



**Secretariaat
Secrétariat**

O./ref.: WIV-ISP/41/BAC/2012/1017

**Evaluation of the environmental risk assessment of application
EFSA/GMO/BE/2009/71 (maize line MON 89034 x MON 88017) submitted
under Regulation (EC) No. 1829/2003:**

Final report of the Belgian Biosafety Advisory Council

Table of contents

Table of contents	2
Introduction	3
1. Background information	4
1.1. Recipient or parental plant	4
1.2. Genetic modification	4
1.3. GM plant.....	4
1.3.1. Traits that have been introduced.....	4
1.3.2. Sequences actually inserted.....	5
1.3.3. Expression of the insert	6
1.3.4. How the GM plant differs from the recipient plant in reproduction, dissemination and survivability	7
2. Potential changes in the interactions of the GM plant with the biotic environment.....	9
2.1. Persistence and invasiveness	9
2.2. Selective advantage and disadvantage	9
2.3. Potential for gene transfer.....	9
2.4. Interactions between the GM plant and target organisms	10
2.5. Interactions of the GM plant with non-target organisms	10
2.6. Effects on human and animal health	12
2.7. Effects on biogeochemical processes	12
2.8. Impacts of the specific cultivation, management and harvesting techniques.....	12
2.9. Potential interactions with the abiotic environment.....	13
Monitoring	14
Overall conclusions.....	15
References (not present in application).....	16
Acknowledgements.....	18

Annex I. BAC_2010_0117: Requests for clarification on the ERA

Annex II. BAC_2010_1106: Further requests for clarification on the ERA

Annex III. BAC_2011_0624: Further requests for clarification on the ERA

Introduction

The application under consideration EFSA/GMO/BE/2009/71 concerns the placing on the market of the genetically modified maize line MON 89034 x MON 88017 for cultivation purposes by the Monsanto Company. MON 89034 x MON 88017 expresses Cry1A.105, Cry2Ab2, Cry3Bb1 and CP4 EPSPS, rendering the maize line resistance to the European corn borer and corn rootworm, and tolerance to glyphosate. Food, feed, import and processing of MON 89034 x MON 88017 are covered by EFSA/GMO/BE/2007/39¹.

The application was submitted to the European Food Safety Authority (EFSA) in accordance with Regulation (EC) No. 1829/2003. EFSA launched a call on June 24, 2009 in accordance with Article 6.3(c) and 18.3(c) to the competent authorities to carry out the initial evaluation of the environmental risk assessment (ERA) of MON 89034 x MON 88107 carried out by the applicant. The Belgian competent authority under Directive 2001/18/EC was designated by EFSA to carry out the ERA. The application was declared valid on November 4, 2009 and subsequently assessed by the Belgian Biosafety Advisory Council on request of the Belgian competent authority. On February 18, 2010 (Annex I), November 29, 2010 (Annex II) and June 30, 2011 (Annex III), the Belgian Biosafety Advisory Council sent its requests for additional information on the ERA and the post-market environmental monitoring plan to the Belgian competent authority.

This evaluation report focuses on issues related to the stacked event MON 89034 x MON 88017. The evaluation of MON 89034 is covered in BAC (2012) and of MON 88017 in BAC (2010a) and EFSA (2011).

The Belgian Biosafety Advisory Council conducted its evaluation of the ERA based on the information received by the applicant on MON 89034 x MON 88017, the information found in peer-reviewed studies (see References) and the scientific comments raised by the member states within the three month consultation period (November 4, 2009 until April 2, 2010).

¹Application EFSA/GMO/NL/2007/39 (MON 89034 x MON 88017) has been positively assessed by the Biosafety Advisory Council (BAC, 2010b) and EFSA (2010).

Environmental risk assessment

1. Background information

1.1. Recipient or parental plant

Maize (*Zea mays L.*) is a highly domesticated annual agricultural crop, originating from Central America and incapable of surviving without human assistance under European conditions. Maize is not considered as having weedy tendencies (Baker, 1974) and is not known as an invasive species in natural ecosystems (CFIA, 1994). Maize is predominantly wind pollinated. There are no other cultivated or wild plant species that are sexually compatible with maize in the EU. Seed survival over-winter is limited under European weather conditions and hence volunteer appearance rare in Europe. Volunteers are generally controlled by farmers, either by the use of herbicides or manual or mechanical removal.

1.2. Genetic modification

MON 89034 x MON 88017 was obtained by traditional breeding of two inbred lines derived from MON 89034 and MON 88017.

1.3. GM plant

1.3.1. Traits that have been introduced

Properties of the introduced genes conferring herbicide tolerance

Expression of CP4 EPSPS confers tolerance to glyphosate (the active ingredient of Roundup). Glyphosate interferes with normal plant metabolism through inhibiting the enzyme 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS) involved in the biosynthesis of aromatic amino acids. As a consequence of the inhibition of aromatic amino acid biosynthesis, protein synthesis is disrupted, resulting in the plant's death. The application of glyphosate to maize line MON 89034 x MON 88017 will be ineffective, as CP4 EPSPS will take over the intrinsic plant EPSPS function (OECD, 1999).

Properties of the introduced genes conferring tolerance to insects

The *cry3Bb1* gene allows controlling certain coleopteran insect pests of the Chrysomelidae family, namely the larvae of the Western corn rootworm (*Diabrotica virgifera virgifera*), but also the Northern (Vaughn *et al.*, 2005), Southern (Donovan *et al.*, 1992) and Mexican corn rootworm and the Colorado potato beetle (Duan *et al.*, 2003). The only corn rootworm species present in the EU is *D. v. virgifera*.

The *cry1A.105* and *cry2Ab2* genes allow protection against lepidopteran pests. In the EU, *cry1A.105* and *cry2Ab2* genes would allow protection of maize against the European corn borer (ECB, *Ostrinia nubilalis*), the Mediterranean corn borer (MCB, *Sesamia nonagroides*) and secondary noctuid lepidopteran pests, such as the cutworms (*Agrotis spp.*) and cotton bollworm (*Helicoverpa armigera*).

With the insertion of the *cry* genes, the plant is able to produce δ -endotoxins (*Bt* toxins). The δ -endotoxin selectively binds to receptors located in the midgut of susceptible species. After binding to receptors, the gut is perforated, causing the insect to die within 48 to 120 hours.

1.3.2. Sequences actually inserted

The genome of MON 89034 x MON 88017 contains the inserts of the parental lines, one derived from MON 89034 and one derived from MON 88017.

MON 88017

MON 88017 has been genetically modified to produce the Cry3Bb1 and CP4 EPSPS proteins and contains in a single locus one intact copy of the following cassettes:

(1) an *epsps* gene derived from *Agrobacterium tumefaciens* sp. strain CP4 (CP4 EPSPS), which imparts tolerance to glyphosate, under the regulation of the rice actin 1 gene promoter (*P-Ract1*) and first intron (*I-Ract1*), the nopaline synthase terminator sequences from *A. tumefaciens* and the chloroplast transit peptide 2 sequence from the *epsps* gene of *Arabidopsis thaliana*.

The CP4 EPSPS protein of MON 88107 has an identical amino acid sequence to the CP4 EPSPS protein in soybean GTS 40-3-2 and in one of the two cassettes of maize NK603;

(2) a synthetic version of the *cry3Bb1* gene derived from *Bacillus thuringiensis* (*Bt*) subsp. *kumamotoensis* strain EG4691 conferring resistance to certain coleopteran pests, under the regulation of the enhanced 35S promoter derived from cauliflower mosaic virus, a 5' untranslated leader of the wheat chlorophyll *a/b*-binding protein (*L-Cab*), the *I-Ract1* and the transcript termination sequence for wheat heat shock protein 17.3 (*T-Hsp17*).

MON 89034

The maize line MON 89034 has been obtained through *Agrobacterium*-mediated transformation of maize cells with plasmid vector PV-ZMIR245 that contains two separate T-DNAs. T-DNA I includes the *cry1A.105* and *cry2Ab2* expression cassettes, T-DNA II the *nptII* expression cassette. The T-DNA II expression cassette was eliminated through breeding.

The T-DNA I cassette contains:

(1) a modified *cry1A*-type gene (*cry1A.105*) conferring resistance to certain lepidopteran pests, under the regulation of the enhanced 35S promoter derived from cauliflower mosaic virus, a 5' untranslated leader of the wheat chlorophyll *a/b*-binding protein (*L-Cab*), the rice actin 1 gene first intron (*I-Ract1*) and the transcript termination sequence for wheat heat shock protein 17.3 (*T-Hsp17*).

