Appendix 5.1. MON 810 Literature Review – Food/Feed

MON 810 literature review (July 2014)

Appendix 5.1 - Food/Feed

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Review of peer-reviewed publications

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Bednarek *et al.*, 2013)	***Objective:*** To evaluate the immune effects of genetically modified (GM) MON 810 maize expressing the *Bacillus thuringiensis* Cry1Ab insecticidal protein (Bt maize) and meal from glyphosate tolerant soybean (40-3-2) when fed to pig, cattle and poultry. ***Experimental Design:*** The study was conducted on 60 pigs (36 fatteners from ca. 30 to 110 kg bodyweight and 24 sows), 20 calves (from ca. 7 to 90 days of age), 40 broilers (from 1 to 42 days of age) and 40 laying hens (from 25 to 54 weeks of age). Each species was divided into four groups: (i) controls, fed conventional feed, (ii) fed GM soybean meal and non-modified maize, (iii) fed non-modified soybean meal and GM maize, (iv) fed GM soybean meal and GM maize. In the experiment on fatteners, two additional groups were formed: (v) fed non-modified soybean meal and bruised grain and (vi) fed GM soybean meal and non-modified bruised grain. Blood samples for analysis of selected parameters characterising immune response were collected twice from all groups before and after administration of feed. Total and differential number of white blood cells (WBC), total number of lymphocytes (LYM), total number of "mid-size" cells such as monocytes, eosinophils and basophils (MID) and polymorphonuclear leukocytes (PMNL) were assayed. Also, immune phenotyping of peripheral blood lymphocytes by the expression of CD2 (T-cell antigen), CD4 (T-helper cell antigen), CD8 or CD8a in poultry (T-cytotoxic/suppressor cell antigen) and WC4 (bovine B-cell antigen) surface marker was performed using immunofluorescent flow cytometry. ***Results:*** No significant effects on the immune response related to feed mixtures containing GM components were observed in any of the treated groups compared to controls. No significant changes in the WBC, leukogram and lymphocyte immune-phenotyping were found. In the case of cattle, WC4 positive cell subpopulation showed no significant differences from controls. The phagocytic activity of bovine leukocytes and humoral immune response was analysed in calves after specific immunisation. The results also did not indicate any significant effect of GM feed in the livestock.	The authors concluded that: *"the obtained results indicate that glyphosate tolerant soybean meal (Roundup Ready) and insect resistant MON 810 maize did not affect the cellular and humoral immunity of fattened pigs, poultry and cattle"*.	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 – Animal Feeding Study

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Swiatkiewicz *et al.*, 2013)	***Objective:*** To evaluate the effect of glyphosate tolerant Roundup Ready 40-3-2 soybean meal and *Bacillus thuringiensis* (Bt) insect resistant MON 810 maize on sow performance and haematological parameters as well as on piglet rearing indices. ***Experimental Design:*** The experiment was conducted on 24 sows (PL x WLP) mated with boar (Du x Pi). After mating sows were divided into four groups: (1) control, conventional soybean meal and conventional maize, (2) genetically modified (GM) soybean meal and conventional maize, (3) conventional soybean meal and GM maize, and (4) GM soybean meal and GM maize. Feed mixtures differed by the presence or absence of MON 810 maize (5% for pregnant and 8% for lactating sows) and/or soybean meal 40-3-2 (4% for pregnant and 14% for lactating sows). Born piglets were allotted to the same group as their mothers. Blood samples for haematological analysis were collected from the jugular vein of six sows per group. The following haematological indices were determined: red blood cell count, mean cell volume, haemoglobin concentration, mean amount of cell haemoglobin, mean cell haemoglobin concentration, haematocrit value, platelet cell count, mean platelet volume, white blood cell count and lymphocyte percentage. The transfer of transgenic DNA from the GM materials to the sows' organisms was also estimated. ***Results:*** Exposure to GM feed did not significantly affect sows and offspring performance. There was no significant difference among groups in feed intake during the whole reproductive cycle. Body weight gains and feed utilisation of piglets were similar in all groups. The number of erythrocytes, leukocytes and thrombocytes, their volume and content of haemoglobin did not differ among groups. The mean level of blood parameters estimated at the beginning of experiment and at the end was similar in both probes. No fragments of transgenic DNA, typical for genetically modified soybean or maize, were detected in blood samples of the sows.	The authors concluded that: *"feeding pregnant and lactating sows feeds containing genetically modified RR soybean meal or/and Bt maize did not significantly affect their reproductive characteristics and offspring performance. There was no effect of dietary treatment on sow haematological status. The lack of transgenic DNA in sow blood was confirmed"*.	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 – Animal Feeding Study

