maize. Whereas the Cry protein in the root of MON 810 maize significantly increased after the first leaf was damaged by <i>O. furnacalis</i> for 6h, as compared to the control treatment.</p> <p>There were no obvious effects on DIMBOA content in the second leaf or roots between the MON 810 maize and the control maize. There were systemic induced effects on the expression of DIMBOA synthesis mediated genes (<i>Bx6</i> and <i>Bx9</i>) in the roots of both conventional control maize and MON 810 maize, but not in the second leaf of the control maize or MON 810 maize.</p> <p>“No remarkable effects were observed on the total phenolics content in the second</p>	The authors conclude that “these findings suggest that <i>Bt</i> gene introduction alters the systematic induced effects of pest damage on the corns, leading to stronger chemical defense response in the second leaf for <i>Bt</i> corns than in conventional corn”	Environment	The feeding damage between MON 810 and the conventional control maize would not be expected to be equivalent, and is not controlled for in this experiment. This difference in insect sensitivity and feeding behavior could account for the few differences that were reported in the expression of measured plant defense genes. Therefore, no conclusion of adverse effects can be drawn from this study. <sup>6</sup>

<sup>6</sup> The authors do not address that *O. furnacalis* is highly sensitive to the Cry1Ab protein of MON 810, with LC<sub>50</sub> (50% lethal concentration) levels reported as low as 0.10 to 0.81 µg/g (Cry1Ab protein/diet; He *et al.*(2005)). The feeding damage between the test (MON 810) and the conventional control maize are not expected to be equivalent. Differences in insect sensitivity or feeding behaviour can affect the plant defence response and should be considered in the design of such studies. These important variables were not considered by the authors, and, injury ratings were not evaluated in this study. Furthermore, the authors’ experiment represents a screening-level test and is not fully designed to make firm conclusions. In addition to the absence of injury ratings, the use of a single and sensitive insect species, the small number of measurements, and the lack of a baseline reference for maize varieties including the control maize used in this experiment, represent limitations of this study.

	<p>leaf and roots of MON 810 after feeding by <i>O. furnacalis</i> for 6h.” In control conventional maize the total phenolics in the roots decreased. However, the second leaf on the control maize was not different after <i>O. furnacalis</i> damage. The phenolics acid mediated gene <i>Pal</i> was induced by <i>O. furnacalis</i> damage in roots of the control maize and MON 810, and in the second leaf of MON 810.</p> <p>The expression of direct defense protein mediated genes <i>MP1</i> and <i>PR-2a</i> were enhanced in the second leaf and roots of the control maize by <i>O. furnacalis</i> damage. In MON 810 <i>MP1</i> and <i>PR-2a</i> were slightly enhanced in the second leaf, and <i>PR-2a</i> was slightly enhanced roots following <i>O. furnacalis</i> damage.</p> <p><i>O. furnacalis</i> feeding systemically induced the expression of <i>Fps</i> and <i>Tps</i> (volatile substances mediated genes) in the roots of conventional maize but had no effects on the expression of <i>Fps</i> and <i>Tps</i> in the second leaf. Expression of <i>Tps</i> was enhanced in MON 810 in the roots and second leaf, but no induced effects of <i>O. furnacalis</i> feeding was detected for expression of <i>FPS</i>.</p>		<p><b>Observed parameter</b></p>	<p><b>Feedback on initial environmental risk assessment</b></p>
			<p>Insect Resistance Management</p>	<p>There are no changes to the conclusions of the safety of the initial risk assessment.</p>

