SCIENTIFIC OPINION

Scientific Opinion on an application from Pioneer Hi-Bred International and Dow AgroSciences LLC (EFSA-GMO-NL-2005-23) for placing on the market of genetically modified maize 59122 for food and feed uses, import, processing and cultivation under Regulation (EC) No 1829/2003[1]

EFSA Panel on Genetically Modified Organisms (GMO)[2,3]

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ABSTRACT

This Scientific Opinion reports on a risk assessment on an application for placing on the market of genetically modified maize 59122 for import and processing for food and feed uses and cultivation. The EFSA GMO Panel considers that maize 59122 is unlikely to have any adverse effect on the environment, except for the possible evolution of resistance to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests. The Panel recommends the implementation of appropriate and diversified insect resistance management strategies and case-specific monitoring to delay and monitor the possible evolution of resistance to Cry34Ab1/Cry35Ab1 in coleopteran target pests, respectively. In addition, the Panel recommends revision of the applicants' insect resistance management plan and the proposed post-market environmental monitoring plan. Although maize 59122 is tolerant to glufosinate-ammonium-based herbicides, the Panel did not assess the potential adverse effects associated with the use of such herbicides on maize 59122, as maize 59122 will not be marketed in the European Union as a herbicide-tolerant crop. This Scientific Opinion updates the previous Panel safety evaluation of the food and feed uses, and import and processing of maize 59122 and derived products. The Panel concludes that the information available for maize 59122 addresses the scientific comments raised by Member States and that maize 59122, as described in this application, is as safe as its conventional counterpart and commercial maize varieties with respect to potential adverse effects on human and animal health. If subjected to appropriate management measures, the cultivation of maize 59122 is unlikely to raise safety concerns for the environment.

KEY WORDS

GMO, maize (*Zea mays*), 59122, *cry34Ab1*, *cry35Ab1*, risk assessment, food and feed safety, environment, cultivation.

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1 On request from the Competent Authority of the Netherlands for an application (EFSA-GMO-NL-2005-23) submitted by Pioneer Hi-Bred International and Dow AgroSciences LLC, Question No EFSA-Q-2005-250, adopted on 6 March 2013.

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SUMMARY

Following the submission of an application (Reference EFSA-GMO-NL-2005-23) under Regulation (EC) No 1829/2003 from Pioneer Hi-Bred International and Dow AgroSciences LLC (referred to hereafter as the applicant), the Panel on Genetically Modified Organisms of the European Food Safety Authority (EFSA GMO Panel) was asked to deliver a Scientific Opinion on the safety of the genetically modified (GM) insect-resistant and herbicide-tolerant maize 59122 (Unique Identifier DAS-59122-7) for food and feed uses, import and processing, and cultivation. As the scope of this application also covers the food and feed uses, and import and processing of maize 59122, this Scientific Opinion updates the previous EFSA GMO Panel safety evaluation of the food and feed uses, and import and processing of maize 59122 and derived products.

In delivering its Scientific Opinion, the EFSA GMO Panel considered the application EFSA-GMO-NL-2005-23, additional information supplied by the applicant, scientific comments submitted by Member States, the environmental risk assessment report of the Competent Authority of the Netherlands (NL CA), and relevant scientific publications.

Maize 59122 expresses the *cry34Ab1* and *cry35Ab1* genes from *Bacillus thuringiensis*, conferring resistance to coleopteran insect pests belonging to the genus *Diabrotica* such as the larvae of western corn rootworm (WCR; *Diabrotica virgifera virgifera*), and the *pat* coding sequence from *Streptomyces viridochromogenes*, which renders maize 59122 tolerant to the herbicidal active substance glufosinate-ammonium. Although maize 59122 is tolerant to glufosinate-ammonium-based herbicides, the EFSA GMO Panel did not assess the potential adverse effects associated with the use of such herbicides on maize 59122, as maize 59122 will not be marketed in the European Union (EU) as a herbicide-tolerant crop.

The EFSA GMO Panel evaluated maize 59122 with reference to its intended uses and the appropriate principles described in its guidelines for the risk assessment of GM plants and derived food and feed, the environmental risk assessment of GM plants, the selection of comparators for the risk assessment of GM plants, and for the post-market environmental monitoring of GM plants. The scientific evaluation of the risk assessment included molecular characterisation of the inserted DNA and expression of target proteins. An evaluation of the comparative analyses of the composition and agronomic and phenotypic characteristics was undertaken, and the safety of the new proteins, both individually and in combination, and the whole food/feed was evaluated with respect to potential toxicity, allergenicity and nutritional quality. An evaluation of environmental impacts and the post-market environmental monitoring plan was undertaken.

