Vitenskapskomiteen for mattrygghet
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Risk assessment of insect-resistant and herbicide-tolerant genetically modified maize Bt11 for cultivation, import, processing, food and feed uses under Directive 2001/18/EC and Regulation (EC)

Opinion of the Panel on Genetically Modified Organisms of the Norwegian Scientific Committee for Food Safety

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(Authors in alphabetical order after chair of the working group)

Assessed and approved

The opinion has been assessed and approved by the Panel on Genetically Modified Organisms. Members of the Panel are: Hilde-Gunn Opsahl-Sorteberg (chair), Knut Helkås Dahl, Knut Tomas Dalen, Olavi Junttila, Richard Meadow, Inger Elisabeth Måren, Kåre M. Nielsen, Monica Sanden and Rose Vikse

(Panel members in alphabetical order after chair of the panel)

Acknowledgment

The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has appointed a working group consisting of VKM members to answer the requests from the Norwegian Environment Agency and the Norwegian Food Safety Authority. Project leaders from the VKM secretariat have been Nana Asare and Merethe Aasmo Finne. The members of the working group, Richard Meadow, Olavi Junttila, Lawrence Kirkendall (Panel on Alien organisms and trade in Endangered species (CITES)) and Inger Elisabeth Måren are acknowledged for their valuable work on this opinion. The Panel on Genetically Modified Organisms is acknowledged for comments and views on this opinion.

Competence of VKM experts

Experts working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

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Summary

The Norwegian Environment Agency (NEA) and the Norwegian Food Safety Authority (NFSA) requested the Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) for an opinion of potential risks to biodiversity and agriculture in Norway associated with import of seeds for sowing and cultivation of insect-resistant and herbicide-tolerant genetically modified maize Bt11 under Directive 2001/18/EC (Notification C/F/96.05.10). The notification is still pending for authorisation in the European Union. VKM is also requested to assess the applicant´s post-market environmental monitoring plan, and the management measures suggested in the draft implementing decision of the European Commission.

As the scope of the notification does not cover food and feed uses of maize Bt11, VKM was not asked for a health risk assessment of maize Bt11. However, VKM has decided to update a previous safety evaluation of the food and feed uses of maize Bt11 and derived products (VKM, 2014).

VKM appointed a working group consisting of members from the Panel on Genetically Modified Organisms, the Panel on Alien Organisms and trade in Endangered Species (CITES) and the VKM staff to answer the requests. The Panel on Genetically Modified Organisms assessed and approved the final report.

The genetically modified maize Bt11 was developed to provide protection against certain lepidopteran target pests, such as the European corn borer (ECB, *Ostrinia nubilalis*), and some species belonging to the genus *Sesamia*. The insect resistence is achieved by the expression of a truncated form of a Cry1Ab protein encoded by a modified cry1Ab gene derived from the soil microorganism *Bacillus thuringiensis* subsp *kurstaki* HD-1.

Maize Bt11 also expresses the *phosphinothricin-N-acetyltransferase* (*pat*) gene, derived from the soil microorganism *Streptomyces viridochromogenes* strain Tu494, which encodes the enzyme: phosphinothricin acetyl transferase (PAT). PAT protein confers tolerance to the herbicidal active substance glufosinate-ammonium. The PAT protein expressed in Bt11 was used as a selectable marker to facilitate the selection process of transformed plant cells and is not intended for weed management purposes. Since the scope of the notification C/F/96.05.10 does not cover the use of glufosinate-ammonium-containing herbicides on maize Bt11, potential effects due to the use of such herbicides on maize Bt11 are not considered by VKM.

In delivering its scientific opinion, VKM considered relevant peer-reviewed scientific publications and information provided by the applicant in the notification C/F/96.05.10, the renewal application EFSA/GMO/RX/Bt11, and scientific opinions and comments from EFSA and other EU-member states.

VKM has evaluated maize Bt11 with reference to its intended uses in the European Economic Area (EEA), and according to the principles described in the Norwegian Food Act, the Norwegian Gene Technology Act and regulations relating to impact assessment pursuant to the Gene Technology Act, Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, and Regulation (EC) No 1829/2003 on genetically modified food and feed.

The Norwegian Scientific Committee for Food Safety has also decided to take account of the appropriate principles described in the EFSA guidelines for the risk assessment of GM plants and derived food and feed (EFSA 2011a), the environmental risk assessment of GM plants (EFSA 2010a), selection of comparators for the risk assessment of GM plants (EFSA, 2011b) and for the post-market environmental monitoring of GM plants (EFSA, 2011c).

The scientific risk assessment of maize Bt11 includes molecular characterisation of the inserted DNA and expression of novel proteins, comparative assessment of agronomic and phenotypic characteristics, nutritional assessments, toxicology and allergenicity. An evaluation of unintended effects on plant fitness, potential for gene transfer, interactions between the GM plant and target and non-target organisms, effects on biogeochemical processes, the post-market environmental monitoring plan and coexistence measures at the farm level has also been undertaken.

It is emphasised that the VKM mandate does not include assessments of contribution to sustainable development, societal utility and ethical considerations, according to the Norwegian Gene Technology Act and Regulations relating to impact assessment pursuant to the Gene Technology Act. These considerations are therefore not part of the risk assessment provided by the VKM.

Molecular characterisation

Appropriate analyses of the integration site, inserted DNA sequence, flanking regions, and bioinformatics have been performed. The molecular characterisation reported by the applicant shows that the DNA-fragment containing the cry1Ab and pat genes, is integrated as a single copy at a single locus in the nuclear genome of maize Bt11 and that it is stably inherited as a dominant trait. VKM considers the molecular characterisation of maize Bt11 satisfactory.

Comparative assessment

Comparative analyses of data from field trials located at representative sites and environments in North America and Europe indicates that maize Bt11 is compositionally equivalent to its conventional counterpart, with the exception of the herbicide tolerance and insect resistance traits, conferred by the expression of the PAT and Cry1Ab proteins. However, data on the amino acid tryptophan, is only given in one out of six studies. Based on current knowledge, VKM concludes that maize Bt11 is compositionally equivalent to conventional maize.

The data provided by the applicant are not sufficient to show that Bt11 maize is phenotypically and agronomically equivalent to conventional near-isogenic maize lines. The agronomic assessment data are provided from one growing season in the North America and one growing season in France. This is not considered to be sufficient for representative testing of agricultural environments.

Food and feed risk assessment

Whole food feeding studies have not indicated any adverse health effects of maize Bt11. These studies further support that maize Bt11 is nutritionally equivalent to conventional maize. The Cry1Ab and PAT proteins do not show sequence resemblance to other known toxins or IgE allergens, nor have they been reported to cause IgE mediated allergic reactions. Some studies have however indicated a potential role of Cry-proteins as adjuvants in allergic reactions. Based on current knowledge, the VKM concludes that maize Bt11 is nutritionally equivalent to conventional maize varieties. It is unlikely that the Cry1Ab and PAT proteins will introduce a toxic or allergenic potential in food or feed based on maize Bt11 compared to conventional maize.

Environmental risk assessment

Maize is the only representative of the genus *Zea* in Europe, and there are no cross-compatible wild or weedy relatives outside cultivated maize with which maize can hybridise and form backcross progeny. Vertical gene transfer in maize therefore depends on cross-pollination with other conventional or organic maize varieties. In addition, unintended admixture of genetically modified material in seeds represents a possible way for gene flow between different crop cultivations. The risk of pollen flow from maize volunteers is negligible under Norwegian growing conditions. Since maize Bt11 has no altered agronomic and phenotypic characteristics, except for the specific target insect resistance and herbicide tolerance, the likelihood of unintended environmental effects as a consequence of spread of genes from maize Bt11 is considered to be extremely low.

There are no reports of the target lepidopteran species attaining pest status on maize in Norway. Since there are no Bt-based insecticides approved for use in Norway, and lepidopteran pests have not been registered in maize, issues related to resistance evolution in target pests are not relevant at present for Norwegian agriculture.

Published scientific studies showed that the likelihood of negative effects of Cry1Ab protein on non-target arthropods that live on or in the vicinity of maize plants is low.

In Norway, the maize cultivation is marginal. The total crop area of forage maize is estimated to 2000-2800 decares, equivalent to less than 0.1 % of the areas with cereal crops. The area of individual fields is limited by the topography such that the quantity of maize pollen produced under flowering is also limited. The potential exposure of Cry1Ab-containing maize pollen on non-target lepidopteran species in Norway is therefore negligible.

Cultivation of maize Bt11 is not considered to represent a threat to the prevalence of red-listed species in Norway.

Exposure of nontarget organisms to Cry proteins in aquatic ecosystems is likely to be very low, and potential exposure of Cry proteins to non-target organisms in aquatic ecosystems in Norway is considered to be negligible.

VKM concludes that, although the data on the fate of the Cry1Ab protein and its potential interactions in soil are limited, the relevant scientific publications analysing the Cry1Ab protein, together with the relatively broad knowledge about the environmental fate of other Cry1 proteins, do not indicate significant direct effects on the soil environment. Despite limited number of studies, most studies conclude that effects on soil microorganisms and microbial communities are transient and minor compared to effects caused by agronomic and environmental factors. However, data are only available from short-term experiments and predictions of potential long-term effects are difficult to deduce.

Coexistence

VKM concludes that separation distances of 200 meters most likely will ensure coexistence between genetically modified maize and conventional and organic maize varieties in Norway.

Overall conclusion

Based on current knowledge, VKM concludes that maize Bt11 is nutritionally equivalent to conventional maize varieties. It is unlikely that the Cry1Ab and PAT proteins will introduce a toxic or allergenic potential in food or feed derived from maize Bt11.

VKM likewise concludes that cultivation of maize Bt11 is unlikely to have any adverse effect on the environment or agriculture in Norway.

Keywords

Maize, Zea mays L., genetically modified maize Bt11, C/F/96.05.10, EFSA/GMO/RX/Bt11, insect-resistance, herbicide-tolerance, Cry1Ab, PAT, glufosinate ammonium, cultivation, food/feed risk assessment, environmental risk assessment, Directive 2001/18, Regulation (EC) No 1829/2003

Sammendrag på norsk

Miljødirektoratet og Mattilsynet har bedt Vitenskapskomiteen for mattrygghet (VKM) om en vitenskapelig vurdering av risiko for negative konsekvenser for biologisk mangfold og norsk landbruk ved import og omsetning av såvare og dyrking av den insektresistente og herbicidtolerante maislinjen Bt11. Maislinjen er søkt godkjent til dyrking under direktiv 2001/18/EC (Notifisering C/F/96.05.10), men søknaden er fortsatt under vurdering for godkjenning i EU.

VKM er også bedt om å vurdere søkers miljøovervåkingsplan, og om tiltakene som er beskrevet i vedlegget til EU-kommisjonens utkast til implementeringsbeslutning ivaretar hensynet til norsk natur og landbruk. Notifiseringen C/F/96.05.10 omfatter ikke bruk av mais Bt11 som mat og fôr, og VKM er derfor ikke bedt om å vurdere disse bruksområdene. VKM har imidlertid besluttet å oppdatere en tidligere helserisikovurdering av mais fra 2014 (VKM, 2014).

VKM nedsatte en prosjektgruppe bestående av medlemmer fra faggruppen for genmodifiserte organismer, faggruppen for fremmede organismer og handel med truede arter (CITES), og ansatte i VKMs sekretariat for å besvare oppdragene. Faggruppen for genmodifiserte organismer har gjennomgått utkastet fra prosjektgruppen og godkjent den endelige rapporten.

Maislinjen Bt11 har fått innsatt et *cry1Ab*-gen fra jordbakterien *Bacillus thuringiensis* var*. aizawai* og et *pat*-gen, isolert fra jordbakterien *Streptomyces viridochromogenes*. *Cry1Ab*-genet koder for et δ-endotoksin som gir resistens mot enkelte arter i sommerfuglordenen Lepidoptera, eksempelvis maispyralide (*Ostrinia nubilatis*) og enkelte arter i slekten *Sesamia*. *Pat*-genet koder for enzymet fosfinotricin acetyltransferase (PAT), som acetylerer og inaktiverer glufosinat-ammonium (fosfinotricin), virkestoffet i fosfinotricin-herbicider. PAT-proteinet er kun benyttet som markør for seleksjon av transformerte planteceller under utviklingen av maislinjen, og bruksområdet for søknaden omfatter ikke sprøyting med dette herbicidet. Potensielle helse- og miljøeffekter ved bruk av glufosinat-ammonium er derfor ikke vurdert av VKM.

Risikovurdering av mais Bt11 er basert på fagfellevurderte, vitenskapelige publikasjoner, informasjon fra søker i notifikasjonen C/F/96.05.10 og fornyingssøknaden EFSA/GMO/RX/Bt11, samt vitenskapelige vurderinger og kommentarer fra EFSA og andre EU-medlemsland.

Vurderingen er gjort i henhold til tiltenkt bruk i EU/EØS-området, og i overensstemmelse med miljøkravene i genteknologiloven med forskrifter, først og fremst forskrift om konsekvensutredning etter genteknologiloven. Videre er kravene i EU-forordning 1829/2003/EF, utsettingsdirektiv 2001/18/EF (vedlegg 2,3 og 3B) og veiledende notat til

Annex II (2002/623/EF), samt prinsippene i EFSAs retningslinjer for risikovurdering av genmodifiserte planter og avledete næringsmidler (EFSA 2010a, 2011a,b,c) lagt til grunn for vurderingen.

Den vitenskapelige vurderingen omfatter transformeringsprosess og vektorkonstruksjon, karakterisering og nedarving av genkonstruksjonen, komparativ analyse av ernæringsmessig kvalitet, mineraler, kritiske toksiner, metabolitter, antinæringsstoffer, allergener og nye proteiner. Videre er agronomiske egenskaper, potensiale for utilsiktede effekter på fitness, genoverføring, effekter på målorganismer og ikke-målorganismer og biogeokjemiske prosesser vurdert. VKM har også vurdert søkers miljøovervåkingsplan og tiltak for å sikre sameksistens fram til og med høsting av avlingen.

Det presiseres at VKMs mandat ikke omfatter vurderinger av etikk, bærekraft og samfunnsnytte, i henhold til kravene i den norske genteknologiloven og dens konsekvensutredningsforskrift. Disse aspektene blir derfor ikke vurdert av VKMs faggruppe for genmodifiserte organismer.

Molekylær karakterisering
Data fra den molekylære karakteriseringen indikerer at det kun er integrert en kopi av ekspresjonskassetten med genene *cry1Ab* og *pat* i genomet til mais Bt11, og at genene og egenskapene er dominant og stabilt nedarvet. Bioinformatikk- og sekvensanalyser er utført av integreringssetet i plantens genom, og innsatt og flankerende DNA. VKM vurderer den molekylære karakteriseringen av mais Bt11 som tilfredsstillende.

Komparative analyser
Data fra feltforsøk i Nord-Amerika og Europa indikerer, med unntak av insektsresistens og herbicidtoleranse, ekvivalens mellom genmodifisert mais Bt11 og korresponderende, nær-isogene kontrollinjer med hensyn på ernæringsmessige karakterer. VKM peker imidlertid på at aminosyren tryptofan kun er analysert i ett av de seks studiene som er vedlagt søkers dokumentasjon. VKM vurderer også at søkers dokumentasjon knyttet til fenotypiske og agronomiske karakterer er ufullstendig og av for dårlig kvalitet til å kunne vurdere ekvivalens med konvensjonelle, nærisogene maislinjer.

Helserisiko
Fôringsstudier utført på rotter og broiler har ikke indikert helseskadelige effekter av mais Bt11. Cry1Ab-proteinet viser ingen likhet til kjente toksiner eller allergener, og er heller ikke rapportert å ha forårsaket IgE-medierte allergiske reaksjoner. Det er heller ikke dokumentert at noen av proteinene kan utløse IgE-medierte allergiske reaksjoner. Enkelte studier har derimot indikert at noen typer Cry-proteine potensielt kan forsterke andre allergiske reaksjoner (virke som adjuvans).

Ut i fra dagens kunnskap konkluderer VKM med mais Bt11 er næringsmessig vesentlig lik konvensjonell mais, og at det er lite trolig at de nye proteinene vil introdusere et toksisk eller allergent potensiale i mat og fôr basert på mais Bt11 sammenliknet med konvensjonelle

maissorter.

Miljørisiko

Det vurderes ikke å være økt risiko knyttet til spredning, etablering og invasjon av maislinjen Bt11 i naturlige habitater, eller utvikling av ugraspopulasjoner av mais i dyrkingsmiljø sammenlignet med konvensjonelle sorter. Det er ingen stedegne eller introduserte viltvoksende arter i den europeiske flora som mais kan hybridisere med, og vertikal genoverføring vil være knyttet til krysspollinering med konvensjonelle og eventuelle økologiske sorter. I tillegg vil utilsiktet innblanding av genmodifisert materiale i såvare representere en mulig spredningsvei for transgener mellom ulike dyrkingssystemer. En slik spredning vurderes som ubetydelig.

Målorganismene for den genmodifiserte maislinjen Bt11 er ikke rapportert som skadegjørere i mais i Norge. Siden det ikke er godkjente Bt-produkter til bruk i mais i Norge, og det ikke er registrert Lepidoptera-arter som skadegjørere i mais, er problematikken knyttet til resistens i målorganismene ikke relevant i norsk sammenheng.

Publiserte vitenskapelig studier viser at sannsynligheten for negative effekter av Cry1Ab-proteinet på ikke-målartropoder som lever på eller i nærheten av maisplanter er lav.

Maisdyrkingen i Norge er marginal. Det totale dyrkingsarealet av fôrmais er estimert til 2000-2800 dekar, tilsvarende under 0,1% av det totale kornarealet. Arealet av enkeltfelt er dessuten begrenset av topografiske forholdt og mengden av maispollen som produseres under blomstring er begrenset. Den potensielle eksponeringen av maispollen med Cry1Ab-protein på ikke-målorganismer av lepidoptera i Norge er derfor ubetydelig.

Det vurderes ikke å være risiko for rødlistede arter ved dyrking av Bt11 i Norge.

Det er kunnskapsmangler med hensyn på effekter av Cry-proteiner på vannlevende organismer. Konsentrasjonene av Cry-proteiner er imidlertid vist å være svært lave i akvatiske systemer og eventuell eksponering av toksinene på disse organismene vil være marginal i Norge.

Det er publisert få studier som har undersøkt mulige effekter av Cry1Ab-protein på økosystemer i jord, mineralisering og næringsstoffomsetning eller effekter på jordsamfunn som bidrar til dette. De fleste studiene konkluderer med at effektene av Cry1Ab er små og forbigående sammenlignet med effekter av dyrkingsmessige og miljømessige forhold. Tilgjengelige data er imidlertid basert på kortvarige studier, og mulige langsiktige effekter er derfor vanskelig å predikere.

Selv om datagrunnlaget er begrenset, indikerer relevante vitenskapelige studier av Cry1Ab-proteinet og kunnskapen om andre Cry-proteiners skjebne i jord at Cry1Ab ikke har direkte effekter på jordmiljøet.

Sameksistens
VKM konkluderer med at dyrkingsavstander på 200 meter mest sannsynlig vil sikre sameksistens mellom genmodifisert mais og konvensjonelle og økologiske maissorter i Norge.

Samlet konklusjon
Ut i fra dagens kunnskap konkluderer VKM med at maislinje Bt11 er ernæringsmessig ekvivalent med konvensjonell mais. Det er videre lite trolig at Cry1Ab og PAT vil introdusere et toksisk eller allergent potensiale i mat eller fôr basert på mais Bt11.

VKM finner det lite trolig at dyrking av maislinje Bt11 vil medføre negative effekter på miljø eller landbruk i Norge.

Abbreviations and glossary

ALS	Acetolactate synthase, an enzyme that catalyses the first step in the synthesis of the branched-chain amino acids, valine, leucine, and isoleucine
AMPA	Aminomethylphosphonic acid, one of the primary degradation products of glyphosate
ARMG	Antibiotic resistance marker gene
BC	Backcross. Backcross breeding in maize is extensively used to move a single trait of interest (e.g. disease resistance gene) from a donor line into the genome of a preferred or "elite" line without losing any part of the preferred lines existing genome. The plant with the gene of interest is the donor parent, while the elite line is the recurrent parent. BC_1, BC_2 etc. designates the backcross generation number.
BLAST	Basic Local Alignment Search Tool. A software that is used to compare nucleotide (BLASTn) or protein (BLASTp) sequences to sequence databases and calculate the statistical significance of matches, or to find potential translations of an unknown nucleotide sequence (BLASTx). BLAST can be used to identify evolutionary relationships between sequences and to group genes with homology in gene families.
bp	Basepair
Bt	*Bacillus thuringiensis*
CaMV	Cauliflower mosaic virus
Codex	Set by The Codex Alimentarius Commission (CAC), an intergovernmental body to implement the Joint FAO/WHO Food Standards Programme. Its principle objective is to protect the health of consumers and to facilitate the trade of food by setting international standards on foods (i.e. Codex Standards).
Cry	Any of several proteins that comprise the crystal found in spores of *Bacillus thuringiensis*. When activated by enzymes in the insects midgut, these proteins may attack the cells lining the gut and subsequently kill the insect.
Cry1Ab	Cry1 class crystal protein from *Bacillus thuringiensis* subsp. *kurstaki.* The protein provides protection against certain lepidopteran target pests, such as the European maize borer (*Ostrinia nubilalis*), and species belonging to the genus *Sesamia*
CTP	Chloroplast transit peptide
DAP	Days after planting
DNA	Deoxyribonucleic acid
DT50	Time to 50% dissipation of a protein in soil

DT90	Time to 90% dissipation of a protein in soil
dw	Dry weight
dwt	Dry weight tissue
EC	European Commission
ECB	European corn borer, *Ostrinia nubilalis*
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ERA	Environmental risk assessment
E-score	Expectation score
EU	European Union
fa	Fatty acid
FAO	Food and Agriculture Organisation
FIFRA	US EPA Federal Insecticide, Fungicide and Rodenticide Act
Fitness	Describes an individual's ability to reproduce successfully relative to that of other members of its population.
fw	Fresh weight
fwt	Fresh weight tissue
GAT	Glyphosate N-acetyltransferase
GLP	Good Laboratory Practice
Glufosinate-ammonium	Broad-spectrum systemic herbicide
Glyphosate	Broad-spectrum systemic herbicide
GM	Genetically Modified
GMO	Genetically Modified Organism
GMP	Genetically Modified Plant
H	Hybrid
ha	Hectare
ILSI	International Life Sciences Institute
IPM	Integrated Pest Management
IRM	Insect Resistance Management
Locus	The position/area that a given gene occupies on a chromosome
LOD	Limit of detection
LOQ	Limit of quantification
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time Of Flight. A mass spectrometry method used for detection and characterisation of biomolecules, such as proteins, peptides, oligosaccharides and oligonucleotides, with molecular masses between 400 and 350,000 Da.
MCB	Mediterranean corn borer, *Sesamia nonagrioides*
mEPSPS	Modified 5-enolpyruvylshikimate-3-phosphate synthase
mRNA	Messenger RNA
MT	Norwegian Food Safety Authority (Mattilsynet)

NDF	Neutral detergent fibre, measure of fibre used for animal feed analysis. NDF measures most of the structural components in plant cells (i.e. lignin, hemicellulose and cellulose), but not pectin.
Northern blot hybridisation	Northern blot hybridization is a technique used to study gene expression by detection of RNA or mRNA separated in a gel according to size.
NTO	Non-target organism
Nicosulfuron	Herbicide for maize that inhibits the activity of acetolactate synthase
Near-isogenic lines	Term used in genetics/plant breeding, and defined genetic lines that are identical except for differences at a few specific locations or genetic loci.
OECD	Organisation for Economic Co-operation and Development
ORF	Open Reading Frame, in molecular genetics defined as a reading frame that can code for amino acids between two stop codons (without stop codons).
OSL	Over season leaf
OSR	Over season root
OSWP	Over season whole plant
pat	*Phosphinothricin-Acetyl-Transferase* gene
PAT	Phosphinothricin-Acetyl-Transferase protein
PCR	Polymerase chain reaction, a technique to amplify DNA by copying it
PMI	Phosphomannose Isomerase enzyme. Metabolizes mannose and allows positive selection for recovery of transformed plants.
R0	First transformed generation, parent
Rimsulferon	Herbicide, inhibits acetolactate synthase
RNA	Ribonucleic acid
RP	Recurrent parent
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis. Technique to separate proteins according to their approximate size
SAS	Statistical Analysis System
SD	Standard deviation
Southern blot hybridistion	Method used for transfer of electrophoresis-separated DNA fragments to a filter membrane and possible subsequent fragment detection by probe hybridisation
T-DNA	Transfer DNA, the transferring DNA of the tumour-inducing (Ti) plasmid of some species of bacteria such as *Agrobacterium tumefaciens* and *A. rhizogenes*, into plant's nuclear genome. The T-DNA is bordered by 25-base-pair repeats on each end. Transfer

	is initiated at the left border and terminated at the right border and requires the *vir* genes of the Ti plasmid.
TI	Trait integrated
TMDI	Theoretical Maximum Daily Intake
U.S. EPA	United States Environmental Protection Agency.
Maize growth stages	*Vegetative*
	VE: emergence from soil surface
	V1: collar of the first leaf is visible
	V2: collar of the second leaf is visible
	Vn: collar of the leaf number 'n' is visible
	VT: last branch of the tassel is completely visible
	Reproductive
	R0: Anthesis or male flowering. Pollen shed begins
	R1: Silks are visible
	R2: Blister stage. The kernels are filled with a clear nourishing endosperm fluid and the embryo can be seen
	R3: Milk stage. The kernels endosperm is milky white.
	R4: Dough stage. The kernels endosperm has developed to a white paste
	R5: Dent stage. If the genotype is a dent type, the grains are dented
	R6: Physiological maturity
Western blot	Technique used to transfer proteins separated by gel electrophoresis by 3-D structure or denatured proteins by the length of the polypeptide to a membrane, where they might be identified by antibody hybridization.
WHO	World Health Organisation
ZM	*Zea maize* L.
ZM-HRA	A modified version of the native acetolactate synthase protein from maize. Confers tolerance to the ALS-inhibiting class of herbicides

Background

Notification C/F/96.05.10

The competent French authorities received the maize Bt11 application in 1996, C/F/96.05.10, under Council Directive 90/220/EEC, from the then Novartis Seeds (now Syngenta Seeds SAS). Syngenta Seeds SAS delivered an updated application (2003) under Directive 2001/18/EC of the European Parliament and Council. In both cases, the French authorities concluded that they found no scientific basis that maize Bt11 would result in increased risk for humans, animals or the environment when compared with its conventional maize variety.

On 16 June 2003, the European Commission received from the Competent Authority of France a notification (reference C/F/96/02.10) under Part C of Directive 2001/18/EC, for authorisation of the insect-resistant and herbicide tolerant genetically modified maize Bt11 for cultivation, feed use and industrial processing.

On 20 April 2005, the EFSA GMO Panel issued a scientific opinion on the notification (EFSA 2005), and concluded that there was no evidence indicating that placing maize Bt11 on the market is likely to cause adverse effects on human or animal health or the environment in the context of its proposed uses. At the time, the EFSA GMO Panel also recommended that maize Bt11 cultivation should be accompanied by appropriate risk management strategies to delay the potential evolution of resistance to the Cry1Ab protein in target insects and to minimise exposure of non-target Lepidoptera (EFSA, 2005).

In both 2006 and 2008, the European Commission requested the EFSA GMO Panel to consider whether new evidence published in the scientific literature required a revision of the conclusions of its 2005 Scientific Opinion on maize Bt11 (EFSA 2005). Following these requests, the EFSA GMO Panel evaluated the available new scientific information, and found no new evidence for adverse effects caused by the cultivation of maize Bt11 (EFSA 2006, 2008). The EFSA GMO Panel concluded that no new scientific information had been made available that would invalidate its previous risk assessment conclusions.

On 8 December 2010, the European Commission requested the EFSA GMO Panel to consider whether new scientific elements might require a revision of the conclusions of its previous Scientific Opinion on maize Bt11 in particular in the light of the mathematical model applied to the risk assessment of the insect resistant maize 1507. On 30 November 2011, the EFSA GMO Panel adopted a Statement supplementing the environmental risk assessment conclusions and risk management recommendations on maize Bt11 cultivation. In its Statement, the EFSA GMO Panel concluded that: “subject to appropriate management measures, maize Bt11 cultivation is unlikely to raise additional safety concerns for the environment compared to conventional maize” (EFSA 2011a).

The EFSA GMO Panel further supplemented its previous risk management recommendations on maize Bt11 for cultivation by reapplying the mathematical model developed by Perry et al.

(2010, 2011, 2012), in order to consider additional hypothetical agricultural conditions, and to provide additional information on the factors affecting the insect resistance management plan (EFSA, 2012a).

Following a request from the European Commission, the EFSA GMO Panel re-evaluated previous risk assessment conclusions and risk management recommendations on maize Bt11, in light of new relevant scientific publications (EFSA, 2012b).

Thus, EFSA assessed the application in the 2005, 2006, 2008, 2011, and 2012 and concluded consistently that based on the requested areas of application, it is unlikely that Bt11 has negative consequences on human and/or animal health and/or the environment. After the publication of a study by Hofmann et al. regarding maize pollen deposition in 2014, EFSA updated its advice on risk management in an opinion published in 2015. In 2016, EFSA evaluated a new study by Hofmann et al. (2016), and concluded in its technical report that its previous risk management recommendations for the cultivation of MON810, Bt11 and 1507 remained valid and applicable. In September 2016, EFSA published a technical report on the findings of teosinte and its impact on risk management recommendation regarding the cultivation of MON810, Bt11 and 1507 (EFSA, 2016).

The Commission presented a draft Commission Implementing Decision for the approval of the placing on the market for cultivation of genetically modified maize Bt11 (SYN-BT011-1) seeds to the Standing Committee in 2016. The application, however, received an unqualified majority of votes at the Committee meeting on January 27th, 2017. On 27 March 2017, the Appeal Committee voted on the draft implementing acts concerning the authorisation for the cultivation of GM maize crops Bt11 and 1507, and the re-authorisation of GM maize MON810). However, no qualified majority was achieved.

Terms of reference from the Norwegian Environment Agency and the Norwegian Food Authority

The European Commission has proposed in their draft decision to grant consent to the application to market seeds of genetically modified maize Bt11 for cultivation (notification C/F/96.05.10 under Directive 2001/18/EC). The draft decision has been up for a vote in the Regulatory Committee of competent authorities under Directive 2001/18/EC, and subsequently an Appeal Committee. Both committees delivered no opinion. The Commission will therefore make the final decision. In preparation for the potential approval of the application, the Norwegian Environment Agency has initiated the process of final assessment of the application in Norway under the Gene Technology Act.

The Norwegian Environment Agency

With reference to the letter of assignment for 2017, The Norwegian Environment Agency requests the Norwegian Scientific Committee for Food Safety (VKM) to prepare a final environmental risk assessment of application C/F/96.05.10 regarding approval of seeds of genetically modified maize Bt11 for cultivation. Maize Bt11 is genetically modified with resistance to certain insects of the order Lepidoptera and to herbicides containing the active ingredient glufosinate-ammonium. The assessment shall specifically consider Norwegian conditions. The risk assessment shall be in line with the conditions set out in the Gene Technology Act, and shall identify eventual adverse effects on the Norwegian environment. EFSA's risk assessment of the application may be used as justification for the conclusions of the environmental risk assessment, however, conditions specific to Norway, must be addressed and evaluated in the assessment. VKM is also requested to assess the applicant's post-market environmental monitoring plan and if this is sufficient to capture identified and potentially un-identified adverse effects of the product. VKM shall evaluate if the control measures suggested in the draft decision of the Commission, amongst others but not limited to, the given isolation distances between cultivated area and protected habitats with potential populations of non-target Lepidoptera, is consistent with the protection level needed for species and nature in Norway.

The Norwegian Food Safety Authority

The Norwegian Food Safety Authority requests the Norwegian Scientific Committee for Food Safety to undertake environmental risk assessments for the cultivation applications C/F/96.05.10 and C/ES/01/01, on cultivation of GM maize, in accordance with our current order.

The EU Commission has made drafts for the implementing decisions concerning the placing on the market of genetically modified maize Bt11 for cultivation. The EU-drafts have enclosed annexes, which include conditions or restrictions on the placing on the market, use or handling of the products, among other management measures. These management measures, in addition to the measures for securing co-existence, may consequently effect the cultivation system of these GM plants in Norway, as well as a potential regulation of such cultivation. The Norwegian Food Safety Authority requests the Norwegian Scientific Committee for Food Safety to assess whether the management measures described in the annexes of the EU-drafts requires adjustments to our previous recommended co-existence measures, and if so, which adjustments that should be.

Assessment

1 Introduction

Genetically modified maize Bt11 (Unique Identifier SYN-BTØ11-1) has been developed to provide protection against certain lepidopteran target pests, such as the European maize borer (*Ostrinia nubilalis*), and species belonging to the genus *Sesamia* (in particular the Mediterranean maize borer *Sesamia nonagrioides*). Protection from feeding damage by pest larvae is provided by expression in the tissues of Bt11 maize of a truncated form of a Cry1Ab protein encoded by a modified *cry1Ab* gene derived from the soil microorganism *Bacillus thuringiensis* subsp *kurstaki* HD-1. The mode of action of the Cry1Ab protein and other Cry proteins is to bind selectively to specific receptors on the epithelial surface of the midgut of larvae of susceptible insect species, leading to death of larvae through pore formation, cell burst and subsequently septicaemia. None of the target pests for maize Bt11 are present in the Norwegian agriculture.

Bt11 was also genetically modified to express the enzyme phosphinothricin acetyl-transferase (PAT), encoded by the *pat* gene from the soil actinomycete *Streptomyces viridochromogenes*. Expression of PAT confers tolerance to the herbicide glufosinate-ammonium, only used for selection of GM cells with the transferred *cry1Ab* gene. Glufosinate-ammonium acts by inhibiting the plant enzyme glutamine synthetase, the only enzyme in plants that detoxifies ammonia by incorporating it into glutamine. Inhibition of this enzyme leads to an accumulation of ammonia in the plant tissues, which kills the plant within hours of application. PAT catalyses the acetylation of the herbicide phosphinothricin and thus detoxifies glufosinate-ammonium into an inactive compound. The PAT protein expressed in maize Bt11 has been used as selectable marker to facilitate the selection process of transformed plant cells, and is not intended for weed management purposes.

The genetic modification in maize Bt11 is intended to improve agronomic performance only and is not intended to influence the nutritional properties, the processing characteristics and the overall use of maize as a crop. Event Bt11 has been crossed into both field maize and sweet maize varieties.

Maize Bt11 has been evaluated with reference to its intended uses in the European Economic Area (EEA), and according to the principles described in the Norwegian Food Act, the Norwegian Gene Technology Act and regulations relating to impact assessment pursuant to the Gene Technology Act, Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, and Regulation (EC) No 1829/2003 on genetically modified food and feed.

VKM also considers the appropriate principles described in the EFSA guidelines for the risk assessment of GM plants and derived food and feed (EFSA, 2011a), the environmental risk assessment of GM plants (EFSA, 2010), the selection of comparators for the risk assessment of GM plants (EFSA, 2011b), and for the post-market environmental monitoring of GM plants (EFSA, 2011c).

The food/feed and environmental risk assessment of the genetically modified maize Bt11 is based on a review of relevant peer-reviewed scientific publications and information provided by the applicant in the notification C/F/96.05.10 and the renewal application EFSA/GMO/RX/Bt11, and scientific opinions and comments from EFSA and other EU-member states. The risk assessment is also based in part on a risk analysis report of Bt11 from the Australia New Zealand Food Authority (ANFZA, 2000).

It is emphasised that the VKM mandate does not include assessments of contribution to sustainable development, societal utility and ethical considerations, according to the Norwegian Gene Technology Act and Regulations relating to impact assessment pursuant to the Gene Technology Act. These considerations are therefore not part of the risk assessment provided by VKM.

2 Literature

2.1 Search strategy

Literature searches was performed to retrieve publications addressing putative environmental and heath effects of genetically modified maize Bt11. Even though no systematic review of the literature is carried out in this scientific opinion, VKM adhered to some general principles for performing systematic review, in order to ensure methodological rigour and coherence in the retrieval and selection of publications, transparency, and reproducibility of the performed literature search (EFSA, 2010 c).

