**Bioveiligheidsraad**
**Conseil de Biosécurité**

***7 December 2012***

**Secretariaat**
**Secrétariat**

O./ref.: WIV-ISP/41/BAC/2012/1018

# 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 report of the Belgian Biosafety Advisory Council

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## Introduction

The application under consideration EFSA/GMO/BE/2011/90 concerns the placing on the market of the genetically modified maize line MON 89034, resistant to the European corn borer, for cultivation purposes by the Monsanto Company. Food, feed, import and processing of MON 89034 are covered by EFSA/GMO/BE/2007/37[1].

The application was submitted to the European Food Safety Authority (EFSA) in accordance with Regulation (EC) No. 1829/2003. EFSA launched a call on January 19, 2011 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 handed in 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 11 May 2012 and subsequently assessed by the Belgian Biosafety Advisory Council on request of the Belgian competent authority.

The Belgian Biosafety Advisory Council had started the evaluation of MON 89034 in 2009 in the context of application EFSA/GMO/BE/2009/71 at that time still covering MON 89034 and MON 89034 x MON 88017. On request of EFSA, MON 89034 was handed in as a separate application in 2011. On February 18, 2010 (Annex I) and November 29, 2010 (Annex II) the Belgian Biosafety Advisory Council sent its requests for additional information on the ERA of application EFSA/GMO/BE/2009/71 to the Belgian competent authority. The assessment of the information in EFSA/GMO/BE/2011/90 resulted in some further requests on the ERA of MON 89034 (Annex III), which were forwarded to the Belgian competent authority on June 12, 2012. This report describes the whole evaluation process of MON 89034 which started in 2009.

The Belgian Biosafety Advisory Council conducted its evaluation of the ERA based on the information received by the applicant on MON 89034, the information found in peer-reviewed studies (see References) and the scientific comments raised by the member states within the three month consultation period of EFSA/GMO/BE/2009/71 (November 4, 2009 until April 2, 2010).

[1]Application EFSA/GMO/NL/2007/37 has been positively assessed by the Belgian Biosafety Advisory Council (BAC, 2009) and EFSA (EFSA, 2008).

**Environmental risk assessment**

## 1. Background information

### 1.1. Recipient or parental plant

Maize (*Zea mays L.*) is a highly domesticated annual agricultural crop, incapable of surviving without human assistance under European conditions and originating from Central America. 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 has been obtained through *Agrobacterium*-mediated transformation of maize cells with plasmid vector PV-ZMIR245, containing the Cry1A.105 and Cry2Ab2 expression cassette.

### 1.3. GM plant

#### 1.3.1. Traits that have been introduced

The *cry1A.105* and *cry2Ab2* genes allow protection against lepidopteran pests (see 2.4). 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 maize line MON 89034 has been obtained through *Agrobacterium*-mediated transformation of maize cells with plasmid vector PV-ZMIR245 containing 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 ribulose 1,5-biphosphate carboxylase small subunit and first intron, and nopaline synthase terminator sequences (T-NOS) from *A. tumefaciens*.

Data demonstrating the molecular and functional equivalence of the Cry1A.105 and Cry2Ab2 proteins produced by *E. coli* or *B. thuringiensis* (only the case for Cry2Ab2) and in MON 89034 were originally lacking and requested (see Annex I & II). The information provided on the equivalence of the Cry proteins produced by *E. coli* to those produced in maize had been evaluated in the food/feed application of MON 89034 (BAC, 2009; EFSA, 2008); the information on the equivalence of *B. thuringiensis*- and MON 89034-produced Cry2Ab2 proteins was novel and therefore assessed in detail.
As there are uncertainties on the precise *N*-terminus of the plant-produced Cry2Ab2 protein, the *E. coli*-produced Cry2Ab2 is either identical to the plant-produced protein or differs in 3 amino acids at the *N*-terminus; the *B. thuringiensis*-produced protein differs either by a single amino acid or by 4 amino acids from the plant-produced protein at the *N*-terminus. The studies demonstrating the equivalence of the biological activity of the *E. coli*-produced and *B. thuringiensis*-produced Cry2Ab2 (Levine & Uffman, 2006) were positively evaluated by the Biosafety Advisory Council and found to be sufficient to prove that small differences at molecular level at the *N*-terminus do not influence the biological activity.