The molecular characterisation data establish that maize 59122 contains a single insert of the T-DNA. No vector backbone sequences are present in the transformed plant. Bioinformatic analyses of the open reading frames spanning the junction sites within the insert or between the insert and genomic DNA did not raise safety issues. The levels of the Cry34Ab1, Cry35Ab1 and PAT proteins in various plant parts collected from field trials performed in Europe have been sufficiently analysed. The stability of the inserted DNA and phenotypes was confirmed over several generations.

Based on the results of compositional analysis of samples from a representative range of environments and seasons, the EFSA GMO Panel concludes that forage and kernels of maize 59122 are compositionally equivalent to those of conventional maize, except for the presence of Cry34Ab1/Cry35Ab1 and PAT proteins. In addition, results from field trials did not show indications of unexpected changes in agronomic performance and phenotypic characteristics.

The Cry34Ab1/Cry35Ab1 proteins induced no adverse effects in acute and repeated dose oral toxicity studies in rodents. In addition, these proteins are rapidly degraded in simulated gastric fluid and inactivated during heat treatments.

A 90-day feeding study of rats fed a diet including kernels from maize 59122 at a level of 35 % indicated no adverse effects. A feeding study of broilers did not indicate differences in the nutritional

value of maize 59122 versus the conventional comparator. These animal studies support the findings of the compositional analysis and indicate no effect beyond the intended introduction of the Cry34Ab1/Cry35Ab1 and PAT proteins.

Diets formulated with 59122 were shown to be as nutritious as those formulated with commercial non-GM maize varieties.

In a previous Scientific Opinion on maize 59122 for food and feed uses, the EFSA GMO Panel concluded that maize 59122 is unlikely to have any adverse effect on human and animal health in the context of its intended uses. The applicant performed for the current application a screening of the literature with respect to publications that could be considered as relevant to the risk assessment of food and feed uses of maize 59122 and that were not considered as part of the risk assessment in the previous EFSA GMO Panel Scientific Opinion. The literature search revealed several new publications, the assessment of which did not change the previous conclusion that maize 59122 is unlikely to have any adverse effect on human and animal health in the context of its intended uses.

As the scope of the current application covers cultivation, the environmental risk assessment considered the environmental impact of full-scale commercialisation of maize 59122.

The NL CA provided to EFSA its report on the environmental risk assessment of maize 59122 on 13 May 2008 in line with Articles 6.3(c) and 18.3(c) of Regulation (EC) No 1829/2003. The report on the environmental risk assessment of the NL CA is provided in Annex H of the EFSA overall opinion, and has been considered in this EFSA GMO Panel Scientific Opinion.

Maize 59122 has no altered agronomic and phenotypic characteristics, except for the specific target insect resistance and herbicide tolerance. The likelihood of unintended environmental effects due to the establishment, survival and spread of maize 59122 is considered to be extremely low, and will be no different from that of conventional maize varieties.

It is highly unlikely that the recombinant DNA will transfer and establish in the genome of bacteria in the environment or human and animal digestive tracts. In the rare but theoretically possible case of transfer of the *cry34Ab1*, *cry34Ab1* and *pat* genes from maize 59122 to soil bacteria, no novel property would be introduced into the soil bacterial community and thus no positive selective advantage that would not have been conferred by natural gene transfer between bacteria would be provided.

WCR has the ability to evolve resistance to the Cry34Ab1/Cry35Ab1 proteins, especially if maize 59122 is used repeatedly and exclusively, and the WCR infestation levels are high. The possible resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests is identified by the EFSA GMO Panel as a concern associated with the cultivation of maize 59122, as resistance evolution may lead to altered pest control practices that may cause adverse environmental effects. The EFSA GMO Panel therefore recommends that appropriate risk management strategies are implemented to delay and monitor resistance evolution.

Based on the evidence provided by the applicant and relevant scientific literature on maize 59122, there are no indications of adverse effects on non-target organisms due to unintended changes in maize 59122.