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Buzoianu *et al.*, 2013a)	***Objective:*** To assess the effects of feeding genetically modified (GM) MON 810 maize expressing the *Bacillus thuringiensis* Cry1Ab insecticidal protein (Bt maize) to sows during gestation and lactation and to their offspring from weaning to 115 days post-weaning on offspring growth and health. ***Experimental Design:*** Bt (PR34N44) and non-GM (isogenic) control (PR34N34) maize were grown side by side in Navarra, Spain in 2007. Twenty-four nulliparous sows, ca. 28 days old, were fed a non-GM diet until reaching around 165 kg body weight (bw) and were then assigned to the following dietary treatments: (i) control maize from service to weaning (iso) or (ii) Bt maize from service to weaning (Bt). Offspring were assigned to a control or Bt maize diet for 115 days, resulting in 4 dietary groups: (i) control maize-fed sow/control maize-fed offspring (iso/iso); (ii) control maize-fed sow/Bt maize-fed offspring (iso/Bt); (iii) Bt maize-fed sow/control maize-fed offspring (Bt/iso) or (iv) Bt maize-fed sow/Bt maize-fed offspring (Bt/Bt). Growth performance of the offspring was recorded at intervals until harvest (Day 115 post-weaning). Blood samples were taken for biochemical analysis on Days 0, 30, 70, 100 and 115 post-weaning. At harvest, carcass weight, back fat depth and selected organ weights were recorded. Kidney, liver, lymph nodes and small intestine were collected for histological analysis. ***Results:*** Offspring from Bt group sows were heavier than the iso group counterparts on Days 30, 100 and 115 post-weaning and had greater overall average daily body weight gain. Overall average daily feed intake was greater for offspring from Bt group sows and for Bt group pigs. Offspring from Bt group sows also had greater carcass and lighter spleen weights. Dressing percentage was greater for Bt group pigs than for iso group pigs and livers were lighter for pigs in the Bt/Bt group than pigs in the iso/Bt or Bt/iso group. Furthermore, offspring from Bt group sows had greater duodenal crypt depths and lower villus height/crypt depth ratios. No pathology was observed in the organs and serum biochemistry values generally remained within normal limits. No overall differences were observed, with the exception of γ-glutamyltransferase, which was lower for pigs on the Bt/Bt treatment than pigs on the iso/Bt and Bt/iso ones.	The authors concluded that: "*these results indicate that trans-generational consumption of Bt maize diets is not detrimental to pig growth and 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.