The Cry1A.105 protein present in MON 89034 is a chimeric protein derived from Cry1Ab, Cry1Ac and Cry1F. Cry1A.105 shares an identity of 93,4% to Cry1Ac (*Bacillus thuringiensis* subsp. *kurstaki*), 90% to Cry1Ab (*B. thuringiensis* subsp. *kurstaki*) and 76,7% to Cry1F (*B. thuringiensis* subsp. *aizawai*).

(2) a modified version of the *cry2Ab2*² gene derived from *B. thuringiensis* subsp. *kurstaki* HD-1 (Donovan, 1991; Widner and Whiteley, 1989) conferring resistance to certain lepidopteran pests, under the regulation of the promoter derived from figwort mosaic virus (*P-FMV*), the wheat heat shock gene 70 intron (*I-Hsp70*), a chloroplast transit peptide of maize

² Cry2Ab2 produced in MON 89034 is identical to that of Cry2Ab2 produced in MON 115985 cotton

ribulose 1,5-biphosphate carboxylase small subunit and first intron, and nopaline synthase terminator sequences (T-NOS) from *A. tumefaciens*.

Equivalence at molecular and functional level of the Cry1A.105 and Cry2Ab2 proteins produced by *B. thuringiensis* (*Bt*), *E. coli* and MON 89034 has been demonstrated and positively assessed by the Biosafety Advisory Council in the context of application EFSA/GMO/BE/2011/90 (BAC, 2012). Equivalence of the Cry3Bb1 protein produced by *E. coli* and the GM plant MON 88017 has been demonstrated and positively assessed in the context of application EFSA/GMO/CZ/2005/27.

1.3.3. Expression of the insert

The expression level of Cry1A.105, Cry2Ab2 and Cry3Bb1 was determined in various tissues of MON 89034 x MON 88017, MON 89034 and MON 88017 maize (leaf (over season), root (over season and senescent), grain, silk and pollen). Samples were taken from field trials in the USA (5 sites) during the 2005 growing season (Hull, 2006; Hartman *et al.*, 2006b,c) and from field trials in the EU (7 sites in Germany and Spain) in 2007 (De Billot, 2008; Niemeyer and Silvanovich, 2008b,c). A randomised complete block design with three replications was used at all sites. The production plan for the US field trials (Hull, 2006) was missing in the original application and requested.

In the evaluation of the data by the Biosafety Advisory Council, main focus was put on the expression values obtained from MON 89034 x MON 88017, MON 89034 and MON 88017 grown in EU fields, as of the expression data provided these were considered the most relevant in the context of the ERA. The analyses of the expression levels in the EU field trials were considered to be well-performed and the data from the trials conducted in 2007 at different locations (Germany and Spain) were considered sufficient. Cry1A.105, Cry2Ab2, Cry3Bb1 and CP4 EPSPS proteins were found to be expressed in all tissues tested (silk was not tested for *cp4 epsps* expression).

For MON 89034 x MON 88017 samples taken from EU field trials (considering all sites), the range of mean Cry1A.105 protein expression levels was 49-290 µg/g dry weight (dw) for leaves (depending on growth stage before harvest), 16-71 µg/g dw for root (depending on growth stage), 2.1-3.8 µg/g dw for grain, 6-32 µg/g dw for silk and 3.9-6.3 µg/g fw for pollen; the range of mean Cry2Ab2 protein expression levels were 61-620 µg/g dw for leaves (depending on growth stage before harvest), 17-70 µg/g dw for root (depending on growth stage), 0.5-3.2 µg/g dw for grain 14-72 µg/g dw for silk and 0.18-1.1 µg/g fw for pollen; the range of mean Cry3Bb1 protein expression levels was 220-570 µg/g dw for leaves (depending on growth stage before harvest), 56-530 µg/g dw for root (depending on growth stage before harvest), 9.8-25 µg/g dw for grain, 64-250 µg/g dw for silk and 6.8-11 µg/g fw for pollen; the range of mean CP4 EPSPS protein expression levels 48-390 µg/g dw for leaves (depending on growth stage before harvest), was 8.8-69 µg/g dw for root (depending on growth stage), 0.63-5.0 µg/g dw for grain and 66-170 µg/g fw for pollen (Niemeyer and Silvanovich, 2008b,c).

For MON 89034 samples taken from EU field trials (considering all sites), the range of mean Cry1A.105 protein expression levels was 45-280 µg/g dw for leaves (depending on growth

stage before harvest), 15-66 µg/g dw for root (depending on growth stage before harvest), 1.7-5.9 µg/g dw for grain, 4.9-22 µg/g dw for silk and 12-18 µg/g fw for pollen; the range of mean Cry2Ab2 protein expression levels was 35-400 µg/g dw for leaves (depending on growth stage before harvest), 4.4-65 µg/g dw for root (depending on growth stage before harvest), 0.58-3.0 µg/g dw for grain, 14-59 µg/g dw for silk and 0.12-0.85 µg/g fw for pollen (Niemeyer and Silvanovich, 2008b,c).

For MON 88017 samples taken from EU field trials (considering all sites), the range of mean Cry3Bb1 protein expression levels were 100-550 µg/g dw for leaves (depending on growth stage before harvest), 63-480 µg/g dw for root (depending on growth stage before harvest), 7.9-28 µg/g dw for grain, 160-330 µg/g dw for silk and 6.2-14 µg/g fw for pollen; the range of mean CP4 EPSPS protein expression levels 63-350 µg/g dw for leaves (depending on growth stage before harvest), were 19-130 µg/g dw for root (depending on growth stage before harvest), 1.7-6.6 µg/g dw for grain and 150-360 µg/g fw for pollen (Niemeyer and Silvanovich, 2008b,c).

The Cry proteins and CP4 EPSPS were found to be expressed in all tissues of MON 89034 x MON 88017 tested. Overall the range of the protein levels for Cry1A.105, Cry2Ab2, Cry3Bb1 and CP4 EPSPS in MON 89034 x MON 88017 samples taken in the EU were spanning the ranges observed in the single events, except in pollen, where the expression levels of *cry1A.105* in MON 89034 x MON 88017 was lower in comparison to MON 89034. On the basis of the data measured in the EU field trials, the Biosafety Advisory Council concluded that the Cry doses applied in the diets to test toxicity effects on non-target organisms were at least 10 times higher than the maximum expected environmental concentration of MON 89034 x MON 88017. Historically, a 10-fold factor has been used in laboratory studies as an uncertainty factor to address between species extrapolation and lab to field extrapolation (Rose, 2007).

1.3.4. How the GM plant differs from the recipient plant in reproduction, dissemination and survivability

MON 89034 x MON 88017 has been tested in the USA (5 locations) in 2004 (Sammons & Leafgren, 2006b) and in 2007 in the EU (3 locations in Germany and 5 locations in Spain, De Billot, 2009a) to compare phenotypic characteristics of maize line MON 89034 x MON 88017 to conventional maize. Potential changes in reproduction (seedling vigour, silking, pollen shed) and agronomic characteristics, i.e. early and late stand count, stalk lodging, root lodging, plant and ear height, dropped ears, stay green, ear/kernel/stalk rot, yield and grain moisture, were studied. A randomised complete block design with three replications was used at each site in the field studies.

The field study conducted in the USA has been evaluated by EFSA in the context of application EFSA/GMO/NL/2007/39 (EFSA, 2010). EFSA concluded *that no agronomic differences specific for maize MON 89034 x MON 88017 as compared to its conventional counterpart and commercial non-GM maize varieties have been observed except for the introduced herbicide tolerance and insect resistance traits*. The data from field trials in the EU and Argentina were evaluated by the Belgian Biosafety Advisory Council. Statistically across-site differences were found for ear height and stalk lodging in Spain. For

MON 89034 x MON 88017 ear height was lower compared to the control (91.3 vs. 97.6 cm) and the number of stalk lodged plants lower compared to the control (0.0 *versus* 0.5). No across-site differences were found in the field trials carried out in Germany. As all the differences observed fell within the range of what is observed for the reference varieties (conventional maize hybrids currently cultivated), the Biosafety Advisory Council agreed with the conclusion of the applicant that the differences detected are not considered biologically meaningful with respect to plant weed potential.

