Appendix 5.1. MON 810 Literature Review – Food/Feed

MON 810 literature review (July 2015)

Appendix 5.1 - Food/Feed

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Area of the environmental risk assessment: Food/Feed Safety – Animal Feeding Study

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Furgal-Dieriuk *et al.*, 2015)	***Objective:*** To determine whether feed containing grains from genetically modified (GM) MON 810 maize expressing the *Bacillus thuringiensis* Cry1Ab insecticidal protein, and GM MON 40-3-2 herbicide tolerant soybean meal affect milk composition and production, serum metabolite profiles, and transfer of transgenic DNA (tDNA) into the milk of cows. ***Experimental Design:*** The experiment was conducted in Poland from the 3rd week before parturition to the 305th day of lactation. 40 Polish Holstein-Friesian cows were assigned to 4 groups of 10 animals according to body weight, milk yield and parity. They were fed a total mixed ratio (TMR), containing 35% of concentrate mixture in dry matter. This mixture was different for each group: it contained either GM maize and GM soybean meal, non GM near-isogenic maize and GM soybean meal, GM maize and non GM near-isogenic soybean meal, or non GM near-isogenic maize and non GM near-isogenic soybean meal. Samples of each feed were taken three times to determine chemical composition. Effective rumen degradability of dry matter and crude proteins were determined on 3 permanently fistulated cows. The body weight of the cows in lactation was regularly determined. Milk yield was estimated daily according to standard procedures. Milk composition was determined in daily samples collected from each cow every two weeks throughout lactation, starting approximately 10 days after calving. On Days 120, 150 and 220 after calving, a total of 72 milk samples were collected from 6 cows of each group and analysed for the presence of the transgenic DNA by PCR. From the first week after calving to the 4th week of lactation, blood samples were taken on Days 7, 10, 17 and 24 from the jugular vein about 4 h after the morning feeding. These samples were used to measure metabolite parameters (e.g., β-hydroxybutyric, free fatty acids, glucose, insulin and progesterone). Statistical analysis was performed using one-way analysis of variance. ***Results:*** There were no significant differences between transgenic and non-transgenic feed with respect to milk yield and composition, dry matter intake, body weight and blood metabolite profiles. Although numerically small differences were observed in the composition of the feed, they were within the normal expected range and comparable to the feed used in Poland. Transgenic DNA sequences from GM maize and GM soybean meal were not detected in the cow milk.	The authors concluded that: *'The current results conform to earlier work with plants of the "first generation", e.g., without substantial changes. There is, however, a need for new, quality studies using new/ other plants, e.g. bio-fortified plants or plants with substantial changes in composition, and new, more sensitive, analytical methods'.*	Animal health **Observed parameter** Dietary fate of the DNA	No adverse effects were determined in this study **Feedback on initial environmental risk assessment** There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Gu *et al.*, 2014)	***Objective:*** To investigate the response of Atlantic salmon (*Salmo salar* L.) juveniles exposed to genetically modified (GM) insect resistant maize (MON 810) in a 99-day feeding trial. ***Experimental Design:*** *Bacillus thuringiensis* (Bt) maize (MON 810) and its near-isogenic non-GM line were derived from PR34N44 and PR34N43 varieties, respectively. Fish diets were balanced regarding vitamins and minerals and optimized to achieve equal protein:energy ratios of 25g/MJ. Four experimental diets were prepared, each containing approximately 20% maize. One pair was fishmeal-based while the other pair included standard soybean meal (SBM; 16.7% inclusion level). Three replicate tanks of fry (0.17 ± 0.01 g) were fed one of the four diets and samples were taken on Days 15, 36, 48 and 99. Survival, growth performance, whole body composition, digestive