Literature searches were performed from January 2013 to April 2017 using the scientific databases ISI Web of Science, Medline, EMBASE, Scopus, Agris and CAB Abstracts. Each database was searched individually. Searches in ISI Web of Science, Medline, Embase and Scopus were conducted by a librarian at the Norwegian Institute of Public Health (NIPH). Searches in CAB Abstracts and Agris were conducted by librarians at the Norwegian University of Life Sciences. Additionally, PubMed searches were conducted by the VKM staff.

The literature was searched and screened in a stepwise manner. As a first step, a combination of generic keywords being both trait- and event-specific was used to retrieve all references for further consideration (TOPIC FIELD: maize AND Bt11 OR MON810 or Cry1Ab) The search by keywords using the topic field, enabled to retrieve publications that contain these keywords, either in the publications title, list of keywords, or abstract.

The following search terms were employed:

1. Gene flow/co-existence

(Organisms, Genetically Modified/ or Plants, Genetically Modified/) and Zea Mays/
((gmo or (Genetically adj1 (Modified or engineered)) or Transgenic or bt) adj2 (zea or corn or maize or teosinte)).tw.
(("Bt crop?" or "Bt GM crop?") and (zea or corn or maize or teosinte)).tw.
or/1-3
Gene Flow/
("gene flow" or "pollen dispersal" or "cross pollination?" or crosspollination? or "out crossing" or outcrossing or "co existence" or coexistence).tw.
5 or 6
4 and 7

limit 8 to yr="2013 -Current"
limit 9 to english language

2. Potential effects of non-target organisms

(Organisms, Genetically Modified/ or Plants, Genetically Modified/) and Zea Mays/
((gmo or (Genetically adj1 (Modified or engineered)) or Transgenic or "Bt11") adj2 (zea or corn or maize or teosinte)).tw.
("Bt11" or “MON810” or "cry1Ab").tw.
(("Bt crops" or "Bt GM crops") and (zea or corn or maize or teosinte)).tw.
or/1-4
Soil Microbiology/
(predator? or parasitoid? or lepidoptera or "pollinating insect?" or pollinator? or "pollen feeder?" or “natural enemy”? or “beneficial insect” or "soil arthropod?" or "aquatic arthropod?" or earthworm? or "enchytraeid worm?" or nematod* or isopod? or collembolan? or diplopod? or "non target organism?" or "nontarget organism?" or biodiversity or "soil microorganism?" or "soil microbiology" or "soil microbial" or rhizosphere).tw.
6 or 7
5 and 8
limit 9 to english language

3. Potential effects of target organisms

(Organisms, Genetically Modified/ or Plants, Genetically Modified/) and Zea Mays/
((gmo or (Genetically adj1 (Modified or engineered)) or Transgenic or "Bt11" or "mon810" or bt) adj2 (zea or corn or maize or teosinte)).tw.
(“Bt11” or "MON810" or "cry1Ab") tw.
(("Bt crops" or "Bt GM crops") and (zea or corn or maize or teosinte)).tw.
or/1-4
(resistance or susceptibility or sensitivity or crossresistance or tolerance).tw.

Lepidoptera/
(sesamia? or ostrinia? or "european corn borer?" or lepidoptera?).tw.
7 or 8
5 and 6 and 9
limit 10 to english language

4. Food and Feed

(bt[All Fields] AND 11[All Fields] AND ("zea mays"[MeSH Terms] OR ("zea"[All Fields] AND "mays"[All Fields]) OR "zea mays"[All Fields] OR "maize"[All Fields])) AND ("2011/12/01"[PDat] : "2016/11/28"[PDat]) bt[All Fields] AND 11[All Fields] AND ("zea mays"[MeSH Terms] OR ("zea"[All Fields] AND "mays"[All Fields]) OR "zea mays"[All Fields] OR "maize"[All Fields]) (bt[All Fields] AND 11[All Fields] AND ("zea mays"[MeSH Terms] OR ("zea"[All Fields] AND "mays"[All Fields]) OR "zea mays"[All Fields] OR "maize"[All Fields])) AND ("2012/04/29"[PDat] : "2017/04/27"[PDat]) ("bacillus thuringiensis"[MeSH Terms] OR ("bacillus"[All Fields] AND "thuringiensis"[All Fields]) OR "bacillus thuringiensis"[All Fields]) AND ("zea mays"[MeSH Terms] OR ("zea"[All Fields] AND "mays"[All Fields]) OR "zea mays"[All Fields] OR "maize"[All Fields]) AND cry[All Fields] AND 1ab[All Fields]

2.2 Relevance screening

The titles of all hits were scanned, and for those that were of potential relevance, the abstracts were also inpected. The relevance screening was performed by the members of the project group.

Citations were excluded if they did not relate to the terms of reference. Publications related to detection, quantification, labelling, traceability and socio-economics were excluded, as these topics are not in the remit of VKM. Only full-text, peer-reviewed articles published in English were included in this assessment.

3 Molecular characterisation

3.1 Information related to the genetic modification

3.1.1 Description of the methods and vectors used for the genetic modification

Bt11 maize was generated by transformation of a proprietary inbred maize line, H8540, with the vector pZ01502 to insert two new genes, a truncated *cry1ab* gene (referred to as the *cry1Ab* gene) and the *pat* gene. The line was transformed using protoplast transformation. The protoplasts were transformed with a single DNA fragment obtained by a restriction digest of the plasmid pZO1502 with the enzyme *Not* I. According to the applicant, the *Not* I fragment contained part of the plasmid backbone DNA sequences. Regenerated plants were backcrossed to a selected line resulting in the plant named Bt11 maize.

3.1.2 Nature and source of vector(s) used including nucleotide sequences intended for insertion

The vector used for transformation was named pZO1502. The pZO1502 vector is a derivative of pUC18 isolated from the host *E. coli*. A detailed vector map of pZO1502 is presented in Figure 1. The *Not*I restriction fragment, which consists of the expression cassette and a part of the backbone sequence was used for transformation. The NotI restriction fragment intended for insertion consists of the *cry1Ab* and the *pat* genes and regulatory elements needed for their expression, ColE1 ori from the vector backbone, but did not contain the ampicillin resistance gene.

A description of the vector components and their origins are presented in Table 1 and 2 below. The elements intended for insertion within the *Not*I restriction fragment of plasmid pZO1502 is schematically shown in Figure 2. The source, size and intended function of each constituent in the Not I fragment are shown in Table 2.

Table 1. Vector backbone components of pZO1502

Vector Component	Description
Lac	A partial lacI coding sequence, the promoter plac, and a partial coding sequence for β-galactosidase or LacZ proteins (Yanisch-Perron *et al.,* 1985)
Bla	The TEM type β-lactamase gene from *E. coli* plasmid pBR322 confers resistance on bacterial cells to ampicillin and other penicillins (Sutcliffe, 1978). The gene is under the control of its native bacterial promoter.
ColE1ori	The origin of DNA replication from the *E.Coli* high copy plasmid pUC19 (Yanisch-Perron *et al.,* 1985)

Table 2. Source, size and intended function of constituents intended for insertion in Bt11 maize

Vector component	Approx Size (kb)	Source	Intended function
35S promoter	**0.509**	*cauliflower mosaic virus 35S* gene	Promoter of high level constitutive gene expression in plant tissues (Gardner *et al.*, 1981)
IVS6 enhancer	0.471	intron from maize *alcoholdehydrogenase 1S* gene	regulatory sequence that enhances *cryIAb* gene expression in the plant (Freeling and Bennet, 1985)
cry1ab gene	1.848	*Bacillus thuringiensis* subsp. *kurstaki* strain HD-1	encodes a truncated version of the full-length *cryIAb* gene which confers tolerance to certain lepidopteran species (Perlak *et al.*,1991).
Nos'3 terminator	0.253	*A. tumefaciens* nopaline synthase gene	Contains the signal for the termination of transcription and directs polyadenylation (Bevan *et al.*,1983)
35S promoter	**0.418**	*cauliflower mosaic virus 35S* gene	Promoter of high level constitutive gene expression in plant tissues (Gardner *et al.*, 1981)
IVS2 enhancer	0.180	intron from maize *alcoholdehydrogenase 1S* gene	regulatory sequence that enhances *pat* gene expression in the plant (Freeling and Bennet, 1985)
pat gene	0.552	Phosphinothricin acetyl transferase from *Streptomyces viridochromogenes*	encodes for a phosphinothricine acetyl-transferase enzyme which confers tolerance to glufosinate ammonium herbicides (Strauch *et al.*,1988)
Nos'3 terminator	0.253	*A. tumefaciens* nopaline synthase gene	Contains the signal for the termination of transcription and directs polyadenylation (Bevan *et al.*,1983)

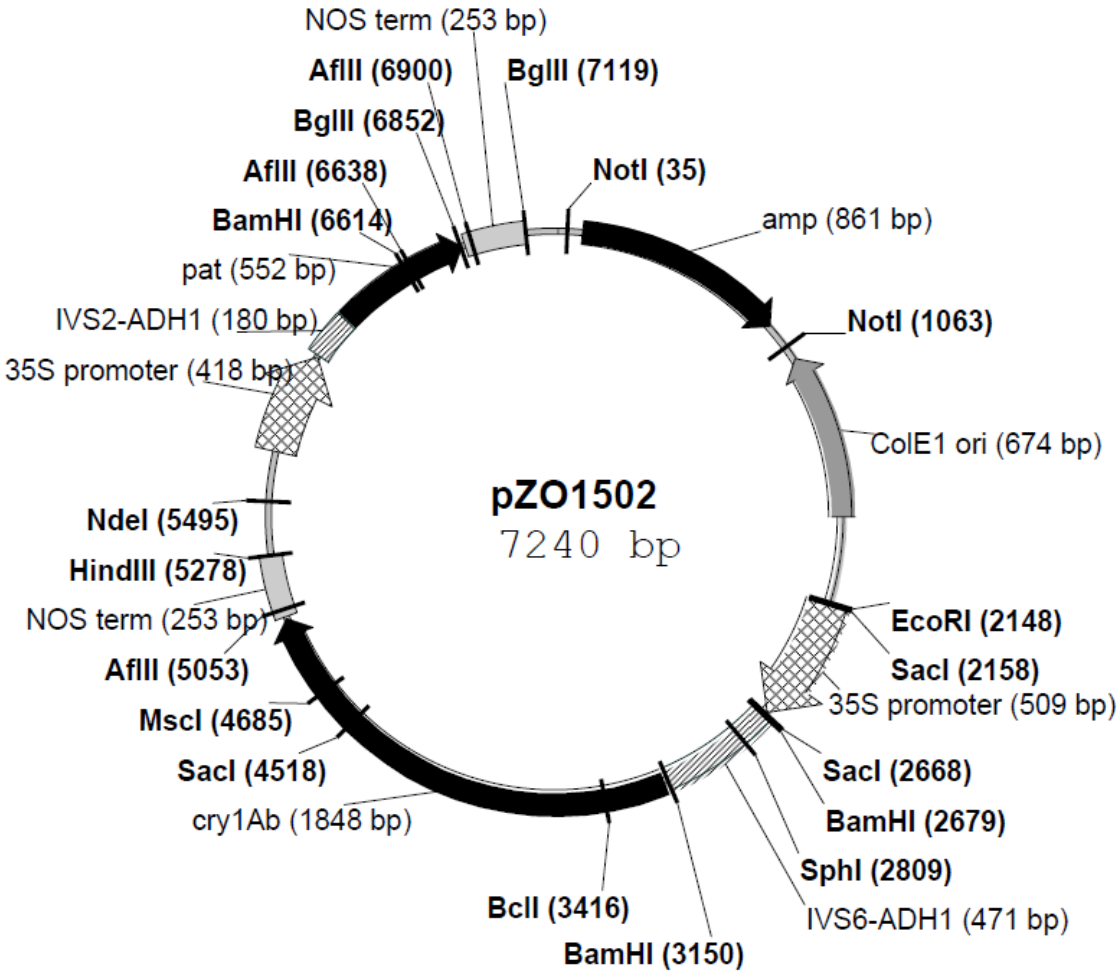

Figure 1. Plasmid map of transformation vector pZO1502

3.2 Information relating to the GM plant

3.2.1 General description of the trait(s) and characteristics which have been introduced or modified

The introduced genetic elements in the Bt11 maize gives protection from certain Lepidopteran insect pests (including *Ostrinia nubilalis* (European Maize Borer) and *Sesamia* spp.) and tolerance to glufosinate-ammonium herbicides. Protection from feeding damage by pest larvae is provided by expression of the Cry1Ab protein encoded by the engineered *cry1Ab* gene. The *cry1Ab* gene is a truncated version of the δ-endotoxin *cry1* gene derived from the soil microorganism *Bacillus thuringiensis* subsp *kurstaki* HD-1. In the *cry1Ab* gene, the DNA sequence of the *cry1* gene has been truncated at the 3' end and codon optimised to increase the level of expression in maize without changing the amino acid sequence of the remaining expressed truncated protein (Perlak et al., 1991). The tolerance to glufosinate ammonium

herbicides is provided by expression of the *pat* gene, derived from the soil microorganism *Streptomyces viridochromogenes* strain Tu494, which encodes the enzyme phosphinothricin acetyl transferase (PAT), capable of detoxifying the glufosinate-ammonium herbicides.

According to the applicant, expression of the Cry1Ab and PAT proteins within maize tissues protects the plant from feeding damage by first and second brood ECB larvae.

3.2.2 Information on the sequences actually inserted/deleted or altered

Bt11 maize was subjected to molecular analysis in order to determine the number of integration sites within the maize genome, the copy number (the number of integrated copies of the DNA fragment), the integrity of the inserted cassettes, and the absence of backbone sequences outside the NotI fragment of the vector. Southern hybridization analyses were performed with a variety of DNA probes including sequences from the *pat, cry1Ab,* and *amp* genes as well as the entire pZO1502 vector to search for unintended insertions in the maize genome.

Sequence analysis of the entire insert present in Bt11 maize indicate overall integrity of the insert and that the contiguousness of the functional elements has been maintained. PCR analysis and DNA sequencing were used to establish a detailed transgene locus structure and to verify the 5' and 3' junction sequences of the insert with the plant genome. These analyses indicate intactness of the 5' and 3' ends of the inserted cassettes. The sequence of the inserted fragment was obtained by sequencing PCR fragments amplified directly from the Bt11 maize genomic DNA. According to the applicant, the resulting sequence was identical to the sequence of the corresponding fragment in the plasmid. This supports that no rearrangements occurred within the *Not* I fragment during gene transfer and integration into the plant genome. Sequence information indicates that no vector unintended backbone fragments, including any *amp* sequences, are inserted and fused to the inserted *Not* I fragment.

3.2.2.1 Size and copy number of all detectable inserts, both complete and partial

Data from Southern analysis indicate that single copies of the *cry1Ab* gene, *pat* gene and ColE1 origin of replication derived from the transformation plasmid pZO1502 are present in the Bt11 maize. According to the applicant, the Bt11 maize insert contains two copies of the 35S promoter, corresponding to the two copies of the promoter present in the transformation plasmid pZO1502. The applicant also concludes that the Bt11 maize insert does not contain any unintended backbone sequences from the transformation plasmid pZO1502.

The structure of the Bt11 maize locus is represented in Figure 2. As described previously, the DNA inserted in the maize genome is the fragment obtained by *NotI* restriction of the plasmid pZO1502 derived from pUC18 (Figure 1). This fragment contains two gene cassettes: the CaMV35S/intron/*Bt/nos* cassette for the *cry* gene and the CaMV35S/intron/*pat*/*nos* cassette

for the *pat* gene. Additionally, it contains vector backbone upstream from the *Bt* cassette, between the two cassettes and downstream from the *pat* cassette.

The size of the plasmid is 7,24 Kb, and the fragment integrated in the Bt11 maize is 6,2 Kb.

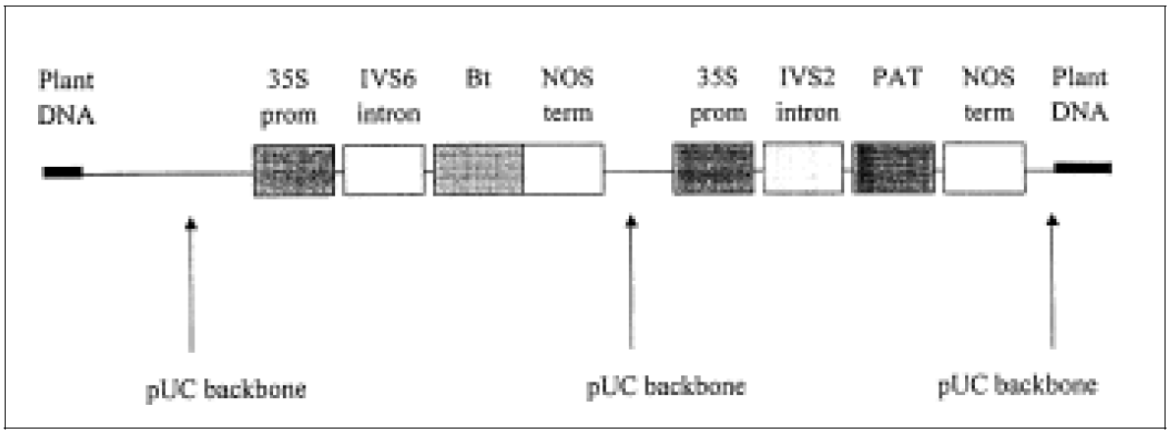

Figure 2. Structure of the inserted Bt11 maize locus.

3.2.2.2 The Organization and sequence of the inserted genetic material at each insertion site

According to the applicant, the entire insert and flanking regions inserted in Bt11 maize have been sequenced and the sequences flanking the fragment inserted in Bt11 maize have been identified. DNA sequences at the junctions between the insert and the parent genome were further analysed. At the 5' flank, approximately 350 bp of the plant DNA adjacent to the insert was sequenced. At the 3' flank, approximately 540 bp of the plant DNA adjacent to the insert was sequenced. The identified 5' and 3' flanking sequences were used to search for homologies with sequences found in public databases with blast analysis. According to the applicant, BLAST analysis of both the 5' and 3' regions of the Bt11 maize insert revealed homology primarily to the *Zea mays* 180 bp knob-associated tandem repeat. Knobs are components of the maize heterochromatin, a class of chromatin believed not to encode for expressed sequences. Based on these findings, the applicant concludes that the insertion of the *Not* I fragment in the maize genome does not disrupt any endogenous maize open reading frame(s).

An updated bioinformatic analysis performed in 2008 confirmed the original analysis carried out by the applicant and supports the conclusion that the genomic sequences in the 5' and 3' regions flanking the insert of maize Bt11 show homology to highly repetitive knob-associated sequences.

3.2.2.3 In the case of deletion(s), size and function of the deleted region(s)

Not applicable.

3.2.2.4 Sub-cellular location(s) of insert(s) and methods for its / their insertion

According to the applicant, the inserted DNA is located on the short arm of chromosome 8. The insert is stably integrated into the plant chromosome and is inherited as a single dominant gene in a classic Mendelian manner.

3.2.3 Information on the expression of the inserted/modified sequence

To characterize the range of expression of Cry1Ab and PAT proteins in Bt11maize plants, the concentrations of Cry1Ab and PAT proteins in different plant tissues and whole plants at various growth stages (from whorl to senescence) from various hybrids of field and sweet maize, were determined by ELISA.

According to the applicant, the Cry1Ab protein was found in all tissues examined, with a decrease in concentration with plant maturation and senescence. The concentrations of Cry1Ab protein (the active insecticidal principle) were determined in several plant tissues and whole plants at four developmental stages (whorl, anthesis, seed maturity and senescence) in two different field maize hybrids. The quantity of Cry1Ab protein was also estimated on a per-acre and a per-hectare basis. Levels in pollen were below the lower limit of quantification, < 0.08 µg/g fresh wt. pollen and < 0.15 µg/g dry wt. pollen. Across all plant stages, mean Cry1Ab levels measured in leaves, roots and whole plants ranged from ca. 10 - 22 µg/g fresh wt. (12 – 154 µg/g dry wt.), 2 – 4 µg/g fresh wt. (9 – 22 µg/g dry wt.), and 4 – 9 µg/g fresh wt. (6 – 70 µg/g dry wt.), respectively. Mean Cry1Ab levels measured in grain at seed maturity and senescence were 1 – 2 µg/g fresh wt (2 µg/g dry wt.). The level of Cry1Ab was generally similar between hybrids for each tissue type at each time point.

The application also cover marketing and trading on the European market of Bt11 sweet maize for immediate consumption, for the consumption of tinned and frozen sweet maize, and for further processing into sweet maize powder. The applicant has therefore performed a specific analysis to determine the level of the Cry1Ab protein in tissues from three Bt-11 sweet maize hybrid varieties and control lines with a similar genetic background from those used in field tests in 1996. The Cry1Ab protein levels in grains tested at prime harvest stage was also assessed in these sweet maize hybrids that had been canned. The level of the Cry1Ab protein in Bt11 sweet maize hybrids grain at prime harvest was 1.97 ± 0.36 µg/g fresh weight, and at 21 days post prime harvest 2.98 ± 1.12 µg/g fresh weight. The range of Cry1Ab in grain for all three sweet maize hybrids was 0.51 to 3.80 µg Cry1Ab/g fresh weight. Cry1Ab protein was not detectable in any of the canned maize samples tested.

Based on the findings that the Cry1Ab protein was not detected in canned maize and that the levels of Cry1Ab in grain for all Bt11 maize varieties (field and sweet maize) were low, the applicant concludes that dietary exposure to the Cry1Ab protein is expected to be very low.

According to the applicant, the level of the PAT protein was determined using Bt11 field maize plants; measurable levels (ng/g) were only found in leaves, silk and tassel. For grain, pollen, root and stalk, concentrations were below the limits of detection. The PAT protein is present at less than 0.000008% fresh weight and 0.00016% of the total maize grain protein.

3.2.4 Genetic stability of the inserted / modified sequence and phenotypic stability of the GM plant

The genetic stability of the inserted DNA-fragment in Bt11 maize was verified by both a classical approach and a molecular approach with Southern analysis of genomic DNA. The Bt11 maize was subjected to a backcrossing program with the elite line H8540. The lines BC3 and BC6, developed as part of this program, were used to evaluate genetic stability. BC3 was developed from 3 backcrosses with H8540 and BC6 from 6 backcrosses. According to the applicant, no differences in banding pattern were observed between the DNA from these generations demonstrating the stability of the inserted DNA-fragment.

Segregation data for glufosinate ammonium tolerance and European maize borer resistance were collected at different points in the backcrossing experiment. BC3 and BC6 plants identified to contain the *cry1Ab* and *pat* genes were subjected to selfing experiments. According to the applicant, the results indicate heritability and stability of the two genes in cultivated Bt11 maize. Data support the presence of a single insertion that segregates according to Mendelian inheritance patterns.

Restriction fragment length polymorphism (RFLP) mapping was used to determine the location of the inserted DNA-fragment in Bt11. The progeny of Bt11 plants crossed with two inbred maize lines were screened with RFLP probes corresponding to different regions of the corn genome. Comparison of the genotypes of the progeny with isogenic controls demonstrated that the site of integration for the DNA-fragment in Bt11 maize is located on the long arm of chromosome 8.

According to the applicant, no differences in the agronomic and phenotypic characteristics were observed between the Bt11 maize and the non-transgenic counterpart during field trials at different locations (Spain, France, Italy and Portugal) conducted between 1994 and 2006 that would indicate unexpected pleiotropic effects of the genetic modification (Final reports at JRC web page).

The stability of the inserted DNA in Bt11 maize was confirmed to follow a Mendelian inheritance pattern. The inheritance of the *cry1Ab* and *pat* genes and their phenotypic traits was followed over multiple generations to determine a possible segregation of these. F1 plants (first generation hybrids) identified to contain the *cry1Ab* and *pat* genes were self-fertilised to produce the S1 population. This S1 population was screened for protection against the European corn borer and for tolerance to glufosinate- ammonium. The S1 plants were again self-fertilised. The insect protection and herbicide tolerance traits were then

backcrossed into two genetic backgrounds (H8540 and 977), and in some cases, followed by further self-fertilisation.

Seeds from maize plants representing different backcross stages were planted in various fields and samples collected for analysis in 1994 and 1995. Plant materials were tested for protection against the European corn borer and tolerance to glufosinate ammonium. All tested plants were either both tolerant to the herbicide and protected against insect attack, or susceptible to both. A lack of segregation of the two traits is consistent with the molecular characterization, which concluded with insertion of the DNA fragment into a single locus.

3.3 Conclusions

Appropriate analyses of the integration site, inserted DNA sequence, flanking regions, and bioinformatics have been performed. The molecular characterisation performed by the applicant support that the DNA-fragment containing the *cry1Ab* and *pat* genes are integrated as a single copy at a single locus in the nuclear genome of maize Bt11 and is stably inherited as a Mendelian dominant trait.

4 Comparative assessments

4.1 Production of material for comparative assessment

The application EFSA/GMO/RX/Bt11 for food and feed use, import and processing of maize Bt11 within the EU presented compositional data on seed and forage material collected in field trials performed in the USA in 1995, 1996 and 1998, and in France in 1998. These studies have been conducted on Bt11 seeds and major components in inbred and hybrid lines at different stages of maturity are assessed and compared with their respective near-isogenic controls. In addition, the applicant has provided data from compositional analysis of Bt11 maize plants grown in greenhouses in Europe in 1999.

The applicant has provided data on the original transformant (H8540 and hybrids) and different maize hybrids widely used in food and feed production. This includes several field maize and sweet maize lines that have been developed from conventional breeding of the original transformed line. (See Table 1, Appendix 1 for a complete summary of the lines analysed).

Studies submitted by Syngenta Seeds Inc.

- Compositional analysis of Bt11 maize: determination of the substantial equivalence — chemical composition analysis done with Bt-11 maize with a European background. *(Greenhouse study in Europe in 1999)*

- Compositional analysis of Bt11 maize: determination of the substantial equivalence — chemical composition analysis done with Bt-11 maize with a US background. Part 1: Properties of grain produced from ECB protected maize hybrids; Part 2: Characterization of grain attributes of normal, wild-type maize hybrids and the Bt11 converted iso-hybrid counterparts; Part 3: Analyses of fatty acid and amino acid profiles of grain from Bt-11 maize. Report No. NSB-004-97.
  (Field trials conducted in the USA in 1995 (6 sites) with six field maize lines developed from conventional breeding with the original transformant)

- Comparison of vitamin and mineral composition of Bt11 maize and non-modified maize hybrids. Report No. NSB- 004-97. Novartis Seeds.
  (Field trials conducted in the USA in 1995 (3 sites) with six field maize lines developed from conventional breeding with the original transformant)

- Comparison of nutritional composition of fresh and canned grain prepared from Attribute insect protected and control sweet maize hybrids. Report No. NSV-002-98. Novartis Seeds Inc.
  (Field trials conducted in the USA in 1996 (1 site) with six sweet maize lines developed from conventional breeding with the original transformant)

- Goy PA (1999) Novartis Seed's genetically modified Bt11 maize: biochemical composition of grain from plants treated with a glufosinate ammonium herbicide.
  (Field trials conducted in France in 1998 (2 sites) with three field maize hybrids)

- Goy PA (2000) Novartis Seed's genetically modified Bt11 sweet maize: further determination of the biochemical composition of kernel- analysis of secondary metabolites.

  (Field trials conducted in the USA in 1998 (1 site) with three sweet maize hybrids)

4.2 Compositional analysis

4.2.1 Study 1: Analysis of Bt11 maize grown in greenhouse in Europe (1999)

The components of several lines and hybrids of the Bt11 maize, derived through backcrossing from the original transformant, were analyzed and compared to isogenic non-modified lines and hybrids. The following greenhouse grown plants were analysed: an inbred line (H8540-Bt), a hybrid line (hybrid Bt+/Bt-) and their respective controls (isogenic non-modified H8540 and control hybrid). Between 45 and 56 ears were taken from each plant. Ears were harvested and dried four months after sowing and 500 g samples were analysed.

The following parameters were analysed: moisture, total nitrogen, ash, starch, cellulose, xanthophyll, fatty acid and amino acid composition. Statistical comparison with STATITCF software was made on the values of two replicate analyses, except in the case of xanthophyll, fatty acids and amino acids, where data points are the result of a single analysis.

Proximates

All values for proximates were within the range, except total nitrogen content (Table 1). The total nitrogen content of both Bt11 maize and non-transgenic maize were higher than the spectrum ranges found in the literature. As the protein content is influenced by the available soil nitrogen, the increase could be caused by the fertilizer used in culturing of the plants in the greenhouse.

Table 1. Proximate composition for Bt11 and control maize (ANZFA, 2000).

	Inbred line H8540-Bt	***Isogenic control H8540***	**Hybrid Bt+/Bt-**	***Control hybrid***	**Normal range[2]**
Total nitrogen[3]	13.18 ± 0.07	12.35 ± 0.06	12.28 ± 0.03	12.30 ± 0.07	7.7–10[4]
Moisture	12.3	12.6	12.6	13.3	7–23
Ash	1.47 ± 0.04	1.79 ± 0.007	1.70 ± 0.02	1.6 ± 0.02	1.1–3.9
Starch	68.02 ± 0.4	67.57 ± 0.4	70.83 ± 0.81	70.25 ± 0.48	61–78
Cellulose	2.99 ± 0.007	2.9 ± 0.05	2.67 ± 0.28	2.92 ± 0.05	3.3–4.3 1.93–2.5[4]
Xanthophyll	24.2	21.0	21.6	19.1	19.2–33.1[4]

[1]Samples are 500g of kernels from: Bt^+/Bt^+ H8540 ears n=54, Control H8540 n=56, Bt^+/Bt^- hybrid n=50, Control hybrid ears n= 45. Each data point represents the mean of two replicate analyses made with the 500g sample. Data from AGPM. All data except moisture (% H_2O) and xanthophyll (mg/kg dry weight basis) are presented on a % dry weight basis.
[2]Wright, 1987 in Corn chemistry and technology, 1987, Watson SA and Ramstad PE (eds), American Association of Cereal Chemists, St. Paul, Minesota, USA.
[3]All values from control and genetically modified lines are significantly different to range.
[4]Data from AGPM

Fatty acids

A single analysis was done on 500g samples of grains from Bt+/Bt+ H8540 (number of ears n=54), isogenic control H8540 (number of ears n=56), Bt+/Bt- hybrid (number of ears n=50), control hybrid (number of ears n= 45). No range is available for arachidic acid, gadoleic acid or behenic acid. All other values regarding the fatty acids were within the range (Table 2). There were no differences in these values greater than 10% (which allows for experimental error) between the modified maize and controls. Literature ranges were available for most of the common fatty acids.

Table 2. Fatty acid composition for Bt11 and control maize (ANZFA, 2000).

Fatty acid composition	Bt^+/Bt^+ line H8540	*Control H8540*	Bt^+/Bt^- hybrid line	*Control* hybrid line	Range
C16 palmitic acid	15.1	14.5	15.3	14.6	6–22[2]
C18 stearic acid	1.7	1.6	1.6	1.5	1–15[2]
C18:1 oleic acid	20.6	21.9	21.8	21.8	14–64[2]
C18:2 linoleic acid	58.9	58.2	58.1	60	19–71[2]; 56–65[3]
C18:3 linolenic acid	1.7	1.7	1.2	1.1	0.5–2[2]
C20 arachidic acid	0.5	0.4	0.4	0.4	
C20:1 gadoleic acid	0.2	0.2	0.2	0.2	
C22: behenic acid	0.2	0.2	0.1	0.1	

[1]Samples are 500g of kernels from: Bt^+/Bt^+ H8540 ears n=54, Control H8540 n=56, Bt^+/Bt^- hybrid n=50, Control hybrid ears n= 45. Values are expressed as % of the analysed fatty acid relative to the total amount of fatty acids.
[2]From Weber, "Lipids of the kernel", Chapter 10 in Corn chemistry and technology, 1987, Watson SA and Ramstad PE (eds), American Association of Cereal Chemists, St. Paul, Minesota, USA. data
[3]AGPM

Amino acids

A single analysis was done on 500g samples of grain from Bt+/Bt+ H8540 (number of ears n=54), isogenic control H8540 (number of ears n=56), Bt+/Bt- hybrid (number of ears n=50), control hybrid (number of ears n= 45). The experimental data regarding the amino acid composition are generally higher than the bibliographical ones (Table 3). According to the applicant, the reason is the high nitrogen content of the analysed material. The bibliographical values originated from maize grains have total nitrogen content of approximately 9.5%. According to the applicant these data, therefore, need to be corrected to give comparable values. Protein levels were higher than the normal range for all plants assessed. The data were multiplied by 1.33, the corrective factor shown in the total nitrogen analysis (Table 1). As protein content is affected by soil nitrogen, it is possible that the fertiliser used caused the high level of nitrogen for all plants in the study.

After correction and taking in account experimental error (10%) the values are included in the range (Table 3). Comparison between Bt11 maize and control maize indicate no difference except for glutamic acid, which is in the limit of the allowed variation for the hybrid (experimental error of 10%).

The levels of glutamine, asparagine and tryptophan were not determined. Tryptophan is one of the essential amino acids and one of the limiting amino acids in maize. No spectrum or literature ranges were available for some of the amino acids, because some of these analyses are not routinely carried out by the laboratory assaying these samples.

Table 3. Amino acid composition for Bt11 and control maize (ANZFA, 2000).

Amino acid composition	Bt^+/Bt^+ line H8540	*Control H8540*	Bt^+/Bt^- hybrid line	*Control hybrid line*	Range[2]
Aspartic acid	9.8	9	8.7	8.4	
Threonine	5.2	5	4.9	5	3.2–3.4
Serine	6.6	6.4	6.1	6.1	
Glutamic acid	28.1	25.7	26.2	25.1	
Proline	12	12.5	12	11.4	
Glycine	4.1	4.2	4	4	
Alanine	11.5	10.8	10.8	10.1	
Cysteine	2.4	2.4	2.5	2.7	
Valine	6.5	6.1	5.9	6.2	4.2–4.6
Methionine	2.5	2.5	2.7	2.9	1.8–1.9
Isoleucine	5.2	4.8	4.6	4.6	3.4–3.7
Leucine	19.4	17.5	17.7	17.3	10–11.3
Tyrosine	5.4	4.9	5	4.7	
Phenylalanine	7.2	6.5	6.4	6.3	4.4–4.5
Lysine	3.2	3.3	3.1	3	2.45–2.6
Histidine	3.5	3.4	3.4	3.5	
Arginine	4.4	4.8	4.9	4.8	4.1–5.2

[1]Values are expressed as g/Kg dry matter.
[2]Data from L'alimentation des animaux monogastriques: porc, lapin, volailles. INRA 1989, Feedstuffs ingredient analysis table, edition 1996, AEC Table and 1995 UCAAB data.

4.2.2 Study 2: Analysis of Bt11 field maize grown in USA (1995)

Grain property studies were conducted on Bt11 maize hybrids and non-transgenic maize hybrids grown in 3-6 field locations in the USA in 1995. The following traits were considered: grain size, density and the content of proximates: starch , protein , oil and fiber expressed as percentage of dry weight, and fatty acid,amino acid, and mineral and vitamin profiles.

Dataset 1:
An analysis of the major components and nutritional qualities of two elite Bt11 field maize lines (X6534CBR, X7634CBR) developed from conventional breeding with the original transformant, has been assessed. In this trial, the Bt11 hybrids and their near-isogenic controls (X6534 and X7634) were grown in three field locations in the USA (Table 1, Appendix 1).

Grains from the Bt11 hybrids and the conventional counterparts were analysed for percentage of starch, protein, oil and fibre. No significant differences were detected between the Bt hybrids and the control hybrids ($p>0.05$) (Table 4). The grains from insect-resistant maize hybrids were comparable to control hybrids regarding the content of starch, protein, oil and fibre and fell within the normal ranges expected for these components.

Table 4. Proximate composition for Bt11 and control maize (dataset 1) (ANZFA, 2000).