The potential adverse effects of maize 59122 due to the expression of the Cry34Ab1, Cry35Ab1 and PAT proteins on non-target terrestrial (plant- and ground-dwelling), soil and aquatic arthropods, as well as non-target organisms that are not arthropods, are expected to be negligible in the context of its intended uses, except for chrysomelids. The risk of maize 59122 to non-target chrysomelid species in the field is low due to their low occurrence and abundance in maize fields and because of the low likelihood of encountering harmful amounts of pollen from maize 59122 in and around maize fields. Non-target adult chrysomelids, which may occasionally feed on maize 59122 plants, are not expected to be affected due to the low activity of the Cry34Ab1/Cry35Ab1 proteins on adults. Furthermore, the

only protected chrysomelid species (*Macroplea pubipennis*) considered to be at risk across the EU (under Directive 92/43/EEC on conservation of natural habitats and of wild fauna and flora) does not occur in maize fields.

The apparent activity of Cry34Ab1/Cry35Ab1 at high concentrations against the lepidopteran species (e.g., *Ostrinia nubilalis* and *Sitotroga cerealella*) was not expected based on the known spectrum of activity (Coleoptera only) of these binary proteins. The EFSA GMO Panel considers that there are indications of a potential hazard to Lepidoptera owing to cross-order activity at high Cry34Ab1/Cry35Ab1 protein concentrations. However, based on the submitted toxicity data and a theoretical exposure assessment, no risk to non-target Lepidoptera is expected from exposure to maize 59122 pollen in the field.

Despite the limited sequence similarity between Cry35Ab1 and dipteran-active binary toxins from *Lysinibacillus sphaericus*, no hazard to *Culex quinquefasciatus* and no risk to Diptera are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 and maize 59122.

Based on general knowledge of the degradation of plant-produced *Bt*-proteins in soils and the overall concentrations of Cry34Ab1/Cry35Ab1 proteins in maize 59122, it is unlikely that the Cry34Ab1/Cry35Ab1 proteins will reach soil concentrations that would affect non-target organisms, in context of the intended uses of maize 59122. Although no risk was identified in the short term, scientific uncertainties pertaining to the specific potential of Cry34Ab1/Cry35Ab1 to accumulate and persist in soil during subsequent years of cultivation of maize 59122 remain, owing to the lack of experimental evidence. Therefore, the EFSA GMO Panel recommends that the remaining scientific uncertainties can be resolved with data acquired during post-market environmental monitoring.

Potential effects on soil microorganisms and microbial communities, as well as the ecosystem services they provide, due to the cultivation of maize 59122, if they occur, will be transient and minor, and are likely to be smaller or within the range currently caused by other agronomic and environmental factors.

The conclusions of the EFSA GMO Panel are consistent with those of the NL CA, which concluded that "*cultivation of line 59122 poses a negligible risk to human health and the environment*" (Section 8 of the environmental risk assessment report of the NL CA).

The EFSA GMO Panel evaluated the efficacy and made recommendations on the scientific quality of the insect resistance management plan proposed by the applicant. While caution must be exercised when extrapolating laboratory and greenhouse results to field conditions, evidence indicates that several conditions contributing to the success of the high dose/refuge strategy are not met for maize 59122 and WCR. Scientific uncertainties related to the appropriateness of the proposed strategy in delaying resistance evolution in WCR remain. Therefore, the EFSA GMO Panel does not accept the high dose/refuge strategy as the sole insect resistance management strategy, and requires that the applicant's insect resistance management plan should be complemented with additional resistance management measures. Stewardship agreements should prescribe diversity in cropping and WCR management practices, and recommend: (1) rotating fields to crops that are not hosts of WCR larvae; (2) alternating maize 59122 with other *Bt*-maize events that express one or more different *Bt*-protein(s) active against WCR; and (3) using additional pest management measures, such as insecticides or biological control agents, only when and where necessary in maize 59122 fields. The additional recommendations made by the EFSA GMO Panel to revise the applicant's insect resistance management plan in terms of refuge requirements should also be implemented by the applicant.

The EFSA GMO Panel recommends that resistance and compliance monitoring is conducted to allow the periodic evaluation of the adequacy and efficacy of the revised insect resistance management strategy.

If appropriate insect resistance management measures are implemented, the EFSA GMO Panel concludes that resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests could be successfully delayed.

The NL CA acknowledged the potential for resistance to the Cry34Ab1/Cry35Ab1 proteins to evolve within the Diabrotica spp. population. The NL CA noted that "*the insect resistance management approach proposed by the applicant will only be adequate in case of recessive inheritance of Bt-resistance*", but did not assess the appropriateness of the insect resistance management plan further (Section 7.1 of the environmental risk assessment report of the NL CA).