Further, potential changes in ecological interactions of MON 89034 x MON 88017 in the EU with insect pests and diseases naturally present in the managed sites, and abiotic stressors were observed at 4 growth stages during field trials (De Billot, 2008; 2009; 2010). Disease incidence was recorded for 4 out of 10 biotic stressors at very few sites (one for ECB, cutworm and frit fly; two for aphids). As biotic stress was low in the field trials, the Biosafety Advisory Council doubted that firm conclusions could be drawn on potential differences in disease incidence and insect damage for MON 89034 x MON 88017 compared to its comparators. The Biosafety Advisory Council is of the opinion that these studies merely give an indication of no increased pest potential, but disagrees that they “*support a conclusion of no increased pest potential*”.

2. Potential changes in the interactions of the GM plant with the biotic environment

In its evaluation of the ERA, the Biosafety Advisory Council agreed with the assessment provided by the applicant on the issues related to (2.1) persistence and invasiveness, (2.2) selective advantage and disadvantage, (2.3) potential for gene transfer, (2.6) effects on human and animal health, (2.7) effects on biochemical processes, (2.8) impacts of specific cultivation, management and harvesting techniques and (2.9) issues of interactions with the abiotic environment. On the issues of target (2.4) and non-target (2.5) interactions the Biosafety Advisory Council formulated requests for clarification or additional information (see Annex I, II & III). For the latter issues, an overview of the evaluation conducted by the Biosafety Advisory Council is given below. To complete the evaluation report, a summary of the assessment provided by the applicant is given for the former issues.

2.1. Persistence and invasiveness

The applicant assessed whether MON 89034 x MON 88017 maize is any more likely to become a weed than the non-transgenic control or other maize currently cultivated in the EU. The assessment took into account the biology of maize, the agronomic and phenotypic characteristics of MON 89034 x MON 88017 and the newly introduced traits.

Given (a) the biology of maize (see 1.1), (b) the information that maize line MON 89034 x MON 88017 does not exhibit characteristics that would cause it to be more weedy than other maize hybrids (see 1.3.4) and (c) that the traits conferred are not expected to change the persistence and invasiveness of maize, it can be concluded that the likelihood of MON 89034 x MON 88017 to become more persistent or invasive is negligible.

2.2. Selective advantage and disadvantage

The insect-resistance traits Cry1A.105, Cry2Ab2, Cry3Bb1 and the herbicide-tolerance trait CP4 EPSPS confer specific advantages to **maize in the field**, namely resistance to certain lepidopteran and coleopteran pests, and tolerance to glyphosate.

Comparison of MON 89034 x MON 88017 with conventional maize did not reveal any biological meaningful changes in reproduction, dissemination and survivability (see 1.3.4). The effect of the transgenes on the biology of maize is therefore negligible. Taking the today's hybrid seed production and agronomic management practices for maize production into account, it is highly unlikely that the introduced traits in MON 89034 x MON 88017 will confer any meaningful selective advantage or disadvantage.

As maize does not survive **outside the agricultural environment** and has no wild relatives in the EU, the question of selective advantage and/or disadvantage to plants outside the agricultural environment is not applicable.

2.3. Potential for gene transfer

As there are no wild relatives of maize in the EU, **vertical gene flow** through cross-pollination from GM maize fields is restricted to plants of the same species. Gene flow might also result from the adventitious presence of GM maize kernels in conventional maize seeds, or less likely from seed spillage during transport. Gene transfer might thus result in the occurrence of GM volunteers. As the control of these volunteers will be the same as for non-GM volunteers,

the occurrence of GM volunteers will not raise any novel environmental concerns compared to non-GM volunteers.

The possibility of **horizontal gene transfer** between the GM plant and micro-organisms is considered as a rare event under natural conditions (Keese, 2008; EFSA, 2009). In the very unlikely case of transfer, maintenance and functional expression of the *cp4 epsps* or *cry1A.105*, *Cry2Ab2* or *cry3Bb1* in micro-organisms of the receiving environment, no impact on the ecology of micro-organisms communities and no adverse effect on human/animal health or to the environment are expected.

2.4. Interactions between the GM plant and target organisms

The toxicity of Cry3Bb1 has been shown to be restricted to certain coleopteran pests, namely to the Chrysomelidae family (leaf beetles): the biological activity of Cry1A.105 and Cry2Ab2 to lepidopteran pests (EFSA, 2011; BAC, 2012). These data were confirmed by efficacy trials in the USA with MON 89034 x MON 88017 in 2005 (Headrick *et al.*, 2006b).

The activity of the Cry proteins and CP4 EPSPS is not likely to be affected by potential interactions of the proteins with one another, as their mode of action is different (see also EFSA, 2005a,b). Hence, the Biosafety Advisory Council considered testing of the combined effects of these two types of proteins not necessary. In the context of the evaluation of MON 89034 it was demonstrated that Cry1A.105 and Cry2Ab2 do not interact (EFSA, 2011; BAC, 2012). Concerning the potential interactions of the Cry1A.105 and Cry2Ab2 with Cry3Bb1 proteins, the Belgian Biosafety Advisory Council agreed that activity towards Lepidoptera was not enhanced by the addition of Cry3Bb1, but was initially of the opinion that one could not firmly state that Cry3Bb1 activity was not enhanced by either the addition of Cry1A.105 or Cry2Ab2 or a combination of both. Therefore, data corroborating the applicant's statement were requested (see Annex I, II & III). On the basis of the additional information provided, a dietary toxicity test showing no effects of the three Cry proteins on *Poecilus cupreus* larvae (data available at <http://www.gmo-safety.eu/topic/36>), and a 3-year long field trial conducted in Germany demonstrating that the abundance of ground beetles was not affected, the Council agreed with the applicant that the toxicity of Cry3Bb1 to coleopteran species is not enhanced in MON 89034 x MON 88017.

In conclusion, the Biosafety Advisory Council agrees that the target specificity of MON 89034 x MON 88017 will remain the same compared to the single events.

2.5. Interactions of the GM plant with non-target organisms

The potential of maize line MON 89034 x MON 88017 to have direct or indirect adverse effects on non-target organisms was evaluated by the Biosafety Advisory Council (see also 2.6). Impacts on non-target organisms due to unintended changes of composition or morphology of the GM maize are not expected to occur, as no compositional and phenotypic differences have been found between the GM maize and its non-GM comparators.

Given that (1) the range of the protein levels for Cry1A.105, Cry2Ab2, Cry3Bb1 and CP4 EPSPS in MON 89034 x MON 88017 samples taken in the EU was spanning the ranges observed in the single events (see 1.3.3) and (2) no interaction between the Cry proteins and

CP4 EPSPS (see 1.3.1), nor among the Cry proteins (see 2.4) resulting in synergistic effects is expected, the Biosafety Advisory Council is of the opinion that studies to evaluate the impact on non-target organisms (insects living above- and on-ground species, including endangered Lepidoptera & chrysomelids, of the soil community and other organisms than insects) of the single events are also valid for the stacked event.

Recent dietary toxicity tests on honey bee (*Apis mellifera*) larvae with MON 89034 x MON 88017 pollen and the three purified proteins (Hendriksma et al., 2011; 2012) and *P. cupreus* (as described in 2.4; <http://www.gmo-safety.eu/topic/36>) revealed no adverse effects. Further a 3-year field study in Germany showed no adverse effects on the abundance of ground beetles, spiders, herbivorous insects and ladybirds (<http://www.gmo-safety.eu/topic/36>).

As Cry proteins may affect insects belonging to the same order/family of the envisaged pests, it was assessed whether the cultivation of MON 89034 x MON 88017 might impact non-target chrysomelids and Lepidoptera, including endangered ones. The risk to non-target Chrysomelidae and Lepidoptera which do not directly feed on maize, but might ingest pollen passively when deposited on their host plants located within the field or field edge, was assessed by the applicant. The provided theoretic quantitative risk assessment was based on the worst-case estimated environmental exposure to the three Cry proteins. The worst-case exposure concentration was calculated to be 8 times less than the one needed to cause 50% mortality of the known most sensitive lepidopteran species (*O. nubilalis*) and 8 times less than the one needed to cause 50% mortality of the known most sensitive coleopteran species (Colorado potato beetle). Hence, the margins of safety were calculated to be 8 times and up to 187 times the maximum estimated exposure of non-target Lepidoptera and chrysomelids to pollen within the maize field and at 4-5 m from the field edge, respectively.