### 1.3.3. Expression of the insert

The expression level of Cry1A.105 and Cry2Ab2 was determined in various tissues of MON 89034 (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; Hartmann *et al.*, 2006a,b), in Argentina (5 sites) in 2004 (see EFSA/GMO/NL/2007/37), and from field trials in the EU (7 sites in Germany and Spain) in 2007 (De Billot, 2008; Niemeyer and Silvanovich, 2008a,b). 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 (see Annex II).

In the evaluation of the data by the Biosafety Advisory Council, main focus was put on the expression values obtained from MON 89034 grown in the 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 and Cry2Ab2 proteins were found to be expressed in all tissues tested.

[2] Cry2Ab2 produced in MON 89034 is identical to that of Cry2Ab2 produced in MON 15985 cotton (Bollgard II).

For MON 89034 samples taken from the 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, 2008a,b).

On the basis of data measured in the EU field trials, the Biosafety Advisory Council calculated 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. 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

Both laboratory experiments (Woodrum 2005, 2006) and multi-site field trials have been conducted with MON 89034 in 2004 (USA: 9 sites, Kendrick *et al.*, 2005a,b; Leafgren, 2005), 2005 (USA: 9 sites, Kendrick & Clark, 2006a,b,c,d), 2005/2006 (Argentina: Clark *et al*., 2007) and 2007 (EU: 8 sites, De Billot, 2008; 2009; 2010) to compare phenotypic characteristics of maize line MON 89034 to conventional maize. Potential changes in reproduction (germination, dormancy, pollen morphology and viability, seedling vigour, silking and pollen shed) and agronomic characteristics, i.e. early and final stand count, stalk lodging, root lodging, plant and ear height, dropped ears, stay green, ear/kernel/stalk rot and yield were studied. A randomised complete block design with three replications was used at each site in the field studies. The studies on seed germination, seed dormancy, pollen morphology and viability (Woodrum 2005, 2006) were missing in the original application and asked for (see Annex III).

The field studies conducted in the USA in 2004 and 2005 and the information in the studies of Woodrum (2005, 2006) have been evaluated by EFSA in the context of application EFSA/GMO/NL/2007/37 (EFSA, 2008). EFSA concludes in its opinion "... *field trials did not show changes in phenotypic characteristics and agronomic performance except for the introduced traits*." The data from field trials in the EU and Argentina were evaluated by the Belgian Biosafety Advisory Council. For the field study conducted in the EU in 2007, comparison of MON 89034 maize with a non-transgenic maize with a similar genetic background as MON 89034 across field sites in Spain did not reveal any across-site phenotypic differences, except for the number of stalk lodged plants. There were fewer stalk lodged plants in MON 89034 compared to the control (0.0 *versus* 0.5, respectively). The mean values for stalk lodging for MON 89034 fell within the range of the reference varieties (conventional maize hybrids currently cultivated). No across-site differences were found in the German field trials. Comparison of the values for phenotypic and agronomic characteristics of MON 89034 grown in Argentina with the control (Clark *et al*., 2007) also revealed few

statistical differences. Days after planting to 50% pollen shed and yield were higher for MON 89034 compared to the control, but were within the references ranges.
The Biosafety Advisory Council agreed with the conclusion of the applicant that the relatively small differences detected were not considered biologically meaningful with respect to plant weed potential.

Further, potential changes in ecological interactions of MON 89034 in the EU with insect pests and diseases naturally present in the managed sites, and abiotic stressors were recorded at 4 growth stages during the 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 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, (2.7) 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 (2.4) target and (2.5) non-target interactions, and (2.6) effects on human and animal health 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 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 and the newly introduced traits.

Given (a) the biology of maize (see 1.1), (b) the information that maize line MON 89034 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 to MON 89034 are not expected to change the persistence and invasiveness potential of maize, it can be concluded that the likelihood of MON 89034 to become more persistent or invasive is negligible.