	X6534CBR	Isogenic control X6514	X7634CBR	Isogenic control X7514	Normal range[2]
Protein	9.89 (9.40-10.60)	9.96 (9.10-11.40)	10.55 (10.24-11.00)	9.68 (8.90-10.94)	6-12
Oil	4.09 (4.00-4.16)	4.11 (4.10-4.13)	4.02 (4.00-4.02)	4.07 (3.80-4.31)	3.1-5.7
Starch	70.09 (68.80-71.07)	70.19 (67.80-71.50)	69.32 (68.60-70.36)	70.36 (69.07-71.40)	61-78
Fibre	2.95 (2.86-3.00)	2.97 (2.92-3.00)	2.93 (2.89-3.0)	2.91 (2.90-2.92))	2.5[3]

[1]Values presented as % dry weight. Values are means of 3 samples taken from 3 locations (ie 1 sample/location), ranges are given in brackets. Genetically modified corn lines are denoted CBR and are isogenic to their controls except for the presence of the novel genes.

Dataset 2:

A second nutritional study on Bt11 field maize that included additional hybrids was conducted. Three to five ears were picked from the center two rows of a four row strip plot for each hybrid per two sites within three geographical regions to give a total of six locations per hybrid. Two of the hybrids had a 'northern' (early-season) genetic background and two had a 'southern' (mid-late-season) genetic background. The hybrids were derived from separate backcross conversion processes using the same original transformation event.

Proximates

The compositional data for the Bt11 maize and control maize plants were analysed for significant differences by Analysis of Variance. The components measured were % protein, oil, starch and fibre (Table 5). Grain from the early season (northern hybrids) Bt11 maize hybrids (X4334CBR and X4734CBR) have a significantly lower protein content than grain from the control maize lines ($p<0.05$ and $p<0.01$), respectively). All other components were comparable between the Bt11 maize hybrids and their respective control maize lines.

Although the protein was lower in the northern hybrids, there is a lack of consistent differences between the non-modified hybrids and their genetically modified equivalents. These results may indicate that the effects observed, are not likely to be a result of the genetic modification itself but more likely from differences arising out of an incomplete backcross conversion in the normal breeding process. Values for all measured parameters fell within the literature ranges.

Table 5. Proximate composition for Bt11 and control maize (dataset 2) (ANZFA, 2000).

Northern / Early	X4334CBR	Control N4242	X4734CBR	Control N4640	Normal range[2]
Protein	8.65[3] (8.03-9.11)	9.25 (8.63-9.63)	8.19[4] (7.74-9.16)	8.96 (8.28-9.53)	6-12
Oil	3.17 (2.81-3.73)	3.23 (3.04-3.50)	3.34 (3.36-3.48)	3.30 (3.12-3.68)	3.1-5.7
Starch	72.93 (71.8-73.2)	72.57 (71.7-73.4)	72.73 (71.5-73.7)	72.62 (71.3-73.2)	61-78
Fibre	2.69 (2.66-2.83)	2.75 (2.67-2.93)	2.77 (2.68-2.83)	2.77 (2.69-2.83)	2.5[5]
Southern / Mid-late	**X6534CBR**	**X6514**	**X7634CBR**	**X7514**	
Protein	9.52 (8.35-10.60)	9.93 (9.10-11.40)	9.85 (8.63-11.00)	9.87 (8.67-10.94)	6-12
Oil	3.80 (3.63-4.16)	3.93 (3.27-4.13)	3.37 (2.59-4.00)	3.48 (2.70-4.31)	3.1-5.7
Starch	70.77 (68.8-72.5)	71.07 (67.8-72.7)	71.33 (68.6-74.3)	71.12 (69.1-73.9)	61-78
Fibre	2.78 (2.55-3.00)	2.80 (2.61-3.0)	2.74 (2.53-3.00)	2.72 (2.46-2.92)	2.5[5]

[1]Values presented as % dry weight. Values are means of a total of 6 samples taken from 2 sites in 3 locations (ie 2 distinct samples from each of the 3 locations), ranges are given in brackets.
[2]From Corn chemistry and technology, 1987, Watson SA and Ramstad PE (eds), American Association of Cereal Chemists, St. Paul, Minesota, USA.
[3]Values are significantly different to that of control value at 5% level of probability.
[4]Values are significantly different to that of control value at 1% level of probability.
[5]Average value

Fatty acids and amino acids

Fatty acid and amino acid analyses were performed on twenty grain samples obtained from Bt11 and non-transgenic control maize. Two separate analyses were performed. In the first analysis, variation between Bt11 maize and non-transgenic control maize were analysed to determine whether there were significant differences between Bt11 maize and the control maize. In the second analysis, only the replicates of Bt11 maize versus the genetically similar near isogenic non-transgenic control maize were analysed.

Amino acids

Amino acid analysis was performed on twenty grain samples from Bt11 maize (6534CBR and 4734CBR) and non-transgenic control maize (N6800, N4640, N4242, N5220, N5666, N6223, N6822, N7070 and N7590). Significant difference between Bt11 maize and non-transgenic control maize were found for cysteine and arginine ($p < 0.05$). For cysteine Bt11 maize (4734CBR) was lowest in content at 0.170. The range among the maize plants was from 0.170 to 0.230. This difference is not consistent for all genetically modified maize hybrids and is consistent with the variability that is observed between lines. Some variability may arise as a result of incomplete backcrossing. This variation is not considered to be a result of the genetic modification nor is it biologically significant.

	X6534-CBR	N6800	X4734-CBR	N4640	N4242[4]	N5220[5]
Tryptophan	0.05-0.06	0.05-0.06	0.05[2]	0.05-0.06	0.05[2]	0.07
Aspartic Acid	0.61-0.67	0.60-0.66	0.54[2]	0.55-0.57	0.55[2]	0.64
Threonine	0.35-0.38	0.35-0.38	0.29-0.30	0.30-0.31	0.30-0.32	0.36
Serine	0.50-0.55	0.50-0.55	0.42-0.43	0.43-0.44	0.43-0.44	0.52
Glutamic Acid	1.54-1.72	1.55-1.79	1.17-1.25	1.22-1.30	1.30-1.32	1.63
Proline	0.77-0.88	0.83-0.91	0.68-0.70	0.63-0.68	0.61-0.66	0.84
Glycine	0.34-0.37	0.35[2]	0.29-0.30	0.31-0.33	0.32-0.34	0.36
Alanine	0.75-0.82	0.75-0.87	0.60-0.62	0.58-0.63	0.61-0.63	0.74
Cysteine[3]	0.21-0.22	0.22-0.23	0.17[2]	0.20-0.21	0.18-0.21	0.22
Valine	0.41-0.43	0.40-0.45	0.32-0.33	0.32-0.34	0.32-0.36	0.43
Methionine	0.19-0.21	0.19-0.22	0.17-0.20	0.20-0.23	0.19-0.21	0.24
Isoleucine	0.28-0.32	0.28-0.33	0.23-0.25	0.24-0.26	0.23-0.27	0.32
Leucine	1.23-1.37	1.23-1.45	0.93-0.98	0.96-0.98	0.92-1.01	1.32
Tyrosine	0.14-0.18	0.14-0.16	0.13[2]	0.13-0.14	0.14[2]	0.17
Phenylalanine	0.44-0.49	0.44-0.51	0.37-0.39	0.36-0.40	0.35-0.38	0.50
Histidine	0.32-0.35	0.34-0.37	0.26-0.27	0.28-0.29	0.27-0.28	0.31
Lysine	0.25-0.26	0.24-0.26	0.23-0.24	0.24-0.25	0.23-0.25	0.27
Arginine[3]	0.36-0.37	0.37-0.38	0.31-0.32	0.32-0.34	0.33[2]	0.39

	N5866[5]	N6223[5]	N6822[5]	N7070[5]	N7590[5]	Range[6]
Tryptophan	0.06	0.07	0.06	0.06	0.08	
Aspartic Acid	0.58	0.68	0.59	0.71	0.67	
Threonine	0.34	0.38	0.34	0.40	0.39	0.32-0.34
Serine	0.47	0.55	0.45	0.53	0.56	
Glutamic Acid	1.54	1.83	1.53	1.61	1.83	
Proline	0.77	0.93	0.79	0.75	1.03	
Glycine	0.34	0.36	0.33	0.40	0.36	
Alanine	0.73	0.85	0.70	0.90	0.83	
Cysteine	0.22	0.22	0.20	0.21	0.23	
Valine	0.40	0.45	0.39	0.48	0.47	0.42-0.46
Methionine	0.23	0.26	0.24	0.27	0.34	0.18-0.19
Isoleucine	0.31	0.35	0.30	0.33	0.34	0.34-0.37
Leucine	1.24	1.46	1.20	1.28	1.47	0.10-0.11
Tyrosine	0.15	0.17	0.16	0.15	0.16	
Phenylalanine	0.47	0.54	0.46	0.46	0.54	0.44-0.45
Histidine	0.31	0.35	0.30	0.37	0.32	
Lysine	0.26	0.26	0.25	0.32	0.25	0.25-0.26
Arginine	0.36	0.40	0.36	0.36	0.38	0.41-0.52

[1]Values are ranges for three samples taken from 3 field sites (ie 1 sample/site) and are expressed as g/100g dry weight.
[2]The same value was obtained for all three samples.
[3]Values for genetically modified corn plants are significantly different to those of control corn plants.
[4]Range is obtained from two values
[5]Single value only.
[6]Data from L'alimentation des animaux monogastriques: porc, lapin, volailles. INRA 1989, Feedstuffs ingredient analysis table, edition 1996, AEC Table and 1995 UCAAB data.

Fatty acid

Fatty acid analyses were also performed on the grain sampled as described above. The grain was sampled from two locations, three samples per line from two Bt11 hybrid maize lines X6534CBR and X4734CBR and their genetically equivalent controls (N6800 and N4640 respectively). Additionally, grain from another seven non-modified reference hybrids was analysed (N4242, N5220, N5666, N6223, N6822, N7070 and N7590). As outlined above for the amino acid analysis, two separate statistical analyses were performed. The first analysed the variation between hybrids to determine whether there were significant differences

between them. The second study analysed differences specifically between genetically modified hybrids and their isogenic controls. The results are shown in Table 7. A statistical analysis to determine the variation between hybrids, as described above for the amino acid analysis, found no significant differences between the hybrids for fatty acid values ($p>0.05$).

A second statistical analysis of the fatty acid values investigated specifically differences between the genetically modified maize hybrid plants versus the non-modified control hybrids. Significant different between Bt11 maize and non-transgenic control maize were found for palmitic- and stearic acid. According to the applicant, Bt11 maize falls within the range of non-transgenic control maize for palmitic- and stearic acid content (Table 7).

Table 7. Fatty acid composition for Bt11 and control maize (ANZFA, 2000).

	Palmitic	Stearic	Oleic	Linoleic	Linolenic
X6534CBR	10.99-11.14	1.99-2.16	27.15-27.36	56.88-57.31	1.16-1.25
N6800	10.78-11.11	2.11-2.24	26.85-26.90	56.81-57.07	1.29-1.43
X4734CBR	10.76-10.97	2.38-2.41	25.93-26.04	57.62-57.86	1.61-1.67
N4640	10.61-10.65	2.45-2.52	26.31-27.06	56.69-57.59	1.56-1.59
N4242[2]	10.76-11.27	2.15-2.31	25.51-25.89	57.32-57.85	1.59-1.66
N5220[3]	13.14	1.89	26.55	55.13	1.40
N5866[3]	9.17	2.18	21.05	64.53	1.28
N6223[3]	11.53	2.01	26.58	57.04	1.24
N6822[3]	12.05	2.27	18.79	64.30	1.18
N7070[3]	10.11	1.77	25.49	59.77	1.19
N7590[3]	9.86	2.17	20.59	64.68	1.18
Range[4]	6–22	1–15	14–64	19–71 56–65[5]	0.5–2

[1]Values are ranges for three samples taken from 3 field sites (ie 1 sample/site) unless otherwise indicated and are expressed as % of fatty acid as a proportion of total fatty acid.
[2]Values are the range for two samples.
[3]Single values given only.
[4]From Weber, "Lipids of the kernel", Chapter 10 in Corn chemistry and technology, 1987, Watson SA and Ramstad PE (eds), American Association of Cereal Chemists, St. Paul, Minesota, USA. data
[5]Data from AGPM.

Dataset 3:

An analysis of the major components and nutritional qualities of elite Bt11 field maize lines has also been assessed. These lines are derived from the original transformant. Two genetically modified Bt11 hybrid maize lines and their near-isogenic controls were grown in three field locations in the USA in 1995.

Vitamins and minerals

One-pound (2.24 kg) samples of grain were taken from each of three locations from the two Bt 11 maize hybrids N4242-Bt and N4640-Bt and their corresponding near-isogenic non-modified hybrids and analysed for their vitamin and mineral content.The data were analysed to determine if there were significant difference between the Bt11 maize and the non-transgenic control maize. For each analysed parameter, means and standard deviation were calculated. Transgenic maize was compared to non-transgenic control by *t-test* ($p = 0.05$).

The grain was analysed for the minerals copper, magnesium, manganese and zinc as well as the vitamins folic acid, niacin, vitamin B1 and vitamin B2 (Table 8). No significant differences (p=0.05) between Bt-11 maize hybrids and their corresponding control hybrids were observed for any of the selected components.

Table 8. Vitamin and mineral composition for Bt11 and control maize (ANZFA, 2000).

	N4242Bt	Control N4242	N4640Bt	Control N4640
Copper	0.17 ± 0.06	0.17 ± 0.06	0.20 ± 0.0	0.20 ± 0.0
Magnesium	95.7 ± 1.15	91.7 ± 5.51	90.0 ± 1.73	86.3 ± 4.73
Manganese	0.47 ± 0.06	0.43 ± 0.06	0.40 ± 0.0	0.33 ± 0.06
Zinc	1.93 ± 0.06	2.03 ± 0.29	1.77 ± 0.12	1.70 ± 0.10
Folic acid	0.051 ± 0.010	0.045 ± 0.002	0.57 ± 0.03	0.57 ± 0.03
Niacin	8.62 ± 1.32	8.03 ± 0.14	8.96 ± 0.21	9.49 ± 0.41
B_1	1.44 ± 0.10	1.37 ± 0.21	1.26 ± 0.23	1.48 ± 0.15
B_2	0.71 ± 0.04	0.70 ± 0.09	0.72 ± 0.04	0.71 ± 0.02

[1]Values are means of 3 samples, one from each of 3 locations. Minerals are expressed as % and vitamins are expressed in mg/lb.

4.2.3 Study 3: Comparison of nutritional composition of fresh and canned Bt-11 sweet maize (North America 1996)

A fifth analysis of Bt-11 maize lines was done, specifically to assess the nutritional value of three Bt-11 sweet maize varieties.

Maize was harvested from the Bt-11 sweet maize hybrids, Bt 98- 0943, Bt 95-0937 and Bt 95-0941, and from their corresponding near-isogenic non-modified hybrids, grown in 1996 at one location in the North America. The field trials were designed following a random block design with three replicate plots of each genotype. Data for each genotype were subjected to analysis of variance. For each analyte the statistical significance of the genotype effect was determined using a standard F-test at the 5% probability. The significance of the location x genotype interaction was also assessed using an F-test. The results were compared to compositional analysis data for grain and forage published in the literature and in compositional analysis databases.

Ten ears of each of the hybrids were harvested at prime harvest and analysed as fresh maize on the cob. Maize from each hybrid was canned and also analysed (processed maize analysis). Fresh and canned sweet maize was analysed for moisture, protein, fat, ash, carbohydrates, fibre, vitamins and minerals (Table 9). Given that there was only duplicate analysis of one sample taken for each line, no statistical analysis was performed. Comparable nutritional composition was observed between Bt11 sweet maize (Bt 95-0943, Bt 95-0937 and Bt 95-0941) and corresponding non-transgenic hybrids (Jubilee, Bonus and Empire) for the selected compounds (Table 9). According to the applicant, the values obtained in the study were consistent with reported literature values.

Table 9. Compositional analysis for fresh and canned sweet Bt11 maize and control maize (ANZFA, 2000).

Fresh	Bt 95-0943	Jubilee	Bt 95-0937	Bonus	Bt 95-0941	Empire
Moisture (g)	69.88 – 69.78	69.67-69.70	73.65	72.20-72.24	71.15-71.28	70.34-70.56
Protein (g)	3.7-4.09	3.20-4.35	3.75-3.37	3.89-4.06	3.75-3.83	4.17-4.26
Fat (g)	0.76-1.34	1.10-0.97	0.75-0.91	0.81-0.88	0.85-1.18	0.91-1.13
Ash (g)	0.90-0.93	0.91	0.99-1.05	1.00-1.03	1.01-1.02	0.91-0.95
Carbohydrates - total[2] (g)	24.28	24.63	20.94	22.06	22.89	23.36
Calories[2]	111	112	93	100	105	110
Calories[2] from fat	10	9	7	7	10	9
Sugars[2] (g)	6.8	6.31	4.14	4.38	5.21	4.86
Other Carbohydrates[2] (g)	14.71	15.59	13.77	15.01	14.81	16.04
Total Dietary Fibre (g)	2.83-2.71	2.93-2.54	2.61-3.44	2.64-2.70	2.36-3.38	2.38-2.54
Vitamin A[2] (IU)	230	137	280	211	95.8	160
Vitamin C[2] (mg)	0.869	1.63	7.35	6.53	7.25	7.69
Sodium (mg)	9.9-14.2	5.9-7.2	10.0-13.0	3.9-5.3	5.8-7.2	4.9-8.6
Potassium (mg)	293.5-286.2	326.0-322.6	287.6-307.4	292.6-306.7	372.7-391.8	255.6-322.9
Calcium (mg)	3.4-8.6	1.6	0.7-7.1	0.0-0.4	7.1-8.0	0.7-7.1
Iron (mg)	0.49-0.85	0.49-0.56	0.57-0.61	0.6-0.90	0.54-0.63	0.71-0.74
Canned	**Bt 95-0943**	**Jubilee**	**Bt 95-0937**	**Bonus**	**Bt 95-0941**	**Empire**
Moisture (g)	77.81 – 77.83	76.81-76.85	77.66-77.76	77.77-77.80	76.44-76.52	77.80-77.96
Protein (g)	2.95-2.99	2.62-2.97	2.95-3.00	3.09-3.18	2.85-2.94	2.93-3.02
Fat (g)	0.85-1.77	1.02-1.90	1.01-1.09	0.68-0.75	0.83-0.96	0.62-0.85
Ash (g)	0.97-1.01	1.01	0.84-0.85	0.85-0.87	0.85-0.87	0.83-0.83
Carbohydrates - total[2] (g)	16.91	17.92	17.42	17.5	18.87	17.59
Calories[2]	83	87	81	79	86	79
Calories[2] from fat	12	13	9	6	8	7
Sugars[2] (g)	1.8	1.92	1.54	1.3	1.89	1.53
Other Carbohydrates[2] (g)	12.99	13.85	13.38	13.72	14.65	13.56
Total Dietary Fibre (g)	1.99-2.23	2.01-2.29	2.47-2.55	2.41-2.54	2.19-2.48	2.18-2.82
Vitamin A[2] (IU)	175	209	192	185	175	206
Vitamin C[2] (mg)	2.07	2.32	2.25	2.31	2.15	1.99
Sodium (mg)	262.8-285.0	266.1-304.1	245.9-248.0	212.5-230.2	225.7-239.6	191.9-235.6
Potassium (mg)	199.9-202.8	212.2-262.4	210.3-228.4	191.4-202.6	181.1-205.3	176.3-200.2
Calcium (mg)	3.1-8.8	2.4-4.2	0.0-1.8	5.1-8.2	3.7-10.2	5.2-8.2
Iron (mg)	0.29-0.55	0.289-0.614	0.31-0.25	0.23-0.34	0.348-0.387	0.31-0.37

[1]Values are expressed per 100 g serving basis.
[2]Only one sample determined.

4.2.4 Study 4: Analysis of Bt11 field maize and sweet maize grown in North America (1998)

Compositional analyses of three Bt11 maize hybrids and non-transgenic control maize, grown in one location, were analysed. An *F test* was conducted to see whether the values obtained for the Bt11 sweet maize were statistically significantly different from the values obtained for the isogenic hybrids, taking the various hybrids as "repetitions" (P = 5%). The values obtained were compared to those listed for maize grain in the *NOTIS plus* database, when available (Table 11). The following traits were considered: furfural, *p*-coumaric acid, ferulic acid, *myo*-inositol and raffinose.

Secondary metabolites

There are no generally recognised anti-nutrients in maize at levels considered to be harmful, but for the purposes of assessment of substantial equivalence, the OECD has asked for analytical data for the following secondary metabolites in maize: ferulic acid, p-coumaric acid, furfural, inositol, phytic acid, raffinose and trypsin inhibitor.The study indicate that grains from Bt11 sweet maize are substantially equivalent to grain from non-transgenic maize, by showing that the levels of furfural, *p*-coumaric acid, ferulic acid, *myo*-inositol and raffinose are similar in both types of maize (Table 10). The values were not significantly different at P = 5%, for any of the parameters measured. One striking difference observed are the higher amount of myo-inositol in one hybrid pair (GS0975 2330 and the corresponding isogenic GSS9377 322), compared to the other hybrids. Phytic acid and trypsin inhibitor were not analysed.

Table 10. Secondary metabolites composition for Bt11 sweet maize and control maize

Hybrid	furfural (ppm*)	*p*-coumaric acid (ppm*)	ferulic acid (ppm*)	*myo*-inositol (ppm*)	raffinose (ppm*)
GH0937 2132	11	11800	940	21	280
GS0975 2330	12	10900	860	79	260
GH0913 2117	12	7900	650	23	230
mean ± sd	**11.7 ± 0.5**	**10200 ± 1667**	**817 ± 122**	**41 ± 27**	**257 ± 21**
Bonus 2133	22	10800	840	31	150
GSS9377 322	8	11900	720	72	390
Sprint 2118	3	11100	730	22	480
mean ± sd	**11.0 ± 8.0**	**11267 ± 464**	**763 ± 54**	**42 ± 22**	**340 ± 139**

*: mean of the three replicate analyses

The values obtained in Table 10 were compared to those listed for maize grain in the NOTIS plus database, when available. The comparison is shown in Table 11. Significant differences were observed. According to the applicant, these differences are most likely due to the fact that the values reported in the NOTIS plus database are for "field maize", whereas the analyses reported here were conducted on sweet maize. Sweet maize has a different biochemical composition than field maize, the major difference being that energy is stored in the endosperm of the sweet maize grain in the form of various "sugars", whereas field maize accumulates starch. According to the applicant, differences are not unexpected. No literature values for these compounds of sweet maize were found.

Table 11. Comparison of the values measured in maize samples compared to the values in the NOTIS database

Compound	NOTIS database (ppm)			Novartis Seeds' results (mean, ppm)	
	mean	minimum	maximum	Bt-maize	isogenic maize
p-coumaric acid	167.3	18.9	222.9	10200	11267
ferulic acid	294.1	241.4	380.5	817	763
raffinose	11733.3	2100.0	30000.0	257	340

4.2.5 Study 5: Analysis of Bt11 field maize lines treated with herbicide in Europe (1998)

An additional study was done to assess the potential effects of herbicide treatment on the major components of the maize grain. Three Bt-11 hybrids representing different maturity types (Madera, Manuel and Magister) and their isogenic controls were grown in open fields at two locations in France in 1998.

An *F test* was conducted to see whether the values obtained for the "treated Bt11 hybrids", the "untreated Bt11 hybrids" and the "non-modified control hybrids" were statistically significantly different at $p<0.05$, taking the various hybrids as "repetitions". The evaluation

of the data was done by comparing the values obtained for the “treated Bt11 hybrids” to those obtained for the “untreated Bt11 hybrids” and for the “control hybrids”.
Proximate analysis (carbohydrate, protein, fat and fibre), fatty acids and amino acid composition were compared between transgenic crops treated with a glufosinate ammonium herbicide (Liberty®) at a rate of 2.25 L/ha active ingredient at the 3 and 6–7 leaf stages and untreated transgenic and isogenic controls (Table 12). Values presented in this experiment are not directly comparable to values for other experiments because they have been performed by a different laboratory using slightly different methods.

Proximates

No significant differences in the content of proximates were observed between the different groups of hybrids, for none of the parameters measured (Table 12).

Table 12. Proximate composition for Bt11 and1 maize and control (treated or untreated with glufosinat ammonium) (ANZFA, 2000).

	Treated	Untreated	Control
Energy	1441 ± 37	1430 ± 35	1433 ± 29
Carbohydrate	70.0 ± 2.0	69.5 ± 1.5	68.8 ± 1.5
Protein	7.6 ± 0.9	8.2 ± 0.8	8.4 ± 0.8
Fat	3.3 ± 0.6	3.0 ± 0.6	3.3 ± 0.8
Fibre	8.0 ± 1.0	8.0 ± 0.8	7.7 ± 0.2

[1]Values are means of 3 samples, one from each of the hybrids Madera, Manuel and Magister. Values are all expressed as a % except for energy (KJ/100g). *(ii)*

Amino acids

Bt11 hybrids, untreated or treated with glufosinate ammonium, as well as control hybrids gave comparable results, with the exception of proline and alanine (Table 13). When comparing the three “treatments” using an *F test*, the values obtained for glutamic acid, proline, alanine, isoleucine, leucine and phenylalanine were statistically different at P = 5%. However, when comparing only the “treated Bt11 hybrids” to the “control hybrids”, the values for glutamic acid, isoleucine, leucine and phenylalanine were not statistically significantly different. According to the applicant, concerning proline and alanine, the differences observed are unlikely to be due to the genetic modification itself in interaction with the glufosinate ammonium treatment, as not all three hybrids showed this. The difference between the treated modified and non-modified line was not consistent for all lines and may be a result of variability between the lines. This difference is not considered to raise safety or nutritional concerns.

The values for proline and alanine for each of the three hybrids is shown in detail in Table 14. The difference between the treated modified and non-modified line was not consistent for all lines and may be a result of variability between the lines. This difference is not considered to raise safety or nutritional concerns. The levels of glutamine and tryptophan were not determined. Tryptophan is one of the essential amino acids and one of the limiting amino acids in maize.

Table 13. Amino acid composition for Bt11 and control maize (treated or untreated with glufosinate-ammonium) (ANZFA, 2000).

	Treated	Untreated	Control
Aspartic Acid	4690 ± 406	5033 ± 439	4703 ± 142
Threonine	2690 ± 423	2850 ± 165	2690 ± 423
Serine	3537 ± 353	3750 ± 260	3537 ± 353
Glutamic Acid	14533 ± 1595	16233 ± 1626	15700 ± 625
Proline	6967 ± 1154	8367 ± 234	8590 ± 769
Glycine	3047 ± 238	3187 ± 111	2920 ± 26
Alanine	5057 ± 415	5760 ± 606	5500 ± 207
Valine	2963 ± 552	3327 ± 654	3210 ± 183
Methionine	1030 ± 183	1270 ± 122	1170 ± 30
Isoleucine	1717 ± 315	2320 ± 368	2013 ± 42
Leucine	8153 ± 918	9320 ± 1105	8787 ± 420
Tyrosine	3800 ± 573	4240 ± 455	3957 ± 172
Phenylalanine	3163 ± 440	3540 ± 243	3363 ± 280
Histidine	1867 ± 376	2147 ± 170	1853 ± 169
Lysine	1967 ± 228	2223 ± 228	1967 ± 163
Arginine	3257 ± 319	3443 ± 119	3160 ± 236

[1]Values are means of 3 samples, one from each of a different maturity type. Values are all expressed as mg/kg.
[2]Data from L'alimentation des animaux monogastriques: porc, lapin, volailles. INRA 1989, Feedstuffs ingredient analysis table, edition 1996, AEC Table and 1995 UCAAB data.

Table 14. Significant differences in amino acid composition between treated Bt11 and control maize (ANZFA, 2000).

Hybrid	Proline (mg/kg)		Alanine (mg/kg)	
	Bt11 hybrid[2]	Control hybrid	Bt11 hybrid[2]	Control hybrid
Madera	5640	7730	4720	5330
Manuel	7520	9210	5520	5730
Magister	7740	8830	4930	5440

[1]Values are all expressed as mg/kg.

Fatty acids

The results of the determination of palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid are presented in Table 15. The values obtained for the "treated Bt11 hybrids", the "untreated Bt11 maize" and the "non-modified control maize" was comparable. An *F test* confirmed the lack of significant differences at P = 5%.

Table 15. Fatty acid composition for Bt11 and control maize (treated or untreated with glufosinate- ammonium) (ANZFA, 2000).

	Treated	Untreated	Control
Palmitic	12.4 ± 1.9	12.3 ± 1.2	11.2 ± 1.2
Stearic	2.3 ± 0.2	2.4 ± 0.3	2.2 ± 0.2
Oleic	28.0 ± 1.9	27.4 ± 2.0	27.2 ± 1.3
Linoleic	55.1 ± 2.7	55.8 ± 3.0	57.0 ± 2.3
Linolenic	1.1 ± 0.2	1.1 ± 0.2	1.2 ± 0.2

[1]Values expressed as a % of total fatty acids. Values are means of 3 samples, one from each of the hybrids Madera, Manuel and Magister.

4.2.6 Determination of DIMBOA levels in Bt11 maize (field maize and sweet maize)

DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) is a naturally occurring hydroxamic acid, a benzoxazinoid. DIMBOA is a powerful antibiotic. In maize, DIMBOA functions as natural defense against European corn borer larvae and other damaging pests. MBOA (on 6-methoxybenzoxazolin-3-one (MBOA) is a degradation product of DIMBOA and was analysed in Bt11 maize. Bt11 maize has been back-crossed into several field maize and sweet maize varieties through traditional breeding methods. Therefore, "Bt11 field maize" and "Bt11 sweet maize" represents the same transformation event. The report presents data on the level of MBOA in seedlings of 4 different Bt11 maize hybrids (N6800Bt, N4640Bt, N4242Bt and Attribute), compared to their (near) isogenic hybrids (N6800, N4640, N4242, Attribute non-modified control). One of the hybrid pair tested was a sweet maize variety. No statistically significant differences were found.

Considerable plant-to-plant variability was observed, as reflected by the large standard deviation. A t-test was conducted and indicates that the DIMBOA levels (as determined after conversion to MBOA) were not significantly different between the Bt11 maize plants and the isogenic controls, for any of the hybrid pairs analyzed. An F-test was also conducted, taking all data together and considering the "Bt11-trait" as a "treatment". No statistically significant difference was identified this way either.

No significant differences in the levels of DIMBOA were observed between the genetically modified Bt11 hybrids and their isogenic counterparts, which could be linked to the genetic modification itself.

4.3 Agronomic and phenotypic characters

According to the applicant, agronomic and phenotypic data on maize Bt11 and its non-transgenic counterpart were collected from field trials conducted in Spain, France, Italy and Portugal between 1994 and 2006, and in the USA and Canada in 1994. Appendix 4 of the technical dossier, however, contains only a draft summary of the results obtained in the USA and a visual comparison of Bt11 to its non-modified isogenic hybrid in France. Data on the mentioned field trials in Spain, France, Italy and Portugal are not presented in the application EFSA-GMO-RX-Bt11.

North America 1994

Four generations of backcrossing (to BC_4) supplemented by selection, were used to transfer the chromosome fragment containing Btk coding sequence into the genetic background of elite maize inbred lines. A series of three independent conversions of the inbred 2043 were derived through parallel backcrossing schemes. Likewise, two independent conversions of the inbred 2044, were used to obtain a Btk version of the original 2044.These BC_4 derivates

were then subjected to extensive experimental analysis to assure that they represented adequate recoveries of the essential features of the original elite lines.
The two 2044 conversions, along with the original 2044, were crossed to a series of five, unrelated, elite inbred lines to produce F_1 hybrid seed for evaluation in replicated trials in 1994. Eighteen hybrids were produced for evaluation. Evaluation consisted of planting each hybrid at each of 17 locations which were distributed across eight US States and one Canadian providence. The trial design at each site was a randomized complete block design with two replicates. Each plot was represented by two rows (paired) of 20 feet in length, including three foot alleys. The plant population varied within the range of 25.000 to 27.000 plants per acre at each location. Notes and measures were taken on the plots during the growing season, as is usual for commercial variety trials. At harvest, a grain combine, which is adapted to harvest of maize plot trials, was used to determine plot yield and grain moisture at harvest.

Data were collected for the following agronomic and phenotypic characteristics: yield, grain moisture, stalk lodging rating, root lodging rating, ear height, plant height, heat units to silking, heat units to pollen shed and intactness rating (data not shown). The parameter “intactness rating” was, however, not specified and the method for its evaluation not indicated.Data was subjected to factorial analysis of variance using the SAS GLM Procedure. Contrast was used to test the significance of the difference between the original, non-transformed, 2044 line effects and those of the transformed derivatives. An identical process was used in testing the Btk conversions of the elite inbred 2043. Btk-converted lines were tested in combination with 6 elite inbreds and resulting hybrids were evaluated in 17 locations.

VKM considered the data presented for the USA/Canada trial inadequate for a complete evaluation, as information on the varieties assessed and their relationship (Bt hybrids and conventional control), use of the herbicides, pesticides and the applied fertilizer regime is missing. Furthermore, information is missing on environmental conditions during trials. A complete breeding history of the maize varieties analysed in the trials should be provided as well as a complete description of the agricultural management applied in each location. In particular, as this GMO is tolerant to glufosinate-ammonium, a herbicide-treated and a herbicide untreated variant must be included and potential differences assessed, which are due to the different treatments.

Apart from the incomplete presentation of material and methods used in the USA/Canadian field trials in 1994, only a summary of results is contained in the submitted study. No raw data are presented or data on a per location basis.

According to the applicant, statistically significant differences were observed between the elite line 2044 and the Bt11 conversions for the parameters stalk lodging ($p<0.001$), heat units to silking and pollen shed ($p<0.05$) and intactness rating ($p<0.01$) (data not shown). Similarly for 2043 and its derivates, differences were observed for moisture ($p<0.05$), stalk lodging ($p<0.001$) and intactness rating ($p<0.001$). The parameters stalk lodging rate and

intactness rating (general performance) are expected to be affected by maize borer damage, and thus the results are as expected. According to the applicant, the significant differences between the test line and control for heat units to silking and pollen shed and moisture at harvest are related to different genetic backgrounds and different maturities of the elite lines.

France 1995

During the field trial in one growing season in France, visual observations were taken on maize Bt11 (H8540 Bt11) and the non-transgenic counterpart (near-isogenic line H8540). The following agronomic and phenotypic parameters were assessed: anthocyanin coloration at the level of the ear, tassel, leaf, internodes and glumes; plant and tassel length, grain type, resistance to pests and diseases, number of primary lateral branches, height of insertion of ears, length of peduncle, shape/length of ears and number of rows of grain.

According to the applicant, the data from the field in France confirmed the equivalence of Bt11 maize phenotype to its non-transgenic counterpart. Furthermore, no differences in the agronomic and phenotypic characteristics were found between the Bt11 maize and the non-transgenic counterpart during field trials at different locations in Spain, France, Italy and Portugal conducted between 1994 and 2006 that would indicate unexpected pleiotropic effects of the genetic modification.

The data presented for the 1995 France trial are however, insufficient for an assessment of agronomic behavior of maize Bt11. Appendix 4 contains only an overview over the parameters assessed and, visual comparison of Bt11 to its near-isogenic hybrid. No data from the analyses of phenotypic characters from these field trials is provided in the application. Further, no information is contained on the location of the sites, the methods applied to assess the indicated parameters, the agronomic management conditions (herbicide, pesticide, fertilizer regime), the environmental conditions, the field trial design (replication, plot size etc) and the breeding history of the GM and non-GM varieties used.

Data on the mentioned field trials in Spain, France, Italy and Portugal are not presented in the dossier. Appendix 4 contains only a draft summary on results obtained in the USA and a visual comparison of Bt11 to its non-modified isogenic hybrid in France.
Due to the insufficient data provided by the notifier, no conclusions can be made on the agronomic behavior and characteristics of the GM maize Bt11 as well as the related phenotypic characteristics such as reproduction, dissemination and survivability.

4.4 Effect of processing

Food manufacturing of Bt11 field maize includes many harsh processing steps, e.g. cooking, heating, high pressures, pH treatments, physical shearing, extrusion at high temperatures etc. under which the majority of proteins are denatured, which also applies to CRY1Ab1 and PAT proteins (Hammond et al. 2011).