function, morphology of intestine, liver and skeleton, and mRNA expression of some immune and stress response parameters in the distal intestine were evaluated. Diets and whole fish (at the end of the 99 days feeding trial) were analysed for composition of dry matter, crude protein and crude lipid. Activities of pancreatic enzymes trypsin and amylase, brush border membrane enzymes leucine aminopeptidase, maltase and bile acid concentration were analysed in 10-15 whole fish on each sampling days. ***Results:*** After 99 days of feeding, survival was enhanced and the intended SBM-induced inflammatory response in the distal intestine of the two groups of SBM-fed fish was absent, indicating that the juvenile salmon were tolerant to SBM. Mortality, growth performance and body composition were similar in fish fed the two maize varieties. The Bt-maize fed fish, however, displayed minor but significantly decreased digestive enzyme activities of leucine aminopeptidase and maltase, as well as decreased concentration of gut bile salts, but significantly increased amylase activity at some sampling points. Histomorphological, radiographic and mRNA expression evaluations did not reveal any biologically relevant effects of Bt-maize in the gastrointestinal tract, liver and skeleton.	The authors concluded that: *"the Cry1Ab protein or other compositional differences in GM Bt-maize may cause minor alterations in intestinal responses in juvenile salmon, but without affecting overall survival, growth performance, development or health"*.	Animal health	No adverse effects were determined in this study.
			Observed parameter	**Feedback on initial environmental risk assessment**
			Animal performance	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
(Andreassen *et al.*, 2015b)	***Objective:*** To determine whether intranasal exposure to either pollen from genetically modified (GM) MON 810 maize expressing the *Bacillus thuringiensis* Cry1Ab insecticidal protein, MON 810 leaf extracts, Cry1Ab protoxin or trypsinized protoxin Cry1Ab elicits immune and/or allergic responses in mice. ***Experimental Design:*** Four different sources of Cry1Ab protein were used: 1) pollen from MON 810 and non-GM maize, 2) leaf extracts from MON 810 and non-GM plants, 3) purified Cry1Ab protoxin isolated from *B. thuringiensis* spores, and 4) trypsinized Cry1Ab (trypCry1Ab) protein to intranasally expose 6-7 week old BALB/c female mice on days 0, 1 and 2, and boostered intranasally on days 21, 22 and 23. Blood samples were collected from the *vena saphena lateralis* from each animal on Day 0 and 21 prior to exposure. The mice were terminated and blood and broncho-alveolar lavage fluid (BALF) were collected. In Experiment 2, mediastinal lymph nodes (MNLs) were also collected and single cell suspension was obtained according to a well-established procedure.Anti-Cry1Ab IgG1, IgG2a and IgE were detected in mouse sera by ELISA. Cytokine levels in BALF and in supernates from MNL cells were determined by Cytometric Bead Array. BALF was also used to perform the differential cell count of macrophages, eosinophils, neutrophils, lymphocytes and epithelial cells. ***Results:*** The MON 810 plant material did not elicit humoral immune responses in mice after airway exposure. However, the mice produced specific IgG1 and IgE against the two purified protein versions.	The authors concluded that production of specific IgG1 and IgE antibodies indicate the ability of Cry1Ab protein to induce immune responses and trigger pro-allergic responses in mammals and that the airway exposure of Cry1Ab proteins (e.g. trough pollen and dust) is a relevant route of exposure and the results therefore warrant further studies.	Animal health	The article shows that the MON 810 plant material did not elicit immune response after exposure. This study reports Cry1Ab protein to be pro-allergic based on production of IgE antibodies against the 2 purified versions; the relevance of these findings to the use of Cry1Ab in GM crops is not clear [1], [2], [3].
			Observed parameter	**Feedback on initial environmental risk assessment**
			Allergenicity and toxicity	There are no changes to the conclusions of the safety of the initial risk assessment.