The Biosafety Advisory Council regarded the assessment as appropriate as a conservative scenario overestimating the risks was considered, but noted that the assessment only covers lethal (and not sublethal) effects. Sublethal effects may also be of importance in case of endangered lepidopteran and chrysomelid species. Further, the Council is of the opinion that a more detailed assessment taking into account true exposure to maize pollen (instead of the maximum estimated exposure) and the abundance of non-target Lepidoptera and Chrysomelidae in European maize fields, would increase the safety margins. Recently carried out field experiments substantiate the theoretic quantitative assessment. Rauschen *et al.* (2010) showed that Chrysomelidae are one of the most abundant families of Coleoptera in maize fields in Germany, but that their occurrence is mainly restricted to the chrysomelid pests *Phyllotreta* sp. and *Oulema lichenis*. Due to the low abundance of non-pest chrysomelids in maize fields (Meissle *et al.*, 2012) the risk to these non-target species in field is expected to be minimal. Schuppener *et al.* (2012) showed that the amount of pollen from maize MON 89034 x MON 88017 found on host plants is unlikely to adversely affect a significant proportion of larvae of *Aglais urticae*. The Council therefore agreed with the assessment of the applicant that no unacceptable harmful effects on non-target Lepidoptera and Chrysomelidae, including endangered ones, are expected due to cultivation of MON 89034 x MON 88017.

In conclusion, the Biosafety Advisory Council is of the opinion that adverse effects on non-target organisms, including Lepidoptera and Chrysomelidae, are negligible and substantiate that no harm is to be expected to valued insects and the ecosystem services they provide from the cultivation of MON 89034 in the EU.

2.6. Effects on human and animal health

For the evaluation of effects on animal and human health due to accidental consumption, we refer to the food/feed assessment of maize line MON 89034 x MON 88017 (EFSA, 2010) and bio-informatic updates provided by the applicant for MON 89034 (BAC, 2012).

It was demonstrated that there is a lack of structurally relevant similarity between the Cry1A.105, Cry2Ab2, Cry3Bb1 and CP4 EPSPS protein and any known allergen, toxic or pharmacologically active proteins relevant for human and animal health. Hence, the likelihood for any adverse effects due to contact with MON 89034 x MON 88017, was regarded as negligible.

2.7. Effects on biogeochemical processes

The applicant assessed whether MON 89034 x MON 88017 is any more likely to affect biogeochemical processes than the non-transgenic control or other maize currently cultivated in the EU. As MON 89034 x MON 88017 is shown to be compositionally equivalent to conventional maize, there is no reason to believe that MON 89034 x MON 88017 maize would be any different from conventional maize regarding its direct influence on nutrient levels in the soil.

Taking the information on the lack of observed effects on detritivores and decomposers into account (see 2.5) the Biosafety Advisory Council agreed that immediate or delayed adverse effects on biogeochemical processes due to interaction of MON 89034 x MON 88017 with non-target organisms is expected to be negligible.

2.8. Impacts of the specific cultivation, management and harvesting techniques

In the application it is stated that the specific cultivation, management and harvesting techniques used for MON 89034 x MON 88017 are comparable to those used for other commercially available maize, with the exception of the environmental monitoring plan. Additionally, the possibility of using glyphosate 'over the top of the crop' in the cultivation of MON 89034 x MON 88017 is added to the farmer's weeding options.

It is acknowledged by the applicant that the use of glyphosate 'over the top of the crop' in the cultivation of MON 89034 x MON 88017 has effects³ in maize agro-ecosystems (i.e. effects on biodiversity due to reduction of weeds), but notices that these might also occur in existing intensive production environments. However, the Biosafety Advisory Council is of the opinion

³ The potential (direct) effects of the herbicide use on the environment are considered under Directive 91/414/EEC (a.o. direct effects on non-target organisms (insects, soil organisms, birds, fish and mammals) and development of weed resistance). Indirect effects (e.g. effects on biodiversity due to reduction of weeds), however, are (currently) not addressed under Directive 91/414/EEC and need to be taken into consideration in point 2.8 on impacts of the specific cultivation, management and harvesting techniques.

that the applicant did not provide sufficient information to substantiate that any indirect effects associated with the use of MON 89034 x MON 88017 maize in the EU, will be within the range of currently accepted indirect effects. It is postulated that 'over the top of the crop'-use of glyphosate might result in more effective weed control compared to existing cultivation practices, which in turn might result in biologically relevant adverse effects for maize agro-ecosystems. The Biosafety Advisory Council is of the opinion that the use of glyphosate 'over the top of the crop' must not indirectly interfere with biological functions of non-target organisms (such as biological control and decomposition). First field studies in Spain do not indicate an impact of NK603 (expressing *cp4 epsps*) and its associated weed management on biological control (Albajes *et al.*, 2009, 2011).

In conclusion, given the fact that management and utilisation of a GM crop may vary from region to region, farm to farm and over time, the Biosafety Advisory Council acknowledges the difficulty to predict the range of farming practices that will be deployed with the GM crop and the consequences for biological functions. The risk assessment should have taken this unpredictability of farm management and its consequences for biological functions better into account, e.g. by relating this to monitoring. The Biosafety Advisory Council is of the opinion that the farmer questionnaires are a good tool to detect changes in biological functions, but that the questionnaire should be adapted to cover this issue (for requests see general surveillance).

2.9. Potential interactions with the abiotic environment

Expression of the introduced traits, of which the wild type variants are naturally present in the soil environment, will not alter the natural interactions of maize plants with the abiotic environment.

Monitoring

As no potential adverse effects were identified for the environment and human health, the Biosafety Advisory Council agrees with the applicant that case-specific monitoring to verify the assumptions made in the ERA is not considered necessary during the cultivation of MON 89034 x MON 88017. However, to take the unpredictability of farm management and its potential consequences for biological control functions better into account, the current general surveillance plan needs to be adapted to cover this uncertainty (see below).

As there were no environmental concerns identified in the ERA that urged the need of a management strategy, the proposed refuge strategy for ECB and WCR was not evaluated. The evaluation of the monitoring plan was therefore restricted to the scientific quality of the general surveillance plan. However, the need for a refuge strategy for agricultural and economical concerns was acknowledged.

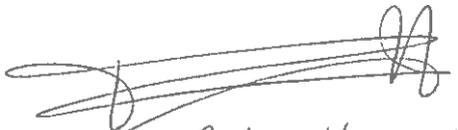
The applicants will conduct general surveillance of MON 89034 x MON 88017 maize throughout the period of validity of the authorisation. In particular, the general surveillance will take into consideration and be proportionate to the extent of cultivation of MON 89034 x MON 88017 maize in the Member States. It will focus on areas where MON 89034 x MON 88017 is grown, but also considers the broader agricultural environment. The Biosafety Advisory Council is of the opinion that the current general surveillance plan needs to be adapted to allow identification of unanticipated adverse effects on non-target organisms involved in biological control (see 2.8 of ERA section), and of management regimes that do not have an environmental performance at least as good as current regimes. The following is advised:

- Under 3.1 to include a question on the biological control measures used in MON 89034 next to the questions on insecticide, herbicide and fungicide use.
- Under 3.7 to clarify better what is meant with insects, i.e. whether the term refers to pests or insects other than pests. In contrast to the current question format, this will allow determining if the farmer has, or has not, looked at insects (other than pests) in the field.
- In the farmer questionnaire, insect control treatments applied on the MON 88017 refuges should be requested in order to check the efficacy of the IRM measures.

The Biosafety Advisory Council wants to point out to the member states' authorities the applicant's commitment to develop a "Technology Use Guide for the European MON 89034 x MON 88017 markets", describing recommendations for the use of their product, including more detailed weed control recommendations specific to each region and local needs.