Bt11 sweet maize is able to consumers as fresh vegetable (maize on the cob) to be eaten as steamed cooked, or roasted on a barbecue. Sweet maize is also processed for freezing, canning and dehydrated powder. The canning process includes a cooking/sterilisation process at or above 110 ^{O}C, and duration of approximately 10 minutes. The freezing process includes a short bleaching process. Generally, the grain are exposed to steam (100 ^{O}C) from a few second to one minute.

4.5 Conclusion

Comparative analyses of data from field trials located at representative sites and environments in North America and Europe indicate that maize Bt11 is compositionally equivalent to its conventional counterpart, with the exception of the herbicide tolerance and insect resistance traits, conferred by the expression of the PAT and Cry1Ab proteins. However, data on the amino acid tryptophan, is only given in one out of six studies. Based on current knowledge, VKM concludes that maize Bt11 is compositionally equivalent to conventional maize.

The data provided by the applicant are not sufficient to show that Bt11 maize is phenotypically and agronomically equivalent to conventional near-isogenic maize lines. The agronomic assessment data are provided from one growing season in the North America and one growing season in France. This is not considered to be sufficient for representative testing of agricultural environments according to EFSA guidelines.

5 Food and feed safety assessment

5.1 Product description and intended use

The genetic modification in Bt11 field maize will not impact the existing production processes used for maize. All Bt11 maize products will be produced and processed for use in food, animal feed and industrial products in the same way as other commercial maize. The Bt11 field maize and all food, feed and processed products derived from Bt11 field maize are expected to replace a portion of similar products from commercial maize, with total consumption of maize products remaining unchanged. The total anticipated intake/extent of use of maize and all food, feed and processed products derived from maize will remain the same.

The Bt11 sweet maize is grown exclusive (100 %) for human consumption. By-products from processing are used as animal feeds. Sweet maize to be used as fresh vegetable is harvested at an immature stage, around 24 % dry matter. According to Syngenta about 12 million hectares Bt11 sweet maize were planted in USA in 2012 (information on Syngenta web-page).

5.2 Toxicological assessment

The potential toxicity of Bt11 maize expressing the Cry1Ab and PAT protein has been assessed in studies within rodents, broilers, pigs and cattle.

5.2.1 Toxicological assessment of the newly expressed protein(s)

The *cry1Ab* gene was originally obtained from *Bacillus thuringiensis*. There is no evidence of Cry1Ab having harmful effects on the health of humans or animals (EPA 1995a; McClintock et al. 1995; EPA 1996). The Cry1Ab protein has a very specific mode of action and selective toxicity against certain lepidopteran and coleopteran insect pests (target organisms).

Test for possible binding of Cry1Ab to proteins of bovine intestinal cells

Shimada et al. (2006b) performed immunohistochemical and ligand blot analysis to investigate the interaction of Cry1Ab protein with bovine intestinal epithelium. Shimada et al. (2006) found that Cry1Ab binds to actin, but not to aminopeptidase N, cadherin, or alkaline phosphatase, all which are cell membrane receptors or candidate proteins for development of toxicity in susceptible insects cells. The results indicate that Cry1Ab toxin is able to bind to cytoskeletal actin, but that the bovine intestinal epithelial cells lack membrane receptors, which are necessary for the toxin to exert its toxicity on cells.

Test for possible binding Cry1Ab to mammalian cellular brush-border membrane vesicle (BBMV)

Shimada et al. (2006a) investigated the affinity of Cry1Ab protein, a lepidopteran-specific Cry1-type protein, to the cellular BBMV of two mammalian intestinal cells as well as the effect of the toxin on the membrane potential of three mammalian intestinal cells compared to its effects on the silkworm midgut cell. Shimada et al. (2006) found that Cry1Ab toxin did bind to the bovine and porcine BBMV, but far more weakly than it did to the silkworm midgut BBMV. Although the silkworm midgut cells developed severe membrane potential changes within 1 h following the toxin treatment at a final concentration of 2 µg/ml, no such membraneous changes were observed on the bovine, porcine, and human intestinal cells. The present *in vitro* results suggest that, although Cry1Ab protein may bind weakly or nonspecifically to certain BBMV components in the mammalian intestinal cell, it does not damage the cell's membrane integrity, thus exerting no subsequent adverse effects on the cell.

Test for possible toxic potential of Cry1Ab on porcine culture intestinal cells

Bondzio et al. (2013) performed *in vitro* testing on porcine culture intestinal cells (IPEC-J_2) with Cry1Ab protein. For comprehensive risk assessment, they used WST-1 conversion and ATP content as metabolic markers for proliferation, lactate dehydrogenase release as indicator for cells with compromised membrane and transepithelial electrical resistance as parameter indicating membrane barrier function. The results were compared to the effects of valinomycin, a potassium ionophore, known to induce cytotoxic effects in most mammalian cell types. Whereas no toxicity was observed after Cry1Ab treatment, valinomycin induced a decrease in IPEC-J2 viability. Two dimensional differential in-gel electrophoresis was performed on the cell proteins. Only three proteins were differentially expressed. The functions of these proteins were associated with responses to stress. The up-regulation of heat shock protein Hsp70 was verified by Western blotting as well as by enzyme-linked immunosorbent assay and may be related to a protective function.

The present study shows that cultured porcine gastrointestinal cells can tolerate Cry1Ab even in a dose range that greatly exceeds any amount that may accumulate in the gastrointestinal tract of pigs. No influence on viability of IPEC-J2 cells was found using a screening with different assays including real-time monitoring of cell viability (Bondzio et al 2013). Consistent with a previously published studies in fish fed maize MON810 [Sagstad et al; 2007], the proteomic data at 24 h were indicative of a mild stress response to Cry1Ab.
According to the authors long-term investigations are needed to determine whether increased Hsp70 expression is only a transient short-term adaptive response to Cry1Ab or may be the cause of further unintended side effects of this protein.

Test for possible toxic potential of Cry1Ab on human culture kidney cells

Mesnage et al. (2012) performed *in vitro* testing on human culture kidney cells (HEK293) with Cry1Ab- and Cry1Ac protein. Time- and dose dependent effects of relatively high

concentrations of Cry1Ab on viability of HEK293 cells, respiration inhibition and plasma membrane alterations were detected. When treating the cells with 100 ppm of Cry1Ab the mitochondrial succinate dehydrogenase activity was decreased by 11%. Lower doses were tested from 10 ppb to 10 ppm, but significant effects were not observed. The adenylate kinase (AK) activity, when released in the medium, reveals possible membrane alterations. A Cry1Ab concentration of 100 ppm increased AK leakage in the medium 2-fold, revealing plasma membrane alterations. Lower doses were tested from 10 ppb to 10 ppm, but significant effects were not observed. Mesnage et al. argue that modified Cry proteins are not inert on non-target human cells, and that they can present combined side effects with other residues of pesticides specific to GM plants.

PAT

The *pat* gene was originally obtained from *Streptomyces viridochromogenes* strain Tü494 which has no known toxic or pathogenic potential. The PAT protein is enzymatically active but it has high substrate specificity to the active ingredient L-phosphinothricin (L-PPT) of glufosinate-ammonium. The PAT protein has already been found safe to human health during the assessment of glufosinate-ammonium tolerant maize (OECD, 1999).

5.2.2 Acute toxicity study

Acute oral exposure of PAT protein in rodents

Syngenta has rights for citing the acute oral toxicity PAT study on mice conducted by Monsanto. The acute oral study was conducted in compliance with EPA Guidelines no. 81-1, and with the following good laboratory standards: US Environ. Protection agency FIFRA: Good laboratory standards, 40 CFR 160 and 40 CFR 158, Japan Ministry of Agric., Forestry and Fisheries, Notf. No. 59 Noshan 3850, Director –General of Agricultural Production Bureau, August 1984 and OECD Principles of GLP, Annex 2, C(81)30.

PAT Microbial Protein (FL), which was 51% pure microbial protein, was evaluated for acute oral toxicity. Group I animals (five male and five female Harlan Sprague Dawley (HSD:ICR) mice received 5050 mg/kg (51 % pure) of the test material as a 26% w/v suspension in aqueous 2% carboxymethylcellulose (CMC), group II (5 males and 5 females) received heath inactivated PAT (52 % pure) and group III received 19.42 ml/kg bw doses of 2 % CMC only. Parameters evaluated during the two-week observation period included body weights and detailed clinical observation. All animals were examined for gross pathological changes. One male in Group I died during the study. Necropsy finding in the animal that died on test pertained to material logged in the esophagus. The identity of the material blocking the esophagus was not determined. There were no treatment-related clinical observations in the rest of the mice. There were no gross pathological lesions for any other animal in the study. Under the condition of this study, the acute oral LD_{50} of PAT Microbial protein in male and female HSD:ICR mice was greater than 2575 mg/kg bw.

Acute oral exposure of Cry1Ab protein in rodents

An acute oral toxicity study was performed with the Cry1Ab protein in mice. An acute oral study was considered appropriate since toxic proteins are only known to exert acute effects. The Cry1Ab protein was administered orally by gavage to three groups of ten male and female mice; additionally, one group of mice was dosed with a vehicle control lacking the Cry1Ab the protein. The targeted doses of Cry1Ab protein administered to mice were 0, 400, 1000, and 4000 mg/kg. A mice control group was dosed with Bovine serum albumin (BSA) at 4000 mg/kg. At the time of sacrifice, 7 days after dosing, there were no statistically significant differences in mortality, body weights, cumulative body weight or total food consumption between the BSA control groups and Cry1Ab protein-treated groups. Results from this study demonstrated that the Cry1Ab protein is not acutely toxic to mammals.

The acute oral toxicity test performed on mice did not indicate toxic effects of *E. coli* produced CryAb1 and PAT protein. However, acute tests do not provide enough information to conclude on possible adverse health effects of maize Bt11. In whole food the concentrations of the protein is low and acute toxic effects in humans and animals will most probably be negligible. Acute toxicity testing of the newly expressed protein is of little additional value to the risk assessment of the repeated human and animal consumption of food and feed derived from GM plants. EFSA discourages the use of acute studies in risk assessments of GMO (EFSA Journal 2011; 9(5):2150).

5.2.2.1 Repeated-dose 14- and 28-day oral toxicity study with the newly expressed proteins in rodents

14-day oral toxicity study of PAT protein in rats

Bayer Crop Sciences has performed a sub-chronic oral toxicity study of the PAT-protein in rats (Pfister et al., 1996). The study was performed in accordance with the principles of Good Laboratory of O.E.C.D. (Organization for Economic Cooperation and Development) and Principles of Good Laboratory Practice, 1992. Good Laboratory Practice (GLP) in Switzerland, Procedures and Principles, March 1986 and the Japanese Ministry of Agriculture, Forestry and Fisheries: On Good Laboratory Practice Standards for Toxicological Studies on Agricultural Chemicals, Agricultural Production Bureau, 59 NohSan Notification Number 3850, August 10, 1984. Test guidelines: The study procedures mostly conform to OECD Guidelines for Testing of Chemicals, number 407 "Repeated Dose 28-day Oral Toxicity Study in Rodents", adopted by the Council on July 27, 1995.

The study comprised four groups of five male and five female Wistar rats in each group. The rats in group 1 received a standard diet without PAT protein, whereas rats in group 2, 3 and 4 received diets with the inclusion of PAT and/or soybean protein: group 1 (standard diet), group 2 (0.5 % PAT + 4.5 % soybean), group 3 (5 % PAT), group 4 (5 % soybean), for a period of 14 days. According to the OECD guidelines the duration of exposure should normally be 28 days although a 14-day study may be appropriate under certain circumstances; justification for use of a 14-day exposure period should be provided. The duration of this

repeated dose oral toxicity study was 14-days. No justification for using 14-days has been found in the dossier from the applicant.

The mean intake of PAT-protein in group 2 over the treatment period was 712 mg/kg body weight/day for males and 703 mg/kg body weight/day for females. In group 3 the mean intake of PAT-protein was 7965 mg/kg body weight/day for males and 7619 mg/kg body weight/day for females.

The results showed no unscheduled deaths or clinical signs. Food consumption and body weights were unaffected by treatment. No treatment-related changes were seen in haematology or urinalysis parameters. Organ weight data, macroscopical and microscopical findings did not distinguish treated groups from controls.

The only changes which might be attributed to treatment were observed in clinical biochemistry parameters. They consisted of a slightly lower glucose level in males of group 4, slightly higher total cholesterol and phospholipid levels in males of groups 2, 3 and 4 and slightly higher triglyceride levels in females of group 4 when compared with rats of group 1. Animals of group 4 received no PAT-protein but - with respect to the protein content - a diet most similar to that of groups 2 and 3. The changes mentioned above were considered to reflect differences in the dietary composition and not related to the PAT protein itself. Further, comparing the increased total cholesterol and phospholipid levels between group 3 (5 % PAT) and group 4 (5 % soybean) they were found to be within similar range, which may suggest a similar nutritional value of the proteins.

28-day toxicity study in F344 male rats with impaired gastrointestinal function and gut damage, administered Cry1Ab protein

The study comprised four groups of eight male F344 rats in each group (Onose et al. 2008). In the control group rats were fed basal diet alone. The other rats were divided a Bt –group fed 10 ppm Cry1Ab-protein in feed, a GI-group with impaired gastrointestinal function and gut damage induced by treatment with famotidine and indomethacin and a GI + Bt-group. During the experimental period, general condition was checked daily, and body weights and food consumption were recorded every week.

The concentration for the Bt dosage was selected based on the content of the Cry1Ab-protein in Bt maize (Betz et al. 2000), assuming that all the food consumed by the animals was Bt maize.

At the end of the experiment, all animals were fasted overnight, anesthetized with ether and euthanized by exsanguination. Blood samples were collected from the abdominal aorta. The blood was applied for hematology and serum biochemistry. The red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT) and white blood cell count (WBC) were determined with an automatic hematology

analyzer (K-4500, Sysmex, Hyogo, Japan). Differential leukocyte counts and the reticulocyte count were obtained with an automatic blood cell analyzer (Microx HEG-120A, Tateishi Electronic, Tokyo, Japan). For serum biochemistry, total protein (TP), albumin (Alb), albumin/globulin ratio (A/G), total bilirubine (T-Bil.), triglycerides (TG), total cholesterol (T-Cho), blood urea nitrogen (BUN), creatinine (CRE), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), cglutamyl transpeptidase (c-GTP), calcium (Ca), inorganic phosphorus (IP), sodium (Na), chloride (Cl) and potassium (K) were measured at the medical laboratory SRL, Inc. (Tokyo, Japan). The medical laboratory SRL, Inc. was certified by the College of American Pathologists.

All animals were subjected to a complete necropsy, and the brain, thymus, lungs, heart, spleen, liver, adrenals, kidneys and testes were resected and weighed. These organs and the following organs and tissues were fixed in 10% neutral buffered formalin and paraffin-embedded sections were cut and stained with hematoxylin and eosin (HE) for histopathological examination: skin, mammary gland, sternum with marrow, femur with marrow, submandibular and mesenteric lymph nodes, salivary glands, aorta, trachea, tongue, esophagus, stomach, small and large intestines, pancreas, urinary bladder, epididymides, seminal vesicles, prostate, bulbourethral gland, pituitary, thyroids, parathyroids, spinal cord, trigeminal nerve, sciatic nerve, nasal cavity, Harderian gland, eyes and thigh muscle.

The twenty-eight-day study in rats showed no adverse effects that can be attributed to diet containing Cry1Ab. The administration of diet containing Cry1Ab protein (10 ppm Cry1Ab) had no significant effect on any physiological or biochemical parameter, except a lower level of AST in the serum of animals fed such maize, when compared with control. However, no changes in organ weights or histo-pathological changes were observed in organs like heart, liver, and kidneys. Also, serum AST levels are usually elevated with tissue injury, but the interpretation of relatively small changes in AST in toxicology studies should be done carefully, since the range of variation of this parameter can be broad in healthy animals. The decrease of AST in this experiment, therefore, is not considered to be toxicologically significant.

5.2.3 Toxicological assessment of the whole plant

42-day feeding study on broiler chickens

The wholesomeness and safety of the Bt11 maize has been shown in a 42-day feeding study using broiler chickens. A poultry feeding study was conducted to confirm the nutritional equivalence of the Bt11 maize with its non-GM commercial maize equivalent (Brake et al. 2003). Broilers were fed over a 42-day period.

Four kinds of maize grain were used in this study:

(1) grain from the Bt-expressing field maize hybrid N7070Bt,
(2) grain from the N7070Bt hybrid that had been sprayed with Liberty brand herbicide (glufosinate) according to manufacturer's instructions (N7070Bt + Liberty),
(3) grain from standard N7070 (non-Bt isoline of N7070Bt) grain,
(4) grain from North Carolina grown the 2000 growing season (NC2000).

Poultry studies are considered to be very useful because they utilize a fast growing organism that can eat a high percentage of maize in the diet, thus, it is very sensitive to potentially toxic effects of dietary components (OECD 2003).

At day 41 of the experiment, there was a sudden and extreme increase in ambient temperature and humidity. The heat index exceeded 43°C during the afternoon, and birds began to die. All mortalities were weighed as quickly as possible, and on the morning of 42 day, the decision was made to terminate the growth performance portion of the experiment by weighing all live birds and total feed consumed. For completeness, BW of live and dead birds are presented; however, group differences were minor and unimportant. Access to feed was limited on 42 and 43 day of the experiment to allow broilers to lose body heat. After the ambient heat stress subsided, the broilers were returned to full feed for 44 day through 47 day of the experiment prior to processing.

The results of the study show that there were no significant differences in percentage survivors for birds that received the two transgenic maize diets and those that received the N7070 isoline and commercial NC2000 maize diets on an overall basis at any age.

However, there were significant differences due to sex, with males exhibiting higher mortality during the finisher phase (35 to 42 d) and cumulatively (0 to 42 day). This should be expected in extremely hot weather conditions as males are well known to be more susceptible to heat stress. There was a significant interaction of sex and maize source for the combined starter-grower periods (0 to 35 day) due to some erratic mortality during the grower period (21 to 35 day) that was not evifield on a cumulative basis.

According to the authors this interaction did not follow any logical or explainable pattern and probably represents chance occurrences.

The diets prepared with transgenic maize did not have any adverse effects on performance of broiler chickens when compared to diets prepared with nontransgenic (isogenic) control and commercial maize. Only minor differences were found due to maize source; performance was poorer for birds fed the commercial NC2000 maize diets.
According to the applicant this study shows that Bt11 maize is nutritionally equivalent to non-GM maize. These findings also provide further confirmation of the safety of the proteins Cry1Ab and PAT expressed in the Bt11 maize. The applicant concludes that the Bt11 maize is nutritionally equivalent to, and as safe as, non-GM commercial maize.

28-day feeding study in pigs

Ten castrated pigs weighing 42 kg (Large White/Duroc cross) were fed diets containing 60% Bt11 or non-Bt isoline maize grain for four weeks (Shimada et al. 2008). At the end of the experiment, the liver, spleen, kidney, heart, lung, lymph nodes, thymus, tonsils, stomach, duodenum, pancreas, jejunum, ileum, ileocecum, cecum, colon, rectum, and spinal cord were sampled after slaughter. These tissue samples were examined histopathologically. There were no significant differences in histopathological observation between Bt and control groups.

28-day feeding study in poultry

Ten one-week-old male White Leghorns were fed diets containing 61.22% of Bt11 or non-Bt isoline maize for four weeks (Shimada et al. 2008).

At the end of the experiment, the liver, spleen, kidney, heart, lung, thymus, thyroid, glandular stomach, gizzard, duodenum, jejunoileum, and spinal cord were sampled after slaughter. These tissue samples were examined histopathologically. No significant differences in histopathological observation were found between Bt and control groups.

Mice reproduction toxicity study over five generations.

A long term performance of multi-generations or life span was assessed using genetically modified (GM) insect-resistant Bt11 maize (Haryu et al. 2009). Diet containing 68% of GM Bt11 or non-Bt isoline with sufficient nutrient composition was fed to male and female ICR mice through 5 generations. The study was performed according to OECD test guideline408 Repeated dose 90-day oral toxicity study in rodents (OECD 1998), OECD test guideline 43 Guidance Document On Mammalian Reproductive Toxicity Testing and Assessment (OECD 2008) and OECD Environmental Health and Safety Publications: Considerations for the Safety Assessment of Animal Feedstuffs Derived from Genetically Modified Plants (OECD 2003).

Parent generation (F0) consisted of 31 females and 16 males. They were cross-bred at the age of 60-70 days. The rate of vaginal plug formation, delivery rate and period, number of fetuses, sex ratio of fetuses, weaning period and feed intake and individual body weight were recorded. F1 mice were bred in the same manner to generate the follow-up generations. One day before delivery of F4 fetuses, the pregnant mice were sacrificed to count the number of fetuses, determine possible abnormalities as well as size and weight of placenta and ovaries. The results of growth, mating, gestation, milking periods, reproduction and life span were not different between the GM Bt11 and non-Bt fed groups. The percentage of embryonic death, litter size, newborn sex ratio and body weight (21-60 days after birth) were not different between these groups. The life span of the third-generation mice did not differ over 1,072 days of observation. In addition, there was a tendency for a weight decrease among each group as the generations progressed, but there was no significant difference in performance among each group in each generation of mice. There was no relevant difference in growth, reproduction performance and life span between the GM and control groups. (Haryu et al., (2009).

Short term and multigenerational mouse studies.

A short term and a multigenerational study were performed on mice (Brake et al., 2004). *Bt* maize (Bt11 (38PO6)) was studied with mouse testes as a sensitive bio-monitor of potential toxic effects. Pregnant mice were fed a Bt maize (38PO6) or a non-transgenic (conventional maize 38PO5) diet during gestation and lactation.

Short term study.
Bt or conventional maize diets were fed to 20 randomly selected female mice (10 for each diet). Following breeding, gestation, and parturition, three male progeny of the same age (by day) were chosen at random for each of six time points, 8, 16, 26, 32, 63, and 87 days after birth. Time points were selected to show the various stages of cellular proliferation and differentiation that occur during the development of the testes and ongoing cycles of spermatogenesis. The relative percent of testicular cell populations reflects the kinetics of the developing testes.

After they were weaned, young male mice were maintained on the respective diets. At 8, 16, 26, 32, 63, and 87 days after birth, three male mice and an adult reference mouse were killed, the testes were surgically removed, and the percentage of germ cell populations was measured by flow cytometry. No differences between conventional and Bt maize-fed mice were seen at 87 days after birth as ongoing cycles of spermatogenesis ensued.
General health parameters of mice fed the Bt maize diet showed no differences in body weight through day 63 ($p < 0.1074$) as compared to those on the conventional maize diet suggesting that the Bt maize diet caused no negative impact on body growth. However, the 87 day old conventional diet mice were fatter. However, the 63 day old mice were the exact opposite (Bt maize diet weighed more), and this was attributed to individual eating behavior after adulthood is achieved. Average litter sizes in the two groups were similar (Bt = 7.2 ± 1.0, n = 9; conventional = 7.3 ± 1.0, n = 9).

Multigenerational studies.
Multigenerational studies were conducted in the same manner. Transgenic or conventional diets were fed to 16 randomly selected male and female mice (two of each sex and strain for each diet). Following breeding, gestation, parturition, and weaning, six female and three male 2nd generation progeny of each strain and diet were randomly selected for growth, development, and breeding to obtain the 3rd generation. Surplus mice from each generation were culled. When all 3rd generation mice were at least 6 weeks old, six C57Bl/6J females and three C3H/HeJ males were randomly chosen and bred to obtain 4th generation cross-bred progeny. Three male progeny of the same age (by day) were chosen at random for each of five time points. Time points and sampling followed the same procedure as the subchronic study, except that the extended adult time point at 87 days postpartum was deleted.

General Health Parameters. Mean body weights showed differences between conventional and transgenic maize-fed mice at 26 ($p < 0.0001$) and 63 ($p < 0.0100$) days (Figure 3B). At both time periods, the Bt maize-fed mice were heavier than the control mice. Mean body weight results suggest that ingestion of a Bt maize diet resulted in no multigenerational impacts on animal growth. Average litter sizes were comparable (Bt = 8.2 ± 1.9, n = 5; conventional = 6.6 ± 2.1, n = 5).

There were no apparent differences in percentages of testicular cell populations (haploid, diploid, and tetraploid) between the mice fed the Bt maize diet and those fed the conventional

diet. Because of the high rate of cell proliferation and extensive differentiation that makes testicular germ cells highly susceptible to some toxic agents, it was concluded that the Bt maize diet had no measurable or observable effect on fetal, postnatal, pubertal, or adult testicular development. If data from this study were extrapolated to humans, Bt maize is not harmful to human reproductive development.

Nakajima et al. (2007) characterised the IgE antibodies of human sera by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. For the ELISA assay, a soluble form of the Cry1Ab protein, purified from *E. coli* transformed with a DNA sequence from maize Bt11, was used as antigen. All tested sera from patients allergic to major food allergens did not contain IgE antibodies directed against Cry1Ab.

Nakasuji et al. (2008) monitored the presence of common enterobacterial genes, a ubiquitous plant chloroplast gene, maize intrinsic zein (*Ze1*) and recombinant *cry1Ab* gene in the gastrointestinal contents, peripheral blood mononuclear cells and visceral organs of mice and their progeny fed with maize Bt11. The enterobacterial and chloroplast genes were detected inconsistently in peripheral blood mononuclear cells, visceral organs, milk or liver of suckling mice, while *Ze1* and *cry1Ab* were not detected. These results suggest that feed-derived enterobacterial and plant DNAs were incompletely degraded in the gastrointestinal tract, and part of them were absorbed into organs or milk as a source of transfer into suckling mice, but the *cry1Ab* gene was not transferred.

14-day feeding study in high producing dairy cows

Three groups of 4 dairy cows were fed fresh chopped whole plant maize (ca. 22.7 kg of dry matter per animal and per day) of either Bt11 maize, another insect tolerant transgenic maize (Bt 176) and the non-transgenic, near isogenic counterpart of Event 176. Both Bt11 maize and Bt 176 have been modified with the Cry1Ab and PAT proteins. Bt 176 derived from plants contained intermediate levels, and plants from the Bt11 maize variety contained relatively high levels of Cry1Ab protein. While the aim of this study was to determine whether transfer of Cry1Ab and PAT to milk from cows fed transgenic maize would occur, it also provides information on animal performance. Milk production, feed intake, milk composition, and udder health were similar for all study groups. Cry1Ab and PAT proteins could not be detected in milk of cows fed the genetically modified maize lines.

Cry1Ab protein and DNA fragments of the *cry1Ab* gene in the gastrointestinal content of animals fed Bt11 maize

Chowdhury et al. (2003a) examined the presence of maize intrinsic and recombinant *cry1Ab* gene by PCR, and the Cry1Ab protein by immunological tests in the gastrointestinal contents of five genetically modified maize Bt11-fed and five non-genetically modified maize-fed pigs. Fragments of maize zein (242 bp), invertase (226 bp) and of ribulose-1,5-bisphosphate carboxylase/ oxygenase genes (1,028 bp) were detected in the gastrointestinal contents of both Bt11 and nongenetically modified maize-fed pigs. Fragments of recombinant cry1Ab gene

(110 bp and 437 bp) were detected in the gastrointestinal contents of the Bt11-fed pigs but not in the control pigs. Neither maize intrinsic nor cry1Ab gene fragments were detected in the peripheral blood by PCR. The gastrointestinal contents were positive for Cry1Ab protein by ELISA, immune chromatography, and immunoblot; however, these methods did not work for blood and precluded conclusions about any potential absorption of the protein. These results suggest that ingested maize DNA and Cry1Ab protein were not totally degraded in the gastrointestinal tract, as shown by their presence in a form detectable by PCR or immunological tests.

Chowdhury et al. (2003b) examined the fate of insecticidal Cry1Ab protein in the gastrointestinal (GI) contents and visceral organs of calves fed insect-resistant genetically modified maize Bt11. Twelve cross-breed (Japanese black x Holstein) calves were fed either Bt11 or non-genetically modified isoline maize for 90 days. Peripheral blood, rumen juice and feces were collected fortnightly, and GI contents and visceral organs were collected at slaughter at the end of the experiment. Samples were checked for Cry1Ab protein by immunological methods, and visceral organs were examined pathologically. Trace amounts of Cry1Ab protein were detected in the GI contents but not in the liver, spleen, kidney, muscle or mesenteric lymph nodes. No lesions were observed pathologically. Cry1Ab protein in the feces was degraded quickly at atmospheric temperature. These results suggested that only a trace amount of Cry1Ab protein survived passage through the GI tract but was not transferred to liver, spleen, kidney, lymph nodes or muscles.

Chowdhury et al (2004) examined the presence of maize intrinsic and recombinant cry1Ab genes in the gastrointestinal (GI) contents, peripheral blood mononuclear cells (PBMC), and visceral organs of calves fed genetically modified Bt11 maize during a subchronic 90-day performance study. Samples were collected from six Japanese Black/Holstein calves fed Bt11 maize and from six calves fed non-Bt maize. DNA-fragments of maize *zein (Ze1)*, *invertase*, *chloroplast*, and *cry1Ab* measured by PCR were detected inconsistently in the rumen fluid and rectal contents 5 and 18 h after feeding. The chloroplast DNA fragments of *ribulose-1,5-bisphosphate carboxylase/oxygenase* and tRNA were detected inconsistently in the PBMC, the visceral organs, and the longissimus muscle, while the *cry1Ab* gene was never detected in PBMC or in the visceral organs. These results suggest that feed-derived maize DNA was mostly degraded in the GI tract but that fragmented DNA was detectable in the GI contents as a possible source of transfer to calf tissues. These results also suggest that the recombinant *cry1Ab* genes were not transferred to the PBMC and tissues of calves fed Bt11 maize.

The applicant has provided several animal studies; some of these are taken into account in the evaluation. However, other studies are either performed with only dose or with too short exposure time.

90-day feeding study in calves

Twelve healthy four-month-old cross-breed calves (Japanese Black × Holstein) were fed 43.3% Bt11 or 43.3% non-genetically modified isoline maize grain as dry matter for 90 days,

according to the feeding experiment procedure for safety assessment of feeds recommended by the Ministry of Agriculture, Forestry and Fisheries of Japan (Shimada et al. 2006c).

At the end of the experiment, tissues from the liver, spleen, kidney, mesenteric lymph nodes, and musculus longissimus were sampled after slaughter. These tissue samples were examined histopathologically and measured for hematological and biochemical parameters, i.e. red blood cells, white blood cells, hematocrit, hemoglobin, aspartate aminotransferase, γ-glutamyltransferase, alkaline phosphatase, total-bilirubin, total protein, albumin, total cholesterol, triacylglycerol, blood urea nitrogen, creatinine, calcium, inorganic phosphorus, magnesium, glucose, sodium, potassium, and chlorine in peripheral blood and ruminal pH, volatile fatty acid, lactic acid, ammonia nitrogen, and free lipopolysaccharides in rumen juice.

No significant gross or histopathological lesions and no discernible clinical, hematological, biochemical, or ruminal abnormalities were found in calves fed Bt11 maize as compared with control calves fed non-Bt maize.

5.3 Allergenicity

Most food allergies are mediated by Immunoglobulin E (IgE, type-I reactions). The strategies used when assessing the potential allergenic risk focuses on the characterisation of the source of the recombinant protein, the potential of the newly expressed protein to induce sensitisation, or to elicit allergic reactions in already sensitised individuals and whether the transformation may have altered the allergenic properties of the modified food. A weight-of-evidence approach is recommended, taking into account all of the information obtained with various test methods, since no single experimental method yields decisive evidence for allergenicity (EFSA 2010a).

Most of the major food and respiratory IgE-allergens have been identified and cloned, and their protein sequences incorporated into various databases. As a result, novel proteins can be routinely screened for amino acid sequence homology and structural similarity to known human IgE-allergens using an array of bioinformatics tools. Sequence homology searches comparing the structure of novel proteins to known IgE-allergens in a database are conducted using various algorithms such as FASTA to predict overall structural similarities. According to FAO/WHO (2001) in cases where a novel protein and a known IgE-allergen have more than 35% identity over a segment of 80 or greater amino acids, IgE cross-reactivity between the novel protein and the allergen should be considered a possibility.

5.3.1 Assessment of allergenicity of the newly expressed protein

A weighted risk analysis based on the decision tree approach has been performed by the applicant. The individual steps of this analysis starts by analyzing the primary amino sequence of the novel protein and looking for similarity with sequences to known allergens, followed by specific or targeted serum screens for IgE cross-reactions to known allergens, digestibility

studies of the proteins in simulated gastric and/or intestinal fluids, and animal studies (FAO/WHO, 2001, Codex Alimentarius, 2003, König et al., 2004, Poulsen 2004).

These assessments have previously been described by the applicant for Cry1Ab and PAT, and were based on the following aspects:

Cry1Ab and PAT

i) The sources of the transgene genes: *B. thuringiensis* (*cry*-genes) and *S. viridochromogenes* (*pat*) have no history of causing IgE-mediated allergy
ii) History of safe use of Cry proteins as microbial pesticides (US EPA, 1998), no indications of Cry proteins originating from *Bacillus thuringiensis* having harmful effects on the health of humans and animals (US EPA, 1996).
iii) The PAT protein has been subjected to previous safety assessments for genetically modified plants and found to have no IgE-mediated allergenic potential
iv) The PAT protein have no homology to known toxins or allergenic proteins
v) The microbially produced Cry1Ab and PAT proteins were rapid degraded in simulated gastric fluids *in vitro*. No degradation assay in gastrointestinal fluids has been performed by the applicant.
vi) PAT and Cry1Ab does not resemble any characteristics of known IgE-allergens, and no significant homologies between the amino acid sequences of the PAT and Cry1Ab proteins and IgE-allergenic proteins have been found (Fard et al, 2013, Kim et al, 2010, Randhawa et al 2011, Meyer, 1999, US EPA, 2010).
vii) The PAT and Cry1Ab protein is not glycosylated (Raybold et al, 2013, US EPA, 2010)
viii) Cry1Ab and PAT are considered heat labile (US EPA 2010)

5.3.2 Assessment of allergenicity of the GM food or feed

Allergenicity of the maize Bt11 could be increased as an unintended effect of the random insertion of the transgene in the genome of the recipient, e.g. through qualitative or quantitative modifications of the expression of endogenous proteins. However, given that no biologically relevant agronomic or compositional changes have been identified in field maize Bt11 or sweet maize Bt11with the exception of the introduced traits, no increased allergenicity is anticipated for maizeBt11. Moreover, maize is not considered a common allergenic food.

It is the opinion of VKM that a possible over-expression of any endogenous protein, which is not known to be allergenic, in maize Bt11 would be unlikely to alter the overall allergenicity of the whole plant or the allergy risk for consumers.

5.3.3 Assessment of adjuvanticity

According to the EFSA Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed (EFSA ,2010a) adjuvants are substances that, when

co-administered with an antigen increase the immune response to the antigen and therefore might increase the allergic response. In cases when known functional aspects of the newly expressed protein or structural similarity to known strong adjuvants may indicate possible adjuvant activity, the possible role of these proteins as adjuvants should be considered. As for allergens, interactions with other constituents of the food matrix and/or processing may alter the structure and bioavailability of an adjuvant and thus modify its biological activity.

Only two of the ~ 10 Cry proteins that are currently used in genetically modified plants, Cry1Ab and Cry1Ac, have been studied experimentally regarding adjuvant effects. To the knowledge of VKM, adjuvant effects have not been investigated for the other Cry proteins normally used in GM plants, or other groups of Cry proteins.

Studies with immunological mapping of the systemic and mucosal immune responses to Cry1Ac have shown that mice produce both systemic IgM and IgG and secretory IgA following intraperitonal (i.p.), intragastric (i.g.) or intranasal (i.n.) immunisation, and that the adjuvant effects of Cry1Ac has been reported to be comparable to that of cholera toxin (CT) (Guerrero et al., 2004; Vazquez-Padron et al., 1999a, b; 2000; Moreno-Fierros et al., 2003). The adjuvant effect of CT is thus a relevant basis for comparison in a risk assessment of Cry1Ac. It is uncertain whether this applies to the same extent to other Cry proteins. A possible immunogenicity and adjuvanticity of Cry proteins has been considered by EFSA and VKM (EFSA, 2009, VKM, 2012).

“Bystander sensitisation”

"Bystander sensitisation” can occur when an adjuvant in food, or an immune response against a food antigen, results in an increased permeability of the intestinal epithelium for other components in food. Traditionally it was assumed that the epithelial cells of the intestine were permanently "glued together" by the so-called "tight junctions". Studies have however shown that these complex protein structures are dynamic and that they can be opened up by different stimuli.