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Ghimire <i>et al.</i> , 2011)	<p><b>Objective:</b> Evaluate performance of current <i>Bt</i> maize hybrids in against Cry1Ab-susceptible and resistant <i>Diatraea saccharalis</i>; evaluate performance of MON 89034 against both strains to determine if MON 89034 could overcome Cry1Ab resistance.</p> <p><b>Experimental Design</b> Used Cry1Ab susceptible (S:S), Cry1Ab-resistant (RR) and backcrossed Cry1Ab-resistant (RS )strains. Bioassays used maize leaf tissue or intact plants. All insect strains tested against eight commercial maize hybrids (non-<i>Bt</i>(2), MON 810(3), TC1507(2) and <i>Bt</i>-11(1); six experimental maize lines (non-<i>Bt</i> (2), 2 MON 810(2) and MON 89034 (2. Leaf bioassays: 25 neonates/well, 8-wells/ tray. Mortality assessed on day 12. Four replications. Glasshouse plant tests Commercial hybrids tested in 2 trials, exp. hybrids in 1 trial. Trial: 16-20 neonates/maize whorl, and dissected 21-25d post infest. 8 plants/treatment replicated 4 times. Data analysis: Percentages of larval survivorship transformed using arcsine (x0.5). Data was analyzed with a two-way ANOVA using GLM.</p> <p><b>Results:</b> Cry1Ab resistant <i>D. saccharalis</i> were resistant to MON 810 tissue and plants as well as TC1507 suggesting cross resistance between Cry1Ab and Cry1F. None of the 4 Cry1Ab commercial varieties contain a high dose against resistant heterozygotes. Resistance in <i>D. saccharalis</i> is incompletely dominant. MON 89034 provided 100% mortality to all insects of all genotypes. There also were no maize tunnels in the two MON 89034 varieties for any of the insect genotypes.</p>	The authors conclude “Larvae of Cry1Ab-RR and -RS also caused significant plant injury to most of the commercial <i>Bt</i> corn hybrids, especially to the Cry1Ab corn. Cry1Ab resistance in <i>D. saccharalis</i> was incompletely dominant on commercial <i>Bt</i> corn hybrids”.	Environment	This article suggest that there is a relatively high potential for resistance development in <i>D. saccharalis</i> to single gene (Cry1Ab) maize. However, field resistance of this pest to Cry1Ab has not been detected. Therefore, no adverse effects can be concluded from this study. <sup>7</sup>
			Observed parameter	Feedback on initial environmental risk assessment
			Insect Resistance Management	There are no changes to the conclusions of the safety of the initial risk assessment.