Overall conclusions

Based on the information in the application, the additional information received by the applicant, the information found in peer-reviewed studies and the scientific comments raised by the member states, the Belgian Biosafety Advisory Council considers that no risks concerning the environment and human and animal health were identified as a result of cultivation of MON 89034 x MON 88017.



p.o. Dr. Philippe Heron
Prof. D. Reheul

President of the Belgian Biosafety Advisory Council

References (not present in application)

- Albajes, R., Lumbierres, B., Pons, X. 2009. Responsiveness of arthropod herbivores and their natural enemies to modified weed management in corn. *Environmental Entomology* 38, 944-954.
- Albajes, R., Lumbierres, B., Pons, X. 2011. Two heteropteran predators in relation to weed management in herbicide-tolerant corn. *Biological Control* 59, 30-36.
- BAC (Biosafety Advisory Council). 2010a. Evaluation of the environmental risk assessment of application EFSA/GMO/CZ/2008/54 (maize line MON 88017) submitted under Regulation (EC) No. 1829/2003. Final evaluation report of the Belgian Biosafety Advisory Council. http://www.bio-council.be/bac_advices.html.
- BAC (Biosafety Advisory Council). 2010b. Advice of the Belgian Biosafety Advisory Council on the application EFSA/GMO/NL/2007/39 (maize MON 89034 x MON 88017) from Monsanto under Regulation (EC) No. 1829/2003 http://www.bio-council.be/bac_advices.html.
- BAC (Biosafety Advisory Council). 2012. Evaluation of the environmental risk assessment of application EFSA/GMO/BE/2011/90 (maize line MON 89034) submitted under Regulation (EC) No. 1829/2003. Final evaluation report of the Belgian Biosafety Advisory Council. http://www.bio-council.be/bac_advices.html.
- Baker, H.G. 1974. The evolution of weeds. *Annual Review of Ecology Systematics* 5: 1-24.
- CFIA (Canadian Food Inspection Agency). 1994. Regulatory Dir94-11: The Biology of *Zea mays* L. (corn/maize). <http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9411e.shtml>
- EFSA (The European Food Safety Authority). 2005a. Opinion of the Scientific Panel on Genetically Modified Organisms on an application (Reference EFSA-GMO-UK-2004-06) for the placing on the market of glyphosate-tolerant and insect-resistant genetically modified maize MON 863 x NK603, for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Monsanto. *The EFSA Journal* 255, 1-21.
- EFSA (The European Food Safety Authority). 2005b. Opinion of the Scientific Panel on Genetically Modified Organisms on an application (Reference EFSA-GMO-UK-2004-01) for the placing on the market of glyphosate-tolerant and insect-resistant genetically modified maize NK603 x MON 810, for food and feed uses under Regulation (EC) No 1829/2003 from Monsanto. *The EFSA Journal* 309, 1-22.
- EFSA (The European Food Safety Authority). 2009b. Scientific Opinion of the Panel on Genetically Modified Organisms on applications (EFSA-GMO-RXMON810) for the renewal of authorisation for the continued marketing of (1) existing food and food ingredients produced from genetically modified insect resistant maize MON810; (2) feed consisting of and/or containing maize MON810, including the use of seed for cultivation; and of (3) food and feed additives, and feed materials produced from maize MON810, all under Regulation (EC) No 1829/2003 from Monsanto. *The EFSA Journal*, 1149: 1-85.
- EFSA (The European Food Safety Authority). 2010. Scientific opinion on application (EFSA-GMO-NL-2007-39) for placing on the market of insect resistant and herbicide tolerant genetically modified maize MON 89034 x MON 88017 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Monsanto. *EFSA Journal* 8, 1564.
- EFSA (The European Food Safety Authority). 2011. Scientific opinion on application (EFSA-GMO-CZ-2008-54) for placing on the market of genetically modified insect resistant and

- herbicide tolerant maize MON 88017 for cultivation under Regulation (EC) No 1829/2003 from Monsanto. EFSA Journal 9, 2428.
- Hendriksma, H.P., Härtel, S., Steffan-Dewenter, I. 2011. Testing pollen of single and stacked insect-resistant Bt-maize on *in vitro* reared honey bee larvae. PLoS ONE, 6, e28174.
- Hendriksma, H.P., Härtel, S., Babendreier, D., von der Ohe, W., Steffan-Dewenter, I. 2012. Effects of multiple Bt proteins and GNA lectin on *in vitro*-reared honey bee larvae. Apidologie 43, 549-560.
- Keese, P. 2008. Risks from GMOs due to horizontal gene transfer. Environmental Biosafety Research 7: 123-149.
- Rauschen, S., Schaarmschmidt, F. & Gathmann, A. 2010. Occurrence and field densities of Coleoptera in the maize herb layer: implications for environmental risk assessment of genetically modified *Bt*-maize. Transgenic Research 19: *in press*. DOI 10.1007/s11248-009-9351-3.
- Rose R.I. (ed.) 2007 White paper on tier-based testing for the effects of proteinaceous insecticidal plant-incorporated protectants on non-target invertebrates for regulatory risk assessment. USDA-APHIS and US Environmental Protection Agency, Washington, DC, USA. <http://www.epa.gov/pesticides/biopesticides/pips/non-target-arthropods.pdf>
- Schuppener, M., Mühlhause, J., Müller, A.-K., Rauschen, S. 2012. Environmental risk assessment for the small tortoiseshell *Aglais urticae* and a stacked Bt-maize with combined resistances against Lepidoptera and Chrysomelidae in central European agrarian landscapes. Molecular Ecology, DOI: 10.1111/j.1365-294X.2012.05716.x.

Acknowledgements

The Biosafety Advisory Council would like to thank the experts Patrick De Clercq (UGent), Adinda De Schrijver (SBB/WIV), Patrick du Jardin (FUSAGx) and Joerg Romeis (Reckenholz-Tänikon Research Station ART, Switzerland) for their valuable contributions; Dirk Reheul and Adinda De Schrijver for coordinating the evaluation of the ERA; the Secretariat of the Council; the GMO Panel and its Secretariat for their scientific support.



**Secretariaat
Secrétariat**

O./ref.: WIV-ISP/41/BAC/2010_0177

**Environmental Risk/Safety Assessment (ERA)
of maize line MON 89034 x MON 88017 (EFSA-GMO-BE-2009-71):**

**Requests of the Belgian Biosafety Advisory Council for clarification on
the ERA**

Mandate for the Group of Experts: mandate of the Biosafety Advisory Council of
6 November 2009

Coordinator: Dirk Reheul (UGent)

Experts: Patrick De Clercq (UGent), Adinda De Schrijver (SBB), Patrick du Jardin (Ulg),
Joerg Romeis (Reckenholz-Tänikon Research Station ART, Switzerland)

Secretariat (SBB): Didier Breyer, Adinda De Schrijver, Martine Goossens, Philippe Herman,
Katia Pauwels

Request 1. The applicant is requested to clarify issues on the Cry2Ab2 proteins used in the NTO studies.

In MacRae et al. (2005, footnote p.11) it is mentioned that the Cry2Ab2 protein used in the study is produced in *E. coli* and is referred to as Cry2Ab2.820.

Apart from the study of MacRae et al. (2005) several other non-target studies have been carried out using an *E. coli*-produced Cry2Ab2 (Paradise, 2000b; 2006b,c; Teixeira, 2006c). From the information present in these studies it is not clear whether the *E. coli*-produced Cry2Ab2 is the same as the Cry2Ab2.820. We request the applicant to clarify this.

The Cry2Ab2 used in the studies of Palmer & Krueger (2000a,b) and Maggi et al. (2000a,b,c) does not originate from *E. coli*, but from *Bacillus thuringiensis* strain EG7699. We request the applicant to clarify the sequence of the Cry2Ab2 protein produced by *B. thuringiensis*, and to provide all available references and reports.

In addition, we request the applicant to provide the study proving the equivalence of the biological activity of the *E. coli*- and MON 89034-produced Cry2Ab2. This study is lacking in the application.

Finally, a summary table, indicating all the studies and their conclusions on the Cry2Ab2 produced by *E. coli* and *B. thuringiensis*, would certainly be welcome!

Request 2. We request the applicant to argue the results of some non-target studies in order to be able to take these studies up as confirmatory evidence that no risks are expected on non-target organisms from the exposure to Cry2Ab2 and/or Cry1A.105.

General comment:

In many laboratory studies performed with Cry1A.105 and/or Cry2Ab2, the applicant states that the concentrations were chosen to ensure a "safety factor of at least 10x". Although expression levels are mentioned in the technical dossier on p. 35 and following, it would be useful to explain how this "safety factor of at least 10x" was derived, in other words, how these test concentrations (of a protein suspension in a diet, in water etc.) relate to concentrations expressed by the plant parts in the field (measured per fresh weight or per dry weight?).