Both *in vitro* and *in vivo* experiments have demonstrated that when an IgG response which can result in a complement activation (among other) is not balanced by an IgA response, the epithelial barrier can be opened and unwanted proteins are able to enter the body (bystander-penetration) and lead to allergic sensitisation (Brandtzaeg & Tolo 1977; Lim & Rowley 1982).

Additional information can be found in the report by VKM on Cry proteins and adjuvanticity: “Health risk assessment of the adjuvant effects of Cry proteins from genetically modified plants used in food and fodder” (VKM, 2012).

5.4 Nutritional assessment of the GM food and feed

Compositional analyses of maize Bt11 and sweet maize Bt11 indicate nutritional equivalence to the non-GM control maize with comparable genetic background and to the published range of values in the literature. The nutritional equivalence between Bt11 maize and non-GM control

maize has been further shown by the results of a poultry feeding study, feeding study in dairy cows, beef steers, pigs and calves.

Intake information/exposure assessment

Net import of maize staple, e.g. flour, starch and mixed products, in Norway in 2007 was 7600 tons, corresponding to 4.4 g dry weight/person/day or an estimated daily energy intake for adults to be 0.6 % (Vikse 2009). The estimated median daily intake of sweet maize is 3.25 g/day, with a 97,5 % percentile of 17.5 g/day. The production of maize porridge for children in 2007 was about 37.5 tons, corresponding to a daily intake of 1.7 g/day or an estimated daily energy intake to be 0.6 % for a 6 month child (Vikse, 2009). The comparable composition and nutritional value of the maize, together with the results of the assessment of dietary intake and nutritional impact, indicate that food products derived from Bt11 maize are nutritionally equivalent to food products derived from commercial maize. Hence, anticipated dietary intake is not expected to change.

Since most foods and foodstuffs from maize are derived from field maize grains, an estimated maximum daily intake for a Norwegian adult of Cry1Ab protein from maize staple is calculated to be 8.8 µg, based on grain dry weight, and the estimated maximum daily intake of Cry1Ab from sweet maize is calculated to be 67 µg, based on a daily intake of 17.5 g fresh sweet maize/day (97.5 % percentile) These levels are several orders of magnitude below the levels shown to have no effect in laboratory toxicology testing. Also, these levels are considerably below the proposed threshold of toxicological concern (TTC) level of 1800 µg/person/day (Class 1, oral exposure) for chemicals considered to have a low potential for toxicity based on metabolism and mechanistic data (Vermeire et al., 2010). Transgenic proteins produced by genetically modified plants are generally not considered toxic to humans. VKM notes that farm (production) animals e.g. pigs and poultry often are fed diets with a substantial inclusion of unprocessed maize grain, and that the exposure to transgenic proteins from maize Bt11 may be higher for these animals.

This dietary exposure assessment is very conservative as it assumes that all maize consumed comes from maize Bt11 and that the transgenic proteins are not denatured by processing.

5.4.1 Interpretation of relevance of animal studies

Utilization of Bt maize residue and maize silage for growing beef steers and dairy cows.

a) Utilization of Bt maize residues by grazing beef steers and Bt maize silage and grain by growing beef cattle and lactating dairy cows has been reported by Folmer et al. (2002). Sixteen lactating dairy cows received diets containing silage of an early- and late-maturing variety of Bt11 maize or a control with the corresponding non-transgenic near isogenic maize line during 21-day feeding periods. No differences were observed between Bt11 maize and control maize for feed intake, body weight, milk production, and milk composition (lactose, protein, fat), as

well as ruminal pH and volatile fatty acids. In addition no effects either were observed of the transgenic trait on in situ ruminal digestion of neutral detergent fibre of maize.

b) The same silages as those used for the dairy cow study were used in a beef cattle study which lasted for 101 days. Measurements included feed intake and body weight. Dry matter intake was significantly higher in steers fed early- and late-maturing Bt11 maize when compared with those fed diets containing non-GM silage. In addition, average daily weight gain in early maturing Bt11 maize-fed steers was higher than in control-fed steers, while final body weight and feed efficiency was decreased in steers fed late maturing Bt11 maize compared with steers fed control maize. In conclusion, the slightly higher dry matter intake was not associated with other effects on performance of beef cattle fed Bt11 maize that would be consistent for diets of both Bt11 maize lines. (see Appendix 8.4, technical dossier).

According to the applicant, the maize and derived feed products are substantially equivalent to, nutritionally equivalent to and as safe as commercial maize and derived feed products. This is based on the compositional analyses comprising proximates, minerals, fatty acids, amino acids, vitamins, secondary metabolites and anti-nutrients of forage and grain samples from; nutritional equivalence shown in a poultry feeding study; and, safety evaluation of the Cry1Ab and PAT proteins expressed in maize.

To the best of our knowledge, PubMed searches performed on November 28th 2016, and recently on April 27th, 2017 as well as additional searches in the EFSA journal and google scholar, among others, did not identify newly published information deemed relevant for human and animal health effects of Bt11, other than previously described in VKMs report published in 2014, herein presented.

5.5 Conclusion

Whole food feeding studies have not indicated any adverse health effects of maize Bt11. These studies also indicate that maize Bt11 is nutritionally equivalent to conventional maize. The Cry1Ab and PAT proteins do not show sequence resemblance to other known toxins or IgE allergens, nor have they been reported to cause IgE mediated allergic reactions. Some studies have however indicated a potential role of Cry-proteins as adjuvants in allergic reactions.

Based on current knowledge, the VKM concludes that maize Bt11 is nutritionally equivalent to conventional maize varieties. It is further unlikely that the Cry1Ab and PAT proteins will introduce a toxic or allergenic potential in food or feed based on maize Bt11 compared to conventional maize.

6 Maize Crop Production in Norway

There is no official agricultural statistics of the total crop area of maize in Norway. Most of the maize in Norway is grown for feed, where the whole plant is harvested for silage before grain ripening. Information from various seed companies indicates annual cropping areas of forage maize of about 2000-2800 decares (Netland et al., 2013). This is equivalent to less than 0.1% of the areas with cereal crops.

In the period 2010-2016, the area of sweet corn for human consumption varied between 84 and 709 decares (Statistics Norway, 2017). According to Debio, the Norwegian control body for organic crop production, there are no cropland under organic management certified for maize production in Norway (Lene Nilssen pers. com. 2017). So far, no maize areas are in the process of conversion to organic farming.

The maize crop production is mainly located in the southeastern Norway, with the largest areas located in the counties of Østfold and Vestfold. There is also some cultivation of fodder maize in Agder and Rogaland.

There is a growing interest in commercial cultivation of forage maize in Norway (Netland et al., 2013). Silage of maize is especially suitable for cattle, and yields of 800-1000 kg dry matter per decare provide a profitable production and an energy-rich and palatable feed supplement which can replace traditional forage and concentrates for livestock. Maize is not labor intensive production, and when the growth season is long enough, maize provides a digestible and nutritious feed that can increase the forage intake. However, if the growing season is too short, and the maize cobs do not get time to evolve, the feed unit concentration becomes very low (0.75 FEu/kg TS; http://www.grovfôrnett.no).

Results from Norwegian field trials demonstrate large differences with respect to yields and qualities of forage maize, both between experimental years and field sites. In a field study from Nord- Trøndelag, Nesheim (2008) reported high dry matter yields of forage maize when growing maize under a plastic film cover (1100 kg t.s. per decare). Other studies have, however, denoted maize crop production in Trøndelag and Rogaland with the current varieties as risky, also if intensive farming methods as establishing maize under plastic cover are adopted (Bakken et al. 2005). In this experiment, Bakken et al. tested a selection of early maturing varieties at different locations in the South and Middle- Norway. The authors concluded that even in the best agricultural areas in the Oslofjord region, maize production will imply risk of crop failure and yields of varying quality. These results are consistent with recently, unpublished studies (T. Lunnan pers. com., 2012).

In the traditional livestock districts in Norway, the growing season is too short, such that forage maize can be a real alternative to other forage productions (Netland et al., 2013). The major prerequisites for a significant increase in the maize cultivation are therefore a higher proportion of lifestock production in the southeastern Norway, improved varieties and technology that enables earlier sowing (Bakken et al., 2005; T. Lunnan, pers. com 2012).

Climate change, which entails a longer growing season and higher average temperatures, however, may expand the maize cultivation area in Norway in the long term.

7 Environmental risk assessment

7.1 Unintended effects on plant fitness due to the genetic modification

Cultivated maize (*Zea mays* L.) is a member of the grass family *Poaceae*. Maize is presumed to have derived from teosinte (*Z. mexicana),* a plant native to Central America, and was introduced into Europe in the sixteenth century.

Maize is a highly domesticated annual plant and generally unable to survive in the environment without management intervention (Eastham & Sweet, 2002). Maize propagates entirely by seed produced predominantly by cross-pollination (OECD, 2003). In contrast to weedy plants, maize has a pistillate inflorescence (ear) with a cob enclosed with husks. Due to the structure of the cob, the seeds remain on the cob after ripening and natural dissemination of the kernels rarely occurs. In Norway, practically all maize is grown for feed, where the whole plant is harvested for silage before grain ripening. There is only a very limited production of sweet maize for human consumption (see section 6.0).

Maize (*Zea mays* L.) is an annual plant and member of the grass family Poacea. The species, originating from Central America, is highly domesticated and generally unable to survive in the environment without management intervention (Eastham & Sweet, 2002). Maize propagates entirely by seed produced predominantly by cross-pollination (OECD, 2003). In contrast to weedy plants, maize has a pistillate inflorescence (ear) with a cob enclosed with husks. Due to the structure of the cob, the seeds remain on the cob after ripening and natural dissemination of the grain rarely occurs.

The survival of maize in Europe is limited by a combination of absence of a dormancy phase resulting in a short persistence, high temperature requirements for germination, low frost tolerance, low competitiveness and susceptibility to plant pathogens, herbivores and climatic conditions (van de Wiel et al., 2011). Maize plants cannot survive temperatures below 0ºC for more than 6 to 8 hours after the growing point is above ground (OECD, 2003), and in Norway and most of Europe, maize grain and seedlings do not survive the winter cold (Gruber et al. 2008). Observations made on cobs, cob fragments or isolated grains shed in the field during harvesting indicate that grains may survive and overwinter in some regions in Europe, resulting in volunteers in subsequent crops. The occurrence of maize volunteers has been reported in Spain and other European regions (e.g. Gruber et al., 2008). However, maize volunteers have been shown to grow weakly and flower synchronously with the maize crop (Palaudelmás et al. 2009). Cross-pollination values recorded were extremely variable among volunteers, most

probably due to the loss of hybrid vigour and uniformity. Overall cross-pollination to adjacent plants was estimated as being low.

Despite cultivation in many countries for centuries, seed-mediated establishment and survival of maize outside cultivation or on disturbed land in Europe is rare (BEETLE Report, 2009). Maize plants occasionally grow in uncultivated fields and by roadsides. However the species is incapable of sustained reproduction outside agricultural areas in Europe and is non-invasive of natural habitats (Eastham & Sweet, 2002; Devos et al., 2009). There are no native or introduced sexually cross-compatible species in the European flora with which maize can hybridise and form backcross progeny (Eastham & Sweet, 2002; OECD, 2003). The only recipient plants that can be cross-fertilised by maize are other cultivated maize cultivars.

It is very unlikely that the establishment, spread and survival of maize Bt11 is increased due to the insect resistance and herbicide tolerance traits. The insect protection against Lepidoptera is not regarded to provide a significant selective advantage to maize plants in Europe, except under high infestation conditions in cultivated fields. In Norway, there have been only a few reports of the target pests (section 7.3), and this trait cannot be regarded as a potential selective advantage to maize Bt11. Moreover, we consider it very unlikely that maize Bt11 plants and their progeny will differ from conventional maize varieties in their ability to survive as volunteers until subsequent seasons, or to establish feral populations under present European environmental conditions. The herbicide tolerant trait can only be regarded as providing a selective advantage for the GM maize plant where and when glufosinate ammonium-based herbicides are applied. Glufosinate ammonium- containing herbicides have been withdrawn from the Norwegian market since 2008, and the substance will be phased out in the EU in 2017 due to reproductive toxicity.

Due to the insufficient data provided by the notifier, no conclusions can be made on the agronomic behavior and characteristics of the GM maize Bt11 as well as the related phenotypic characteristics such as reproduction, dissemination and survivability.

There are, however, no indications of altered fitness of maize Bt11 relative to its conventional counterpart and VKM is not aware of any scientific reports indicative of increased establishment or spread of maize Bt11, or changes to its survivability (including over-wintering), persistence or invasive capacity. Because the general characteristics of maize Bt11 are unchanged, insect resistance, glufosinate tolerance are not likely to provide a selective advantage outside of cultivation in Europe. VKM is of the opinion that the likelihood of unintended environmental effects based on establishment and survival of maize Bt11 will not differ from that of conventional maize varieties.

7.2 Potential for gene transfer

A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either through horizontal gene transfer of DNA, or vertical gene flow via pollen or

seed dispersal. Exposure of microorganisms to transgenic DNA occurs during decomposition of plant material remaining in the field after harvest or comes from pollen deposited on cultivated areas or the field margins (Ryffel, 2014; Trtikova et al., 2015). Transgenic DNA is also a component of a variety of food and feed products derived from maize Bt11. This means that micro-organisms in the digestive tract in humans and animals (both domesticated animals and other animals feeding on fresh or decaying plant material from the transgenic maize line) may be exposed to transgenic DNA.

Maize is the only representative of the genus *Zea* in Europe, and there are no cross-compatible wild or weedy relatives outside cultivation with which maize can hybridise and form backcross progeny (Eastham & Sweet, 2002; OECD, 2003). Vertical gene transfer in maize therefore depends on cross-pollination with other conventional or organic maize varieties. All maize varieties that are cultivated in Europe can interbreed. In addition, unintended admixture/adventitious presences of genetically modified material/transgenes in seeds represent a possible way for gene flow between different production systems.

7.2.1 Plant to micro-organisms gene transfer

Experimental studies have shown that gene transfer from transgenic plants to bacteria rarely occurs under natural conditions and that such transfer depends on the presence of DNA sequence similarity between the DNA of the transgenic plant and the DNA of the bacterial recipient (Nielsen et al., 2000; De Vries & Wackernagel, 2002, reviewed in EFSA 2004, 2009a; Bensasson et al. 2004; VKM, 2005b).

Based on established scientific knowledge of the barriers for gene transfer between unrelated species and the experimental research on horizontal transfer of genetic material from plants to microorganisms, there is today little evidence pointing to a likelihood of random transfer of the transgenes present in maize Bt11 to unrelated species such as bacteria.

It is, however, pointed out that there are limitations in the methodology used in field scale studies of HGT (Nielsen & Townsend, 2004) and that experimental studies of limited scale should be interpreted with caution given the scale differences between experimental investigation and commercial plant cultivation.

Experiments have been performed to study the stability and uptake of DNA from the intestinal tract in mice after M13 DNA was administered orally. The DNA introduced was detected in stool samples up to seven hours after feeding. Small amounts (<0.1%) could be traced in the blood vessels for a period of maximum 24 hours, and M13 DNA was found in the liver and spleen for up to 24 hours (Schubbert et al., 1994). Oral intake of genetically modified soybean has shown that DNA is more stable in the intestine of persons with colostomy compared to a control group (Netherwood et al., 2004). No GM DNA was detected in the faeces from the control group. Rizzi et al. (2012) provides an extensive review of the fate of feed-derived DNA in the gastrointestinal system of mammals.

In conclusion, VKM considers it unlikely for the introduced genes in maize Bt11 to transfer and integrate with the genome of microorganisms in the environment or in the intestinal tract of humans or animals. In the rare, but theoretically possible case of transfer of the *cry1Ab* and *pat* genes from Bt11 to soil bacteria, no novel property would be introduced into or expressed in the soil microbial communities; as these genes are already present in other bacteria in soil. Therefore, no positive selective advantage that would not have been conferred by natural gene transfer between bacteria is expected.

7.2.2 Plant to plant gene flow

7.2.2.1 Reproduction biology

Maize is a tall, monoecious, annual grass with separate male and female flowers on the same plant. The functional staminate flowers are borne in male tassels located terminally on the stems, and the female cobs are borne in the axils of the middle leaves.

Maize is predominantly a protandrous and out-crossing species, where the male inflorescence appears around two to four days before silk emergence (Sleper & Poehlman, 2006). There is however usually some overlap of pollen shedding and silk emergence on the same plant that can account for up to 5 % self-pollination (Eastham & Sweet, 2002). Maize is predominantly wind-pollinated, although there is evidence to suggests that honeybees and other insects collect pollen from maize (Treu & Emberlin, 2000). However, the female flowers of maize produce no nectars and pollinating insects usually do not contribute to fertilisation and cross-pollination of maize plants (Eastham & Sweet, 2002; Malone & Burgess, 2009; OGTR, 2008; Tolstrup et al., 2003).

Pollen is released from the tassels in large quantities. It has been estimated that for each ovule developing into a kernel an individual plant delivers from 9000 to 50000 pollen grains. Assuming an average ear of maize grows approximately 500 kernels, a plant will yield between 4.5-25 million pollen grains (Eastman & Sweet 2002). Compared to pollen of other wind-pollinated species, pollen grains of maize are relatively large (diameter 90-125 µm) and heavy (0.25 µg) (Aylor et al., 2003; Di-Giovanni et al., 1995; Raynor et al., 1972).

The longevity of maize pollen viability strongly differs according to air temperature and humidity, and published data on the length of time that maize pollen remains viable under natural conditions varies from about 24 hours to several days (Eastman & Sweet, 2002). Dehydration is the main factor in maize pollen mortality and water loss in pollen grains during dispersal reduces their ability to germinate on the stigma (Aylor, 2004). In exceptionally hot and dry weather the viability could be reduced to a few hours, and extended up to nine days in cooler, humid conditions (Emberlin et al., 1999; Luna et al., 2001).

7.2.2.2 Pollen-mediated gene flow

Numerous studies have been conducted on pollen dispersal and outcrossing in maize (for a review, see BEETLE report, 2009; Brookes et al., 2004; Devos et al., 2005; Eastham & Sweet, 2002; Feil & Schmid, 2002; Sanvido et al., 2008; Nieh et al., 2014; Nadal et al., 2016; Ricci et al., 2016; Stachow et al., 2013). However, a general interpretation of the results is often difficult because of significant methodological differences and experimental conditions between studies and various environmental factors that are known to influence cross-fertilizations rates (Ingram, 2000; Devos et al., 2005; Trtikova et al., 2015). In addition to direct measurements of pollen concentration at different distances from the pollen source, various qualitative and quantitative methods have been used to estimate the actual outcrossing in maize (phenotypic markers, protein analysis, molecular markers, quantitative DNA analysis) (Devos et al., 2005). More recent studies are based on different mathematical models for simulation of the potential for outcrossing under different growing conditions.

The field trial data are often restricted to small- scale releases and the range of studied conditions (landscape patterns, climate, crop management) is limited. Gene flow modelling at the landscape level makes it possible to take into consideration additional factors affecting gene flow. It also allows the prediction of adventitious presence under different environmental and agronomic conditions, including large- scale adoption (Schenkelaars & Wesseler, 2016).

A number of abiotic and biotic parameters are known to influence outcrossing rates in maize (Hüsken et al., 2007; Sanvido et al., 2008; Palaudelmás et al., 2009). These factors include size, shape and orientation of both pollen source and recipient field, as well as distance, topography and vegetation between pollen source and recipient field. The size of the experimental donor and receptor fields determines the amount of competing pollen (Ingram, 2000; Devos et al., 2005), e.g. a high donor to receptor ratio (large donor field, small receptor field) leads to a higher amount of pollen from the donor field resulting in high cross-fertilisation rates in the receptor field due to low competition with incoming pollen. The shape of the fields is another factor that may influence cross-pollination. The amount of cross-fertilisation is clearly higher in elongated recipient fields than in rectangular ones of the same surface area when the long side of the field faces the source (Messeguer et al., 2006). Using SSR analysis to identify the origin of pollen showed that while changes in the size of the donor field clearly influences the percentage of GMO detected, this effect is moderate (Palaudelmás et al., 2012). This study demonstrated that doubling the donor field size resulted in an approximate increase of GM content in the receptor field by 7 %. This indicates that variations in the size of the donor field have a smaller influence on GM content than variations in the size of the receptor field. Similarly, a buffer zone with the same competitive agricultural crop will produce pollen, as well as being a physical obstacle to wind-dispersed pollen between fields, and reduce the outcrossing effectively (Devos et al., 2009, 2014).

The rate of cross-fertilisation between fields also depends on pollen viability and longevity, male fertility and/or sterility, synchrony in flowering between anthesis of the pollen donor and silking of the recipient field, wind direction and velocity and weather conditions. However, distance between the fields, flowering coincidence and orientation to prevailing horizontal wind speed have been identified within the EU-project SIGMEA as the major factors affecting cross pollination in maize (Hüsken et al., 2007; SIGMEA, 2009).

When assessing the frequencies of outcrossing, it is also important to take the intended use of the maize plant into consideration (Tolstrup et al., 2007). In forage maize, harvested as whole plants for ensilage or direct feed, the vegetative tissue that is not affected by cross-pollination will constitute a major part of the yield (depending on cultivar and maturity level).

The basic pattern of outcrossing in maize is described by the leptokurtic pollen dispersal curve (Eastham & Sweet, 2002; Messeguer et al., 2006). The highest pollen concentrations and most of the crossing and fertilisation occur close to the pollen source with a strong exponential decrease near the source field followed by a very slow decline with increasing distance (e.g. Eastham & Sweet, 2002). Due to its pollen characteristics (size/weight), maize pollen has a high settling speed and usually has a short flight range, and pollen concentrations decline rapidly with the distance from the source (Jarosz et al., 2005). Most of the pollen falls within 5 m of the fields' edge and approximately 95-99 % of the released pollen is deposited within about 30 m from the pollen source (Devos et al., 2005). At distances further than 30-50 m, the levels of pollen dispersion are very low. There is, however, no clear cut-off distance beyond which these levels reach zero.

Under suitable meteorological conditions, maize pollen can be lifted high up in the atmosphere and distributed over significant distances up to kilometers (Jarosz et al., 2005; Hofmann et al., 2010). However, vertical wind movements or gusts during pollen shedding only lead to very low levels of cross-fertilisation over longer distances (Palaudelmás et al., 2012). Most cross-pollination events occur within 40 m of the pollen source (reviewed by Eastham & Sweet, 2002; Brookes at al., 2004; Devos et al., 2005, 2014; Hüsken et al., 2007; Sanvido et al., 2008; Riesgo et al., 2010; Palaudelmás et al., 2012)

In a recent study, Hofmann et al. (2014) aimed to analyse data on maize pollen deposition in relation to the distance from the nearest maize field. The authors employed a standardised method to record maize pollen grains at 216 sites in Germany, Switzerland and Belgium from 2001 to 2010, using a pollen mass filter (PMF) sampler. The study confirms that the highest pollen deposition is within the nearest maize field and decreases with increasing distance from this field. Maize pollen was sampled up to 4.45 km from the nearest maize field and this made it possible to gather an extended dataset on pollen dispersal. The 95 % confidence interval for a predicted value of pollen deposition spans almost two orders of magnitude. Hofmann et al. (2014) also discussed the implications of their study on previous risk assessments of Bt-maize and the associated recommendations for mitigation measures. The pollen dose–distance distribution curve used by Perry et al. (2010, 2011, 2012) differs significantly from that used by Hofmann et al. (2014) for long distances. According to

Hofmann et al. (2014), this difference leads to the underestimation of maize pollen deposition over long distances.

Sanvido et al. (2008) reviewed existing cross-fertilisation studies in maize and established relevant criteria for the evaluation of these studies and applied criteria to define science-based separation distances. The results of their analysis showed that an separation distance of 20 m for silage maize, and 50 m for grain maize, respectively, is sufficient to keep GM-inputs from cross-fertilisation below the arbitrary level of 0.5 % at the border of a conventional/non-GM maize field. The proposed separation distances represent a rather conservative approach leaving an additional safety margin up to the current legal threshold of 0.9 % in the final product.

Occasionally, however, and particularly in the case of small fields less than 0.5 ha and/or of long, narrow fields that are located downwind from a larger GM maize field, the separation distance may need to be extended to 50 m or more (Devos et al., 2005; Hüsken et al., 2007). Based on a statistical analysis of different datasets on cross-fertilisation rates, Riesigo et al. (2010) concluded that a separation distance of 40 m is sufficient to reduce admixture in maize cultivation to below the legal threshold of 0.9 % in the EU.

Cross-pollination in maize has been examined in detail in several European countries in the EU Program 'Sustainable Introduction of GM Crops into European Agriculture' (SIGMEA, 2007, 2009). These studies indicate that a separation distance of 20-50 m is enough to maintain the labelling threshold below 0.9 %. In certain cases, where there are particular spatial conditions and agricultural practices (e.g. small scale production systems, average field size smaller than one hectare and/or long and narrow fields), the separation distances may have to be extended.

Like separation distances, pollen barriers of maize plants effectively reduce out-crossing between neighbouring maize fields. Barrier plants located adjacent to the recipient field act on the one hand as a pollen trap and on the other as an additional source of pollen that dilutes the transgenic airborne pollen. Studies in Germany and Switzerland confirmed the high interception of pollen by the first few maize rows when open ground or low growing intervening crops separate maize fields. The removal of the first 10-20 m of a non-transgenic field facing a GM crop might therefore be more efficient for reducing the total level of cross-fertilisation in a recipient population than to recommend separation distances (Hüsken et al., 2007).

7.2.2.3 Seed mediated gene flow

In spite of extensive cultivation in many countries and accidental seed spillage, seed mediated establishment of maize and its survival outside cropped areas in Europe is rare (see section 7.1). Maize is incapable of sustained reproduction outside cultivation and is non-invasive of natural habitats (ref. Eastham & Sweet, 2002), but maize plants occasionally grow in uncultivated fields and by roadsides. The probability of a volunteer maize crop appearing in subsequent (maize) crops and then contributing to gene flow via cross

pollination from the volunteer to a maize crop in Europe is very low due to the inability of the maize plant to shed seed naturally, a limited dormancy period, low competitiveness, the susceptibility to plant pathogens and herbivores, the common use of mechanical pre-planting soil preparation practices and the inability of maize seed to survive low winter temperatures (Hüsken et al. 2007). In addition, maize is mainly harvested as whole plants for silage. Since these characteristics are not altered in maize Bt11, it is considered very unlikely that the transgenic maize line or its progeny will differ from conventional maize varieties in their ability to establish feral populations in Europe.

Although seeds from the previous crop year can overwinter and germinate the following year, the plant cannot persist as a weed. Based on the observations in central Europe (Grüber et al., 2008), volunteers may only occur after a warm winter period. Monitoring of maize volunteers after maize cultivation in Spain has shown that the vigour of the volunteer plants is low; they are much shorter than normal plants and rarely have cobs (if produced normally without grains). Tassels were frequently produced, but cross-pollination was estimated to be low, most probably due to loss of hybrid vigour and uniformity in plant size, asynchronous flowering with the cultivated maize crops in which they occur, and amount of fertile pollen etc. (Palaudelmás et al., 2009). The contribution of pollen flow from occasional feral maize plants to agricultural fields with conventional maize varieties is therefore considered to be insignificant.

Field trials in Europe, Chile and the USA do not indicate altered agronomic or phenotypic characteristics of maize Bt11, except for the specific target pest resistance (Pioneer, unpublished data).

Pollen production and pollen viability is not expected to be affected by the genetic modification, and it is therefore not likely that out-crossing frequencies to other maize fields will be different from conventional varieties. VKM is of the opinion that the likelihood of unintended environmental effects as a consequence of gene flow from maize Bt11 is negligible.

7.2.1.1 National proposals for coexistence

Coexistence refers to the choice of farmers and consumers between conventional, organic or GM based crop production, in compliance with the legal obligations for labelling and/or purity standards. Coexistence always refers to GM plants that have passed the authorisation process. Therefore, environmental risks or risks to human or animal health do not concern the formulation of coexistence rules. EU regulations have introduced a 0.9 per cent labelling threshold for the adventitious presence of approved GM material in non-GM products.

A number of the EU member states (MS) have implemented coexistence regulations to ensure that GM and non-GM crops can be cultivated side by side without excluding any agricultural option. As cross-fertilization due to pollen flow between neighbouring field represents the major potential biological source of on-farm mixing in maize (e.g. Sanvido et al., 2008), most member states are currently proposing separation distances as the main

coexistence measure to comply with legal tolerance threshold requirements (EC, 2009). Some MS differentiate additionally between distances to conventional non- GM crops, organic crops and crops for seed production. Separation distance can be used as the unique requirement, as in Germany and Denmark, or in conjunction with buffer zones that often allow a reduction of the distance requirement (Schenkelaars & Wesseler, 2016).

An overview of mandatory separation distances adopted by EU member states shows a considerable range of variation, with respect to separation distances between GM and non-GM maize fields (Schenkelaars & Wesseler, 2016). Spain, the country with the highest share of GM maize cultivation in the EU requires the lowest separation distance for maize (20 m), while Bulgaria has the largest distances for maize (600- 30 000 m). Hungary imposes a minimum distance of 400 m that can be extended on a case- by- case approval process.

In 2007, the Norwegian Food Safety Authority requested VKM to assess coexistence measures at farm level in different GM crop production systems (VKM, 2007d). In this report, VKM concluded that separation distances of 200 m most likely would ensure an upper limit of 1 % of adventitious presence as a result of introgression via pollination in maize. In general, VKM assumed that the adventitious presence, most likely, would be less than 0.3% than in the range of 0.3 to 1.0%.

These assessments were based on the maize used being heterozygote for the inserted genes and that the maize plants are harvested as whole plants for ensilage or direct feed (maize grains constitute maximum 50 % of the silage/yield).

7.3 Interactions of the GM plant with target organisms

Genetically modified maize Bt11 was developed to provide protection against a variety of target pests of the order Lepidoptera. Protection is achieved through expression in the plant of insecticidal Cry protein Cry1Ab, derived from *Bacillus thuringiensis*, a common soil bacterium. Cry1Ab, encoded by the *cry1Ab* gene, is derived from *B. thuringiensis* subspecies *kurstaki*. Two Lepidoptera pests are primarily targeted by Bt11; *Ostrinia nubilalis* (European corn borer, ECB) and *Sesamia nonagrioides* (Mediterranean corn borer, MCB).

The European corn borer is widely distributed in Europe covering the Iberian Peninsula, Czech Republic and Slovakia, southwest of France, northern Italy and the southern regions of Germany and Poland. The Mediterranean corn borer is present in the Mediterranean region (Andreadis, 2011). There are ten reports of *O. nubilalis* in Norway, restricted to the counties of Vestfold, Telemark, Aust-Agder and Vest Agder. *Sesamia* spp. has not been reported in Norway. There are no reports of *O. nubilalis* attaining pest status in Norway, and the Plant Clinic (Planteklinikken) at NIBIO has never received samples of this pest or plant material damaged by this pest (K. Ørstad, pers. com.). Consequently, there are no insecticides authorised or previous applications for registrations of insecticides against this herbivore in Norway.

Aphids are the only pests reported on maize in Norway. Studies have shown that aphids are not affected by the Cry1Ab protein (Bourguet et al., 2002). Under the development of Bt maize expressing Cry1Ab, the noctuid *Agrotis ipsilon* was tested as a target, but there was little or no effect (Pilcher et al., 1997). This species is occasionally a pest in root crops in Norway and it is conceivable that it could become a pest of maize.

7.3.1 Adverse effects due to resistance evolution

Development of resistance to Cry proteins following exposure to Bt plants is an important aspect, with both agronomic and environmental implications (e.g. BEETLE Report 2009; Tabashnik et al., 2009). Resistance evolution to the Cry1Ab protein is not considered a direct environmental harm, but the consequences of the establishment of resistant Lepidoptera pests populations may lead to the use of other pest control tools with greater environmental harm.

Field resistance is defined as a genetically based decrease in susceptibility of a population to a toxin caused by exposure of the population to the toxin in the field (Andow, 2008). When Bt is used as a sprayed insecticide, it is active on the plant for a relatively short time (days) and coverage is never so complete that all of the targets in the treated field will be affected. Development of resistance is expected to go faster in insect-resistant crops, where the Cry proteins are expressed constitutively throughout the growing season. In addition to resistance development in the target pest, polyphagous herbivores feeding on *Bt* maize can develop resistance to the Cry proteins. This in turn will render Bt sprays useless in controlling these herbivores in other crops.

Since there are no Bt insecticides approved for use in Norway, and lepidopteran pests have not been registered in maize, issues related to resistance evolution in target pests are not relevant at present for Norwegian agriculture.

Internationally, much attention has been given to proactively avoiding and delaying the potential development of resistance in Bt crops. Resistance management strategies, relying on a “high dose/refuge strategy” have been endorsed in several countries (Andow, 2008). Current practice is to set off a refuge of non-Bt maize adjacent to the Bt maize crop. This is to provide a habitat where the herbivores are not exposed to the Cry protein and can develop populations that do not inherit the resistance genes. The strategies recommended are either to have 5% of the crop as non-Bt and unsprayed and adjacent to the Bt crop, or to incorporate (embedded) the 5% that are non-Bt into the area with the Bt plants, or else to have 80% of the crop as Bt plants and 20% adjacent non-Bt plants that are sprayed with a non-Bt insecticide (Shelton et al., 2002). The methods using conventional cultivars in adjacent refuges are considered to be more effective than the embedded non-Bt plant method.

The first documented case of field resistance to Bt as a sprayed insecticide was observed in Hawaii, where populations of the diamondback moth (*Plutella xylostella*) showed a reduced susceptibility to Bt-sprays (Tabashnik et al., 1990). The main target for maize Bt11 *O. nubilalis* has also developed resistance to Dipel® insecticide containing *B. thuringiensis* subsp. *kurstaki* (Li et al., 2005). When larvae from Dipel®-resistant populations were fed diet containing Cry1Ab, they were also resistant to the diet (Li et al. 2005). With Bt maize, the herbivores ingest the protein whenever they feed on the plant. This has obvious implications for the development of resistance to the protein.

Monitoring data from five continents reported in 41 studies that evaluate responses of field populations of 11 lepidopteran pests to four Cry proteins produced by Bt maize and cotton, have been analysed (Tabasnik et al., 2008; 2009). After more than a decade since initial commercialisation of Bt crops, most target pest populations remain susceptible, whereas field-evolved resistance has been documented in some populations of the noctuid species; *Spodoptera frugiperda, H. zea* and *Busseola fusca.* Recent studies indicate increased frequency of field-evolved resistance to Cry1Ac in *H. armigera* in China (Zhang et al., 2011; Wan et al.,2012).

The first instance of field resistance to Bt-maize has been reported in a population of the African stem borer (*B. fusca*) in South Africa, where some larvae were able to survive on Cry1Ab-expressing maize (ref. EFSA ,2011d). It appeared that the field resistance in stem borer in this area has resulted from a combination of a late general planting date with consequent increased levels of infestation and variance in time of planting providing a continuous supply of moths (Kruger et al., 2009). The survey by Kruger et al. (2011) revealed that compliance with refugia requirements in the region was low especially during the initial 5-7 years after release and high number of farmers applied insecticides as preventative sprays on Bt-maize and refugia irrespective of stem borer infestation levels.

The second instance concerns *S. frugiperda*. Larvae surviving on Cry1F-expressing maize in some fields on an isolated tropical island in the USA (Puerto Rico) were collected and exposed to high concentrations of the Cry1F protein in laboratory bioassays, where no mortality was observed (Matten et al., 2008; Moar et al., 2008; Tabashnik, 2008; Tabashnik et al., 2008). Storer et al. (2010) and Velez et al. (2013), confirmed via laboratory bioassays that *S. frugiperda* collected from the affected area exhibited lower sensitivity to the Cry1F protein compared with typical colonies from other regions, and that the resistance was shown to be autosomally inherited and highly recessive. The unusual combination of biological, geographic, and operational factors (such as high selection pressure for resistance by continuous silage maize production with sequential year-round plantings, high level of overall *S. frugiperda* pest pressure during the year of observing its damage on Cry1F expressing hybrids, drought conditions reducing availability of alternative host plants that encouraged movement of the adult and larval populations into irrigated agricultural maize fields) led to *S. frugiperda* evolving resistance to the Cry1F protein in Puerto Rico. Moreover, no insect resistance management (IRM) measures were put in place at that time in Puerto Rico.