**2.2. Selective advantage and disadvantage**

The insect-resistance traits Cry1A.105 and Cry2Ab2 confer specific advantages to **maize in the field**, namely resistance to certain lepidopteran pests. Comparison of MON 89034 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 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 maize.

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, 2009a). In the very unlikely case of transfer, maintenance and functional expression of the *cry1A.105* or *cry2Ab2* gene in micro-organisms of the receiving environment, no impact on the ecology of microbial 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**

An issue taken into consideration in the evaluation of the ERA was the equivalence of the plant and bacterial derived Cry1A.105 and Cry2Ab2 toxin used in the target (and non-target) toxicity studies. The Biosafety Advisory Council concluded that the Cry1A.105 and Cry2Ab2 proteins produced from different sources were functionally equivalent (see 1.3.2).

The biological activity of Cry1A.105 and Cry2Ab2 has been tested via dietary laboratory studies on a range of herbivorous insects feeding on maize (MacRae *et al*., 2006a,b). The studies were considered to be well-conducted, but additional information on the diet composition and statistical power of the studies was requested (see Annex I & II). MacRae *et al*. (2006a,b) showed that the activity of Cry1A.105 and Cry2Ab2 is restricted to lepidopteran pests of maize, namely the European corn borer (Crambidae: *O. nubilalis*), black cutworm (Noctuidae: *Agrotis ipsilon*), corn earworm (Noctuidae: *Helicoverpa zea*) and fall armyworm (Noctuidae: *Spodoptera frugiperda*). Avilla *et al*. (2005) showed that Cry2Ab2 acts against the *H. armigera* (Noctuidae).
The dietary toxicity data were confirmed by efficacy trials in Argentina, Puerto Rico and USA with MON 89034 (Headrick *et al*., 2006a,b) and quantitative observations of target pests in field trials with MON 89034 conducted in Argentina (Clark *et al*., 2007). Field studies showed that MON 89034 is active against additional lepidopteran pests, namely the sugarcane borer (Crambidae: *Diatraea saccharalis* - Clark *et al*., 2007; Wu *et al*., 2009) and southwestern corn borer (Crambidae: *Diatraea grandiosella* - Headrick *et al.*, 2006a). Further Cry2Ab2 produced in MON 15985 cotton, which is identical to Cry2Ab2 produced in MON 89034, has been shown to be active against several noctuid and gelechiid Lepidopteran cotton pests (Sivasupramaniam *et al*., 2008).

The Biosafety Advisory Council does not subscribe the statement of EFSA of 2008 concerning Cry2Ab2 "*The chimeric cry1A.105 gene and the cry2Ab2 gene both code for proteins that are well known to be toxic to lepidopteran or lepidopteran/dipteran insects*" (EFSA, 2008). No evidence could be found in the literature indicating that Cry2Ab present in MON 89034 and isolated from *B. thuringiensis* subsp. *kurstaki* HD-1 is also active towards dipteran species (Widner and Whiteley, 1989; 1990; Dankocsik et al., 1990; Donovan, 1991; Liang et al., 1994; Morse et al., 2001). Taken the scientific literature and the equivalence of *B. thuringiensis*-produced and plant-produced Cry2Ab2 (see 1.3.2) into account, it was postulated that Cry2Ab from *B. thuringiensis* HD-1 would not impact Diptera more than any other insect order outside the Lepidoptera.

The activity of Cry1A.105 and Cry2Ab2 protein is not likely to be affected by potential interactions of Cry1A.105 and Cry2Ab2 with one another. The applicant demonstrated through the use of sensitive organisms that the activity of Cry1A.105 and Cry2Ab2 together

was additive (MacRae *et al*., 2005). Hence, the Biosafety Advisory Council considered studies combining the two Cry proteins to assess the impact on target and non-target organisms not necessary.

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

The potential of maize line MON 89034 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. As mentioned in 2.4, the equivalence of the plant- and bacterial-derived toxin used in the toxicity studies was taken into account in the evaluation. The potential of non-target effects due to the expression of the traits is further discussed in this section.