Comments on non-target insect and soil organism studies:

Toxicity study with Myzus persicae (McRae et al., 2006a,b)

For a good understanding of the results from the dietary exposure studies in McRae et al. (2006a,b), it would be imperative that the exact composition of the diets used for each test insect would be provided as an annex. Some of the references given here are too general and do not enable the reader to understand exactly what diet was used (e.g. King & Hartley 1992). For the green peach aphid (*Myzus persicae*) a "sucrose-based diet" was used and reference is made to Walters et al. (1990). In latter study, several sucrose solutions (with different pHs, colours) were used to feed last (fourth) instar and adult potato aphids (*Macrosiphum euphorbiae*), so it is not clear which diet was used in MacRae et al. (2006a,b).

The applicant states that in McRae et al. (2006a,b) "adults and nymphs" were exposed to the contaminated and control diets, but does not specify what nymphal stage was used. Only late instar aphid nymphs may be able to reach adulthood on sugar alone, but younger nymphs would require a protein source to successfully complete development (see the work by Mittler cited in Walters et al., 1990).

Furthermore, it is not clear if larviposition by adult aphids occurred during the aphid exposure test and if this was considered in the calculation of survival rates.

In conclusion, we ask the applicant to clarify the exact composition of the diets, the nymphal stage of the aphid nymphs tested and whether measurement of survival also included nymphs born during the assay period.

Toxicity study with Cry1A.105, Cry2Ab2 and Cry3Bb1 (McRae et al., 2006c)

We're not fully convinced by the study of MacRae et al. (2006c). According to the data presented, the single or combined activity of Cry1A.105 and Cry2Ab2 against two lepidopteran insects is not significantly altered by the presence of Cry3Bb1. However, the activity of Cry3Bb1 against the sensitive Colorado potato beetle was consistently enhanced by either the addition of Cry1A.105 or Cry2Ab2 or a combination of both. Given the specific action of the latter two toxins against Lepidoptera this finding is rather surprising and requires

further clarification. We would like to see some evidence that these possible interactions do not expand the spectrum of activity of the Cry3Bb1 toxin to non-target beetles outside the Chrysomelids such as ladybirds for example.

Toxicity studies with *Orius insidiosus* (Teixeira, 2006b,c)

In both studies by Teixeira (2006b,c) second instars developed on bee pollen (mixed with pure Cry protein) to the adult stage in only about 6-7 days (with overall average of 6.2 days) at 24-27 °C, with a survival rate close to 90%. *Orius* bugs are predators and need animal prey for optimal development and reproduction; pollen is an alternative food that supports development and reproduction to some extent. The nutritional quality of pollen for *Orius* bugs appears to vary with plant species. Reported survival rates of *O. insidiosus* on pollen only vary strongly: 3-20% (Lundgren, 2009)¹ or 40-60% on corn pollen (Pilcher et al., 1997) to ca. 90% on bee collected maple pollen (Kiman & Yeargan, 1985)². However, the more striking result in Teixeira (2006b,c) is the very short developmental time (ca. 6 days) from second instar to adult on a pollen diet. In comparison, Kiman & Yeargan (1985) and Pilcher et al. (1997) reported total nymphal developmental periods of *O. insidiosus* on pollen of 19-22 days at similar temperatures of 24-26 °C. As the first instar accounts for ca. 20% of total nymphal development (Isenhour & Yeargan, 1981)³, this implies that in the studies by Kiman & Yeargan (1985) and Pilcher et al. (1997) the development of the predator from second instar to adult on pollen would have taken at least 15 days, i.e. much longer than the 6-day period from second instar to adult reported in Teixeira (2006b,c). It is highly unlikely that the nutritional quality of the honeybee pollen used by Teixeira (the composition of which has not been disclosed) is superior to that of optimal prey for the predator: even on optimal insect prey, like lepidopteran eggs, development from second instar to adult of *O. insidiosus* would require 7-11 days at these temperatures (Isenhour & Yeargan, 1981; Kiman & Yeargan, 1985). In conclusion, this rapid development on pollen only is not in line with previous studies on the biology of this predatory anthocorid and we ask the applicant to clarify this issue.

Our second reservation regards the outcome of the potassium arsenate controls in Teixeira (2006b,c). The results indicate that in these positive controls 40 to 96%(!) of the tested insects survives up to the adult stage, and that overall mortality only starts to increase as compared to the assay and buffer controls from the adult stage on (i.e. after about 6 days, see comment above). The high survival of nymphs on the potassium arsenate treated pollen is quite remarkable, as this broad spectrum poison is likely to also affect *Orius* bugs, and also in their nymphal life. This is confirmed by Duan et al. (2007)⁴ in which the lowest arsenate concentrations tested killed 75 to 96% of the *O. insidiosus* nymphs before they reached the adult stage. This high survival in Teixeira (2006b,c) may indicate that the nymphs of the

¹ Lundgren J. G. (2009). Relationships of natural enemies and non-prey foods. Springer Science + Business Media B.V. 453 pp.

² Kiman Z. B. & Yeargan K. V. (1985). Development and reproduction of the predator *Orius insidiosus* (Hemiptera: Anthocoridae) reared on diets of selected plant material and arthropod prey. *Annals of the Entomological Society of America* 78: 464-467.

³ Isenhour D. J. & Yeargan K. V. (1981). Effect of temperature on the development of *Orius insidiosus*, with notes on laboratory rearing. *Annals of the Entomological Society of America* 74: 114-116.

⁴ Duan J. J., Huesing J. & Teixeira D. (2007). Development of tier-I toxicity assays for *Orius insidiosus* (Heteroptera: Anthocoridae) for assessing the risk of plant-incorporated protectants to nontarget heteropterans. *Environmental Entomology* 36: 982-988.

predator were not effectively exposed to the toxins (potassium arsenate or Cry proteins). How does the applicant explain this high survival in the positive controls of both studies?

Toxicity study with Hippodamia convergens (Palmer & Krueger 2000a)

It is remarkable that in the positive reference the broad spectrum toxin potassium arsenate did not affect the insects (adults) in the 23-day exposure period at a concentration of 1000 ppm and that 10000 ppm was needed to kill part (64%) of the beetles. In comparison, adding a concentration of only 100 ppm to the diet of another ladybird *Coleomegilla maculata* was sufficient to kill nearly 100% of the tested individuals (larvae) (Paradise, 2006a,b). This suggests that the method used in Palmer & Krueger (2000a) may not have sufficiently exposed the adult beetles to the toxins (Cry proteins or potassium arsenate).

This may be related to the fact that the beetles received (untreated) water in addition to the treated honey and/or that there was little feeding by the adults during the experiment as a result of the physiological status of the beetles. Honey is an alternative food for predatory ladybirds that may prolong their survival but it does not allow them to reproduce (Hagen, 1962)⁵. Adults not offered insect prey (e.g. aphids) will thus not be reproductively active. Feeding activity of such adults may be strongly suppressed. Moreover imbibing water alone may sustain their survival, depending on the physiological status of the insects (Hagen, 1962; Galvan et al., 2008)⁶. This implies that the test insects may have ingested very little of the contaminated honey, which may explain the lack of mortality in the positive control at 1000 ppm. The higher mortality at the very high concentration of 10000 ppm potassium arsenate may even be due to the contact activity of this poison (beetles making contact with the contaminated cotton swab). Potassium arsenate has been used in fly paper to speed up kill of trapped flies, which evidently do not need to ingest the poison to get killed.

In conclusion, we are therefore not convinced that the experiment effectively exposed the ladybird adults to the Cry2Ab2 protein and question the relevance of this study.

Toxicity study with Eisenia fetida (Palmer & Krueger, 2000b; Sindermann et al., 2006a)

We don't feel that it is appropriate that the soil incorporated Cry2Ab2 dose of 330 mg/kg soil is given as the NOEC (Palmer & Krueger, 2000b), as a sensitive insect bioassay detected only 7.9 to 46.2% of the expected toxin activity in soil samples. As an explanation for the low bioactivity level in the toxin-spiked soil samples it is stated that this "...*may be related to low bioavailability in the insect bioassay*". It remains unclear what the reasons for this low bioavailability were. Similarly, bioactivity of Cry1A.105 incorporated into test soil was found to be reduced when compared to a reference standard, albeit at a relative low level (Sindermann et al., 2006a).

⁵ Hagen K. S. (1962). Biology and ecology of predaceous Coccinellidae. Annual Review of Entomology 7: 289-326.