The EFSA GMO Panel gave its opinion and made recommendations on the scientific quality of the post-market environmental monitoring plan proposed by the applicant.

The EFSA GMO Panel agrees with the two-pronged approach proposed by the applicant to detect early warning signs indicating increases in tolerance in WCR in the field. This approach consists of: (1) measuring the baseline susceptibility of WCR populations to the Cry34Ab1/Cry35Ab1 proteins and changes in that susceptibility in the EU; and (2) monitoring of unexpected field damage caused by WCR. The EFSA GMO Panel considers these two approaches complementary, because monitoring for WCR susceptibility is more likely to detect changes in susceptibility occurring at a broader spatial scale than reports of unexpected field damage that target the detection of localised resistance. Acquired data will also contribute to resolve the remaining scientific uncertainties related to the appropriateness of the high dose/refuge strategy in delaying resistance evolution in WCR, and allow the periodic evaluation of the adequacy and efficacy of the revised insect resistance management strategy.

The case-specific monitoring plan proposed by the applicant focuses on monitoring resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests only. To resolve the remaining scientific uncertainties pertaining to the modelling predictions of resistance in WCR populations owing to the cultivation of maize 59122, and the potential of the Cry34Ab1/Cry35Ab1 proteins to accumulate and persist in soil following subsequent years of continuous maize 59122 cultivation, the scope of the case-specific monitoring as proposed by the applicant should be extended to include additional studies to address these issues too.

The EFSA GMO Panel accepts the approach of the applicant to general surveillance, but requests that its recommendations to strengthen general surveillance are implemented.

In its evaluation report, the NL CA expressed reservations about the conclusions of the applicant that no negative effects were found in one of the two lower-tier studies with the surrogate coccinellid species *Coleomegilla maculata*. The NL CA noted that "*although laboratory toxicity testing demonstrated a possible adverse effect on the growth of C. maculata larvae; in the field no such effect was observed on ladybird beetles*". Nonetheless, the NL CA was of the opinion that "*the applicant should incorporate specific monitoring for ladybird beetles*" in the frame of case-specific monitoring (Section 7.1 of the environmental risk assessment report of the NL CA). The EFSA GMO Panel agrees that the submitted data show that Cry34Ab1/Cry35Ab1 proteins may be toxic to *C. maculata* at dose levels that exceed field exposure. However, adverse effects were not seen at field dose levels when *C. maculata* larvae were fed a mixture of natural prey and pollen. Because *C. maculata* is not indigenous to Europe, the EFSA GMO Panel requested additional data on a representative European coccinellid species. In response, the applicant provided lower-tier studies (including tritrophic experiments) with the focal species *C. septempunctata*. Based on the additional toxicity data and estimated worst-case expected environmental concentrations, no hazard to *C. septempunctata* and no risk to coccinellids are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122. Therefore, the EFSA GMO Panel concludes that case-specific monitoring of coccinellids is not necessary.

With regard to general surveillance, the NL CA concluded that "*general surveillance will take place through a predefined format that will be provided to the growers and other users of 59122 maize. An example of the format is included in Annex VII. This is considered to be sufficient. It is indicated by the applicant that reporting to the EC will take place immediately if any adverse effects arising from 59122 maize will be reported. Other reporting of results of the case-specific and general surveillance will be according to the requirements of the consent*". The NL CA advised to report results on an annual basis (Section 7.2 of the environmental risk assessment report of the NL CA).

In conclusion, the EFSA GMO Panel considers that maize 59122 is unlikely to have any adverse effect on the environment, except for the possible resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests. The EFSA GMO Panel recommends the implementation of appropriate and diversified insect resistance management strategies and case-specific monitoring to delay and monitor the possible evolution of resistance to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests, respectively. In addition, the EFSA GMO Panel recommends revision of the applicant's insect resistance management plan and the proposed post-market environmental monitoring plan. The remaining non-critical scientific uncertainties pertaining to the modelling predictions of resistance in WCR populations owing to the cultivation of maize 59122, and the potential of the Cry34Ab1/Cry35Ab1 proteins to accumulate and persist in soil following subsequent years of continuous maize 59122 cultivation, are to be resolved with data acquired during post-market environmental monitoring. Although maize 59122 is tolerant to glufosinate-ammonium-based herbicides, the EFSA GMO Panel did not assess the potential adverse effects associated with the use of such herbicides on maize 59122, as maize 59122 will not be marketed in the EU as a herbicide-tolerant crop. This Scientific Opinion also updates the previous Panel safety evaluation of the food and feed uses, and import and processing of maize 59122 and derived products. The EFSA GMO Panel concludes that the information available for maize 59122 addresses the scientific comments raised by Member States and that maize 59122, as described in this application, is as safe as its conventional counterpart and commercial maize varieties with respect to potential adverse effects on human and animal health. If subjected to appropriate management measures, the cultivation of maize 59122 is unlikely to raise safety concerns for the environment.