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Buzoianu *et al.*, 2013b)	***Objective:*** To investigate trans-generational effects of feeding genetically modified (GM) maize expressing *Bacillus thuringiensis* Cry1Ab protein (Bt maize) to sows during gestation and lactation and to their offspring on intestinal microbiota. ***Experimental Design:*** At insemination (Day 0), 24 Large White x Landrace nulliparous sows were randomly assigned to a diet: (i) near-isogenic parent line maize (Pioneer PR34N43) or (ii) Bt maize (Pioneer PR34N44 event MON 810). Sows were treated until weaning. At weaning, offspring were assigned a diet for 115 days: (i) non-GM maize-fed sow/ non-GM maize-fed offspring (non-GM/non-GM), (ii) non-GM maize-fed sow/GM maize-fed offspring (non-GM/GM), (iii) GM maize-fed sow/ non-GM maize-fed offspring (GM/non-GM) and (iv) GM maize-fed sow/ GM maize-fed offspring (GM/GM). Fecal samples were collected at insemination, Day 110 of gestation and Day 28 of lactation, and from offspring at weaning and Days 30, 70 and 100 post-weaning. *Lactobacillus* and *Enterobacteriaceae* were counted in fecal samples and ileal / cecal digesta. Fecal samples at Day 110 of gestation and from offspring at weaning and Day 100 post-weaning and cecal samples from Day 115 post-weaning were sequenced for 16s rRNA gene. ***Results:*** Offspring of GM maize-fed sows had higher counts of fecal total anaerobes and *Enterobacteriaceae* at Days 70 and 100 post-weaning. At Day 115 post-weaning, GM/non-GM offspring has lower ileal *Enterobacteriaceae* counts than non-GM/non-GM or GM/GM offspring and lower ileal total anaerobes than in the other treatments. GM maize-fed offspring had higher ileal total anaerobe counts than non-GM maize-fed offspring, and cecal total anaerobes were lower in non-GM/GM and GM/non-GM offspring than in the non-GM/non-GM treatment. Fecal *Proteabacteria* were less abundant in GM maize-fed sows prior to farrowing and in offspring at weaning. Other differences occurred but they were not observed consistently in offspring, were mostly encountered for low-abundance, low-frequency bacterial taxa, and were not associated with pathology.	The authors concluded that: *"this confirms the lack of adverse effects of GM maize on the intestinal microbiota of pigs, even following trans-generational consumption"*.	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 – Animal Feeding Study

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Sieradzki *et al.*, 2013)	***Objective:*** To assess the possibility of genetically modified (GM) DNA transfer from feed containing MON 810 maize expressing the *Bacillus thuringiensis* Cry1Ab insecticidal protein (Bt maize) or glyphosate tolerant Roundup Ready (RR) soybean (GTS 40-3-2) to animal tissues, gut bacterial flora and food of animal origin. To determine the fate of GM DNA in the digestive tract of exposed animals. ***Experimental Design:*** The study was conducted on broilers, laying hens, pigs and calves. Each species was divided into four groups: (i) controls, fed conventional feed, (ii) fed GM soybean meal and non-modified maize (DKC 3420), (iii) fed non-modified soybean meal and GM maize, (iv) fed GM soybean meal and GM maize. Animals were monitored for standard performance indices. Feed, gastro-intestinal (GI) tract digesta, blood, tissues, stool and eggs were analysed by PCR for the presence of plant-specific genes (invertase for maize, lectin for soybean) and regulatory sequences used in the transformation of MON 810 and GTS 40-3-2 (CaMV 35S promoter for maize and soybean; NOS terminator for soybean). Additionally, samples of ileum/colon content were collected for microbiological analysis. Selected common gut bacteria were isolated, their relative quantity determined, and their DNA investigated for the presence of GM DNA. ***Results:*** All animals achieved satisfactory performance indices, with no significant statistical differences in any of the parameters across the feeding groups. Analyses confirmed that the feed mixtures were prepared according to the planned experimental scheme. No plant reference genes or GM DNA were found in any of the samples analysed. The GM crop diets did not affect bacterial gut flora diversity, quantity of particular bacteria species in the gut or incorporation of GM DNA into the bacterial genome.	The authors concluded that: *"MON 810 maize and RR soybean used for animal feed are substantially equivalent to their conventional counterparts. Genetically modified DNA from MON 810 maize and RR soybean is digested in the same way as plant DNA, with no possibility of its transfer to animal tissues or gut bacterial flora".*	Animal health	No adverse effects were determined in this study
			Observed parameter	**Feedback on initial environmental risk assessment**
			Animal performance and DNA transfer	There are no changes to the conclusions of the safety of the initial risk assessment.