⁶ Galvan T. L., Koch R. L. & Hutchison W. D. (2008). Impact of fruit feeding on overwintering survival of the multicolored Asian lady beetle, and the ability of this insect and paper wasps to injure wine grape berries. Entomologia Experimentalis et Applicata 128: 429-436.

Toxicity study with *Folsomia candida* (Teixeira, 2006a)

In the studies on *Folsomia candida* the collembolans were exposed to lyophilized maize leaf tissue (from conventional or MON 89034 maize expressing Cry1A.105 or Cry2Ab2) mixed at a 50:50 ratio with untreated brewer's yeast. The positive (toxic) reference consisted of the carbamate insecticide thiodicarb mixed with brewer's yeast (without maize tissue).

The problem with this experiment is that it has not shown that the collembolans fed equally on the yeast and the maize leaf powder in the maize leaf treatments. As the diet was provided in excess and half of the diet mixture consisted of yeast, this may have allowed the collembolans to be selective in their feeding. Yeast is a nutritionally superior to lyophilized leaf material for the collembolans and hence the collembolans may have fed more on yeast than on the maize powder. Even if the mixture was finely ground, the small size of the collembolans may have enabled them to still select out the nutritionally superior feed, i.e. the yeast. Moreover, feed selection may have been different in the control (conventional maize) vs. test substance (MON 89034) diet: Bakonyi et al. (2006)⁷ reported that *F. candida* preferred less of Bt-maize than of near-isogenic non-Bt maize.

Consequently, the toxic reference is not fully appropriate to show that the feeding system used was able to detect effects of the Cry proteins on the collembolans. In the toxic reference, maize leaf powder was not available and the collembolans could only feed on the yeast. As such, this toxic reference is not fully equivalent to the exposure in the test substance treatment with MON 89034 leaf tissue.

In fact, because of the presence of the yeast in the feed mixture, this experiment may not be considered a worst-case experiment. The collembolans should be able to survive on maize leaf materials alone, provided that they are aged (to start decomposition). In their study on MON810, Clark & Coats (2006) succeeded in using aged maize leaf material as a test substrate for *F. candida* without the need to add yeast or other additional feed. Their test ran for 28 days and they were able to measure survival, growth and reproduction as endpoints.

Additional requests:

* For several toxicity studies we're not convinced that the power of the test system to detect adverse effects is sufficient. This is particularly the case for the toxicity studies with *Hippodamia convergens* (Palmer & Krueger 2000a), *Ichneumon promissorius* (Sindermann et al., 2006b,c), *Eisenia fetida* (Palmer & Krueger, 2000b; Sindermann et al., 2006a) and *Folsomia candida* (Teixeira, 2006a). The experiments by Palmer & Krueger (2000a) and Sindermann (2006b,c) were conducted in three replications only, with respectively 25 insects and 10 insects per replication. The individuals within a replication cannot be regarded as independent from each other. Although the test with *F. candida* (Teixeira, 2006a) and *E. fetida* (Palmer & Krueger, 2000b; Sindermann et al., 2006a) were conducted in 4 replications per treatment (with 10 individuals per replication), again the individual test organisms cannot be regarded as independent. The statistical power is particularly critical for this study with *F.*

⁷ Bakonyi G., Szira F., Kiss I., Villanyi I., Seres A. & Szekacs A. (2006). Preference tests with collembolas on isogenic and Bt-maize. *European Journal of Soil Biology* 42: S132-S135

candida since the test compounds were tested at the natural concentration (dried GM plant material) and no safety factor could be added. We request the applicant to provide data on the sensitivity of the above-mentioned tests.

* In the studies by Mueth et al. (2006) the recovery levels of Cry2Ab2 (in the tested soils in days 0-1 of the experiment) detected by the Western Blot analyses varied between 29% and 38%; in the insect bioassay from 37% to 65% (Table 6, 7). In the case of Cry1A.105, however, levels were above 100% (between 107% and 176%) in the insect bioassay (Table 8). We ask the applicant to provide an explanation for the high values of Cry1A.105.

* In De Billot (2009a) it is mentioned that fruit flies attack maize. Can the applicant specify which kind of fruit flies?

Request 4. We request the applicant to clarify the following points:

* In the application no data are provided on seed germination, seed dormancy, pollen morphology and viability for MON 89034. As these data are relevant to evaluate potential changes in weed potential, they should be included in the application or at least be referred to.

* In the application (p. 150 of Technical Dossier) is stated "*Field trial data for MON 89034 x MON 88017 have demonstrated that this maize is not different in its phenotypic agronomic, reproductive, seed dormancy and dispersal characteristics, when compared to conventional maize.*" However, data on seed dormancy have not been provided in the field trial studies (Sammons & Leafgren, 2006a,b; De Billot, 2009a) conducted by the applicant.



Prof. D. Reheul
President of the Belgian Biosafety Advisory Council



**Secretariaat
Secrétariat**

O./ref.: WIV-ISP/41/BAC/2010_1106

**Environmental Risk/Safety Assessment (ERA) of maize line MON 89034 x MON 88017
(EFSA/GMO/BE/2009/71):**

Further requests of the Belgian Biosafety Advisory Council for clarification on the ERA

Mandate for the Group of Experts: mandate of the Biosafety Advisory Council of 6 November 2009

Coordinator: Dirk Reheul (UGent)

Experts: Patrick De Clercq (UGent), Adinda De Schrijver (SBB), Patrick du Jardin (Ulg), Jörg Romeis (Agroscope Reckenholz-Tänikon Research Station ART, Switzerland)

Secretariat (SBB): Didier Breyer, Adinda De Schrijver, Martine Goossens, Philippe Herman, Katia Pauwels

Request 1. The applicant is requested to clarify issues on the Cry2Ab2 proteins used in the NTO studies.

We are satisfied with the response, but want to note that there is also no information on biochemical and functional equivalence of the *E. coli*- and MON 89034-produced Cry1A.105 protein present in the application. We request the applicant to provide this information.

Request 2. We request the applicant to argue the results of some non-target studies in order to be able to take these studies up as confirmatory evidence that no risks are expected on non-target organisms from the exposure to Cry2Ab2 and/or Cry1A.105.

We are satisfied with the reply of the applicant on 2.1, 2.2, 2.3, 2.6 and 2.7 and have no further questions. However, we want to note that an explanation as to how the "safety factor of at least 10x" was derived (as provided in 2.1), should be provided for every non-target organism study in each application so that it becomes clear what the margin of exposure and the basis for the calculation of the test concentrations are. The test diet composition (as provided under 2.2) should also be provided with the respective non-target study in future applications.

In addition, we would like to provide some feedback on the reply of the applicant on 2.5, 2.8 and 2.9 and we have further requests on 2.4.

2.4. Toxicity study with Cry1A.105, Cry2Ab2 and Cry3Bb1 (McRae et al., 2006c)

On the basis of the information provided, we cannot agree with the statement that "there is no evidence that the combination of Cry1A.105 and Cry2Ab2 proteins enhances the activity of the Cry3Bb1 protein". Our reasonings are the following:

- (1) The LC₅₀ values and associated 95% confidence intervals only overlap slightly and not at all for Cry3Bb1 spiked with Cry2Ab2, and
- (2) The LC₅₀ values for Cry3Bb1 spiked with Cry1A.105, Cry2Ab2 of both Cry proteins is consistently lower than the LC₅₀ value of Cry3Bb1.

We therefore request the applicant to provide data that corroborate their statement.

2.5. Toxicity studies with *Orius insidiosus* (Teixeira, 2006b,c) - development

The point that was raised by the Belgian Biosafety Advisory Council was that development of *Orius insidiosus* from late second instar to adult stage on bee pollen in ca. 6 days is unlikely, given the data on development of this species at different temperatures and foods available in the literature.