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BACKGROUND

On 21 October 2005, the European Food Safety Authority (EFSA) received from the Competent Authority of the Netherlands (NL CA) an application (Reference EFSA-GMO-NL-2005-23) for authorisation of the genetically modified (GM) insect-resistant and herbicide-tolerant maize 59122 (Unique identifier DAS-59122-7), submitted by Pioneer Hi-Bred International and Dow AgroSciences LLC (referred to hereafter as the applicant) under Regulation (EC) No 1829/2003. The scope of this application covers food and feed uses, import, processing and cultivation of maize 59122 and all derived products. As the scope of this application also covers the food and feed uses, import and processing of maize 59122, this Scientific Opinion also updates the previous EFSA GMO Panel safety evaluation of the food and feed uses, and import and processing of maize 59122 and derived products (EFSA, 2007).

After receiving the application EFSA-GMO-NL-2005-23 and in accordance with Articles 5(2)(b) and 17(2)(b) of Regulation (EC) No 1829/2003, EFSA informed both Member States and the European Commission, and made the summary of the application publicly available on the EFSA website. EFSA initiated a formal review of the application to check compliance with the requirements laid down in Articles 5(3) and 17(3) of Regulation (EC) No 1829/2003. On 28 November 2006, 26 January 2007 and 8 March 2007, EFSA received additional information requested under completeness check (requested on 20 November 2006, 10 January 2007 and 1 March 2007). On 9 March 2007, EFSA declared the application as valid in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003.

On 31 March 2006, following a call for expression of interest among Competent Authorities under Directive 2001/18/EC and in accordance with Articles 6.3(c) and 18.3(c) of Regulation (EC) No 1829/2003, EFSA requested the NL CA to evaluate the initial environmental risk assessment of application EFSA-GMO-NL-2005-23 for the placing on the market of maize 59122 for cultivation. This call was initiated by EFSA on 10 March 2006 and the NL CA gave its conformity on 22 March 2006.

EFSA made the valid application available to Member States and the European Commission, and consulted nominated risk assessment bodies of Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC following the requirements of Articles 6(4) and 18(4) of Regulation (EC) No 1829/2003, to request their scientific opinion. Member States had three months after the date of acknowledgement of the valid application (9 June 2007) within which to make their opinion known.

The NL CA asked the applicant for additional information on maize 59122 on 13 March 2007, 30 July 2007 and 8 October 2007. The applicant provided the requested information on 2 April 2007 and 20 December 2007.

The NL CA provided to EFSA its report on the environmental risk assessment of maize 59122 on 13 May 2008 in line with Articles 6.3(c) and 18.3(c) of Regulation (EC) No 1829/2003.

The Scientific Panel on Genetically Modified Organisms of EFSA (EFSA GMO Panel) carried out an evaluation of the scientific risk assessment of the GM maize 59122 for cultivation in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003. When carrying out the safety evaluation, the EFSA GMO Panel took into account the appropriate principles described in its guidelines for the risk assessment of GM plants and derived food and feed (EFSA, 2006a, 2011b), the environmental risk assessment of GM plants (EFSA, 2010c), the selection of comparators for the risk assessment of GM plants (EFSA, 2011a), and for the post-market environmental monitoring of GM plants (EFSA, 2006b, 2011c); the scientific comments of Member States; the additional information provided by the applicant; the environmental risk assessment report from the NL CA; and relevant scientific publications.

The EFSA GMO Panel asked the applicant for additional information on maize 59122 on 29 May 2008, 1 October 2008, 13 February 2009, 28 May 2009, 30 April 2010, and on 29 February 2012. The applicant provided the requested information on 22 September 2008, 8 January 2009, 28 April 2009, 27 January 2010, 11 March 2010, 18 March 2010, 16 January 2012, and on 2 October 2010, respectively. Additional information was also spontaneously provided by the applicant on 23 January 2013 and 11 February 2013. After receipt and evaluation of the full data package, the EFSA GMO Panel finalised its risk assessment evaluation of maize 59122.