Storer et al. (2012a) provided an update on the status of the previously reported instance of field- evolved resistance to Cry1F-expressing maize in Puerto Rico. Resistant populations in Puerto Rico and susceptible ones in the southern USA were further monitored, showing high levels of Cry1F resistance and full susceptibility, respectively. The authors concluded that the resistant populations have not spread to any measurable extent from Puerto Rico to mainland USA, and that local selection from Cry1F-expressing maize in the southern USA has caused no measurable change in population susceptibility. However, these data indicate that resistance may persist in a population, and that slowing the spread of resistance genes is more practical than eradicating resistance. Therefore, the authors advocated the deployment IRM measures to delay the evolution of resistance, and to manage the sustainable use of Bt-crops.

Monitoring data indicate that neither in the EU, nor in the USA, have populations of resistant *O. nubilalis* or *Sesamia nonagrioides* been found. The field outcomes documented with monitoring data are consistent with the theory underlying the refuge strategy, suggesting that refuges will not prevent the development of resistance but have helped to delay resistance (Tabasnik et al. 2008, 2009; Wan et al. 2012). In addition, other factors like recessive inheritance of resistance and deployment of pyramided *Bt*-crops will potentially delay resistance development. According to Storer et al. (2012b), pyramiding in the same plant of two or multiple Cry proteins, acting independently on target insect pest midgut receptors, is expected to delay the evolution of resistance to either Cry protein effectively when most individuals that are resistant to one Cry protein are killed by the other, and when selection for resistance to one of the Cry proteins does not cause cross-resistance to the other.

A strain of *O. nubilalis*, obtained from field collections throughout the central USA Corn Belt was selected in the laboratory for resistance to Cry1F by exposure to the toxin incorporated into artificial diet (Pereira et al., 2008). The selected strain developed more than 3000-fold resistance to Cry1F after 35 generations of selection and readily consumed Cry1F expressing maize tissue; yet, it was as susceptible to Cry1Ab and Cry9C as the unselected control strain. Only a low level of cross-resistance (seven-fold) to Cry1Ac was observed. This lack of cross-resistance between Cry1F and Cry1Ab suggests that maize hybrids expressing these two toxins are likely to be compatible for resistance management of *O. nubilalis*.

Xu et al. (2010) and Crespo et al. (2011) investigated the potential for cross-resistance between Cry1Ab and Cry1F. Laboratory-selected Cry1Ab-resistant *O. furnacalis* and *O. nubilaris* colonies were shown to exhibit low levels of cross-resistance to Cry1F, ranging between < 4- and 6-fold, respectively.

Several recent laboratory studies have demonstrated the development of resistance and cross-resistance to Cry-proteins in various species including *Ostrinia furnacalis* (Zhang et al., 2014, Wang et al., 2016), *Helicoverpa zea* (Welch et al., 2015) and *Diatraea saccharalis* (Girón-Pérez et al., 2014, Huang et al., 2015).

Omoto et al. (2015) have registered field resistance (decreased susceptibility) to Cry1Ab in Fall armyworm (*Spodoptera frugiperda*) in Brazil. The authors conclude that the Cry1Ab resistance can be the result of exposure to this protein, but that it is indistinguishable from cross-resistance developed from exposure to Cry1F. Jakka et al. (2014) had very similar results in a *S. frugiperda* population from Puerto Rico, USA.

7.4 Interactions of the GM plant with non-target organisms (NTOs)

In agro-ecosystems, non-target organisms (NTOs) provide key ecological functions (including ecosystem services), such as plant pollination, biological control and decomposition, and form important components of farming systems (Arpaia, 2010). Considering that every species cannot be tested, it is important that the main functional groups mediating the ecological functions as well as their response to GM plants are considered in the ERA of GM plants (EFSA, 2010a). Thus, toxicity of Cry proteins is generally tested on a representative subset of NTO species ("focal species") using a tier approach. Lower-tier studies represent a first step to reach reliable risk assessment conclusions, as they give indications of possible hazards associated with the cultivation of GM plants. In case a hazard has been identified in lower-tier studies, a detailed exposure characterization is required to fully characterize the possible risk (EFSA, 2010a).

7.4.1 Effects on pollinating insects

Honeybees and other pollinators can be exposed to any genetically modified products expressed in pollen or nectar. Adult bees consume pollen during their first week after emergence and thus might be exposed to Bt proteins. Bee larvae also ingest pollen but in lesser amounts (e.g. BEETLE report 2009).

Because of their ecological and economic importance, the Western honey bees (*Apis melifera* L.) are often used as test-species in pre-market risk assessment studies to assess direct toxicity on non-target organisms, and are probably the most studied non-target arthropod with respect to potential effects of conventional pesticides. However, relatively few large scale field studies have been conducted to assess the possible ecological impact of transgenic crops on honey bee colonies under realistic agricultural conditions (Rose et al., 2007).

According to the BEETLE Report (2009), no adverse effects of Bt crops on honeybees have been reported so far, and no reports are available regarding harmful effects on other non-target organisms involved in pollination.

In a peer-reviewed paper assessing the impact of the Cry protein on honeybee, Hanley et al. (2003) found that feeding honeybee larvae with the Cry1Ab- or Cry1F-containing maize pollen did not affect larval mortality, pupal mortality, pupal weight or haemolymph protein

concentration, compared with larvae fed regular bee-collected pollen or non-transgenic maize pollen.

A number of studies on effects of purified or pollen-enclosed single Cry proteins demonstrate that there is to date no indication of acute or chronic toxicity either for larvae or adult bees (ref. BEETLE Report, 2009, Duan et al., 2008, Malone & Burgess, 2009, malonema et al., 2011; 2013; Lima et al. ,2011; 2013; Grabowski & Dabrowski, 2012; Dai et al., 2013; Geng et al., 2013; Niu et al., 2013).

Malone & Burgess (2009) have reviewed available scientific data on potential adverse effects on honeybees of Cry proteins or Cry-containing maize pollen gathered either under lower- or higher-tier studies. The authors concluded that none of the Bt-maize events commercially available have significant impacts on the health of honeybees. A meta-analysis of 25 studies that assessed potential effects of Bt proteins on honeybee survival has been published by Duan et al. (2008). No adverse effects on honeybee larvae or adults, in laboratory settings, were reported when looking at studies performed with lepidopteran and coleopteran specific Bt proteins. However, Duan et al. (2008) considered that in field settings, honeybees might face additional stresses, which theoretically could affect their susceptibility to Cry proteins and generate indirect effects.

Hendriksma et al. (2013) examined the response of nurse bees and their gut bacteria to pollen from Bt maize expressing three different insecticidal Cry proteins (Cry1A.105, Cry2Ab2, and Cry3Bb1). Colonies of *Apis mellifera carnica* were kept during anthesis in flight cages on field plots with the Bt maize, two different conventionally bred maize varieties, and without cages, 1 km outside of the experimental maize field to allow *ad libitum* foraging to mixed pollen sources. Honey bee nurses which were forced to cover their full protein demand by pollen from a stacked Bt maize showed no apparent effects on survival rates, body weight and pollen digestibility. The community structure of the gut bacteria significantly responded to the different pollen diets, but differences found with the Bt maize pollen were in the range of those occurring between pollen from conventionally bred varieties or mixed pollen sources. The relatively low Cry protein concentration measurements compared to the high exposure of nurse bees indicate that the recombinant proteins were actively digested. The natural occurrence of Cry proteins in the gut of nurse bees with no exposure to Bt maize and the lack of detectable effects on nurse bees and their gut bacteria give no indication for harmful effects of this Bt maize on honey nurse bees.

Feeding studies performed under controlled conditions with honeybees being fed either with Bt pollen or mixtures of honey and sugar syrup containing purified Cry1Ab protein have indicated no direct adverse effects on foraging activity, learning performance or survival of honeybees (Ramriez-Romero et al. 2005, 2008). Further studies with bees fed purified Bt-proteins, pollen from Bt crops, or bees allowed to forage on Bt crops in the field have confirmed the lack of effects on the mortality of honey bees (Malone & Pham-Delegue 2001; Babendreier et al. 2005; Bailey et al. 2005).

In order to assess the risk that insecticidal transgenic plants may pose for bumblebees, Babendreier et al. (2008) tested whether *Bombus terrestris* (L.) workers are able to detect insecticidal proteins dissolved in sucrose solution and whether consumption of these proteins will affect survival and offspring production. Feeders containing either Cry1Ab, soybean trypsin inhibitor (SBTI) or *Galanthus nivalis* agglutinin (GNA) were offered to bumblebee colonies at different concentrations. No difference was found in the number of visits or the duration of visits among the different concentrations for each of the insecticidal proteins, indicating that bumblebees do not discriminate among the compounds. According to Babendreier et al. Cry1Ab protein did not affect microcolony performance, while the consumption of SBTI and especially GNA affected survival of *B. terrestris* workers and drones and caused a significant reduction in the number of offspring.

In a field study functional colonies of honeybees were exposed to Bt maize pollen (foraging in sweet maize plots, supplied with pollen cakes from Bt maize pollen) expressing Cry1Ab pritein for 28 days (Rose et al., 2007). No significant adverse effects on foraging behavior, bee body weight or colony performance were detected. Offspring development was not affected by exposure to Bt pollen, but significantly reduced by the positive insecticide control.

In a review published by Han et al. (2016), the authors found only six studies of the effects of GM plants on the behavior of arthropod pollinators, four of which involved Cry1Ab maize. Three studies were negative: honeybee foraging, honeybee olfactory learning behavior, and monarch butterfly oviposition behavior. The one positive experiment was conducted with larvae (caterpillars) of monarch butterflies, which species develops exclusively on leaf tissues of milkweeds and thus would not normally be found on maize plants. The study found that larvae exposed to anthers of Bt maize moved more and fed on anthers less, than on related non-Bt maize anthers. The relevance of the two monarch studies (feeding, oviposition behavior) to the behaviors of monarchs in nature is thus unclear.

7.4.2 Effects on natural enemies (predators and parasitoids)

The exposure of natural enemies (predators and parasitoids) to Cry proteins expressed in Bt-plants can occur in different ways: natural enemies can be exposed to Cry proteins by feeding on plant material (including pollen) or honeydew excreted from sap-sucking species, and indirectly through feeding on prey/host organisms which have previously been feeding on Bt plants (ref. EFSA 2009b).

Potential effects of the Bt maize MON810 x MON 88017, expressing Cry1Ab and Cry3Bb1, on ground beetles and spiders were investigated in field and laboratory experiments in Germany in 2008-2011 (Priesnitz et al., 2011). The study compared the GM variety with its isogenic parent and two conventional maize varieties. More than 70 000 predatory arthropods were counted in soil traps and assessed over the three year investigation period. The density of ground beetles and spiders did not differ significantly between the Bt maize plots and the conventional maize plots. By contrast, on a few sampling dates there were clear differences

between the maize MON810 x MON 88017 and the plots with the isogenic variety treated with insecticides. The composition of the ground beetle community varied over the course of the three years, but no differences were found between the different plots. Preliminary results from feeding trials, 600 beetle larva (*Poecilus cupreus*) were tested and fed on CryBb1 protein and a protein mix containing Cry1A.105, Cry2Ab2 and Cry3Bb1, respectively. No negative impacts were found on the pupation rate, hatching rate, development, weight at emergence or fertility of the beetles.

Bourguet et al. (2002) studied the effect of Bt maize on the field abundance of non-target insects. In their experiments with MON810 they looked at the effect on aphids and their predators/parasitoids. There were no significant differences in the abundance of aphids or predators/parasitoids. The predators found were: *Orius insidiosus, Syrphus corollae, Coccinella septempunctata, Chrysoperla carnea* and thrips. The parasitoids were hymenopterans.

In a laboratory study, no effect was found of pollen from Bt maize expressing the Cry1Ab protein on *O. insidiosus, C. carnea* or *Coleomegilla maculata* (Pilcher et al., 1997). This study was followed by a 2-year field study where predators of *O. nubilalis* were monitored before pollen shed, at pollen shed and after pollen shed. The authors concluded that Bt maize pollen did not affect the movement of these predators (Pilcher et al., 1997).

A different *Orius* species, *O. majusculus*, was investigated for non-target effects of Bt maize in a laboratory study (Zwahlen et al., 2000). The predator was fed thrips (*Anaphothrips obscurus*) that were either reared on Bt maize or non-Bt maize. Although the thrips was not sensitive to the Cry protein, it was assumed that the toxin would be in the thrips' body when it was consumed by the predator. The study revealed that there were no differences in mortality or developmental time for the predator.

In a study under laboratory conditions, Lumbierres et al. (2012) examined the effects on the reproduction and nymphal development of O. majusculus caused by feeding on Bt plant materials (Cry1Ab) and on herbivore prey fed on Bt plants in three experiments. To measure the effects of Bt maize plants on O. majusculus fecundity and fertility, nymphs were provided with combinations of Bt or non-Bt pollen and leaf in addition to prey, and egg laying and egg hatching in the resulting adults were measured. Second, the effect of Bt vs. non-Bt pollen and leaf with no additional prey on nymphal development, survival, sex ratio and teneral adult weight and size were measured. Third, prey- mediated effects of Bt protein on nymphal developmental time, survival, sex ratio and teneral adult weight were evaluated using *Tetranichus urticae* Koch fed on Bt and non-Bt maize plants as prey.The study confirmed that ingestion of Bt protein by *O. majusculus* via plant leaves or pollen or via the food web has no negative effects on predator survival, development, fecundity and fertility. On the contrary, in such circumstances a positive effect on fecundity and developmental time of the predator was observed. Fecundity was increased when they feed on Bt plant material, and nymphal development was shortened when nymphs were fed on Bt-

containing spider mites and when they were fed on Bt plant material in the absence of lepidopteran eggs prey.

Torres & Ruberson (2008) studied the effect of Cry1Ac protein on four species of predatory bugs; *Podisus maculiventris, Geocoris punctipes, Nabis roseipennis* and *O.insidiosus*. The bugs were fed with prey from Bt cotton. The authors concluded that the predatory bugs were not adversely affected by eating Cry1Ac-contaminated prey.

Leite et al. (2014) exposed the predator *Podisus nigrispinus* to prey that fed on Bt-maize (Cry1Ab) in laboratory and on potted plants. The predator had delayed development and lower biomass when fed on prey from Bt-maize. The authors attribute this to lower nutritional quality of the prey, as the prey had very short survival time on the Bt-maize.

The effects of the Cry proteins Cry1Ac, Cry1Ab and Cry2Ab on the anthocorid *O. albidipennis* were studied under laboratory conditions (González-Zamora et al., 2007). Tritrophic experiments were performed, in which the nymphs were fed *Helicoverpa armigera* larvae reared on a diet with Cry1Ac, Cry1Ab, or Cry2Ab proteins at different concentrations (0, 1, and 10 microg/ml), when supplemented with *Ephestia kuehniella* eggs. In complementary experiments, the Bt Cry1Ac protein was directly fed to *Orius* nymphs at a very high concentration (1 mg/ml). No effects on prey consumption, developmental time, nymph survival, fecundity, and egg hatching of *O. albidipennis* were found in either experiment. It can be concluded that the toxins tested do not seem to pose a risk for the anthocorid *O. albidipennis*, especially when it is exposed through the prey.

Alvarez-Alfageme et al. (2008) investigated prey-mediated effects of two maize varieties expressing a truncated Cry1Ab protein (Event Bt176 and MON810) on the biology of the ladybird *Stethorus punctillum*. Although immuno-assays demonstrated the presence of Cry1Ab in both prey and predator collected from commercial maize-growing fields, neither transgenic variety had any negative effects on survival of the predator, nor on the developmental time through to adulthood. Furthermore, no subsequent effects on ladybird fecundity were observed. Corresponding results were shown by Alvarez-Alfageme et al. (2009). There were no significant effects on mortality, development time or growth of larvae and pupae of the ground-dwelling predator *Poecilus cupreus* L. fed with *Spodoptera littoralis* larvae reared on Bt176 maize leaves. To elucidate potential detrimental effects due to a reduction in the quality of the prey, the authors assessed the digestive proteolytic activities of *P. cupreus* adults from a laboratory culture and insects collected in commercial Bt and non-Bt maize fields. Field-collected *P. cupreus* adults had higher proteolytic activities than those reared in the laboratory, whereas no significant differences were found between *P. cupreus* adults reared on Bt and non-Bt maize fed *S. littoralis* or between *P. cupreus* adults collected in commercial Bt and non-Bt maize fields.

A comprehensive study using a tritrophic bioassay was conducted to evaluate the potential impact of Cry2Ab- and Cry1Ac-expressing cotton on fitness parameters of the lady beetle *Coleomegilla maculata,* a common and abundant predator found in many cropping systems worldwide (Li et al., 2011). Both larvae and adults of *C. maculata* are predaceous, feeding on

aphids, thrips and lepidopteran eggs and young larvae. In addition to prey, *C. maculata* also feeds on plant tissues, such as pollen. Therefore the species can be directly and indirectly exposed to Cry proteins in several ways when feeding Bt crops. Li et al. (2011) used Bt-susceptible and –resistant larvae of *Tichoplusia ni* as prey. *C. maculata* survival, development time, adult weight and fecundity were not different when they were fed with resistant *T. ni* larvae reared on either Bt or control cotton. To ensure that *C. maculata* were not sensitive to the tested Cry protein independent from the plant background and to add certainty to the hazard assessment, *C. maculata* larvae were fed artificial diet incorporated with Cry2Ab, Cry1Ac or both at >10 times higher concentrations than in cotton tissue. No differences were detected in any life-table parameters between Cry protein-containing diet treatments and the control diet.

Conflicting results regarding potential adverse effects of the Cry1Ab protein to larvae of the ladybird *Adalia bipunctata* have been reported in the literature (Romeis et al., 2012). Hilbeck et al. (2012) reported lethal effects of the protein on larvae of *A. bipunctata* when fed directly to the predator. Corresponding results were found in an earlier feeding study, where *A. bipunctata* suffered increased mortality during the first larval stage when ingesting the Cry1Ab protein (Schmidt et al., 2009). Such toxic effects were not observed in direct feeding bioassays conducted by Porcar et al. (2010) and Alvarez-Alfageme et al. (2011). In the higher tier, tri-trophic study using Bt maize-fed spider mites as prey did not revealed any adverse effects on lethal and sublethal parameters of the predator (Alvarez- Alfageme et al., 2011). This was despite the fact that the larvae had ingested high amounts of biologically-active Cry1Ab protein. Many ladybird species, including *A. bipunctata,* mainly feed on aphis that are known to contain, at best trace amounts of Cry protein when feeding on Bt maize. Romeis et al. (2012) concluded that Bt maize expressing Cry1Ab poses a negligible risk to the predator under realistic worst case exposure conditions.

Adults of common green lacewing (*Chrysoperla carnea*) are prevalent pollen-consumers in maize fields. They are therefore exposed to insecticidal proteins expressed in the pollen of insect-resistant maize varieties expressing Cry proteins. Li et al. (2008) conducted two laboratory experiments to evaluate the impact of Cry1Ab- and Cry3Bb1-expressing transgenic maize pollen on fitness parameters of adult *C. carnea.* Adults were fed pollen from Bt maize varieties or their corresponding near isolines together with sucrose solution for 28 days. Survival, pre-oviposition period, fecundity, fertility and dry weight were not different between Bt or non-Bt maize pollen treatments. In order to ensure that adults of *C. carnea* are not sensitive to the tested proteins independent from the plant background and to add certainty to the hazard assessment, adult *C. carnea* were fed with artificial diet containing purified Cry1Ab or Cry3Bb1 at an approximately 10 times higher concentration than in maize pollen. No differences were found in any life-table parameters between Cry protein-containing diet treatments and control diet.

A preference study was conducted in Switzerland using all three larval stages of the lacewing *C. carnea* and two prey species, the aphid *Rhopalosiphum padi* and the lepidopteran *Spodoptera littoralis*. The Bt maize used expressed Cry1Ab. It was not lethal to either of the

prey species. In choice tests involving only one prey species, the predator showed a preference for the *S. littoralis* larvae feeding on non-Bt maize, but no preference for aphids based on food plant type (Meier & Hillbeck, 2001). When given a choice of *S. littoralis* or *R. padi,* the lacewing preferred the aphids. The authors speculate that the aphids did not contain the toxin, as it is not present in the plant phloem on which they feed. If this is the case, then Bt maize should not pose a problem for *C. carnea*. Laboratory studies that showed that the aphids do not take up the Cry protein from the phloem were conducted by Dutton et al. (2002). These studies also showed that when *C. carnea* are fed *S. littoralis* from Bt maize, they have an increase in mortality and a delay in development. However, this may be of little importance if the non-preference that *C. carnea* showed for *S. littoralis* in the lab also holds true for the field.

Similar studies were conducted to examine the effect on the Ichneumonid parasitoid *Campoletis sonorensis* when its host *O. nubilalis* was fed on Bt maize or non-Bt maize (Sanders et al., 2007). This study found that when the parasitoid developed in hosts feeding on Bt maize, the emerging adults were significantly smaller. The size of the adults was directly related to the size of the host at oviposition by the parasitoid, and the host's subsequent growth rate. When the new generation of adult parasitoids were analyzed, no Cry1Ab was found. This indicated that the smaller size was entirely host-mediated and not a direct effect of the toxin on the parasitoid. This study included a choice test where the parasitoid could choose hosts from Bt maize or non-Bt maize. No obvious preference were observed.

In a Chinese study *Helicoverpa armigera* was fed with a diet containing Cry1Ac protein (Ding et al., 2009). The effect on the Braconid parasitoid *Microplitis mediator* was a result of the host's growth rate and size. No adverse effects of the Cry protein itself were found.

Romeis et al. (2004) fed Cry1Ab protein directly to *C. carnea* larvae at concentrations that were approximately 10,000 times greater than the concentration in lepidopteran prey fed on Bt maize. This resulted in no direct toxic effect of the toxin on the lacewing. The authors concluded that the previously reported negative effects of Bt maize could be attributed to prey-mediated effects and not the Cry protein. In a subsequent study of Lawo & Romeis (2008) no adverse effects were observed of Cry1Ac and Cry1Ab on larvae of *C. carnea.*

A field study was conducted comparing Bt maize expressing Cry1Ab (MON810) with near isogenic maize (Daly & Buntin, 2005). They found a reduction in sap beetles (*Carpophilius* spp.) and an otitid fly (*Euxesta stigmatis*), which they attributed to less ear damage from the target species, the corn earworm (*H. zea*), as the damaged ear is what attracts these insects to the maize. They also found a reduction in predatory damsel bugs (*Nabis* spp.). The authors comment that the numbers of damsel bugs in both Bt maize and non-Bt maize were so low that no conclusions could be drawn. There are 8 reported species of damsel bugs in Norway (Coulianos & Ossiannilsson 1976).

In Spain, where Bt maize has been grown since 1998, a study was conducted to compare the abundance of predatory arthropods in Bt maize (Cry1Ab) and non-Bt maize (de la Poza

et al., 2005). The predators were monitored visually on the plants or in pitfall traps. This study found no differences in the abundance of *Anthocoridae, Coccinellidae, Aranea* or *Carabidae* in the Bt maize compared to the non-Bt maize. All of these taxa are common in Norwegian maize fields.

Ludy and Lang (2006) also investigated spiders in their 3-year study in Germany of the effect of Bt maize expressing Cry1Ab (event MON810). They found no significant differences in the numbers of spiders in Bt maize fields or their margins compared to non-Bt maize fields.

Perhaps the most throughout and detailed investigation of the impact of Bt maize on non-target arthropods to date is that of Dively (2005). This field study was over a 3 year period in Maryland, USA. Over 500,000 arthropods were counted, from 13 orders, with 112 families and 203 taxonomic groups. The maize lines had both the *vip3a* and the *cry1Ab* genes. The effects of Bt maize were compared to non-Bt maize with and without insecticide treatment. Arthropods were registered by visual inspection, sticky traps, pitfall traps and emergence traps. Registration was also carried out the following growing seasons to document carry-over effects. All of the families of arthropod predators and parasitoids that are likely to occur in Norwegian maize fields are represented in this study. There were significant differences between the insecticide-treated maize and the other treatments (Bt and non-Bt maize).The author concludes that there were no significant differences in biodiversity and community-level responses caused by the Bt maize. The differences in abundance of certain species between the Bt maize and non-Bt maize that were recorded are regarded by the author to be the result of factors such as lack of prey or lack of plant injury. This is similar to the conclusion of several other studies mentioned above.

Mann et al. (2010) studied relative abundance of non-target insects on Bollgard cotton cultivars expressing Cry1Ac and Cry2A2 proteins over two cropping seasons. Densities of sucking insects (*Amrasca biguttula biguttula, Bemisia tabaci, Aphis gossopy, Trips tabaci*), the foliage feeder *Myllocerus undecimpustulatus* and of the predators *Chrysoperla* spp, *Brumus* spp*., Vespa* spp*., Lycosa* spp. and *Aranews* spp. were similar on the transgenic and conventional cultivars.

Comas et al. (2014) have published a meta-analysis of data from 20 field trials (11 years) in Spain. Data were from visual counts, pifall traps and yellow sticky traps. The authors found no effect of Bt maize on the most common herbivore, predatory and parasitoid arthropods found in the maize ecosystems of southern Europe (*Orius* spp., *Nabis* spp., Carabidae, Chrysopidae, Coccinellidae, Araneae, Dermaptera, Staphylinidae,Collembola, Myriapoda, Cicadellidae, Fulgoroidea, Ichneumonidae, Mymaridae, Chalcidoidea, Chloropidae, Muscoidea, Aphididae).

Bt maize MON810 was grown on the same plots for three years in a study in the Czech Republic (Skokova et al., 2015). No significant differences in numbers of predators (ground beetles, rove beetles and spiders) were captured in plots with MON810 compared to non-GM.

In a large scale experiment in Poland in the 2008-2010 growing seasons, Twardowski et al. (2014) monitored the population density of surface-active invertebrates of the Staphylinidae family. The average number of rove beetle populations in Bt maize expressing Cry1Ab protein were shown not to differ significantly from the number of beetles in conventional maize fields.

A four year on farm study was conducted in the Philippines between 2006 and 2009 to investigate if Bt maize (Cry1Ab) has long term effects on arthropod communities in commercial farms and in adjacent riparian areas (Alcantara, 2012). The arthropod composition was monitored through visual inspection in commercial farms and through sweep sampling in nearby riparian areas. Results of the sampling revealed that the abundance and diversity of arthropods were similar in Bt and conventional farms and in adjacent areas.

In USA, Andow & Zwahlen (2016) tested the six most common putatively omnivorous carabids to see if they acquired Cry1Ab from Bt maize in 10 fields. Three species acquired Cry1Ab from both live plants and residues, two species only from live plants, one species from neither. Laboratory-fed *C. iowensis* acquired Cry1Ab from both. Larger beetles acquired proportionately higher amounts of Cry proteins than smaller. The authors conclude that carabid species vary significantly in their ecological roles in agricultural food webs. Some ground beetle species do acquire Cry proteins in GM maize fields. Which species do so depends on their feeding behavior.

In a field study from Brazil, insects were collected directly from plants, over two months in large fields of conventional and Bt maize over a wide geographic area (Chaves et al., 2016). The study included *Spodoptera frugiperda* (the target of Bt treatment) and insect community (non-target pests, natural enemies of pests, other insects) of maize agroecosystems. Though there were large differences among maize fields, there was no general negative effect of Bt proteins on insect species richness (including on natural enemy richness). Species richness was generally low.

Peterson et al. (2016) collected spiders in a field study in USA. Most collected species and most individual spiders belonged to three functional guilds (ground runners, orb- weavers, sheet-tangle weavers). Some Siders from each of the three Bt fields were positive for Cry proteins, with the highest proportions occurring during and after anthesis (ca 8%). In feeding trials, the wolf spider showed detectable levels of Cry proteins but the sheet-tangle weaver did not. Direct feeding on pollen resulted in transfer of both Cry1Ab and Cry3Bb1, in all three tested species. Cry proteins were also found in 13 insect species, 3 other arthropods, and an earthworm (Lumbricidae). The authors clearly show that at least some individuals of many spider species in GM maize fields do contain measurable amounts of Cry proteins, and that consuming arthropods containing Bt and pollen feeding (only recently documented for spiders) are likely pathways. The treatments were not replicated, but the large sample sizes are convincing, as is the fact that no insects or other arthropods collected in the control field had Bt.

Szenasi et al. (2014) studied the effects of Cry1Ab maize on flea beetle communities, summers of two years, comparing GM and non-GM maize in a field study in Budapest. The authors sampled 51,000 beetles from 26 species. No significant differences between GM and non-GM plots, in abundances or species richness were found.

Han et al. (2016) review published research on the effects of insect-resistant GM crops on the behaviors of phytophagous, predaceous, and parasitic insects and mites. Almost all such studies have been conducted on Bt crops, some of which were Cry1Ab or Cry1F maize. Altered behaviors could have indirect effects on insect or mite fitness and hence could impact non-target the non-target native arthropod fauna. However, the behavioral studies were usually carried out in the laboratory or were otherwise quite artificial (as acknowledged by the authors), so caution must be used in drawing conclusions for the effects of Bt crops on natural populations. Overall, while there are documented effects on some behaviors, most of these effects are unlikely to impact natural populations negatively.

Of 12 reviewed, 10 studies found significant effects on the behaviors of non-target herbivores (phytophages); 22/47 studies on target herbivores found significant effects. With respect to Cry1Ab and Cry1F maize, all studies were on target species and none involved non-target species. The significant results for these maize studies primarily involved studies on spatial distribution (e.g. aphids clustering in sites with lower Cry protein concentrations, on Bt cotton) or movement (e.g. nematodes abandoned Bt maize more frequently). There were also experiments on host preference for feeding or oviposition (Bt vs non-Bt), which gave mixed results.

Overall, 11/50 reviewed studies found significant effects. For Cr1y1Ab or Cry1F maize, only two of 11 reviewed papers on arthropod natural enemies found a significant behavioral effect. In one study, a wasp parasitiod of a target lepidopteran foraged less efficiently in the presence of frass from Cry1Ab maize-fed host; a different study of the same host-parasite system found no significant effects on foraging behavior, though. The majority of studies on parasitiods found no effects on foraging behavior (including two others on Cry1Ab maize), but a few found minor effects. In the other study with significant results, a mite predator of herbivorous spider mites showed that the predator species spent more time near non-Bt maize fed prey than Bt maize fed prey. Experiments using ladybird beetles (Coccinellidae, 2 species), the garden spider *Araneus diadematus*, a ground beetle, a minute pirate bug and a lacewing, found no effects on predatory behavior for target species lepidopteran larval prey; these are all generalist predators found in maize fields. A three-year field study of spiders and ground beetles in Cry1Ab maize found no phenological effects of Bt maize.

7.4.3 Effects on non-target Lepidoptera

Maize plants are not an important resource of food for indigenous Lepidoptera with the exception of a few pest species. Therefore, the main potential risk to non-target Lepidoptera is expected to be the exposure to potentially harmful amounts of pollen deposited on host-plants in or near maize Bt11 fields.

In a laboratory experiment reported in Nature (Losey et al., 1999), the effect of pollen from Bt maize on larvae of the monarch butterfly (*Danaus plexippus*) was studied. This was followed by a paper that considered ecological factors in the field and their influence on the monarch's exposure to natural quantities of Bt maize pollen (Jesse & Obrycki, 2000), where it was concluded that when the monarch fed on its host plant milkweed (*Asclepias syriaca*) with natural dusting of Bt maize pollen it suffered higher mortality than on plants with non-Bt maize pollen. In a later paper, the same authors conclude that MON810 Bt maize pollen and anthers had no measurable effect on the oviposition or survival of the monarch (Jesse & Obrycki, 2003).

The studies on the monarch butterfly were performed in the USA. Similar studies were later done in European laboratories using the common swallowtail butterfly (*Papilio machaon*) and its host *Pastinaca sativa*. When exposed to different densities of pollen from Cry1Ab maize, the larvae had lower weights, longer development time and lower survival, and smaller wing size as adults (Lang & Vojtech, 2006). This result was more pronounced with higher pollen densities. This study used event Bt176, and the paper mentions that MON810 expresses much lower levels of Cry protein in the pollen.

A field study in Germany evaluated the impact of MON810 on non-target lepidopteran larvae (Gathmann et al., 2006). Weed belts were established in plots containing MON810 and non-Bt maize both with and without insecticide treatment. The naturally occurring lepidopteran larvae on the weeds were recorded. The only species that were numerous enough to compare statistically were specialist species on Brassicaceae, *Plutella xylostella* and *Pieris rapae*, both of which were found on *Sinapis alba*. There were no differences detected between the MON810 plots and the untreated non-Bt maize plots.

Schuppener et al. (2012) have assessed the risk posed by event MON89034 × MON88017 to the small tortoiseshell *Aglais urticae*, a butterfly species common in central Europe. The authors assessed the toxicity of Bt maize pollen on butterfly larvae, measured pollen deposition on leaves of the host plant *Urtica dioica* and mapped the occurrence and distribution of host plants and larvae in two arable landscapes in Germany during maize anthesis. The results showed that larvae-fed 200 Bt-maize pollen grains/cm^2 had a reduced feeding activity. Significant differences in developmental time were also detected at pollen densities of 300 Bt-maize pollen grains/cm2 and in survival at 400 grains/cm2. The highest pollen amount recorded was 212 grains/cm2 at the field margin, and the mean densities were much lower. Schuppener et al. concluded that the amount of pollen from maize MON89034 × MON88017 found on host plants is unlikely to adversely affect a significant proportion of larvae of *A. urticae*, and that the risk of event MON89034 × MON88017 to populations of this species is negligible.

Perry et al. (2010) developed an 11-parameter mathematical model of exposure of larvae of non-target Lepidoptera to Bt-maize pollen in Europe. The model was initially developed for maize MON 810 (Cry1Ab) and has later been recalibrated for maize 1507 (Cry1F) (Perry et

al., 2012). This model integrated a relationship between mortality and pollen dose based on laboratory bioassays with a relationship between dose and distance from a maize crop based on field measurements. Hence, Perry et al. (2010) derived predictions of mortality within a Bt-maize crop and at various distances from it into the field margins. The model structure distinguished between parameters relating to worst-case local exposure at small spatial and temporal scales (within-field and within the duration of anthesis) to large-scale effects (within-region; within-season; utilization rate of GM technology; allowance for physical effects and larval behaviour). It provides a novel structure by which exposure may be quantified for other GM crops, a variety of traits and a range of non-target lepidopteran species. The model generated realistic data for three widespread European species, the butterflies *Inachis io* (L.) and *Vanessa atalanta* (L.) and the moth *Plutella xylostella* (L.) in 11 representative maize ecosystems in four European countries and demonstrated that the likely impact of maize MON810 pollen on non-target lepidopteran populations is low.

The EFSA GMO Panel used the mathematical model of Perry et al. to simulate and assess potential adverse effects resulting from the exposure of non-target Lepidoptera to pollen from maize Bt11 under representative EU cultivation conditions (EFSA, 2011a). The GMO Panel concluded that risk mitigation measures may be needed under specific conditions (depending on e.g., sensitivity and occurrence of NT Lepidoptera, acreage of Bt-maize, host-plant density) in order to reduce the exposure of extremely sensitive NT Lepidoptera to maize Bt11 pollen. The EFSA GMO Panel further supplemented its previous risk management recommendations on maize Bt11 for cultivation by reapplying the mathematical model developed by Perry et al. (2010, 2011, 2012), in order to consider additional hypothetical agricultural conditions, and to provide additional information on the factors affecting the insect resistance management plan (EFSA, 2012a).

Based on these model predictions, EFSA provided risk managers with a set of risk mitigation measures (e.g., non-Bt-maize border rows around a Bt-maize crop, isolation distances from protected habitats to nearest Bt-maize field) to limit the exposure of NT lepidopteran larvae to Bt-maize pollen.