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

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Furgal-Dierzuk *et al.*, 2014)	***Objective:*** To determine whether genetically modified (GM) maize (MON 810) and soybean meal (Roundup Ready, MON 40-3-2) used as the main source of feed in a concentrate can have an effect on: 1) the growth of new-born calves, 2) the basal chemical composition of the *musculus thoracis* (MT), 3) the fatty-acid composition of intramuscular fat, 4) the transfer of transgenic DNA (tDNA) to calf tissues, and 5) the results of histological examination of calf organs and tissues. ***Experimental Design:*** 40 forty Polish Black-and-White HF bull calves aged 10±3 days were allocated to 4 groups. Each group received a different feed: non-modified (traditional) maize and soybean meal (group TMG/TS), non-modified maize and GM soybean (group TMG/MS), GM maize and non-modified soybean meal (group MMG/TS) and GM maize and GM soybean meal (group MMG/MS). Each feed contained similar amounts of maize (56%), soybean meal (25%), oat (15%), premix and limestone (1%). The calves were observed daily in terms of general health and the live weight was monitored at the beginning of the experiment, at Days 56 and 90. The experiment was concluded when the calves were aged 90 days. Six calves from each group were slaughtered to take samples of digesta as well as samples of tissues (blood and skeletal muscle) and organs (lungs, liver, kidney, spleen and pancreas). The *musculus thoracis* (MT) chemical composition was measured according to AOAC standard methods. The fatty acid content of MT was measured by chromatography. The DNA was extracted from homogenized samples of gastro-intestinal digesta and tissue samples, and used for PCR analysis to detect the presence of transgenic DNA. Histological analysis was performed on paraffin sections from liver, kidney, spleen, pancreas, duodenum, jejunum, MT and *musculus gracilis*. ***Results:*** There were no major differences in the feed value of GM maize and RR soybean meal and their non-modified isogenic counterparts and feed mixtures. There were no effects of GM components on final live weight, average daily weight gain, MT chemical composition, or fatty-acid profile of intramuscular fat. The calf rumen fluid contained tDNA, but there was no tDNA in the intestinal content, blood, studied organs or meat. Histological analysis of different organs and muscles found no differences among the four groups.	The authors concluded that *'The obtained results show that genetically modified feeds used in feeding calves for 90 days do not show a negative influence on animal health and food quality, even when the calves were fed the concentrate that contained 81% GM feeds in the group provided modified maize MON 810 and modified RR soybean meal'*	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 – Animal Feeding Study

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Sanden *et al.*, 2013)	***Objective: Experimental Design:*** MON 810 (Bt) seeds and the unmodified parent line (nBt) (PR 34N44 and PR 34N43) were grown simultaneously in 2007 in Spain. Zebra fish were fed casein/gelatine-based diets containing either 19% Bt maize or control maize for two generations. F0 larvae were randomly assigned to one of the diets. In the offspring feeding trial, zebra fish larvae (F1) obtained from the parental generation were fed one of the following three regimens: (i) Bt maize Bt-Bt (larvae from the Bt-maize diet-fed parental generation), (ii) nBt-maize diet Bt-nBt (larvae from the Bt-maize diet-fed parental generation) or (iii) nBt-maize diet nBt-nBt (larvae from the nBt-maize diet-fed parental generation). Growth and reproductive performance, liver CuZn superoxide dismutase (SOD) enzyme activity, gene transcript levels targeting important cellular pathways in the liver and mid-intestine, histomorphological evaluation of the intestine, differential leucocyte counts, offspring larva swimming activity and global DNA methylation in offspring embryos were observed in the two generations. ***Results:*** The Bt-Bt offspring exhibited a significantly higher body mass increase, specific growth rate and feed utilisation than fish fed the nBt-nBt diet and /or Bt-nBt diets. Liver and mid-intestinal gene transcript levels of CuZn SOD were significantly higher in fish fed the nBt-nBt diet than in those fed the Bt-Bt diet. Liver gene transcript levels of caspase 6 were significantly lower for the nBt-nBt group than for the Bt-Bt group. No significant effects were observed in the parental generation. Overall, enhanced growth performance was observed in fish fed the Bt diet for two generations compared to those fed the nBt diet for one and two generations. Effects observed on gene biomarkers for oxidative stress and the cell cycle (apoptosis) may be related to the contamination of nBt-maize with fumonisin B1 and aflatoxin B1.	The authors concluded that: *"no adverse performance or reproductive effects in zebra fish fed the Bt-maize diet for two generations. Bt-maize seems to be as safe and nutritious as its nBt-maize control"*.	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 – Crop Compositional Studies