In giving its Scientific Opinion on maize 59122 to the European Commission, Member States and the applicant, and in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003, EFSA has endeavoured to respect a time limit of six months from the acknowledgement of the valid application. As additional information was requested by both the NL CA and the EFSA GMO Panel, the time limit of six months was extended accordingly, in line with Articles 6(1), 6(2), 18(1) and 18(2) of Regulation (EC) No 1829/2003.

According to Regulation (EC) No 1829/2003, this Scientific Opinion is to be seen as the report requested under Articles 6(6) and 18(6) of that Regulation, and thus will be part of the EFSA Overall Opinion in accordance with Articles 6(5) and 18(5).

The safety of the food and feed uses, import and processing of maize 59122 itself (EFSA, 2007) or as a component of stacked maize events (59122 x NK603; 59122 x 1507 x NK603 and 1507 x 59122) has been evaluated previously by the EFSA GMO Panel under Regulation (EC) 1829/2003 (EFSA, 2008a, 2009a,b, 2010a, 2011e). The Commission Decision 2007/702/EC authorised the placing on the market of products containing, consisting of, or produced from maize 59122 pursuant to Regulation (EC) No 1829/2003.[4]

TERMS OF REFERENCE

The EFSA GMO Panel was requested to carry out a scientific risk assessment of maize 59122 for food and feed uses, import and processing, and cultivation in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003. Where applicable, any conditions or restrictions which should be imposed on the placing on the market and/or specific conditions or restrictions for use and handling, including post-market environmental monitoring requirements based on the outcome of the risk assessment and, in case of GMOs or food/feed containing or consisting of GMOs, conditions for the protection of particular ecosystems/environment and/or geographical areas should be indicated in accordance with Articles 6(5)(e) and 18(5)(e) of Regulation (EC) No 1829/2003.

The EFSA GMO Panel was not requested to give a Scientific Opinion on information required under Annex II of the Cartagena Protocol, nor on the proposals for labelling and methods of detection (including sampling and the identification of the specific transformation event in the food/feed and/or food/feed produced from it), which are matters related to risk management.

[4] http://eur-lex.europa.eu/LexUriServ/site/en/oj/2007/l_285/l_28520071031en00420046.pdf

ASSESSMENT

1. Introduction

Maize 59122 was developed to provide the following characteristics:

(1) Protection against certain coleopteran target pests belonging to the genus *Diabrotica* such as the larvae of western corn rootworm (WCR; *Diabrotica virgifera virgifera*), northern corn rootworm (*Diabrotica barberi*) and southern corn rootworm (SCR; *Diabrotica undecimpunctata howardi*) by the introduction of the Cry34Ab1/Cry35Ab1 proteins from *Bacillus thuringiensis* strain PS149B1. The *B. thuringiensis cry34Ab1* and *cry35Ab1* coding sequences were modified for optimal expression in maize. The mode of action of *Bt*-proteins is to bind selectively to specific receptors on the epithelial surface of the midgut of larvae of susceptible insect species, leading to death of larvae through pore formation and cell burst and subsequently septicaemia (reviewed by OECD, 2007; Sanahuja et al., 2011; Bravo et al., 2012; Vachon et al., 2012). This effect is significantly enhanced for Cry34Ab1/Cry35Ab1 proteins under acidic conditions, such as those typical of coleopteran guts, as opposed to the alkaline conditions found in the lepidopteran larval midgut (Moellenbeck et al., 2001; Masson et al., 2004). Both the Cry34Ab1 and Cry35Ab1 proteins are required for optimal insecticidal activity against WCR. The activity of the Cry34Ab1 protein was shown to be potentiated by the Cry35Ab1 protein (Herman et al., 2002b). When tested on SCR larvae, the Cry34Ab1 protein alone inhibited insect growth, but its toxicity against SCR was synergised by the Cry35Ab1 protein. Relatively small amounts of the Cry35Ab1 protein are necessary for synergism; for example, a 9:1 ratio of Cry34Ab1:Cry35Ab1 was shown to be sufficient for high SCR mortality. However, an optimal ratio has not been identified (Ellis et al., 2002; Herman et al., 2002b; CERA, 2013). Cry34Ab1 was shown to facilitate Cry35Ab1 binding to WCR midgut brush border membrane vesicles (Li et al., 2013). Several *Diabrotica*-active *Bt*-maize events are currently grown commercially in Argentina, Brazil, Canada and the United States (Devos et al., 2012, 2013). Depending on the region, *Diabrotica*-active *Bt*-maize is used to control chrysomelid beetles such as the western, northern, southern and mexican corn rootworm (*D. v. zea*), and *D. speciosa*. At present, WCR is the only species from the corn rootworm complex present in the EU.