***Non-target effects on insects***

Information on the lack of potential adverse effects on non-target organisms of Cry1A.105 and Cry2Ab2 was obtained from laboratory dietary toxicity studies with insects living above- and on-ground and from field studies. Although mainly non-European species where used in the lower-tier studies, except for honeybee and green peach aphids, the selected species were considered as representative for European genera or functional groups in maize by the Biosafety Advisory Council. Ecologically and economically important functional groups, namely herbivores, beneficial insect predators/parasitoids and pollinators were considered.

During the evaluation, additional information on the statistical power of the studies, on diet composition, test concentrations, life-stages used and exposure to the toxin was requested (see Annex I, II & III). The dietary toxicity tests carried out by the applicant on non-target insects were overall considered to be well-conducted, except for the *Hippodamia convergens* (Palmer and Krueger, 2000a) and the *Orius insidiosus* (Teixera, 2006b,c) study (see Annex I & II). The improbable results on nymphal development on pollen (a very short developmental time of ca. 6 days from second instar to adult stage compared to ca. 15 days in literature) shed doubts on the credibility of the *Orius* test which was therefore not accepted as a confirmatory study. Regarding the *H. convergens* study, the Biosafety Advisory Council was not convinced that the test species was sufficiently exposed to the Cry2Ab2 toxin. The shortcomings of the *H. convergens* study were recognised by the applicant and the study was superseded by a more robust *Coleomegilla maculata* study.

It was shown (and the Biosafety Advisory Council agreed) that Coleoptera, namely larvae and adults of the predatory ladybird beetle *C. maculata* (Paradise, 2006a,b) and Hymenoptera, namely an ichneumonid parasitoid (*Ichneumon promissorius*: Sindermann *et al.*, 2006b,c) and larvae and adults of honeybee (*Apis mellifera*: Maggi, 2000a,b,c; Richards, 2006a,b; Hendriksma et al., 2011, 2012) occurring in maize would not be affected by Cry1A.105 or Cry2Ab2. Further, activity spectrum studies showed that Cry1A.105 and Cry2Ab2 did not affect Hemiptera, namely the western tarnished plant bug (*Lygus hesperus*: MacRae *et al*., 2006a,b) and the green peach aphid (*Myzus persicae*: MacRae *et al*., 2006a,b) and Coleoptera, namely larvae of the boll weevil (*Anthonomus grandis grandis*: MacRae *et al*.,

2006a,b) and the Southern corn rootworm (*Diabrotica undecimpunctata howardi*: MacRae *et al*., 2006a,b).

The conclusions of the laboratory studies were supported by field studies with Cry1A.105- and Cry2Ab2-expressing *Bt* maize plants (MON 89034, MON 89034 x NK603) conducted in Argentina (Clark *et al*., 2007). The field study showed that overall (across-sites) there were no statistical differences in abundances of predators (Coccinellidae, *Chrysopa* spp. and *O. insidiosus*) and the parasitoid *Trichogramma* in Cry1A.105- and Cry2Ab2-expressing maize fields compared to non-*Bt* maize.

As for other *Bt* maize events expressing Cry proteins toxic for Lepidoptera (a.o. MON 810) MON 89034 might impact other lepidopteran species than the target pests, including endangered species. The risk to non-target Lepidoptera larvae 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 both Cry1A.105 and Cry2Ab2. The worst-case exposure concentration in field was calculated to be 3 times less than the one needed to cause 50% mortality of the known most sensitive lepidopteran species (*O. nubilalis*). Hence, the margins of safety were calculated to be 3 and up to 73 times the maximum estimated exposure of non-target Lepidoptera to pollen within the maize field times and at 4-5 m from the field edge, respectively.

The Biosafety Advisory Council regarded the assessment of impacts on non-target Lepidoptera 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 be of more importance in case of endangered lepidopteran species. Further, the Council is of the opinion that an assessment taking into account true exposure to maize pollen (instead of the maximum estimated exposure) and the abundance of non-target Lepidoptera in European maize fields, would increase the safety margins. Recently carried out field experiments substantiate the theoretic quantitative assessment. 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, including endangered ones, are expected due to cultivation of MON 89034.