[1] The observed IgE and IgG production in mice could be a result of Cry1Ab protein over exposure and do not represent relevant levels of exposure for MON 810 (25X and 200X higher than the amount of Cry1Ab present in MON 810 leaf and pollen, respectively).

[2] Cry 1Ab source organism (*Bacillus thuringiensis – Bt*) is not an allergenic source. Cry1Ab protoxin is expressed at very low levels in the GM plant and has no sequence similarity to known allergens. Also, the protein is rapidly digested in simulated gastric and intestinal fluids. These conditions make it almost impossible for the protein to elicit immune response.

[3] BALB-C mice are inbred mice that are prone to produce allergic response to proteins. BALB-C mice cannot accurately predict protein allergenicity in humans and therefore the observations are not relevant and the clinical significance of these observations in humans is questionable.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Andreassen *et al*., 2015a)	***Objective:*** To determine whether exposure to pollen and/or leaf material from genetically modified (GM) MON 810 maize, expressing the *Bacillus thuringiensis* Cry1Ab insecticidal protein, elicits airway immune response or exerts an adjuvant effect on antibody production against the allergen ovalbumin (OVA) in a mouse model of airway allergy. ***Experimental Design:*** Three different sources of Cry1Ab protein were used: 1) pollen from MON 810 maize, 2) leaf extracts from MON 810 plants, and 3) trypsin-activated Cry1Ab protein produced in recombinant *Escherichia coli*. Homologous materials from an unmodified near-isogenic maize variety and the known mucosal Th2 adjuvant, cholera toxin (CT), were also included in the test schemes. Two independent experiments were performed at two different locations: North-West University (experiment 1; Porchefstroom, South Africa) and Norwegian Institute of Public Health (experiment 2; Oslo, Norway). 6- 7 week old BALB/c female mice were intranasally exposed to 35 µl of test solutions on Days 0, 1 and 2. On Days 21,22 and 23, all mice except those of the vehicle control group were exposed to allergen. 100 µl of blood sample were collected from the *Vena saphena lateralis* from each animal on Days 0 and 21 prior to the challenge. The mice were terminated and blood and broncho-alveolar lavage fluid (BALF) were collected. In experiment 2, mediastinal lymph nodes (MNLs) were also collected and single cell suspension was obtained according to a well-established procedure. IgE and IgG1 antibodies in sera were determined by using a capture ELISA; OVA-specific IgG2a antibodies were determined by indirect ELISA. Cytokines in BALF and in the supernatant of MNL cell suspensions were analyzed by Cytometric Bead Array. BALF was also used to perform the differential cell count of macrophages, eosinophils, neutrophils, lymphocytes and epithelial cells. ***Results:*** Immune responses induced by intranasal exposure to OVA in combination with each Cry1Ab protein preparation were compared with those induced by OVA alone or together with CT. A clear proallergic adjuvant effect of CT was observed, as proven by increased specific IgE, eosinophils and Th2 cytokines in MLN cell supernates, while no increase in OVA-specific antibodies or cytokine release from MLN cells after stimulation with OVA was observed in mice receiving Cry1Ab-containing plant materials or the trypCry1Ab protein.	The authors concluded that *'Cry1Ab protein from three different sources did not act as an adjuvant in our mouse model under the experimental conditions used. Although the contents in our MON 810 maize tissues may represent 'relevant doses', long-term exposure to plant Cry1Ab as well as purified plant proteins to mimic the total exposures experienced in real-life situations, should be included in future studies*'.	Animal health	No adverse effects were determined in this study
			Observed parameter	**Feedback on initial environmental risk assessment**
			Allergenicity and toxicity	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
(Reiner *et al*., 2014)	***Objective:*** To assess the adjuvant effect of genetically modified (GM) MON 810 maize expressing the *Bacillus thuringiensis* Cry1Ab insecticidal protein (Bt maize) on the initiation and relapse of ovalbumin (OVA)-induced allergic airways disease in experimental mice. ***Experimental Design:*** Four to six week old BALB/c female mice were provided with a diet containing 33% GM or non GM maize for up to 34 days before inducing either ovalbumin (OVA)-experimental allergic asthma or disease relapse in mice with pre-existing allergy. Three days after the last challenge, the mice were sacrificed to collect bronchoalveolar lavage fluid (BAL), lungs and serum. BAL was used to determine the number of inflammatory cells (eosinophils) as a measure of airway inflammation. Lungs were analyzed for the presence of inflammatory cells and mucus secretion. Serum was tested for the presence of OVA-specific antibodies by ELISA assay. ***Results:*** Feeding GM-maize did not affect airway and lung inflammation, mucus secretion in lung and OVA-specific antibody production at initiation or relapse of OVA-induced allergic asthma. This indicates that Bt-maize has no adjuvant effect on allergic responses in a mouse model of allergic asthma.	The authors concluded that:*'...consumption of a Bt-maize containing diet did not influence allergic responses to the experimental, unrelated OVA-induced disease initiation and relapse of allergic asthma. This study differs from previous studies in that the mice had GM and non GM maize included in their diets, which is physiological and more relevant than administering purified Cry proteins via alternative routes'.*	Animal health	No adverse effects were determined in this study
			Observed parameter	**Feedback on initial environmental risk assessment**
			Animal performance	There are no changes to the conclusions of the safety of the initial risk assessment.

Area of the environmental risk assessment: Food/Feed Safety – Molecular characterisation