WCR is a major coleopteran maize pest and a serious threat to agriculture in North America (Metcalf, 1986; Dun et al., 2010; Tinsley et al., 2013) and the EU (FCEC, 2009; Wesseler and Fall, 2010). WCR overwinters through eggs that are laid during mid-summer till autumn, mainly in maize fields. Larvae hatching in the following spring feed on fine maize root hairs, where they typically burrow into the root tips of maize seedlings. As the larvae grow larger, they move, feed and tunnel into younger nodes of adventitious roots of the nodal root system (Meinke et al., 2009), and negatively affect yield by decreasing nutrient and water uptake and plant stability. Maize plants suffering from moderate to severe root pruning are susceptible to lodging, which can result in additional yield losses due to difficulties in harvesting lodged plants (Levine and Oloumi-Sadeghi, 1991). The bulk of plant damage is caused by second and third instars, but adults feeding on silk and grains can be damaging in seed and sweet corn production (Tuska et al., 2002). WCR has been introduced to the EU from North America (Miller et al., 2005), where it is native and widespread. It was first detected near Belgrade (Serbia) in 1992, but has since spread across the continent (Hummel, 2003; Kiss et al., 2005a; Boriani et al., 2006; Ciosi et al., 2008; Gray et al., 2009; Meinke et al., 2009), resulting in well-established populations in approximately 19 European countries (EC, 2012).[5] It is expected that this invasive pest species will expand further in the EU (Hemerik et al., 2004; Moeser and Vidal, 2005; Ciosi et al., 2011; Aragón and Lobo, 2012).

(2) Tolerance to the herbicidal active substance glufosinate-ammonium by the introduction of a gene coding for the phosphinothricin *N*-acetyltransferase enzyme (PAT) from *Streptomyces viridochromogenes.* Glufosinate-ammonium inhibits glutamine synthetase, leading

[5] http://extension.entm.purdue.edu/wcr/

to glutamine deficiency, ammonia accumulation and eventually to plant death. The PAT protein catalyses the conversion of glufosinate-ammonium to *N*-acetyl glufosinate. *N*-acetyl glufosinate is an inactive form that does not bind to glutamine synthetase (De Block et al., 1987) allowing plants to grow in the presence of glufosinate-ammonium. Although maize 59122 is tolerant to glufosinate-ammonium-based herbicides, the EFSA GMO Panel did not assess the potential adverse effects associated with the use of such herbicides on maize 59122, as maize 59122 will not be marketed in the EU as a herbicide-tolerant crop.[6]

Maize 59122 was assessed with reference to its intended uses and the appropriate principles described in the EFSA GMO Panel guidelines for the risk assessment of GM plants and derived food and feed (EFSA, 2006a, 2011b), the environmental risk assessment of GM plants (EFSA, 2010c), the selection of comparators for the risk assessment of GM plants (EFSA, 2011a), and for the post-market environmental monitoring of GM plants (EFSA, 2006b, 2011c). In delivering its Scientific Opinion, the EFSA GMO Panel considered the information provided by the applicant in its application EFSA-GMO-NL-2005-23, and also: (1) a review of all peer-reviewed scientific literature on maize 59122; (2) updated molecular characterisation, including sequence data for the flanking regions; (3) updated information on allergenicity and toxicology; (4) updated information on environmental issues; (5) the post-market (environmental) monitoring plan; and (6) the additional information submitted by the applicant in reply to questions from both the EFSA GMO Panel and the NL CA.

The risk assessment evaluation presented here is also based on the scientific comments submitted by Member States (Annex G), the environmental risk assessment report of the NL CA (Annex H), and relevant scientific publications.

2. Issues raised by Member States

The scientific comments raised by Member States are addressed in Annex G of the EFSA overall opinion[7], and have been considered throughout this EFSA GMO Panel Scientific Opinion.

3. Molecular characterisation

3.1. Evaluation of relevant scientific data

Unless specifically indicated, the information provided in this application, which is described in the following Sections, has been evaluated previously by the EFSA GMO Panel (EFSA, 2007).