Further, the Biosafety Advisory Council considered potential impacts on Lepidoptera in aquatic environments near maize fields negligible as exposure to Cry1A.105 and Cry2Ab2 is unlikely due to their feeding habits on algae (Carstens *et al*., 2011).

In conclusion, the Biosafety Advisory Council is of the opinion that the studies provided confirm the target specificity of Cry1A.105 and Cry2Ab2 to Lepidopteran species 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.

***Non-target effects on other organisms than insects***

As the toxicity of Cry1A.105 and Cry2Ab2 is specific (see 2.4), no direct effects on other invertebrates than Lepidoptera or on vertebrate organisms are expected (see also 2.6). The toxicity and specificity is associated with the binding to specific cell membrane receptors in the brush border membrane vesicles present in the midgut of susceptible insects.

In the evaluation focus was put on the studies of animals occurring in/around maize fields and that had not been evaluated in the context of application EFSA/GMO/NL/2007/37. During the evaluation, additional information on exposure to the toxin and on validity of the test design was requested (see Annex I, II & III). The tests carried out by the applicant were overall considered to be well-conducted, except for the *Daphnia magna* study (Gallagher & Krueger, 2009). The *D. magna* study was considered to be inconclusive as exposure was not confirmed.

It was shown (and the Biosafety Advisory Council agrees) that the collembolan *Folsomia candida* (Teixeira, 2006a), the worm *Eisenia fetida* (Palmer & Krueger, 2000b; Sindermann *et al.*, 2006a), the ground-dwelling bird bobwhite quail (*Colinus virginianus*: Gallagher & Beavers, 2006) and rodents (for mice and rats see EFSA/GMO/NL/2007/37), would not be affected by Cry1A.105 or Cry2Ab2. Further, the Biosafety Advisory Council is of the opinion that the study on the aquatic invertebrate *D. magna* adds little certainty to the risk assessment as there is little evidence to suggest that *D. magna,* a particle feeder known to feed on algae, will be exposed to significant levels of Cry proteins (Carstens *et al*., 2011). The maize pollen grains (size of ~ 90 µm diameter) are assumed to be too large for daphnids to ingest (Burns, 1968). The Biosafety Advisory Council agreed with the assessment that adverse effects on organisms other than insects are negligible.

**2.6. Effects on human and animal health**

For the evaluation of effects on human and animal health due to accidental consumption, we refer to the food/feed assessment of maize line MON 89034 (EFSA, 2008).

In EFSA (2008) it is indicated that there is a lack of structurally relevant similarity between the Cry1A.105 or Cry2Ab2 protein and any known allergens, toxic or pharmacologically active proteins relevant for human and animal health present in the TOXIN5 database of 2001. The applicant demonstrated this conclusion is still up-to-date when using the toxin database TOX_2011 (Tu, 2011; Tu & Silvanovich, 2011). Also no similarities with known allergens were found for the Cry1A.105 or Cry2Ab2 protein using the more recent allergen database AD_2011 (Tu, 2011; Tu & Silvanovich, 2011). Hence, the likelihood for any adverse effects due to contact with MON 89034, was regarded as negligible.

**2.7. Effects on biogeochemical processes**

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

on whether detritivores and decomposers involved in biogeochemical processes may be affected by Cry1A.105 and Cry2Ab2.

Dietary toxicity studies showed that Cry1A.105 and Cry2Ab2 do not affect springtails and worms (see 2.5). As other *Bt* toxins, Cry1A.105 and Cry2Ab2 can be introduced into the soil via leaching from root exudates and incorporation of plant residues after harvest. A laboratory study (Mueth *et al*., 2006) showed that Cry1A.105 and Cry2Ab2 is degraded rapidly: the half-life of Cry1A.105 in decomposing MON 89034 maize is less than 7 days (the dissipation time for a 90% decrease (DT90) less than 90 days); the half-life of Cry2Ab2 6 days (DT90 less than 14 days). Further, shoot and root tissues of MON 89034 were not shown to pose significant risk to micro-organisms and microbial-mediated carbon and nitrogen mineralisation processes in the soil (Huizinga *et al*., 2007) or structure and function of endophytic microflora (Prischl *et al*., 2012). On the basis of these data, the Belgian Biosafety Advisory Council agreed that immediate or delayed adverse effects on biogeochemical processes due to interaction of MON 89034 with non-target organisms is expected to be negligible.