In its rebuttal, the applicant uses several arguments to support the validity of the studies by Teixeira (2006b,c):

- The applicant clarifies that *O. insidiosus* was obtained from a commercial source as "early stage nymphs" and that tests were started with "late stage 2nd instars". This point is taken.
- In its subsequent argumentation, the applicant uses the studies by Butler and O'Neil (2007) and Isenhour & Yeargan (1981) to show that *O. insidiosus* can be already 6 days old when in the 2nd instar and that it is not unlikely that second instars of *O. insidiosus* develop to adulthood in ca. 6 days at 24-27°C. However, in the applicant's reasoning, the effect of diet is entirely ignored. Indeed, Isenhour & Yeargan (1981)

demonstrated that *O. insidiosus* can develop from the start of third instar (supposing that the insects in Teixeira's studies were late second instars ready to molt to the third instar) to adult emergence in 5.5 days at 28°C to 9.3 days at 24°C. However, these are data for *O. insidiosus* fed on lepidopteran eggs, not pollen like in the Teixeira studies. Based on most literature data, development on pollen alone is expected to be much slower. Kiman & Yeargan (1985) reported total nymphal developmental periods of *O. insidiosus* on pollen of ca. 19 days versus ca. 13.5 days on lepidopteran eggs at a temperature of 24°C. The first and second instar account for ca. 37% of total nymphal development (Isenhour & Yeargan 1981). Given the principle of rate isomorphy (constant allocation of relative times to different stages of development), this implies that if we use the studies by Isenhour & Yeargan (1981) and Kiman & Yeargan (1985) as a basis, the development of the predator from late second instar/early third instar to adult on pollen only would have taken about twice as long at a temperature around 24°C than the 6-day period from late second instar to adult reported in Teixeira (2006b,c). Development may be shorter if the average temperature during the test was higher (25-27°C), but the uncertainty of this extrapolation is high given that it is not known how the insects would perform on the nutritionally suboptimal pollen at these higher temperatures. Also (far) longer development periods than 6 days for late second instars were reported by Pilcher et al. (1997) and Lundgren (2009).

- In the final argument, the applicant states that based on Kiman & Yeargan (1985) "*O. insidiosus* fed pollen with insect supplements had significantly faster development times". The applicant appears to use this argument to show that development of their nymphs may have been faster due to the fact that they received lepidopteran eggs during two days *before* the experiment (whereas they only received pollen *during* the 14-day experiment). First, this is nowhere substantiated by Kiman & Yeargan's study. Latter authors showed that nymphal development of *O. insidiosus* was 1) slower on pollen than on insect prey and 2) not faster when insect prey were supplemented (i.e. offered *simultaneously*) with pollen. Second, We agree that previous diet of an insect may have an effect on development in later stages, but the extent of this effect is uncertain and based on experience and our understanding of the literature cannot explain the finding of fast development in Teixeira (2006b,c).
- Finally we can agree with the applicant's concluding statement that studies using different temperatures, diets and populations will yield different developmental data. In our opinion, however, this type of variability cannot explain the very fast development of the predators fed on bee pollen in Teixeira (2006b,c).

In conclusion, we are of the opinion that the response is of the applicant is not convincing. The study is considered to be of poor quality as it is not clear which type of pollen was used, which nymphs stage was used and what the exact temperature was during experiment. Also the occurrence of mortality late in test remains awkward (see 2.6). We therefore do not accept the test, but do not request a new test as (1) Cry1A.105 & Cry2Ab2 target Lepidoptera, (2) other beneficial arthropods are covered in the application (coccinellids, carabids & parasitic wasp) and (3) other hemipterans have been tested (*Lygus hesperus* & *Myzus persicae*).

2.8. Toxicity study with *Eisenia fetida* (Palmer & Krueger, 2000b; Sindermann et al., 2006a)

We're satisfied with the applicant's response on the earthworm study and agree that the concentrations of Cry1A.105 and Cry2Ab2 that were detected in the soil samples can still be regarded as worst-case. We would, however, suggest that the values that were actually detected are used as the NOEC.

2.9. Toxicity study with *Folsomia candida* (Teixeira, 2006a)

The applicants refer to OECD (2009) for the *Folsomia candida* test guideline. The guideline does, however, not describe how to use plant tissue as test material.

3. Additional requests:

We are satisfied with the reply of the applicant on 3.1, 3.2 and 3.3. However, in analogy with the statistical data requested for the NTO studies, we request the applicant to provide information on the statistical power of the studies conducted with target species (MacRae, 2006a,b,c).

Request 4. We request the applicant to clarify the following points or provide the following information:

We want to note that we did not receive an answer of the applicant to our request 4 in its reply of July 30th, 2010. We therefore request this information again and added some additional requests.

* In the application no data are provided on seed germination, seed dormancy, pollen morphology and viability for MON 89034 (as single event). As these data are relevant to evaluate potential changes in weed potential, they should be included in the application or at least be referred to.

* In the application (p. 150 of Technical Dossier) is stated "*Field trial data for MON 89034 x MON 88017 have demonstrated that this maize is not different in its phenotypic agronomic, reproductive, seed dormancy and dispersal characteristics, when compared to conventional maize.*" However, data on seed dormancy have not been provided in the field trial studies (Sammons & Leafgren, 2006a,b; De Billot, 2009a) conducted by the applicant. The statement should therefore be adjusted.

* Concerning the US field trials (Hartmann et al., 2006a,b), the production plan 05-01-50-02 (Hull, 2006) is missing in the application. We request the applicant to provide this information.

* Concerning the field trials with MON 88017, the breeding tree is missing. We request the applicant to include this information in the application.

* The applicant should address in its ERA if the cultivation of MON 89034 x MON 88017 might impact on non-target Chrysomelids (including threatened and endangered Chrysomelids where relevant) and other herbivores (putative targets) occurring in and around maize fields.

* We agree with the evaluation of the applicant that no phenotypic and agronomic differences between the GM and its comparators have been found. However, the low yield levels for some European field trial locations (e.g. 5.8 t/ha for MON 89034 x MON 88017 and 6.1 for MON 89034 in German field trials, see De Billot, 2009a), raises the question whether the trials allowed drawing the conclusions on expression levels. The applicant is asked to give a rationale for the low values of grain yield and to discuss the consequences of these low yield results for expression data.



Prof. Dr. Philippe Herrewé
Prof. Dr. Reheul

President of the Belgian Biosafety Advisory Council



**Secretariaat
Secrétariat**

O./ref.: WIV-ISP/41/BAC/2011_0624

**Environmental Risk/Safety Assessment (ERA) of maize line MON 89034 x MON 88017
(EFSA/GMO/BE/2009/71):**

Further requests of the Belgian Biosafety Advisory Council for clarification on the ERA

Mandate for the Group of Experts: mandate of the Biosafety Advisory Council of 6 November 2009

Coordinator: Dirk Reheul (UGent)

Experts: Patrick De Clercq (UGent), Adinda De Schrijver (SBB), Patrick du Jardin (Ulg), Jörg Romeis (Agroscope Reckenholz-Tänikon Research Station ART, Switzerland)

Secretariat (SBB): Didier Breyer, Adinda De Schrijver, Martine Goossens, Philippe Herman, Katia Pauwels

We are satisfied with all the responses of the applicant provided in its reply of May 13, 2011, except for request 2.4 on the toxicity study with Cry1A.105, Cry2Ab2 and Cry3Bb1 (McRae et al., 2006c).

The provided data of the *in planta* feeding bioassay using *Bt* maize plants of different events indicate that the combination of the Lepidoptera- and Coleoptera-active Cry proteins does not enhance the toxicity of Cry3Bb1 to larvae of the western corn rootworm (WCR). We're, however, not fully satisfied by the data presented. First, scoring the nodal injury is a less sensitive approach when compared to the artificial diet toxicity tests. Second, larvae of the WCR are less sensitive to Cry3Bb1 when compared to the Colorado potato beetle (Meissle & Romeis, 2009¹). Third it might be the case that WCR avoid feeding on Cry3Bb1 expressing roots (Clark et al. 2006²).

As the study of MacRae et al. (2006c) shows that for WCR the LC₅₀ is lower when the Cry proteins are combined (LC₅₀ = 0.25) compared to the LC₅₀ (0.41) when Cry3Bb1 is administered alone (MacRae et al., 2006c), we request the applicant to assess better whether the increase in toxicity will have no effect on non-target organisms closely related to WCR. We therefore suggest the applicant to provide data of a lab bioassay study on a coleopteran species.


Dr. D. BREYER

p.o. Prof. D. Reheul
President of the Belgian Biosafety Advisory Council

¹ Meissle, M. & Romeis, J. (2009) Insecticidal activity of Cry3Bb1 expressed in *Bt* maize on larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). *Entomologia Experimentalis et Applicata* 13, 308-319.

² Clark, P. L., Vaughn, T. Y. T., Meinke, L. J., Molina-Ochoa, J. and Foster, J. E. (2006) *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) larval feeding behavior on transgenic maize (MON 863) and its isolate, *Journal of Economic Entomology*, 99, 722-727.