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Agapito-Tenfen *et al.*, 2013)	*Objective:* To determine protein expression of genetically modified (GM) MON 810 maize expressing the *Bacillus thuringiensis Cry 1Ab* insecticidal protein and the near-isogenic non-GM hybrid grown in different agroecosystem conditions in Brazil by using a proteomic approach. *Experimental Design:* MON 810 maize and its non-GM counterpart were grown in two Brazilian locations (Campos Novos and Chapecó) with different agroecosystem conditions during one growing season. The experimental field consisted in a 120 m^2 area divided into three replicate blocks (4 rows wide for each hybrid grown side-by-side, 5 m long, row spacing 0.8 m). Plots were sown at a density of 80,000 plants/ha and treated following the standard agricultural practices of the region. Maize leaves were collected at the R0 stage (57 days after sowing) during anthesis. Sampling was performed during early morning in both locations. Three biological replicates were randomly sampled per maize hybrid, each grown in a different plot. These samples were used for proteins extraction. The extracted proteins were separated by two-dimensional gel electrophoresis (2-DE). The 2DE analysis was performed twice for each biological replicate so that a total number of 36 gels were used for the quantitative analysis of maize proteomes by densitometry. Gel spots were then used for protein identification by using mass spectrometry. The main sources of variation in the 2-DE experiment dataset were evaluated by Principal Component Analysis (PCA) using Euclidean distance for quantitative analysis. *Results:* In the first phase of analysis, PCA correlated most of the variation to the different agroecosystem conditions. Comparative analysis within each field revealed a total of 32 differentially expressed proteins between GM and non-GM samples. These proteins were identified and their molecular functions were mainly assigned to carbohydrate and energy metabolism, genetic information processing and stress response.	The authors concluded that: "*our results show that the environment was the major source of influence to the expression of GM maize proteins. Protein differences were observed in MON 810 and non-GM agronomic field-grown maize with Brazilian genetic background.*"	Human and animal health	No adverse effects were determined in this study
			Observed parameter	**Feedback on initial environmental risk assessment**
			Plant protein composition	There are no changes to the conclusions of the safety of the initial risk assessment.

Area of food/feed safety assessment: Toxicity – Human *in vitro* test

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Mesnage *et al.*, 2013)	***Objective:*** To investigate potential effects of Cry1Ab and Cry1Ac *Bacillus thuringiensis* (Bt) proteins[1] in the human embryonic kidney cell line 293 (HEK293), as well as combined activity with the glyphosate-based herbicide Roundup®, [2], on biomarkers of cell death. ***Experimental Design:*** Cry1Ab and Cry1Ac toxins were cloned from the Bt subspecies kurstaki HD-1 strain and expressed in *Escherichia coli* as single gene products. The glyphosate-based herbicide was Roundup GT Plus. HEK293 cells were grown at 37°C during 24 h to 80% confluence. Bt toxins were tested at 10 ppb to 100 ppm and Roundup from 1 to 20,000 ppm[3]. Combined effects were measured by mixing Roundup (at the LC50 dose) with three doses of each Bt protein. After treatment, a mitochondrial respiration assay was conducted via succinate dehydrogenase activity measurement. Optical density was assessed at 570 nm and a bioluminescence bioassay was carried out to determine membrane degradation, via intracellular adenylate kinase (AK) release in the medium. Apoptotic cell death was evaluated with the Caspase-Glo 3/7 assay. Experiments were repeated at least three times in different weeks on three independent cultures. ***Results:*** Cytotoxic effects of Bt proteins were seen in HEK293 cells, alone or in combination with Roundup. Mitochondrial succinate dehydrogenase activity significantly decreased at 100 ppm Cry1Ab. This was not observed with Cry1Ac. Cry1Ab at 100 ppm increased AK leakage in the medium 2-fold, revealing plasma membrane alterations. There were no apoptotic effects of Cry1Ab and Cry1Ac alone on HEK293cells. Cry1Ab induced cytotoxicity via a necrotic mechanism at 100 ppm. Further, Roundup induced necrosis at 57.5 ppm (corresponding to the LC50), as measured by a 15-fold increase of AK release. However, as of 10 ppm, both Bt proteins reduced caspase 3/7 activity by ca. 50% when combined with Roundup at the LC50 concentration. Similarly, there was a non-significant tendency for both toxins to reduce AK leakage and mitochondrial respiration inhibition induced by Roundup.	The authors conclude that: "*...modified Bt toxins are not inert on non-target human cells[4], but can exert toxicity[5], and that they can present combined side-effects with other residues of pesticides specific to genetically modified plants*".	Human health	The authors claim, Cry1Ab can induce cytotoxic effects[6], Roundup is cytotoxic[7], combined effects were observed[8], and non-significant tendency for both toxin to AK leakage and mitochondrial respiration inhibition induced by Roundup[9].
			Observed parameter	**Feedback on initial food/feed safety assessment**
			Cell Viability	There are no changes to the conclusions of safety of the initial food/feed safety assessment[10].