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

The Biosafety Advisory Council agreed with the prognosis of the applicant that specific cultivation, management and harvesting techniques used for MON 89034 will be comparable to those used for other commercially available maize varieties, with the exception of the insect resistance monitoring plan. The opinion of EFSA (2009b) states "*No new specific cultivation practices, management or harvesting techniques are associated to the cultivation of maize MON810. The only difference between maize MON810 and its conventional counterpart is due to fewer insecticide treatments needed to control lepidopteran target pests such as Ostrinia nubilalis and Sesamia nonagrioides (Gómez-Barbero et al., 2008)*." Based on the experience with the cultivation of the Cry1A-expressing MON 810 it can be considered that MON 89034 cultivation, management and harvesting techniques in the EU will be similar to MON 810. The Biosafety Advisory Council considered the farmer questionnaires the means to provide feedback on this issue.

An insect resistance monitoring plan will be adopted by the applicant to prevent resistance evolution. If insect resistance would develop due to MON 89034 cultivation, it is not expected that this would lead to changes in the current use of insecticides to control ECB and MCB, i.e. augmentation of use and type of insecticides used. Taking into account the EU regulatory requirements for the sustainable use of pesticides, including reduction of pesticide usage, presumably from 2014 on (European Parliament, 2009) one can expect that alternative options to insecticides will be promoted to control ECB and MCB.

**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, are not expected to 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 agreed 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.

As there were no environmental concerns identified in the ERA that urged the need of a management strategy, the proposed refuge strategy for ECB 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 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 maize in the Member States. It will focus on areas where MON 89034 maize 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 management regimes that do not have an environmental performance at least as good as current regimes. The following is advised:

- Under 2.5 "*Mark all typical weed and pest control practices in maize at your farm*" to also specifically ask for information on the pesticides. Currently, only information on weed control practices is requested and not on pest control practices, such as insecticides, fungicides and use of biocontrol treatments.
- 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.

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 markets", describing recommendations for the use of their product.

**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.

p.o. Dr. Philippe Herman

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

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**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.

**Bioveiligheidsraad**
**Conseil de Biosécurité**

***18 February 2010***

**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)

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***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 (2000b,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 (2000b,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.

***Tocixity 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

**Bioveiligheidsraad**
**Conseil de Biosécurité**

***29 November 2010***

**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 $LC_{50}$ values and associated 95% confidence intervals only overlap slightly and not at all for Cry3Bb1 spiked with Cry2Ab2, and
(2) The $LC_{50}$ values for Cry3Bb1 spiked with Cry1A.105, Cry2Ab2 of both Cry proteins is consistently lower than the $LC_{50}$ 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 2$^{nd}$ 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 2$^{nd}$ 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. Tocixity 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.

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

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**Bioveiligheidsraad**
**Conseil de Biosécurité**

*12 June 2012*

**Secretariaat**
**Secrétariat**

O./ref.: WIV-ISP/41/BAC/12_0585

**Environmental Risk/Safety Assessment (ERA) of maize line MON 89034 (EFSA/GMO/BE/2011/90):**

**Requests of the Belgian Biosafety Advisory Council for clarifications on the ERA & PMEM**

**Mandate for the Group of Experts:** mandate of the Biosafety Advisory Council of 24 January 2011
**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

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***Request 1. We have noted several references are missing in the application. We ask the applicant to provide these references.***

| | |
|---|---|
| De Billot, 2008 | The production plan for the EU field trials |
| Hartmann, 2005 | Assessment of the Cry1A.105, Cry2Ab2, and Cry3Bb1 Protein Levels in Tissues of Insect-protected Corn MON 89034, MON 89034 x MON 88017, MON 89597, MON 89597 x MON 88017. Monsanto Technical Report MSL- 19982, St. Louis, MO. |
| Hull, 2006 | The production plan 05-01-50-02 for the US field trials |
| Woodrum, 2005 | Data on seed germination and dormancy for MON 89034 |
| Woodrum, 2006 | Data on pollen morphology and viability for MON 89034 |