<sup>7</sup> The populations of *D. saccharalis* used in the experiments were developed in the laboratory using F2 screening which may suggest that resistance alleles are present in the field. However, no field resistance of *D. saccharalis* has been detected in the US to Cry1Ab. In addition, caution should be exercised when pyramiding Cry1Ab with Cry1F against *D. saccharalis* due to the potential for cross resistance. MON 89034 can completely overcome Cry1Ab resistance in *D. saccharalis*, thereby providing a means for *Bt* resistance management in *D. saccharalis* if field resistance to MON 810 occurs.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Lopez <i>et al.</i> , 2010)	<p><b>Objective:</b> To determine the impact of <i>Nosema pyrausta</i> infected <i>Ostrinia nubilalis</i> on growth, development and susceptibility to Cry1Ab and the potential impacts on resistance evolution to Cry1Ab-expressing <i>Bt</i> maize</p> <p><b>Experimental Design:</b> Four populations of <i>O. nubilalis</i> were evaluated for susceptibility and weight gain to 2 doses of Cry1Ab: 1) Cry1Ab resistant, uninfected, 2) Cry1Ab resistant, <i>N. pyrausta</i>-infected, 3) Cry1Ab partially resistant, uninfected, 4) Cry1Ab partially resistant, <i>N. pyrausta</i>-infected. Diet overlay bioassays results recorded from all larvae at 7 days including weight and survival. The experiment designed was a stripped-plot with four replicates. Each 128-cell tray considered a replicate with diet as one strip and F1 population as the other strip. For each replicate, 8, 12, and 12 neonates were exposed to control, “low”, and “high” doses of Cry1Ab, respectively. Weight data were log10-transformed. All data were modeled using restricted-maximum-likelihood estimates for the mixed-model analysis of variance. Diet, disease status, resistance, and their interactions were considered fixed effects. Random sources of variance included replicate effects and their interactions with fixed effects.</p> <p>All data were modeled using restricted-maximum-likelihood estimates for the mixed-model analysis of variance</p> <p><b>Results:</b> Infection of <i>O. nubilalis</i> with <i>N. pyrausta</i> affected survival of both resistant and partially resistant larvae at the high <i>Bt</i> concentration, and development at both low and high <i>Bt</i> concentrations. The combination of <i>N. pyrausta</i> infection and <i>Bt</i> led to lower weights (slower development) in both resistant and partially resistant than either factor alone.</p>	<p>The authors conclude: “it is important to account for this third trophic level when modeling resistance evolution. Because the presence of this microsporidium in the ecosystem may function to slow the evolution of <i>O. nubilalis</i> resistance to <i>Bt</i> maize, efforts to conserve <i>N. pyrausta</i>, and facilitate transmission between insects could help ensure continued effectiveness of the technology”.</p>	Environment	No adverse effects were determined in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Insect Resistance Management	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
(O'Rourke <i>et al.</i> , 2010)	<p><b>Objective:</b> To show differences in non-maize feeding for two separate races of <i>Ostrinia nubilalis</i> (ECB) and potential impacts on IRM as it pertains to refugia.</p> <p><b>Experimental Design:</b> ECB races were collected in E or Z-specific pheromone traps in New York, 2006. The wings of 68 E-race and 71 Z-race insects were analyzed for <math>\delta^{13}\text{C}</math> content. Fischer exact test were used to assess relationship between pheromone race and host history and the effects on host history were tested using ANOVA of a full factorial model. In addition, contrasts were made to test hypotheses that E and Z race weigh the same when developed on C4 plants and less when developing on C3 plants. 138 E-race and 206 Z race insects were collected in Penn Yan, New York 2006 to determine relationship between adult mass pheromone race and host history. Moths from large and small tails of mass distributions were selected for <math>\delta^{13}\text{C}</math> analysis; 11 large E, 11 large Z, 7 small E, 9 small Z. The effects of moth size and pheromone race on host history were analyzed with exact logistic regression and the relationship between pheromone race and host history were further investigated using Fisher exact tests of two-by-two contingency tables. Relationship between adult size (pupal) and egg production were also measured for Z race (3 replicates; N = 66, 37,60 females) as well as the effect of pupal size on fecundity using linear regression. The geographic distribution of ECB were consolidated by summing all E and Z moth counts and the relationship between proportion of E moths/county were analyzed using multiple regression. ANOVA were used to test whether E race ECB was higher in eastern counties.</p> <p><b>Results:</b> Unstructured refuges contribute more to E race (18%) than to Z race (4%) populations. Also feeding on non-host plants is associated with decreased body mass and reduced fecundity although no difference in adult mass were detected between Race E and Race Z feeding on C4 plants. Race E were smaller when feed on C3 vs C4. The geographical range of E race ECB is restricted within the the range of the Z race and that E race are increasingly dominant in regions with increasing non-maize habitats.</p>	The authors conclude that their research shows that “utilization of unstructured refuges differ between the E and Z pheromone races of ECB in the United States” and that “where multiple races of a species have overlapping distributions, IRM strategies should be conservatively based on the race most likely to develop resistance, in this case the Z race of ECB” <sup>8</sup>	Environment	No adverse effects were determined in this study.
			<b>Observed parameter</b>	<b>Feedback on initial environmental risk assessment</b>
			Insect Resistance Management	There are no changes to the conclusions of the safety of the initial risk assessment.

<sup>8</sup> The EU does not allow for the use of unstructured refuges for *Bt* maize, and therefore the current maize structured refuge requirement should be appropriate.

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