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(La Paz *et al.*, 2014)	***Objective***: To compare the immature embryo transcriptome of genetically modified (GM) MON 810 maize expressing the *Bacillus thuringiensis* Cry1Ab insecticidal protein (Bt maize) with the one of non-GM near-isogenic varieties. ***Experimental Design***: Maize seeds of commercial varieties of MON 810 (DK6575, PR33P67 and DKC6041-YG) and the corresponding near-isogenic varieties (Tietar, PR33P66 and DKC6040) were obtained from the Spanish market. The MON 810 homozygous line was obtained by auto-pollination of DKC6575. To perform transcriptome sequencing (RNA-seq), 12 plants of DKC6575 and its near-isogenic counterpart were grown to maturity in the greenhouse under controlled conditions and 100 embryos per plant were collected 20 days after pollination (DAP). Polyadenylated RNA was isolated from 1200 embryos to synthesize cDNA used to prepare a 454-cDNA library, which was titered and sequenced using the 454 GS-FLX (Titanium) pyrosequencing technology. 3'-UTR reads were selected and mapped against the maize genome. Differential expression between libraries was assessed by DEseq and EdgeR statistic packages. To compare gene expression, total RNA from pools of 50 maize embryos of 20 DAP of each variety was used for cDNA synthesis and colour labelling. Labelled cDNA was fragmented and hybridized with the Agilent maize 44K microarray. Data analysis was performed using the Robin software. The expression of 30 differentially regulated genes was confirmed by real-time PCR in different tissues and maize varieties. 60 embryos at 20 DAP and full maturity stages were excised from the mid-part of the cobs from twelve plants of the three MON 810-near isogenic variety pairs. Total embryo area and axis length was calculated using high-resolution images. ABA hormone was quantified by ELISA. ***Results***: 3'UTR-anchored mRNA-seq produced 1,802,571 sequences from DKC6575 and 1,170,973 from Tietar, which mapped to 14,712 and 14,854 unigenes, respectively. Gene expression analysis showed 140 differentially expressed genes mainly involved in carbohydrate metabolism, protein metabolism and chromatin organization. qRT-PCR analysis of 30 selected genes confirmed that most of these genes were differentially expressed in the 3 MON 810 events as compared to the near-isogenic counterparts. Analysis of functional annotation and expression pattern during embryogenesis and in response to ABA of the differentially expressed genes suggest a slight but significant delay in seed and plant maturation for MON 810.	The authors concluded that the overall transcription is similar in 20 DAP embryos of the MON 810 variety DKC6575 and the corresponding near-isogenic variety Tietar. Nevertheless, about 140 genes had altered transcription levels, which is very likely due to small differences in seed development in MON 810 *versus* conventional comparators. In addition, these - differences in transcription are most probably linked to the MON 810 event but are not associated to undesirable changes in the phenotype and plant behaviour, nor in the chemical and nutritional composition.	Environment	No adverse effects were determined in this study
			Observed parameter	Feedback on initial environmental risk assessment
			Plant gene expression	There are no changes to the conclusions of the safety of the initial risk assessment.

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Trtikova *et al*., 2015)	***Objective:*** To explore the relationship between the expression of *Bacillus thuringiensis* (Bt) transgene and Cry1Ab protein content in two MON 810 varieties, and to test whether abiotic environmental stress conditions influence the relationship between transgene expression and protein content. ***Experimental Design:*** Seeds of two MON 810 varieties (white Bt—PAN 6Q-321B and yellow Bt—PAN 6Q-308B) were sown and fifteen plants of each variety were grown in the climate chambers under optimal conditions (16/8 L/D, 25/20°C, 50/65% relative humidity (rh), watered regularly). After six weeks, the plants were either kept under optimal conditions or exposed to stressful environmental conditions for one week. The stressful conditions included a hot/dry treatment in a greenhouse or a cold/wet treatment. Upper leaves were sampled before and after stress conditions and used for RNA extraction and leaf extracts. The RNA was used to determine the *cry1Ab* transgene expression, by performing quantitative RT-PCR with specific primers for the *cry1Ab* transgene. The leaf extracts were used to determine the level of Bt protein by ELISA. Three-way analysis of variance (ANOVA) was used to test for the effects of the variety, stress treatment and the timing of the sampling on the transgene expression and Bt content. ***Results:*** Under optimal conditions, there was no significant difference in the transgene expression between the two Bt maize varieties, whereas Bt protein levels differed significantly in the tissue samples of the two Bt maize varieties, with the yellow Bt maize leaves containing on average 40% more Bt protein than the white Bt maize leaves. In addition, the transgene expression was correlated with Bt protein content only in the white Bt plant. Under cold/wet stress the transgene expression was similar to the expression under optimal conditions, but the expression of the transgene was reduced under hot/dry stress, though this difference was significant only in white Bt maize. Bt content was similar in plants grown under optimal and hot-dry condition. However, a higher Bt content (4-fold increase) was observed in the white Bt maize plant exposed to cold/wet stress as compared to the plants grown under optimal conditions. These results suggest that Bt content is not only controlled by the transgene expression but is also dependent on the genetic background of the maize variety.	The authors found '*large variation in the transgene expression and Bt protein content caused by plant genetic background and environmental conditions. Field-grown Bt maize plants might therefore not always produce high enough dose of Bt protein to kill the intermediate (heterozygous) resistant insect pests. ..... Thus, any assessment of transgenic Bt plants will be incomplete without measuring transgene expression in conjunction with Bt protein content and efficacy".*	Environment	No adverse effects were determined in this study
			Observed parameter	**Feedback on initial environmental risk assessment**
			Plant gene expression	There are no changes to the conclusions of the safety of the initial risk assessment.

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