***Request 2. We request the applicant to argue the results of the non-target study on Daphnia magna (Gallagher & Krueger, 2009) in order to be able to take this study up as confirmatory evidence that no risks are expected on non-target organisms from the exposure to Cry2Ab2 and/or Cry1A.105.***

Our main criticisms are on:

**Exposure**

- There is no proof of exposure in this experiment. No positive control is available to demonstrate effective exposure of the test animals. Pollen of maize (with a diameter of ~90µm) is assumed to be too large for daphnids to ingest (Burns, 1968). In certain experiments, however, daphnids have been reported to show internal coloration which is assumed to be a result of the (yellow) pollen ingestion (Monsanto 2002a cited in OECD, 2007). No such observation was reported in the present study, unless if discolorations mentioned on p.17 of the study refer to this phenomenon. Can the applicant clarify if this is the case?
- Further, has it been verified whether Cry toxins from MON 89034 pollen grains have diffused into the water environment in the test chambers and as such have led to exposure of the daphnids in the present study?

**Variability and validity of the test design**

- The test design of the present study may be questioned given the high mortality of the test organisms in the pollen control group after 14 days (30%) and even higher (but statistically similar) rates in both MON 89034 treatments. These high mortality rates are attributed to physical toxicity of the high concentrations of pollen in the living environment of the daphnids and not to the quality of the test animals, as demonstrated by the very low mortality in the assay control (5%). However, the guidelines for reproduction tests with *Daphnia magna* (OECD, 2008) state that the mortality of the parent animals (female Daphnia) should not exceed 20% at the end of the test and should last for 21 days. Can the applicant clarify why a test of 14 days with a mortality of 30% would be appropriate to assess potential impacts?
- We have reservations concerning the reproduction test, related to the high variability of the measured endpoint "Mean no. of young per reproductive day" in the pollen control: the coefficient of variation of this parameter is 52%. The OECD guidelines for reproduction tests with *Daphnia magna* (OECD, 2008) state that the coefficient of variation of the parameter "mean no. of offspring produced per parent animal" in the controls should be less than 25%. However, endpoints were not expressed in this manner in the study by Gallagher & Krueger. It is not clear how many females were responsible for the production of the brood in the 4 replicates A-D and this complicates a sound interpretation of the results of the reproduction test. Can the applicant clarify this issue?

**References:**

Burns, 1968. The relationship between body size of filter-feeding Cladocera and the maximum size of particle ingested. Limnology and Oceanography, 13, 675.

OECD, 2007. Consensus document on safety information on transgenic plants expressing *Bacillus thuringiensis* – Derived insect control protein, Series on Harmonisation of Regulatory Oversighr in Biotechnology No. 45, OECD Environment Directorate, Paris. (Available on the BioTrack website at www.oecd.org/biotrack/).

OECD, 2008. OECD guidelines for the testing of chemicals. *Daphnia magna* Reproduction test.

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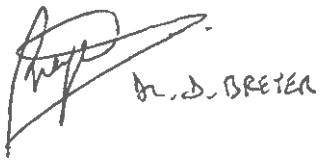
***Request 3. We request the applicant to substantiate the claim that a safety factor of at least 10X is chosen in the studies of Hartmann Paradise (2006a,b), Richards (2006a,b) and Sindermann (2006b,c) by clarifying to which data is referred to and providing these data (references) if not yet available in the application.***

***Request 4. We ask the applicant to clarify an issue on the farmer questionnaire.***

Under 2.5 "Mark all typical weed and pest control practices in maize at your farm" of the farmer questionnaire, only information on herbicides are specifically requested and not information on use of insecticides, fungicides, *etc.* Can it be clarified why in contrast to former farmer questionnaires no information on the pesticides, other than herbicides, is asked for?



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