[1] Cry1Ab and Cry1Ac have a long history of safe use and have been subjected to extensive testing and review by regulatory agencies around the world. Tolerances (permissible levels in foods) for Cry1Ab and Cry1Ac have not been set by regulators due to their low degree of mammalian toxicity. Safety assessments for Monsanto products, including those with Cry1Ab and Cry1Ac, are available on Monsanto.com (http://www.monsanto.com/products/Pages/product-safety-summaries.aspx).

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[2] Glyphosate has an excellent human health and environmental profile and a long history of safe use in more than 130 countries. This has been a key factor in the acceptance of glyphosate products as one of the most widely used herbicides in the world. When used according to label directions, these products present no unreasonable risk of adverse effects to human health or the environment. Glyphosate, the active ingredient in Roundup branded agricultural products, inhibits an enzyme that is essential to plant growth; this enzyme is not found in humans or other animals, which explains the generally low acute toxicity of glyphosate in humans and animals (Franz *et al.*, 1997). Comprehensive toxicological studies in animals have demonstrated that glyphosate does not cause cancer, birth defects, mutagenic effects, nervous system effects or reproductive problems (U.S. EPA, 1993; Williams *et al.*, 2000; Williams *et al.*, 2012; European Commission, 2002; JMPR/WHO, 2004; Mink *et al.,* 2011). In fact, after a thorough review of all toxicology data available, the U.S. EPA concluded that glyphosate should be classified in Category E ("Evidence of Non-carcinogenicity in Humans"), the most favourable category possible (U.S. EPA, 1993).

[3] The tested levels of formulated product containing glyphosate are not relevant to real exposures. Anadon *et al.*, 2009 (cited in a prior publication from this group, Clair *et al.*, link below) dosed rates with 400 mg/kg of glyphosate, a massive dose relative to any environmental exposure, and achieved peak modelled plasma concentrations of approximately 5 ug/ml (5 mg/L or 5 ppm). Assuming linear kinetics, the maximum allowable US daily intake (2 mg/kg/day) would give an approximated blood concentration of 0.025 ppm (25 ppb). McQueen *et al.* (2012) recently evaluated glyphosate exposure to pregnant women and concluded that estimated exposures based on actual measurements in food were only 0.4% of the acceptable daily intake. The "Roundup" LC50 concentration used (57.5 ppm) is more than 2,000-fold higher than the anticipated concentration (based on Anadon *et al.,* 2009) following maximum allowable intake. (It is further worth noting that this allowable concentration is based on a 100-fold safety factor above a NOAEL in animal studies.)

[4] This study used artificial conditions in testing the effects of Cry1Ab, Cry1Ac and glyphosate. Direct exposure to cells in culture bypasses physiologic normal processes, limiting absorption and cellular exposure, and avoids normal metabolism, excretion, serum protein binding and other factors that would protect cells in the intact organism.