Following new information reported by Hofmann et al. (2014) concerning maize pollen deposition in relation to distance from the pollen source (see section 7.2.2.2), EFSA assessed the consequences for its previous risk assessment conclusions and risk management for Bt-maize (EFSA, 2015). In particular, isolation distances to protected habitats were reviewed. For non-target lepidopteran larvae of conservation concern potentially occurring in protected habitats, isolation distances of 20 m were recommended between protected habitats and the nearest fields of maize Bt11 and MON810.

In 2016, the EFSA GMO Panel assessed the relevance of the scientific publications presenting new data on pollen deposition and potential exposure of butterflies in protected habitats by Bt maize cultivation (EFSA, 2016). EFSA concluded that neither Lang et al. (2015) nor Hofmann et al. (2016) provide data indicating the necessity to revise the previous ERA conclusions and risk management recommendations for Bt maize made in EFSA (2015).

In a recent commentary, Kruse-Plass et al. (2017) respond to the EFSA GMO Panel's criticism of the conclusions of two previous articles by their research group (Hofmann et al., 2014; 2016). In their commentary, they reiterate that measurements of pollen dispersal indicate that there is a need for specific environmental impact assessments for Bt-maize cultivation in relation to distance from protected habitats.

Perry et al. (2017) responded to this commentary stating that there are no new data that refute the GMO Panel's criticism. The main points that apply to the Norwegian situation are the proximity to protected habitats for the endangered lepidopteran species in Norway and the degree of exposure to maize pollen grains containing Cry proteins. The latter point is affected by (i) the distance from the Bt maize crop, (ii) the amount of pollen deposited on the host plant of the endangered species and (iii) coincidence of maize pollen spread with the feeding larval stage of the endangered species.

In Norway, the maize cultivation is marginal. The total crop area of forage maize is estimated to 2000-2800 decares, equivalent to less than 0.1 % of the areas with cereal crops. The area of individual fields is limited by the topography such that the quantity of maize pollen produced under flowering is also limited. The potential impact/exposure of Cry1Ab containing maize pollen on non-target lepidopteran species in Norway is therefore negligible.

7.4.4 Effects on non-target soil arthropods

Springtails (Collembola) and mites (Acari) are key indicator organisms of soil fertility and health, as they are important in the breakdown and recycling of crop residues. In well-managed agricultural soils, populations of these microarthropods are generally abundant. Springtails and mites can be exposed to Cry proteins in crop residues, root exudates, live roots and associated fungi in the rhizosphere.

In general, no negative effects of the Cry1Ab, Cry1Ac and Cry2A proteins on springtails have been reported in the scientific literature (reviewed by Icoz & Stotzky, 2008).

Microbially produced purified Bt insecticidal proteins (Cry1Ab, Cry1Ac, Cry2A and Cry3A) were added at concentrations of 200 µ g/g fresh weight to the diet of the species *Folsomia candida* and *Xenylla grisea* for 21 days (Sims & Martin, 1997, ref. Icoz & Stotzky, 2008). In soils in the field, concentrations of Cry proteins in plant material exposed to soil organisms are usually lower and are estimated to be less than 30µ g/g. The results showed no effects on adult survival or reproduction compared with the unamended diet, and are consistent with the findings of Yu et al. (1997).

In a study by Yuan et al. (2013), roots, stems, and leaves of different Bt rice varieties expressing Cry1Ab and Cry1Ac were exposed to *F. candida* under laboratory conditions, with survival, reproduction and growth of the collembolan as ecological fitness parameters. Significant differences in ecological fitness were found among the different treatments, including differences in the plant parts and varieties of non-Bt rice, presumably as the result

of three factors: gene modification, plant parts and rice varieties. The fitness of *F. candida* was less affected by the different diets than by the exposure to the same materials mixed with soil. According to the authors, the results clearly showed that there was no negative effect of different Bt rice varieties on the fitness of *F. candida* through either diet or soil exposure.

In a laboratory toxicity study of the subacute effects of maize expressing Cry1Ab on springtails, Clark & Coats (2006) fed *F. candida* with ground up meal of leaves of Bt maize and corresponding non-Bt isolines. No deleterious effects on survival and reproduction of *F. candida* were observed. However, springtails receiving isoline material had significantly more offspring compared with those in the corresponding Bt line, but no other pairs were different. Time to reproduction of *F. candida* was only affected by the reference control treatment. The authors concluded that differences in growth of springtails were due to nutritional differences in the two varietal lines of maize, and not due to the Cry protein.

Bakonyi et al. (2006) showed that Bt maize was less preferred as food by *F. candida* than near- isogenic maize. However, this was not the case for other species of Collembola, i.e. *Heteromurus nitidus* and *Sinella coeca.* In a laboratory experiment, Heckmann et al. (2006) reported differences in springtail performance when they were reared on baker yeast versus maize, but no significant differences between Bt maize (Cry1Ab) and non-Bt maize. No significant differences in the population density of springtails were found in soils cultivated with Bt and non-Bt maize and between the application of an insecticide and no insecticide (Lang et al., 2006).

Griffiths and his partners in the EU-project ECOGEN investigated the effects of different soils collected from field sites in Denmark and France in which MON810 maize and non-Bt maize were grown. These studies, carried out in a glasshouse included an insecticide treatment, the pyrethroid deltamethrin, which increased the concentration of the Cry protein in MON810 (Griffiths et al., 2006). The reasons for this are unclear. The experiments evaluated effects on the two microarthropod groups collembola and mites by soil extraction at different plant growth stages. To investigate the effect on macroarthropods, swedes (*Brassica napus*) were grown in the soils from the maize and were inoculated with eggs of the cabbage root fly (*Delia radicum*). Neither the micro- nor macroarthropods were affected by the soil from MON810. Corresponding results on soil microarthropods have been published by Cortet et al. (2007). This study was carried out at four European locations (2 in France and 2 in Denmark). The Danish sites are comparable climatically to regions in Norway where maize is grown. Cortet et al. reported some significant negative effects of Bt maize on microarthropods in soils with a high clay content. The authors concluded however, that the slight differences in abundance of some soil microarthropods were most likely due to maize variety and not the Cry protein, and within the normal variation expected in conventional agricultural systems.

Potential effects of Bt maize expressing Cry1Ab on soil microarthropods (Collembola, Actinedida, Arcaridida, Gamasida and Oribatida) were assessed in a 4-month microcosm

study in the ECOGEN project (de Vaufleury et al., 2007). Total soil microarthropod abundance and diversity were similar between the conventional control and the Bt maize microcosms.

Bakonyi et al. (2011) conducted a multiple generation laboratory study to investigate the potential effects of long-term feeding of the springtail *F. candida* on Bt maize MON810 (0,6, 16 and 22 months). Significant differences were found in food consumption, egg production and food preference between the populations in some cases, but no time-response effect was observed. The authors concluded that long-term feeding on maize containing Cry1Ab seems not to have adverse effects on this species.

In a laboratory study of Bt rice expressing Cry1Ab protein, growth, development, reproduction, and superoxide dismutase activity (indicator of environmental stress) of *F. candida* were investigated (Bai et al., 2011). The springtail populations were reared on leaf tissue or leaf-soil mixtures of two CrylAb rice lines and a non-Bt rice isoline in two independent tests. No significant differences between the populations reared on Bt and non-Bt rice leaf tissue were detected in all measured parameters, suggesting no significant effects of the CrylAb protein in Bt rice on *F. candida*.

No negative effects of Cry proteins on mites have been observed (Icoz & Stotzky, 2008). Yu et al. (2007) fed the soil mite, *Oppia nitens,* fresh and old Bt cotton and Bt potato leaves expressing the Cry1Ab/Ac and Cry3A protein, respectively, as well as leaves of isogenic controls. After 7 weeks, no significant effects on oviposition, the number of eggs produced per female or final body length were observed.

The woodlouse *Porcellio scaber* is considered a model decomposer organism and has been a subject of a few studies on the effects of Cry proteins on isopods (Sims, 1997; Escher et al., 2000; Pont & Nentwig, 2005). Sims (1997) observed no effect of purified Cry2A protein on mortality and growth of *O. scaber.* In a laboratory feeding experiment with *P. scaber,* no adverse effects of Bt maize expressing Cry1Ab were found (Escher et al., 2000). *P. scaber* did not differ between Bt and the non- transgenic control in its food preference, and the number of offspring did not differ between the two maize varieties. In the study of Pont & Nentwig (2005), *P. scaber* was fed for 15 days on two different transgenic maize varieties expressing Cry1Ab. The Cry protein was detected in the body and faeces of *P. scaber,* showing that the woodlouse ingested and excreted the protein. No adverse effects of the protein on survival and growth of *P. scaber* were detected.

7.4.5 Effects on non-target aquatic arthropods

Byproducts from genetically modified plants (e.g. pollen, detritus) can be transported in water courses to downstream water bodies where non-target aquatic arthropods can be exposed to transgene products through consumption.

In the current literature, the environmental risk assessment of aquatic environments concerning the cultivation of GM crops is under discussion (BEETLE report, 2009; Carstens et

al., 2012; Holderbaum et al., 2015). So far, few studies have addressed the potential exposure of aquatic ecosystems to GM plant material and transgene products, and the potential impacts of Bt proteins on aquatic organisms (e.g. Douville et al., 2005, 2007; Rosi-Marshall et al., 2007; Griffiths et al., 2009; Jensen et al., 2010; Tank et al., 2010).

Exposure of non-target organisms to Cry proteins in aquatic ecosystems in Canada has been studied by Douville et al. (2005, 2007). In an initial study Douville et al. (2005) aimed to quantify levels of Cry1Ab endotoxin and locate its source in the environment. Agricultural soils and surface waters were spiked with crystals (biopesticide-Dipel®) or with pure Bt-maize endotoxin. Additionally, surface water, soils and sediments were sampled in an area sprayed with *Bt kurstaki* and at a site where maize expressing Cry1Ab protein was grown. The results showed that Bt-endotoxin was degraded more rapidly in water than in soils (4 and 9 days, respectively), while crystals appeared to be more resilient, as expected. The levels of Cry1Ab protein were generally below the detection limit, although it was detected at concentrations ranging from 0.1 to 1 ng/g in sediment and surface water, respectively. In a follow-up study the group spiked surface water and sediment of a surface water body with genomic maize DNA containing the *cry1Ab* gene (Douville et al., 2007). Samples from surface water and sediments were collected and tested for *cry1Ab* residues at different times during the growth season. The gene was detected 40 days after introduction in clay and sand-rich sediment. Persistence of the gene was significantly higher in the sediments than in the open water. Tank et al. (2010) reported occurrence of maize detritus and detectable levels of Cry1Ab protein (0.56 ng/mL) in the water column located less than 500 m from maize fields up to six months after harvest in water streams in the Midwestern USA.

Direct input of pollen and other by products from Bt maize into headwater streams nearby to maize fields cultivated with Bt maize in the Midwest of USA was investigated by Rosie-Marshall et al. (2007). They found evidence for transport of Bt containing maize residues downstream in the water bodies, but with respect to degradation rates of Bt containing plant litter no differences were found between Bt and non Bt-containing litter. On the basis of experimental data under laboratory conditions, Rosie-Marshall et al. claimed that this would reduce growth and increase mortality in larvae of caddisflies (Trichopterans), species that are closely related to Lepidoptera. Concentrations of Cry1Ab protein in leaves and pollen were not measured, so no dose-response relationship with the Bt-protein can be estimated (EFSA, 2009b). Measurement of growth rates of the caddisflies genera *Hydropsyche* and *Cheumatopsyche* in three streams draining fields planted with Bt maize did not show effects of Bt pollen on growth or mortality (Pokelsek et al., 2007).

In a study of exposure and effects of Bt maize on four non-target aquatic arthropods, Jensen et al. (2010) showed that input of maize detritus after harvest was extended over months in a stream adjacent to maize fields in USA. The study documented no bioactivity of Cry1Ab protein in senesced maize tissue after 2 weeks of exposure to terrestrial or aquatic environments, indicating rapid degradation of the protein. No toxic effects were observed on the larvae of caddisflies (*Lepidostoma* ssp. and *Pycnopsyche scabripennis*) when fed senesced leaf tissues of maize expressing Cry1Ab. However, Jensen et al. proved that near-

isolines modified growth and survivorship of crane fly (*Tipula abdominalis*) and the isopod *Caecidita communis* in the control groups. These effects were attributed to tissue-mediated differences among the isogenic line treatments.

Laboratory experiments performed by Bøhn et al. (2008, 2010) revealed that *Daphnia magna* fed a suspension of 100 % maize MON810 flour had a higher mortality and reduced fitness as compared to the control group. However, it is unclear whether the delays in development of the water fleas were caused by nutrient deficiencies related to the feeding regime or the presence of Cry1Ab protein (EFSA, 2012c; Ricroch et al., 2010).

In a similar experiment, Holderbaum et al. (2015), fed a chronic high dose of ground leaf material from MON810 maize, compared to a chronic high dose of a near isogenic maize or the normal diet of algae to *D. magna*. Those that were fed on their normal diet of algae had a mortality rate of 20% at 21 days, while the mortality on both types of maize was higher, indicating that maize is deficient in some nutrients that *D. magna* needs or is unable to assimilate. Comparing only the MON810 maize and the near isoline, there were no significant differences in survival or survival time, although the median survival time of *D. magna* fed MON810 was slightly longer than those fed on the isoline. There was little difference in body size between the two maize diets until the *D. magna* were beyond their median lifespan. After that time, a significant number of *D. magna* fed on MON810 had a slight reduction in body size (<5%) compared to those fed on the near isoline. There were no significant differences in incidence of reproduction or age at first reproduction between the two populations fed on the two varieties. There were significant differences in the stage fecundity, with the *D. magna* that were fed MON810 reaching peak reproduction earlier. There were no significant differences between cumulative fecundity rates at any given time. A small number (ca. 10%) of the *D. magna* produced ephippia (resting stage eggs produced under adverse conditions), 14 of these were from the MON810 diet and 4 were from the near isoline diet. The authors suggest that the differences between the *D. magna* fed MON810 and those fed the near isoline can be due to the Cry1Ab proteins or an effect of nutritional differences in the MON810.

In a case study, Carstens et al. (2012) identified exposure pathways and calculated early tier exposure estimates for Bt maize in aquatic ecosystems. Established models and worst-case assumptions were applied, and the resulting EECs for aquatic organisms were low. The shredders were identified as the functional group most likely to be exposed to insecticidal proteins. However, even using worst-case assumptions, the exposure of shredders to Bt maize was low. The research group concluded that because the potential exposure of aquatic particle feeders, predators and shredders to insecticidal proteins in current Bt crops is very low, additional hazard testing would provide useful information for the environmental risk assessments.

7.4.6 Effects on non-target organisms that are not arthropods

Maize Bt11 may have potential direct or indirect adverse effects on non-target organisms that are not arthropods, as well as the ecological functions they provide. Potential adverse effects on soil microorganisms are considered in section 5.6.2, while this section focuses on earthworms, enchytraeid worms, nematodes and molluscs.

Annelida (earthworms and enchytraeid worms)
Earthworms and enchytraeid worms play an important role in decomposing plant litter, and are responsible for numerous physical changes that affect the biological properties and processes in soil (e.g. structure, quality, functionality) (EFSA, 2011d). These species are considered important organisms in the regulation of nutrient cycling processes. As Cry proteins can enter the soil by root exudates, plant material and by plant residues (Icoz & Stotzky 2008), earthworms and enchytraeid worms can be exposed to Cry proteins.

According to reviews of Icoz & Stotzky (2008) and the BEETLE Report (2009) studies to date have found no or few significant effects of Bt maize on survival, growth and reproduction on the earthworm species *L. terrestris, E.fetida* and *A.caliginosa*.

Impacts of Bt maize expressing Cry1Ab on the earthworm species *L. terrestris* have been studied in the laboratory and under semi-field conditions (e.g. Saxena & Strotzky, 2001b; Zwahlen et al., 2003b; Lang et al., 2006; Zeilinger et al., 2010). None of the studies showed consistent effects on *L. terrestris.* Overall, laboratory experiments with adult earthworms feeding on either Bt- or non-Bt maize litter, showed no significant difference in weight change between the two treatments.

In a study by Saxena & Stotzky (2001b), no significant differences in percent mortality or weight of earthworms were detected after 40 days exposure to root exudates in soils planted with Bt maize (Cry1Ab). Corresponding results were found after 45 days in soil amended with residues from Bt maize. It was nonetheless evident that Cry proteins were taken up as they were detectable in the casts as well as the guts of earthworms. Within two to three days after placing earthworms in fresh soils, the toxins, however, were cleared from the gut.

Zwahlen et al. (2003b) showed that mortality and weight of adult and juvenile earthworms were not significantly different when fed Bt or non-Bt maize residues over 160 days, with the exception that after 200 days, adults fed Bt maize residues had a significant reduction in weight (18 %) compared to those fed non-Bt maize. Under semi-field conditions, no significant differences in growth patterns were observed in immature earthworms feeding on Bt or non-Bt litter (Zwahlen et al., 2003b).

Lang (2006) found no significant differences in population density or biomass of *Lumbricidae* earthworms in soils planted with Bt maize or non-Bt maize and between soils with maize either treated or not treated with insecticide. The field experiment, which was conducted at five sites during four growth seasons, showed that field site and sampling years had greater

effect on population density and biomass of the earthworms than the presence of Cry protein.

Clark & Coats (2006) conducted laboratory toxicity studies to determine the sub-acute effects of Cry1Ab in maize litter on non-target soil organisms. No significant differences in survival and growth of compost worm (*Eisenia fetida*) were detected between transgenic and isogenic maize residue consumption. In a corresponding Danish study, leaf or root exudates from Bt maize had no deleterious effects on survival, growth, development or reproduction of the grey worm *Aporrectodea caliginosa* var. *tuberculata*, probably the most abundant species in agricultural soils in the temperate climate zone (Vercesi et al., 2006). However, a slight, but statistically significant negative effect of Bt maize residues on cocoon hatchability was observed. Field studies in Denmark and France on responses by earthworms to reduced tillage in herbicide tolerant maize and Bt maize cropping systems, did not show significant effects of Bt maize expressing Cry1Ab on biomass and abundance of different earthworm populations (Krogh et al., 2007a).

In a field study conducted in USA over four years, Zeilinger et al. (2010) did not observe significant differences in numbers and biomass of juvenile and adult individuals of four earthworm species (*Aporrectodea caliginosa, A.trapezoides, A.tuberculata* (collectively the *A. caliginosa* complex), and *L. terrestris*) in the soil of Bt maize varieties expressing Cry1Ab and Cry3Bb1 proteins and non-Bt maize. However, Zeilinger et al. underline that only a small number of earthworm species that are likely to be exposed in the field have been investigated in this and previous studies. Considering the difficulty in extrapolating effects and the low species diversity of earthworm communities in maize agroecosystems in temperate climates, these data do not merit any general conclusion on the effects of Bt maize on earthworms.

The fate of insecticidal Cry1Ab protein from crop residues (leaves and roots) of the transgenic maize variety MON 810 expressing Cry1Ab, was studied by Schrader et al. (2008) in the presence and absence of two earthworm species (*L. terrestris* and *Aporrectodea caliginosa*) in soil microcosms (artificial ecosystem). All earthworms survived in the microcosms over a period of 5 weeks, irrespective of whether they received transgenic or non-transgenic plant material. Weight loss was observed for both earthworm species, independent of the plant material. A strong decline of immunoreactive Cry1Ab in plant residues of MON810 was observed in all treatments, but in microcosms with earthworms this decline was significantly higher with less than 10 % of the initial Cry1Ab concentration remaining after 5 weeks. No immunoreactive Cry1Ab protein was found in earthworm tissues.

In a study of Shu et al. (2010), *E. fetida* were bred in substances with stover of Bt maize expressing Cry1Ab protein (MON810, Bt11) and their corresponding near-isogenic varieties. More than 90% of the individuals of *E. fetida* survived over a period of 30 d, irrespective of whether they received Bt or non-Bt maize. ELISA results indicated immunoreactive Cry1Ab in

casts and guts of the earthworms from Bt maize treatments. However, no significant deleterious effects on survival rate or reproduction were reported.

Hönemann & Nentwig (2009) analysed survival and reproduction of the enchytraeid worm *Enchytraeus albidus*, fed with diets containing Bt maize litter (Cry1Ab, Cry3Bb1). For the Cry1Ab treatment, survival was significantly higher than for the treatment with the corresponding near-isoline. In contrast, reproduction was significantly lower for the Cry1Ab compared to the isoline. According to Hönemann & Nentwig the transgenic variety expressing Cry1Ab was less degradable compared to the control, and suggested a variety effect on life history traits of *E. albidus.* Naturally enchytraeids do not feed on a single food source, but take up all degradable organic matter of adequate size in the soil. It is therefore not expected that Cry1Ab-expressing maize will endanger the survival or reproduction of *E. albidus,* provided that organic matter of sufficient quality is available in the soil (Hönemann & Nentwig, 2009). For the Cry3Bb1 treatment, no effect was shown on survival or In a

In a laboratory study with *E. fetida*, no acute toxicity was observed after exposure to senescent Bt maize leaf tissue (Cry1Ab) (Whiting & Lydy, 2015).

Nematodes

Nematodes are considered particularly good bio-indicators for assessing soil quality, due to their great diversity and participation in many functions at different levels of food webs in soil and due to their presence in virtually all habitats with a high population density and a large number of species (ref. EFSA, 2011d).

Studies on the effects of Cry proteins on soil nematodes have shown different results (reviewed by Icoz & Stotzky, 2008). Impacts of Cry1Ab proteins on nematodes were examined in four studies using soil samples from fields planted with Bt maize and near-isogen control (Saxena & Stotzky 2001b; Griffiths et al., 2005; Höss et al., 2008). Results from the study of Saxena & Stotzky (2001b) indicated that there were no significant differences in the number of nematodes between rhizosphere soil of Bt and Bt maize grown in a plant-growth room. In a field experiment comparing Bt maize expressing the Cry1Ab protein with near-isogenic non-Bt maize, Manachini & Lozzia (2002, ref. Icoz & Stotzky, 2008) reported no overall significant influence on communities and biodiversity of nematodes. However, in one of the eight study regions, fungi feeding nematodes were found to be more abundant in the field with transgenic maize, while bacteria-feeding nematodes were more abundant in the field cultivated with the isogenic hybrid.

In field studies over two years conducted in the ECOGEN project covering different soil types and distinct climatic zones (three European sites), MON810, the near-isogenic non-Bt cultivar, a conventional maize cultivar and plots of grass were evaluated (Griffiths et al., 2005). In all sites, nematode numbers, as well as of protozoa, associated with the transgenic variety were reduced. Nematode community structure was different at each site and the Bt effect was not confined to specific nematode taxa. It was concluded that the effect of the Bt maize was small and fall within the normal variation expected in these agricultural systems. In later studies, Griffiths et al. (2006, 2007 a,b) concluded that effects on soil nematode

abundance by Cry1Ab-expressing maize was not related to the Bt trait, but more likely to the effects of agricultural practices, environmental stresses or differences between localities and maize varieties.

In a study of maize MON 810, significant effects were found on reproduction and growth of *Caennorhabditis elegans* in rhizosphere and bulk soil from fields with Bt maize expressing Cry1Ab compared with soils from fields with the near-isogenic variety (Höss et al. 2008). According to the authors, the observed effect of the soil samples on the nematodes could not be explained by a direct toxicity of the Cry1Ab, however, the toxicity of the pure Cry1Ab protein to the reproduction and growth of *C. elegans* was concentration-dependent

Unpublished results from a German study on the effects of Bt maize MON89034 x MON 88017 (Cry1A.105, Cry2Ab2, Cry3Bb1) on nematodes showed that the incidence of nematodes fluctuated slightly on all plots over the course of the study (http://www.gmo-safety.eu). On most of the sampling dates no significant differences between the maize varieties were detected. A significant difference was found between the number of nematodes on the Bt maize plots and on the conventional plots only on the last sampling date. The composition of the nematode communities in the field was assessed by classifying the nematodes according to food type (plants or bacteria) and according to reproductive strategy. The authors reported a change in the composition of the different food types in all plots during the growing season, with one exception, there were no significant differences between the different maize varieties. In terms of reproductive strategy, with one exception, no significant differences were observed between the different varieties. *C. elegans* exposed to aqueous Cry1A.105- Cry2Ab2- and Cry3Bb1-containing solutions and in equimolar (1:1) mixtures showed a dose- dependent inhibitory effect for all three proteins and protein mixtures on growth and reproduction. Cry3Bb1 displayed the highest toxicity, followed by Cry1A.105 and Cry2Ab2.

Molluscs

Slugs can be abundant and play an important role in the food web of maize ecosystems as prey of spiders, carabids, birds and hedgehogs. In a study of effects of Bt maize material (Cry1Ab) on the life cycle of the land snail *Cantareus aspersus,* snails exposed to Cry protein in food and soil had a growth coefficient 25 % lower than unexposed snails after 47 weeks of exposure (Kramarz et al., 2009). After the first period of reproduction (68 weeks) a significant difference remained for body mass between the two groups. Differences in body mass were not significant at the end of exposure (88 weeks).

In a laboratory experiment with two transgenic maize varieties expressing Cry1Ab and Cry3Bb1, a potential impact of Bt maize was examined for the non-target slug *Arion vulgaris* (Hönemann & Nentwig, 2010). Lifespan after field collection, weight change and oviposition was examined for slugs fed with Bt maize, conventional control or dandelion (*Taraxacum offiscinale*). Test parameters were neither significantly different between transgenic and comparator nor among the maize varieties overall over an exposure period of 16 weeks. These results are in compliance with previous studies on effects of Cry1Ab and Cry3Bb1 on

A. lusitanicus and *Deroceras reticulatum* (Zurbrügg & Nentwig, 2009). Cry proteins were detected in the gut and faeces, but no differences in biomass or leaf consumption were observed between the treated and untreated groups.

7.4.7 The Norwegian red list of threatened species

The 2015 Norwegian Red List for species (www.artsdatabanken.no) (Henriksen & Hilmo, 2015) contains 459 Lepidoptera, a decrease of 3 species from the Red List published in 2010. 172 of these taxons are categorised as critically endangered (CR) or endangered (EN), and thus have an extremely or very high risk of extinction. Most of the species are red listed due to a narrow host range, limited distribution range and a reduction in/disappearance of accessible habitats for their host plants. Most species on the Red List live in open habitats, which are either becoming overgrown or being affected by increasing use of monoculture. As the CR and EN species' habitats and host plant range do not include agricultural crops, exposure to the Cry protein in maize Bt11 would be extremely low or none if this maize were to be cultivated in Norway.

Although the Cry protein expressed in maize Bt11 is toxic to a wide range of Lepidoptera, none of the endangered species in Norway feed on maize plants. Among the red listed Lepidoptera categorized as endangered, only two species live on grasses in the vicinity of agricultural areas. *Euthrix potatoria* prefer habitats with open woodlands and wetlands, where the larvae feed on various grass species and reeds. The species are threatened because of severe fragmentation and decline in accessible habitats. Threats to *Coenonympha hero* (the Scarce Heath) are primarily related to changes in farming methods and in land use practices. The species is favoured by lightly managed hay meadows, and is negatively affected by both agricultural intensification and overgrowth (Endrestøl & Bengterson, 2012). The Scarce Heath is listed on the Bern Convention ("strictly protected fauna species-list") and was also protected by law in Norway in 2001.

As none of these endagered species occur in agricultural crops and they do not feed on maize, cultivation of maize Bt11 is not considered to represent a threat to the prevalence of these endangered species in Norway.

7.4.8 Conclusion

Based on a review of available scientific literature VKM concludes that the likelihood of adverse effects of Cry1Ab protein from cultivation of GM maize on non-target organisms in Norway is negligible.

7.5 Impacts of the specific cultivation, management and harvesting techniques

The PAT protein expressed in maize Bt11 has been used as selectable marker to facilitate the selection process of transformed plant cells and is not intended for weed management purposes. As the scope of the notification does not cover the use of glufosinate-ammonium-containing herbicides, and maize Bt11 will not be marketed in the EEA as a herbicide-tolerant crop, potential environmental adverse effects due to the applications of these herbicides and possible changes in weed management has not been assessed by VKM in this opinion.

Apart from changes in insecticide regimes, there are no anticipated changes in cultivation practices, management or harvesting techniques associated with the cultivation of maize Bt11. Bt crops, such as maize Bt11, may reduce the use of insecticides and may cause changes in crop rotations in response to reduced pest pressure (ref. EFSA 2011d). However, this reduction in pesticide use and narrow spectrum of activity of Cry proteins may provide an opportunity for secondary pests, previously controlled by insecticides used against key target pests, to reach damaging levels. This is reported for mirid bugs in Bt-cotton in China (Lu et al., 2010). Natural enemies failing to fully control secondary pests, and reducing competition with target pests might also play a role in secondary pest outbreaks (ref. EFSA, 2011d). Incidence of secondary pests and the environmental consequences of changes in management measures are highly dependent upon farming systems and regional environmental factors. The implementation of insect resistance management strategies is desirable to delay or prevent the potential evolution of insect resistance to Cry1Ab in lepidopteran target pest populations.

As there are no pests which are controlled by Bt-based insecticides, cultivation of maize Bt11 will not lead to changes in cultivation practices in Norway.

7.6 Effects on biogeochemical processes

7.6.1 Fate of Bt-proteins in soil

Cry protein expressed in Bt crops can enter the soil system via root exudates released into the rhizosphere throughout the growth of the plant, and via senescent plant material remaining in the field after harvest and incorporated into the soil during tilling operations (Icoz & Stotzky, 2008; BEETLE Report, 2009). Beside root exudates and plant residues, pollen is another source of Bt proteins entering soils (e.g. Losey et al., 1999). Additionally, Cry proteins are found in the gastrointestinal tract of cows and their feces, as well as in the feces of decomposers (rew. Icoz & Stotzky, 2008).

The stability, persistence and potential accumulation of the Cry proteins in soil are key factors for determining exposure and potential effects on soil biota related to the soil function. Persistence of Cry proteins in soil is primarily dependent on the protein quantity added and on the rate of inactivation and degradation by biotic and abiotic factors (Sanvido et al., 2006; Helassa et al., 2010).

Degradation of Cry proteins are known to be influenced by different factors like type of crop, microbial communities, environmental conditions like the soil surface versus below the soil surface, temperature, pH, moisture, etc. (Sanvido et al., 2006; Icoz & Stotzky, 2008). Furthermore, various salt and hydroxides in soil may alter Cry proteins levels in the ecosystem. The soil accumulation of Cry proteins depends on their absorption onto soil components, and bioavailability. In particular the absorption of Cry to soil components are little understood (Singh & Dubey, 2016). For evaluation of Cry1Ab degradation in soil, these factors should be taken in consideration.

Persistence, degradation and accumulation of Cry proteins in the soil has been assessed in a number of laboratory and field studies. However, reviews of the scientific literature reveal various results with regards to the persistence of Cry proteins. The majority of the studies have been conducted with Bt maize expressing Cry1Ab. From studies dealing with potential impacts of Bt maize on soil processes and communities, some reveal a lower decomposition rate of residues of Bt crops compared to non-Bt crops (e.g. Flores et al. 2005; Saxena & Stotzky 2001a; Zwahlen et al. 2003a,b), while other laboratory and field studies show absence of negative effects of Cry proteins on decomposition processes and microbial community structure (e.g. Hopkins & Greogorich, 2003, 2005; Devare, 2004, 2007; Zwahlen et al, 2007; Hönemann et al., 2008; Zurbrügg et al. ,2010; Gruber et al. 2012).

The Cry1Ab protein released in root exudates of Bt maize persisted in soil microcosms for at least 180 days and for at least three years from biomass of Bt maize (Saxena & Stotzky, 2002; Stotzky 2002, 2004). Zwahlen et al. (2003a) has published the results from two Swiss field studies where the decomposition of the Cry1Ab protein from leaf of Bt11 maize was recorded through autumn, winter and spring for a period of 200 days. At the end of the experimental period, 0.3% of the original proteins were still present in the soil.

Flores et al. (2005) investigated the decomposition of various species expressing Cry 1Ab protein, and discussed the results in relation to the lignin content and potential environmental impacts. The authors concluded that Bt maize had higher lignin content than the conventional counterpart, and decomposed less in soil compared to non-Bt maize. Another study with different maize lines expressing Cry1Ab (MON 810, Bt11), showed no differences in lignin content of 12 Bt maize hybrids and isogenic non-Bt maize (Jung & Scheaffer, 2004).

In the ECOGEN project, Cortet et al. (2006) investigated the effects of Cry1Ab protein on decomposition of wheat straw in three climatically different areas in Europe (Denmark, France). In the field-incubation trial, the Bt-maize and conventional, near-isogenic lines were grown on 3 different soils and according to common cultivation practices. Results after 4 months showed that decomposition and mineralisation of organic matter were mainly driven by climatic parameters with no adverse effect of Bt proteins on these processes.

Devare (2004, 2007) reported no differences in N-mineralising potential, nitrification rates and soil respiration between fields planted with either Bt or non-Bt maize. Corresponding

results have been reported by Hopkins & Gregorich (2003, 2005) and Dubelman et al. (2005). These studies showed that the Cry1Ab protein do not persist in biologically relevant concentrations in soil 3 months after harvest, and they found no evidence of accumulation of the Cry1Ab protein in soil from fields planted for at least 3 consecutive years with Bt maize, regardless of soil type, geographical region or climatic conditions.

In a field experiment, Zurbrügg et al. (2010) studied decomposition of leaf residues from three Bt maize cultivars expressing Cry1Ab and Cry3Bb1, corresponding near-isogenic lines and three conventional hybrids using litterbags. The Cry protein concentrations in maize leaf residues were measured from harvest to the next growing season. The C:N ratios of Bt maize differed from their corresponding near-isolines, but more pronounced differences in C:N ratio, lignin, cellulose and hemicellulose content were present among conventional cultivars. Consequently, the decomposition dynamics of transgenic hybrids were similar to the non-transgenic near-isolines, but varied among conventional hybrids, demonstrating that Bt maize hybrids lie within the variation found in conventional maize agroecosystems. Expression levels and degradation patterns were different for Cry1Ab and Cry3Bb1, but leaf residues and Bt protein concentrations decreased rapidly in all Bt maize hybrids. Thus, non-target soil organism were exposed to relatively low Bt protein concentrations within a few months after harvest, and Zurbrügg et al. concluded that there is no indication of ecologically relevant, adverse effects on the activity of the decomposer community.

Helassa et al. (2010) investigated the adsorption properties, the mobility of the adsorbed protein and the decline of the Cry1Aa protein as a function of time and microbial activity in contact with various soils and soil minerals. No mobility of adsorbed toxin was observed at any pH and at different degrees of surface saturation.

Gruber et al. (2012) investigated the fate of Cry1Ab protein in soil under long-term Bt maize cultivation in an experimental field trial performed over nine growing seasons on four field sites in Germany. The results from this study showed that on any of the four sites the climatic and field conditions led to complete degradation of the Bt-maize plant material containing the recombinant Cry1Ab protein by the following growth season. No persisting immunoreactive Cry1Ab protein was detected in any soil shortly before the next seeding over the experimental period of three years, which comprised the last third of nine years of Bt-maize planting. No experimental evidence for accumulation or persistence of Cry1Ab protein in different soils under long-term Bt-maize cultivation could be drawn from this field study.

Zeng et al. (2014) found that, the Cry1Ab protein concentration in root samples was higher than those in rhizospheric soil and bulk soil samples, although the difference did not reach significant in all samples at any sampling time point. The authors concluded that Cry1Ab protein decreased rapidly in soils treated with Bt maize straw. Another study performed by Zeng et al. (2015) concluded that the Bt protein that was released from the roots of the Bt maize may have been maintained in the soils because of the persistence and activity of Bt protein. However, the much lower Bt protein content in the rhizospheric soils and bulk soils than in the harvested roots suggests that there was no apparent accumulation of the Cry1Ab

protein that had been released from 5422Bt1 and 5422CBCL in the soils. Another reason may be the difficulty of extracting the Cry1Ab protein bound to soil clays and humic acid using the PBST method, which results in the detection of lower concentrations of this protein in soils. The studies performed by Zeng et al. (2014, 2015) lack information regarding the time span regarding sampling and determination of Cry1Ab concentration. Both investigations were field studies and performed in China.