[5] The references cited regarding the *in vitro* toxicity studies of other Bt derived proteins are largely irrelevant. There are many different Bt varieties that produce many different kinds of toxins, and some Bt toxins are known to be toxic to mammalian cells *in vitro*. We utilize the Cry proteins that are closely related to the many kinds of proteins found in commercial Bt microbial pesticides that have been safely used in agriculture around the world for approximately 50 years. The Bt toxins used in GM plants have been subject to extensive safety assessment (Betz, 2000; Federici and Siegel, 2008; OECD, 2007; WHO/IPCS, 1999). The authors cited the work of Ito *et al.* (2004) that reports the effect of a non-insecticidal Bt-derived protein which is cytotoxic to some human cell lines. The authors also cited the work of Nagamatsu *et al.* (2010) that similarly reports on a non-insecticidal Bt protein. The work of Rani and Balaraman (1996) involves a solubilized Cry protein from an insecticidal Bt strain. Oral toxicity is not demonstrated in any case but, more to the point, extensive toxicity studies of Cry1Ab and Cry1Ac in mammalian species indicate to toxic effect at relevant doses and by relevant routes.

[6] The doses of Cry proteins used on this study are irrelevant to real life exposures for several reasons. High-dose animal toxicity testing via the oral routes using Cry1Ab and Cry1Ac demonstrates no toxic effects at doses thousands of times higher than any potential human intake. The only concentration of Cry protein demonstrating any effect on cellular function was 100 ppm, used in an otherwise protein free medium. The concentration of Cry protein in grain is below 1 ppm (see Monsanto product safety data, link above), and these cry proteins are both degraded by cooking and are readily digestible. Studies of meat, milk, and eggs have not demonstrated intact Cry protein detection in animals fed on GM crops containing these proteins. The studies of Aris and Leblanc cited by the author, taken at face value, indicate Cry protein concentration in human blood up to about 0.2 parts per billion- or 500-fold less than the concentrations used by Mesnage *et al.* We would note, however, that the validity of the Aris and Leblanc publication has been seriously questioned by scientists and regulators. Regulatory opinions, original article, and associated correspondence at: http://www.food.gov.uk/multimedia/pdfs/acnfp10308pest, http://www.foodstandards.gov.au/consumerinformation/gmfoods/fsanzresponsetostudy5185.cfm, http://www.sciencedirect.com/science/article/pii/S0890623811000566.

[7] Animal data and human experience contradicts findings of Petri dish experiments with a formulated product containing glyphosate herbicides. Glyphosate has been tested extensively in higher order animals (Giesy *et al.*, 2000; Williams *et al.*, 2000). There is no evidence for developmental or reproductive concerns in multiple species despite numerous high-dose tests by different manufacturers (Williams *et al.,* 2012; EU, 2002; JMPR/WHO, 2004). Furthermore, studies with polyethoxylated tallow amine (POEA), the predominant surfactant in Roundup formulation, have not demonstrated any target organ toxicity or effects on embryos, fetuses or the placenta (Williams *et al.*, 2000; Williams *et al.*, 2012).

[8] The co-application of Cry protein with a formulated product containing glyphosate reduces the apparent degree of cellular injury (as measured by induction of caspase levels). This occurs even at concentrations of Cry1Ab which the authors report to cause cellular injury and membrane disruption. This is worth noting for several reasons. First, it brings into question the toxicity observations with Cry1Ab, as the argument that membrane disruption and impaired mitochondrial function should be protective seems to be highly untenable, especially in view of the studies demonstrating the mitochondrial membrane activity of surfactants (Levine *et al.*, 2007). Second, it should remove any implications of a "synergistic effect" of Cry proteins and a formulated product containing glyphosate herbicides (the direction is, if anything, antagonistic, but one would not argue for any true *in vivo* protective effects as the entire system is fundamentally irrelevant). Third, this probably is demonstrating the artificiality of the system itself. As noted above, this is a protein-free medium. Protein protects cells in culture by multiple mechanisms: binding to toxic materials, binding to potential receptor sites or other non-specific surface-stabilization effects. It appears from Mesnage's own data that simple addition of protein to their system, even at low concentrations (and even if that protein is a Cry protein), protects from toxicity.

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