In a recent study performed by Shu et al. (2017), the authors concluded that the concentration curves of the Cry1Ab protein in soil without earthworms from 5422Bt1 and 5422CBCL treatments were similar over time, where there was a sharp decline from 15 to 30 d and a slow decrease from 30 to 90 d. On the 15th, 60th, 75th and 90th d, Cry1Ab protein concentrations in 5422Bt1 treated soil were significantly higher than those in 5422CBCL treated soil. The study was performed in a greenhouse in China.

As earlier mentioned, degradation of Cry proteins depends on different factors like type of crop, microbial communities and environmental conditions (e.g. temperature, pH, moisture, salt and hydroxides in soil). In the above mentioned studies, sufficient data regarding these factors have not been provided and discussed appropriately. Therefore, we cannot draw any conclusive conclusion regarding stability of the Cry1Ab in these studies, although all three studies confirm a decline of Cry1Ab concentration in soil, which indicate Cry1Ab is not stable in soil.

7.6.2 Effects on soil microorganisms

Microorganisms are the dominant organisms both in terms of biomass and activity in the soil. The soil microbiota is involved in a number of important processes including decomposition of organic matter, nutrient mineralisation, regulation of plant pathogens, decomposition of agricultural chemicals and the improvement of soil structure (ref. Sanvido et al., 2006; BEETLE Report et al., 2009). Due to the close interaction between crop cultivation and soil processes, soil organisms in the rhizosphere are likely to be exposed to the Cry proteins released from root exudates and decaying plant material.

There have been numerous studies, with different methods (e.g. functional and structural composition of soil microbial communities) and different crops on the effects of Bt plants on soil microbial communities. Different effects, ranging from no effect to significant small transient negative effects on rhizosphere organisms (soil protozoa and microorganisms) have been reported (reviews by Sanvido et al., 2006; Icoz & Stotzky, 2008; BEETLE Report, 2009; Stefani & Hamelin, 2010). Data are however only available from short-term experiments and predictions of potential long-term effects are difficult to deduce. Based on available literature, The BEETLE Report (2009) concluded that the likelihood of adverse effects of Bt maize in the EU is low. However, uncertainties remain regarding mycorrhizal fungi.

Fungi

Root exudates of Bt maize (event Bt176) have been shown to reduce presymbiotic hyphal growth of the arbuscular mycorrhizal fungus *Glomus mosseae* compared with root exudates of another Bt maize hybrid (event Bt11) and conventional control (Turrini et al., 2005). A higher level of Cry1Ab protein was measured in the event Bt176 (80.63 Cry1Ab/g protein) that negatively affected *G. mosseae* compared to Bt11 (<0.55 Cry1Ab/g protein) and the authors stated that their findings could possibly be explained by the expression levels of Cry1A. Castaldini et al. (2005) have also reported consistent differences in rhizosphere heterotropic bacteria and mycorrhizal colonisation (including *G. mosseae*) between Bt-maize expressing Cry1Ab (Bt176, Bt11) and its conventional counterpart. In both transformed lines the intraradical colonisation of *G. mosseae* was significantly lower (about 50%) compared to wild type after 8 and 10 weeks of interaction under controlled conditions. The percentage of root length colonised by arbuscular mycorrhizal fungi was significantly lower in *Medigaco sativa* grown for four months in soil containing Bt11 residues. The reasons for which Bt maize were less susceptible to endomycorrhizal colonisation remain unknown (Stefani & Hamelin, 2010).

Cheeke et al. (2012) investigated the impact of several Bt-maize events on symbiotic arbuscular mycorrhizal fungi (AMF) under greenhouse potting conditions. The authors observed lower levels of AMF colonisation in the roots of Bt maize, as compared with the non-Bt-maize (parental) lines. The reduced mycorrhization was not related to the expression of a specific Bt-toxin, but may be the result of other factors such as unintended changes in Bt-maize due the genetic modification process. The authors themselves state that scientific uncertainty remains on how the reported observations translate to the field situation, as low levels of fertilisation had to be applied during the experiment to favour mycorrhization (Verbruggen et al., 2012). With the example of Cry1Ab-expressing maize event Bt11, the authors demonstrated in a previous study that differences between the Bt-maize and non-Bt-maize in fact disappeared when fertilisers were added to soil (Cheeke et al., 2011). For the cultivation of maize, in which normally larger amounts of organic or inorganic fertilisers are added to improve maize yield, the effects as observed by Cheeke et al. (2011) are therefore most likely insignificant (EFSA, 2012b). Furthermore, under common agricultural practices, the contribution of AMF to improve health or increase yield of maize appears to be negligible or not existent (e.g. as reviewed by Ryan & Kirkegaard, 2012).

In 2014, Cheeke et al. published a study of effects of Bt maize cultivation history on arbuscular mycorrhizal fungal colonization, spore abundance and diversity, and plant growth. In this field experiment, the authors used split plots to evaluate the effect of Bt maize or non-Bt maize cultivation history on AMF spore abundance, diversity, root colonization, and growth of seven different genotypes of Bt maize expressing Cry1F, Cry1Ab, Cry34/35Ab and Cry3Bb1 and five corresponding non-Bt parental isolines. Cheeke et al. found that Bt plants had higher leaf chlorophyll content when they were grown in plots that had been cultivated with Bt maize the previous year, and similarly, non-Bt plants had higher chlorophyll content when they were grown in plots with a non-Bt cultivation history, indicative of a positive feedback effect. There was a lower density of AMF spores in plots with a Bt maize cultivation history than in plots where conventional maize had been grown in the previous year, but no

difference in spore diversity.There were no significant differences in AMF colonization or root or shoot biomass between plots with a cultivation history of Bt and conventional maize. The study was a field investigation and was performed in USA.

In a follow-up-study, Cheeke et al. (2015) evaluated the effects of Bt maize and their corresponding non-Bt parental isolines on AMF colonization and community diversity in plant roots. The research group used seven different genotypes of Bt maize that exhibited reduced AMF colonization in previous greenhouse studies. The Bt genotypes differed in the Cry protein expressed (Cry1Ab, Cry34/35Ab1, Cry1F + Cry34/35Ab1, Cry1F, Cry3Bb1). Plants were harvested 60 days after sowing, and data were collected on plant growth and percent AMF colonization of roots. AMF community composition in roots was assessed using 454 pyrosequencing of the 28S rRNA genes, and spatial variation in mycorrhizal communities within replicated experimental field plots was examined. Growth responses, per cent AMF colonization of roots and AMF community diversity in roots did not differ between Bt and non-Bt maize, but root and shoot biomass and per cent colonization by arbuscules varied by maize cultivar. The authors concluded that spatial soil heterogeneity in the field has a greater effect on the structure of AMF communities and plant growth than host plant cultivar or modification by Cry protein genes. The study was a field investigation and was performed in USA.

Hurej et al. published in 2013 results from field experiments in Poland in the time period 2008–2010. Aphids infected with fungi were counted in situ on 18 plants per plot every two weeks through each of the growing seasons, using culturing method. Cry1Ab insecticidal protein had no effect on the incidence of entomopathogenic fungi infecting aphids on Bt maize. The number of fungi-infected aphids and their time of occurrence were similar on the three compared cultivars. The same three or four species of entomopathogenic fungi-infected aphids were found in each treatment.

Verbruggen et al. (2012) studied the potential effects of maize expressing Cry1Ab on mycorrhizal fungal communities via DNA- and RNA-based pyrosequensing molecular fingerprinting. In order to compare AM fungal communities between GM and non-GM plants, seeds were sown into pots that contained soil from a field. The research yielded three major conclusions. First, no consistent differences were detected between AM fungal communities associated with GM plants and non-GM plants. Second, temporal variation in AMF community composition (between two measured time points) was bigger than GM trait-induced variation. Third, natural variation of AMF communities across 15 agricultural fields in The Netherlands, as well as within field temporal variation, was much higher than GM-induced variation. In conclusion, the authors found no indication that Bt maize cultivation poses a risk for AMF. The study was performed in the Netherland.

In a field study from China, Zeng et al. (2014) compared the diversity and composition of the AMF communities between two Bt maize events (Bt11 and MON810) and their conventional counterparts after cultivation for five seasons. The diversities of AMF communities did not consistently differ significantly in soils and roots of subsequently planted

conventional maize grown with Bt maize straw at three sampling stages (seedling, large bell, and maturity stages). Plant growth stage had a greater influence on AMF diversity than Bt traits. In conclusion, cultivation of non-Bt maize on soils previously cultivated with Bt maize for five seasons had minor effects on AMF communities.

A greenhouse experiment was conducted to assess the impact of five seasons of continuous Bt maize cultivation on the colonisation and community structure of AMF in the maize roots, bulk soils and rhizospheric soils using the terminal restriction fragment length polymorphism (T-RFLP) analysis of the 28S ribosomal DNA and sequencing methods (Zeng et al., 2015). AMF colonisation was significantly higher in the two Bt maize lines expressing Cry1Ab (Bt11, MON810) than in the conventional control. A clustering analysis based on the DNA sequence data suggested that the sample types (i.e., the samples from the roots, bulk soils or rhizospheric soils) might have greater influence on the AMF community phylotypes than the maize cultivars. This study indicated that the Cry1Ab protein has minor effects on the AMF communities after five seasons of continuous Bt maize cultivation. The investigation was a field study and was performed in China.

Bacteria and fungi

Results from the ECOGEN project revealed that the small effects of Bt maize or a conventional insecticide on protozoa and microorganisms were less pronounced than effects due to soil and plant growth stage (Griffiths et al., 2006), and less than the variation seen between the eight maize cultivars (Griffiths et al., 2007b). No effects could be attributed to the Bt maize on mycorrhizal fungi in a separate mesocosm experiment (de Vaufleury et al., 2007). These field experiments, point to the conclusion that Bt maize (Cry1Ab) could have a significant, but small and transient, negative effect on soil protozoa and microorganisms (Griffiths et al., 2005, 2007a), but no effects on organic matter (wheat straw) decomposition (Cortet et al., 2006). EGOGEN developed a quantitative model to summarise the effects of the different cropping systems on soil quality (Bohanec et al., 2007). The authors concluded that Bt maize did not have deleterious effects on the soil biota, and that factors such as plant growth stage, season, soil type, tillage, crop type or variety produced larger effects on soil microbial community structures than the Bt maize (Griffiths et al., 2007b; Krogh et al., 2007b).

Cotta et al. (2013) evaluated temporal dynamics of microbial communities in the rhizosphere of two GM maize hybrids in tropical agrosystems using PCR-DGGE profiles. The study aimed to evaluate the possible effects of Bt maize expressing Cry1Ab and Cry1F protein, in comparison to the parental line, on the structure and abundance of microbial communities in the rhizosphere. Moreover, the effect of soil type was addressed. For this purpose, the bacterial and fungal communities associated with the rhizosphere of GM plants were compared by culture-independent methodologies to the near-isogenic parental line. Two different soils and three stages of plant development in two different periods of the year were included. As evidenced by principal components analysis (PCA) of the PCR-DGGE profiles of evaluated community, clear differences occurred in these rhizosphere communities between soils and the periods of the year that maize was cultivated. However, there were no

discernible effects of the GM lines as compared to the parental line. For all microbial communities evaluated, soil type and the period of the year that the maize was cultivated were the main factors that influenced their structures. No differences were observed in the abundances of total bacteria between the rhizospheres of GM and parental plant lines. The investigation was a field experimental study and performed in Brazil.

Bacteria

Prischl et al. (2012) compared the endophytic bacterial communities in plants of the transgenic Bt maize lines MON810, MON 88017 (*cry3Bb1*) and the stacked event MON 88017 x MON810, with those of the respective near-isogenic line and three additional conventional maize lines. The maize plants were grown in a containment system on two different soils that were commonly used for maize cultivation in Lower Austria. 700 bacterial endophytes were obtained and characterised regarding their phylogenetic diversity and specific plant growth promoting functions. Both the soil environment and the plant cultivars had an effect on the phylogenetic diversity of the endophytic communities, but there were no specific effects of the transgenic varieties. Diversity measures of endophytic isolates were not different in Bt-versus non Bt-maize varieties.

Rhizosphere samples were collected from fields in Slovakia during two years and 16S rRNA gene was amplified from metagenomic DNA using universal eubacterial primers (ONDREIČKOVÁ et al., 2014). Differences in the number of terminal restriction fragments between control and GM maize hybrids were not detected. The 16S rDNA clone library creation from rhizosphere sample of MON810 maize followed by DNA sequencing revealed that the Proteobacteria were major group of bacteria and Actinobacteria, Firmicutes, and Chloroflexi were less represented. This study did not confirm any changes in the soil ecosystem, which would have been larger than normal variations caused by external conditions.

In a 90-day microcosom study, Shu et al. (2017) investigated the effects of two hybrids of Bt maize (Bt11 and MON810) straw return on *Eisenia fetida* bacterial community by the terminal restriction fragment length polymorphism (T-RFLP) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) combing with DNA sequencing, compared to near-isogenic non-Bt maize (5422). The study demonstrated the significant differences were shown in soil bacterial community between Bt and non-Bt maize treatments on the 75th and 90th d, which was closely correlated with soil available N, P and K rather than Cry1Ab protein concentrations in straw and soil. The study was performed in a greenhouse in China.

Microbial community structure has also been studied by bacterial and phylum-specific PCR-DGGE and PCR cloning approaches (García-Villaraco Velasco et al., 2013). In general, differences were again more pronounced between sampling times, as opposed to between TG versus WT plants, although marked differences were observed within the Betaproteobacteria between plant lines. For the first time it describes the presence of Iamiaceae family in soil, specifically to TG plant rhizosphere. The study showed that some

important properties of rhizopshere microbes may be impacted by Bt maize cultivation and highlighted the fact that such potential effects need to be viewed within the context of seasonal and spatial variability.

Most published studies indicate that the host plant cultivar or modification has no or minor effect on soil microorganisms. Spatial variation and heterogeneity in the field has probably greater effects on soil microorganisms. There is need for evaluation of the methods regarding effects of Cry proteins on microorganisms in soil. There are lack of in vitro data regarding antibacterial/anti-fungal activity of Cry proteins. Furthermore, there is lack of data regarding the quantity of microbial species in soil, which may be performed using q or RT-PCR. The change of microbial species in non-microbial organisms, living in the soils, is not investigated.

7.7 Conclusion

Maize is the only representative of the genus *Zea* in Europe, and there are no cross-compatible wild or weedy relatives outside cultivated maize with which maize can hybridise and form backcross progeny. Vertical gene transfer in maize therefore depends on cross-pollination with other conventional or organic maize varieties. In addition, unintended admixture of genetically modified material in seeds represents a possible way for gene flow between different crop cultivations. The risk of pollen flow from maize volunteers is negligible under Norwegian growing conditions. Since maize Bt11 has no altered agronomic and phenotypic characteristics, except for the specific target insect resistance and herbicide tolerance, the likelihood of unintended environmental effects as a consequence of spread of genes from maize Bt11 is considered to be extremely low.

There are no reports of the target lepidopteran species attaining pest status on maize in Norway. Since there are no Bt-based insecticides approved for use in Norway, and lepidopteran pests have not been registered in maize, issues related to resistance evolution in target pests are not relevant at present for Norwegian agriculture.

Published scientific studies showed that the likelihood of negative effects of Cry1Ab protein on non-target arthropods that live on or in the vicinity of maize plants is low.

In Norway, the maize cultivation is marginal. The total crop area of forage maize is estimated to 2000-2800 decares, equivalent to less than 0.1 % of the areas with cereal crops. The area of individual fields is limited by the topography such that the quantity of maize pollen produced under flowering is also limited. The potential exposure of Cry1Ab-containing maize pollen on non-target lepidopteran species in Norway is therefore negligible.

Cultivation of maize Bt11 is not considered to represent a threat to the prevalence of red-listed species in Norway.

Cry1Ab protein does not negatively affect honeybee larvae and adults in laboratory settings. Considering that the proportion of maize pollen as a total of all pollen collected and fed to larvae during a summer will be low, VKM does not consider that maize Bt11 will cause reductions to pollinating insects that are significantly greater from those caused by cultivation of conventional maize in Norway.

Few studies have assessed the impact of Cry proteins on non-target aquatic arthropods and the fate of these proteins in senescent and decaying maize detritus in aquatic environments, and no specific lower-tier studies, assessing the impact of the Cry1Ab protein on non-target aquatic arthropods have been reported in the scientific literature so far. However, exposure of non-target organisms to Cry proteins in aquatic ecosystems is likely to be very low, and potential exposure of Cry proteins to nontarget organisms in aquatic ecosystems in Norway is considered to be negligible.

Few studies have been published examining potential effects of Cry1Ab protein on ecosystems in soil, mineralization, nutrient turnover and soil communities. Some field studies have indicated that root exudates and decaying plant material containing Cry proteins may affect population size and activity of rhizosphere organisms (soil protozoa and microorganisms). Most studies conclude that effects on soil microorganisms and microbial communities are transient and minor compared to effects caused by agronomic and environmental factors. However, data are only available from short term experiments and predictions of potential long term effects are difficult to deduce.

VKM concludes that, although the data on the fate of the Cry1Ab protein and its potential interactions in soil are limited, the relevant scientific publications analysing the Cry1Ab protein, together with the relatively broad knowledge about the environmental fate of other Cry1 proteins, do not indicate significant direct effects on the soil environment.

VKM concludes that separation distances of 200 m most likely will ensure an upper limit of 0.9 % of adventitious presence as a result of introgression via pollination in maize.

8 Post-market environmental monitoring

Directive 2001/18/EC introduces an obligation for applicants to implement monitoring plans, in order to trace and ifieldify any direct or indirect, immediate, delayed or unanticipated effects on human health or the environment of GMOs as or in products after they have been placed on the market. Monitoring plans should be designed according to Annex VII of the Directive. According to Annex VII, the objectives of an environmental monitoring plan are 1) to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO or its use in the environmental risk assessment (ERA) are correct, and (2) to ifieldify the occurrence of adverse effects of the GMO or its use on human health or the environment which were not anticipated in the environmental risk assessment.

Post-market environmental monitoring is composed of case-specific monitoring and general surveillance (EFSA 2011c). Case-specific monitoring is not obligatory, but may be required to verify assumptions and conclusions of the ERA, whereas general surveillance is mandatory, in order to take account for general or unspecific scientific uncertainty and any unanticipated adverse effects associated with the release and management of a GM plant. Due to different objectives between case-specific monitoring and general surveillance, their underlying concepts differ. Case-specific monitoring should enable the determination of whether and to what extent adverse effects anticipated in the environmental risk assessment occur during the commercial use of a GM plant, and thus to relate observed changes to specific risks. It is triggered by scientific uncertainty that was identified in the ERA.

The objective of general surveillance is to identify unanticipated adverse effects of the GM plant or its use on human health and the environment that were not predicted or specifically identified during the ERA. In contrast to case-specific monitoring, the general status of the environment that is associated with the use of the GM plant is monitored without any preconceived hypothesis, in order to detect any possible effects that were not anticipated in the ERA, or that are long-term or cumulative.

Summary and evaluation of the monitoring plans from the applicant

8.1 Case-specific GM plant monitoring

When potential adverse effects or important gaps in scientific information or significant levels of critical uncertainty linked to the GM plant and its management have been identified in the environmental risk assessment, then case-specific monitoring should be carried out after placing on the market, in order to confirm assumptions made in the ERA and to further inform the ERA (EFSA 2011c). Case-specific monitoring (CSM) should be targeted at assessment endpoints and environmental protection goals identified in the ERA conclusion as being at risk or where levels of critical uncertainty were identified in relation to potential risks associated

with the GM plant. Monitoring of potentially adverse cumulative long-term or large-scale effects and the resolution of areas of critical uncertainty, identified in the ERA are important objectives of monitoring (EC 2002).

CSM should be put in place, in order (1) to confirm that any assumption in the ERA regarding the occurrence and impact of potential adverse effects is correct, and (2) to determine the efficacy of risk mitigation measures and/or ultimately to allow the modification of risk mitigation measures, so that their efficacy and proportionality can be improved (see EFSA, 2011a).

The environmental risk assessment, conducted by the applicant, support a conclusion that cultivation of Bt11 represent negligible risk the environment. Because no immediate adverse risk effects are expected, the probability of long-term adverse effects is also negligible. The applicant has therefore considered that there is no need for case-specific monitoring. Specific strategies for risk management are however required with regard to the interactions between the GM plant and target organisms. Insect resistance management measures have been put in place in Bt11 cultivation countries to proactively avoid and in any case delay insect resistance development. The applicant submitted an IRM plan consisting of (1) a strategy based on a high dose of Cry protein accompanied by non-Bt refugia in order to delay the potential development of resistance of the target pests (ECB and MCB) to maize Bt11, (2) resistance monitoring and baseline studies on target pests' susceptibility and (3) communication with and education of farmers (e.g. a technical user guide) and a proactive education programme for farmers on compliance with implementation of refugia (e.g. letters, interviews and press articles, leaflets).

EFSA accepted the monitoring plan developed by the applicant to monitor specifically for resistance in corn borers and recommends that cultivation should be accompanied by appropriate risk management strategies to minimise exposure of non-target insects and to delay the development of resistance to the Cry1Ab protein in target insects (EFSA, 2005).
In 2012, the EFSA GMO Panel reiterates that its earlier recommendation that appropriate IRM strategies relying on the 'high dose/refuge' strategy should be employed, in order to delay the potential evolution of resistance to the Cry1Ab protein in lepidopteran target pests (EFSA, 2005, 2011a). The EFSA GMO Panel recommends that there is coordination and integration of IRM and monitoring of maize Bt11 with those of other Cry1Ab-expressing maize events currently grown commercially in the EU and caution when predicting future responses of the European and Mediterranean corn borer in the EU based on experiences elsewhere, as resistance evolution in target insect pests is dependent upon many factors (EFSA, 2011a, 2012a).

Regarding non-target Lepidoptera, the EFSA GMO Panel recommended case-specific monitoring of non-target Lepidoptera only under certain exposure scenarios when maize Bt11 cultivation would present a risk to non-target Lepidoptera that are 'extremely sensitive' to the Cry1Ab protein and when the risk cannot be reduced by appropriate mitigation measures (e.g., non-Bt strips). However, in many cases, e.g., if 'extremely sensitive' species do not exist or

are not present where maize Bt11 might be cultivated, the recommended risk mitigation measures may be disproportionate to the level of risk or uncertainty and put unnecessary burdens on farmers. If applicants, in agreement with risk managers, wish to reduce the proposed risk mitigation measures because they are considered too conservative, then monitoring studies may be required. The EFSA GMO Panel also suggested that, in these latter cases, further studies could be conducted to confirm the estimates of the ERA on the sensitivity of non-target Lepidoptera and whether non-target Lepidoptera larvae, with an extremely high sensitivity to the Cry1Ab protein, are present and feeding on host-plants occurring in and adjacent to maize fields at the time of pollen shed (EFSA, 2011a, 2012a,b).

In Norway, there are no reports of the target lepidopteran species attaining pest status on maize. There have been ten reports of *O. nubilalis*, restricted to the counties of Vestfold, Telemark, Aust-Agder and Vest Agder. *Sesamia* spp. has not been reported in Norway. Since there are no Bt-based insecticides approved for use in Norway, and lepidopteran pests have not been registered in maize, issues related to resistance evolution in target pests are not relevant at present for Norwegian agriculture. Published scientific studies show no or negligible adverse effects of Cry1Ab protein on non-target arthropods that live on or in the vicinity of maize plants. Likewise, cultivation of maize is not considered to represent a threat to the prevalence of red-listed species in Norway. VKM therefore concludes that a case-specific monitoring plan is not necessary in Norway.

8.2 General surveillance (GS) for unanticipated effects

According to the principles and objectives outlined in Annex VII of Directive 2001/18/EC, the objectives of general surveillance is to detect any unanticipated adverse effects on protected and valued entities of the environment, including biodiversity and ecosystem services (EFSA 2011c).

The general surveillance proposed by the applicant is based on four pillars: (1) the use of annual farm questionnaires to feed a general surveillance database; (2) the review of scientific information provided by existing observation networks; (3) the implementation of company stewardship programs; and (4) the follow-up of various information sources such as scientific publications, expert reports etc to identify potential adverse effects associated with the intended uses of maize Bt11. The applicant proposed to conduct general surveillance for maize Bt11 throughout the period of validity of the authorisation.

8.3 Conclusion

VKM agrees with the conclusions and recommendations on general surveillance from the EFSA opinion (EFSA 2011a, 2012).

9 Uncertainty and data gaps

VKM finds that the application follows relevant guidance documents as developed by EFSA. These guidance documents address and seek to reduce measurement, parameter and sampling uncertainty. Moreover, they address various types of model uncertainty. VKM has not identified critical uncertainty remaining after previous assessments done by VKM and EFSA, or specific conditions in Norway that would require in-depth uncertainty analysis. VKM acknowledge the ongoing work in EFSA that focus on developing guidance on systematic and transparent approaches to uncertainty analyses, and seeks to integrate such recommendations as they become available.

Examples of specific sources of uncertainty discussed for this application include the following:

- There are only a limited number of published scientific studies on the environmental effects of Cry1Ab protein.
- Few studies have been published examining potential effects of Cry1Ab protein on ecosystems in soil, mineralization, nutrient turnover and soil communities.
- Few studies have assessed the impact of Cry proteins on non-target aquatic arthropods and the fate of these proteins in senescent and decaying maize detritus in aquatic environments. No specific lower-tier studies, assessing the impact of the Cry1Ab protein on non-target aquatic arthropods have been reported in the scientific literature so far.
- Acute toxicity tests do not provide enough information to conclude on possible adverse health effects of maize Bt11

Adjuvanticity

There are many knowledge gaps related to assessment of adjuvants. Most of the immunologic adjuvant experiments have been performed using Cry1Ac. Whether the other Cry proteins have similar adjuvant properties is unknown. The quantities of Cry proteins in genetically modified maize and soya are marginal compared with the amounts of other adjuvants that are natural components of food. However, the extent to which these naturally occurring adjuvants and Cry proteins contribute to the development of allergies is largely unknown. Determination of their importance is hampered by the lack of validated methods for measuring adjuvant effects.

The possibility that Cry proteins might increase the permeability of the intestinal epithelium and thereby lead to "bystander" sensitization to strong allergens in the diet of genetically susceptible individuals cannot be completely excluded. This possibility could be explored in a relevant animal model. One element of uncertainty in exposure assessment is the lack of knowledge concerning exposure via the respiratory tract and the skin, and also the lack of quantitative understanding of the relationship between the extent of exposure to an adjuvant and its effects in terms of development of allergies.

Herbicide residue levels

Herbicide residue levels on plants with engineered resistance to one or two broad spectrum herbicides could entail higher levels of herbicide residue cocktails compared to plants produced by conventional farming practice.

Since it is difficult to predict the toxicity of cocktails from the toxicity of the single components, there is uncertainty related to risk of confounding effects such as additive or synergistic effects between the residues in herbicide resistant plants. The transgene technology used can possibly lead to different metabolic products of the applied herbicides from what is expected from conventional usage. The risk assessment of herbicides should take into account plants with altered metabolism.

10 Conclusions

Food and feed safety evaluation (updating of the 2014 opinion)

Molecular characterisation

Appropriate analyses of the integration site, inserted DNA sequence, flanking regions, and bioinformatics have been performed. The molecular characterisation reported by the applicant shows that the DNA-fragment containing the *cry1Ab* and *pat* genes, is integrated as a single copy at a single locus in the nuclear genome of maize Bt11 and that it is stably inherited as a dominant trait. VKM considers the molecular characterisation of maize Bt11 satisfactory.

Comparative assessment

Comparative analyses of data from field trials located at representative sites and environments in North America and Europe indicate that maize Bt11 is compositionally equivalent to its conventional counterpart, with the exception of the herbicide tolerance and insect resistance traits, conferred by the expression of the PAT and Cry1Ab proteins. However, data on the amino acid tryptophan, is only given in one out of six studies. Based on current knowledge, VKM concludes that maize Bt11 is compositionally equivalent to conventional maize.

The data provided by the applicant are not sufficient to show that Bt11 maize is phenotypically and agronomically equivalent to conventional near-isogenic maize lines. The agronomic assessment data are provided from one growing season in the North America and one growing season in France. This is not considered to be sufficient for representative testing of agricultural environments.

Food and feed risk assessment

Whole food feeding studies have not indicated any adverse health effects of maize Bt11. These studies also indicate that maize Bt11 is nutritionally equivalent to conventional maize. The Cry1Ab and PAT proteins do not show sequence resemblance to other known toxins or IgE allergens, nor have they been reported to cause IgE mediated allergic reactions. Some studies have however indicated a potential role of Cry-proteins as adjuvants in allergic reactions.

Based on current knowledge, the VKM concludes that maize Bt11 is nutritionally equivalent to conventional maize varieties. It is unlikely that the Cry1Ab and PAT proteins will introduce a toxic or allergenic potential in food or feed based on maize Bt11 compared to conventional maize.

Answers to the Terms of Reference from the Norwegian Environment Agency and the Norwegian Food Authority

Maize is the only representative of the genus *Zea* in Europe, and there are no cross-compatible wild or weedy relatives outside cultivated maize with which maize can hybridise and form backcross progeny. Vertical gene transfer in maize therefore depends on cross-pollination with other conventional or organic maize varieties. In addition, unintended admixture of genetically modified material in seeds represents a possible way for gene flow between different crop cultivations. The risk of pollen flow from maize volunteers is negligible under Norwegian growing conditions. Since maize Bt11 has no altered agronomic and phenotypic characteristics, except for the specific target insect resistance and herbicide tolerance, the likelihood of unintended environmental effects as a consequence of spread of genes from maize Bt11 is considered to be extremely low.

There are no reports of the target lepidopteran species attaining pest status on maize in Norway. Since there are no Bt-based insecticides approved for use in Norway, and lepidopteran pests have not been registered in maize, issues related to resistance evolution in target pests are not relevant at present for Norwegian agriculture.

Published scientific studies showed that the likelihood of negative effects of Cry1Ab protein on non-target arthropods that live on or in the vicinity of maize plants is low.

In Norway, the maize cultivation is marginal. The total crop area of forage maize is estimated to 2000-2800 decares, equivalent to less than 0.1 % of the areas with cereal crops. The area of individual fields is limited by the topography such that the quantity of maize pollen produced under flowering is also limited. The potential exposure of Cry1Ab-containing maize pollen on non-target lepidopteran species in Norway is therefore negligible.

Cultivation of maize Bt11 is not considered to represent a threat to the prevalence of red-listed species in Norway.

Cry1Ab protein does not negatively affect honeybee larvae and adults in laboratory settings. Considering that the proportion of maize pollen as a total of all pollen collected and fed to larvae during a summer will be low, VKM does not consider that maize Bt11 will cause reductions to pollinating insects that are significantly greater from those caused by cultivation of conventional maize in Norway.

Few studies have assessed the impact of Cry proteins on non-target aquatic arthropods and the fate of these proteins in senescent and decaying maize detritus in aquatic environments, and no specific lower-tier studies, assessing the impact of the Cry1Ab protein on non-target aquatic arthropods have been reported in the scientific literature so far. However, exposure of non-target organisms to Cry proteins in aquatic ecosystems is likely to be very low, and potential exposure of Cry proteins to nontarget organisms in aquatic ecosystems in Norway is considered to be negligible.

Few studies have been published examining potential effects of Cry1Ab protein on ecosystems in soil, mineralization, nutrient turnover and soil communities. Some field studies have indicated that root exudates and decaying plant material containing Cry proteins may affect population size and activity of rhizosphere organisms (soil protozoa and microorganisms). Most studies conclude that effects on soil microorganisms and microbial communities are transient and minor compared to effects caused by agronomic and environmental factors. However, data are only available from short term experiments and predictions of potential long term effects are difficult to deduce.

VKM concludes that, although the data on the fate of the Cry1Ab protein and its potential interactions in soil are limited, the relevant scientific publications analysing the Cry1Ab protein, together with the relatively broad knowledge about the environmental fate of other Cry1 proteins, do not indicate significant direct effects on the soil environment.

The PAT protein expressed in maize Bt11 has been used as selectable marker to facilitate the selection process of transformed plant cells and is not intended for weed management purposes. As the scope of the notification does not cover the use of glufosinate-ammonium-containing herbicides, and maize Bt11 will not be marketed in the EEA as a herbicide-tolerant crop, potential environmental adverse effects due to the applications of these herbicides and possible changes in weed management has not been assessed by VKM in this opinion.

As there are no pests which are controlled by Bt-based insecticides, cultivation of maize Bt11 will not lead to changes in cultivation practices in Norway.

Post-market environmental monitoring plan

Since there are no Bt-based insecticides approved for use in Norway, and lepidopteran pests have not been registered in maize, issues related to resistance evolution in target pests are not relevant at present for Norwegian agriculture. Published scientific studies show that the likelihood of adverse effects of Cry1Ab protein on non-target arthropods that live on or in the vicinity of maize plants is low. Likewise, cultivation of maize is not considered to represent a threat to the prevalence of red-listed species in Norway. VKM therefore concludes that a case-specific monitoring plan is not necessary in Norway.

VKM is of the opinion that the general surveillance plan provided by the applicant, with the recommendations from EFSA, is sufficient to observe and register possible unanticipated adverse effects of maize Bt11.

Mangement measures in the draft Annex to the Commission Implementing Decision concerning the placing on the market for cultivation of genetically modified maize Bt11:

Management measures

(a) *Refuge areas to delay the development of resistance and limit exposure of non-target Lepidoptera species to maize pollen expressing Cry1Ab protein*
There are no reports of the target lepidopteran species attaining pest status on maize in Norway. Since there are no Bt-based insecticides approved for use in Norway, and lepidopteran pests have not been registered in maize, issues related to resistance evolution in target pests are not relevant at present for Norwegian agriculture.

(b) *Isolation distances from protected habitats*
Cultivation of maize Bt11 is not considered to represent a threat to the prevalence of red-listed Lepidoptera species in Norway.

(c) *Teosinte control/eradication measures*
Teosinte, the common name for a group of annual and perennial species of the genus *Zea*, is native to Mexico and Central America. In Europe, teosinte has been reported in maize fields in Spain and France. Teosinte control/eradication measures is not considered an issue in Norway.

Coexistence measures

VKM concludes that separation distances of 200 m most likely will ensure an upper limit of 0.9 % of adventitious presence as a result of introgression via pollination in maize.

Overall conclusion

Based on current knowledge, VKM concludes that maize Bt11 is nutritionally equivalent to conventional maize varieties. It is unlikely that the Cry1Ab and PAT proteins will introduce a toxic or allergenic potential in food or feed derived from maize Bt11 compared to conventional maize.

VKM concludes that cultivation of maize Bt11 is unlikely to have any adverse effect on the environment and agriculture in Norway.

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12 Appendix I

Table 1. Summary of lines evaluated (ANFZA 2000).

Lines	Protein Expression	Proximate[2]	Fatty Acids	Amino Acids[3]	Vitamins & Minerals
Initial transformant (greenhouse data)					
H8540 Bt$^+$/Bt$^+$		+	+	+	
Control H8540		+	+	+	
H8540 Bt$^+$/Bt$^-$ hybrid		+	+	+	
Control hybrid		+	+	+	
Dent Corn					
N4640-CBR					+
X4734-CBR	+	+	+	+	
X4334-CBR	+	+			
N4242-CBR					+
N4640		+	+	+	+
NK4242	+	+			+
X6534-CBR	+	+	+	+	
X6514		+			
N6800			+	+	
X7634-CBR	+	+			
X7514	+	+			
Sweet Corn Varieties					
0943	+	+			+
Jubilee	+	+			+
0937	+	+			+
Bonus	+	+			+
0941	+	+			+
Empire	+	+			+
Herbicide treated plants					
Madera-Bt		+			+
Madera		+			+
Manuel-Bt		+			+
Manuel		+			+
Magister-Bt		+			+
Magister		+			+

[1]A "+" indicates the data that was provided for that line. Control lines are in italics and genetically modified corn lines are in bold and are denoted as CBR – corn borer resistant or Bt. Control lines are either corresponding isogenic non-GM lines or are of a similar genetic background.

[2]Proximate components analysed were: *Initial transformants*: Total nitrogen, moisture, ash, starch, cellulose, xanthophyll; *Dent corn*: protein, oil, starch and fibre; *Sweetcorn*: moisture, protein, fat, ash, carbohydrates (total), calories, calories from fat, sugars, other carbohydrates, total dietary fibre; *Treated*: energy, carbohydrate, protein, fat, fibre.

[3]Some analyses did not assess all amino acids.