Upon request of the EFSA GMO Panel, the applicant performed a literature search for the period 2007–2011.[8] For the period 2012 to present, EFSA performed a literature search using the databases Scopus (13 February 2013)[9] and ISI Web of Knowledge (25 January 2013)[10], but did not retrieve additional publications or reports dealing with the molecular characterisation of maize 59122.

3.1.1. Transformation process and vector constructs[11]

Maize 59122 was developed through *Agrobacterium*-mediated transformation of the maize line Hi-II and as a result expresses the *cry34Ab1* and *cry35Ab1* genes conferring resistance to coleopteran insect pests belonging to the genus *Diabrotica*, such as the larvae of WCR, and the *pat* coding sequence resulting in tolerance to glufosinate-ammonium-based herbicides.

6 Communication of applicant to EFSA on 11/03/2010.
7 http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2008-312
8 Additional information received on 16/01/2012/Annex: Ilegems (2012).
9 Search string: (maize or corn or *zea* or mays) in (Title, abstract, keywords), and (59122 or das59122 or "das-59122" or "herculex-rw") in (Title, abstract, keywords), or (cry34ab1 or cry35ab1 or cry34-ab1 or cry35-ab1) in (Title, abstract, keywords) for all document types.
10 Topic field: 59122.
11 Technical dossier/Sections C1, C2, C3 and D1.

The binary vector used, PHP17662, contains between the left and right T-DNA borders the *cry34Ab1* coding sequence combined with the *ubi*1ZM promoter (from *Zea mays*) and the *pin*II terminator (from *Solanum tuberosum*), the *cry35Ab1* coding sequence combined with the wheat peroxidase promoter (from *Triticum aestivum*) and the *pinII* terminator and the *pat* coding sequence (from *S. viridochromogenes*) combined with the 35S promoter and 35S terminator (from cauliflower mosaic virus). The *cry34Ab1* and *cry35Ab1* genes were cloned from *B. thuringiensis* strain PS149B1 and the coding sequence of both genes has been adapted to the codon usage in maize as to optimise expression in the maize plant. The vector backbone portion contains among others a spectinomycin resistance gene, the ColE1 *ori*, *tetA* and *tetR* genes (tetracycline resistance) and several *vir* genes.

3.1.2. Transgene constructs in maize 59122[12]

Molecular characterisation data established that maize 59122 contains a single insert of the transfer (T)-DNA. The structure of the insert in maize 59122 was determined by Southern blot analysis and DNA sequencing. No vector backbone sequences were detected with updated Southern blot analyses.[13]

Bioinformatic analyses revealed that flanking regions of the maize event 59122 show significant identity to maize genomic DNA and EST sequences. Updated bioinformatic analyses indicated that the DNA in 59122 was inserted 1032 bp downstream of the 3′ end of the empty pericarp 4 (*emp*4) gene, coding for a pentatricopeptide repeat (PPR) protein.[14] PPR proteins are thought to be involved in RNA processing within organelles and this particular PPR protein has been shown to be essential for seed development in maize (Gutiérrez-Marcos et al., 2007). In maize 59122, phenotypic analysis did not show any changes, suggesting that the *emp4* gene is functional. Updated bioinformatic analyses of the open reading frames (ORFs) spanning the two junction regions and of all internal ORFs of the insert were performed. No novel ORFs with sequence similarity to known toxins or allergens were identified.

3.1.3. Information on the expression and stability of the insert[15]

The levels of newly expressed proteins Cry34Ab1, Cry35Ab1 and PAT were analysed in various tissues of event 59122 by enzyme-linked immunosorbent assay (ELISA). Tissue samples for analysis were collected from five field trials conducted in Chile (2002–2003), the United States and Canada (2003) and Europe (2003 and 2004). The field trials in Europe were conducted in three locations in Bulgaria (2003 and 2004) and three locations in Spain (2004).[16] Each trial included appropriate comparators. The ranges of levels of Cry34Ab1, Cry35Ab1 and PAT proteins in various plant parts obtained from the EU trials at the developmental stages where the expression was the highest are summarised in Table 1 (see below).

The levels of Cry34Ab1, Cry35Ab1 and PAT in maize 59122 and stacked maize events containing 59122 (59122 × NK603, 1507 × 59122, 59122 × 1507 × NK603 and MON 89034 × 1507 × MON 88017 × 59122) have also been reported for grain and other tissues including forage, and reviewed by the EFSA GMO Panel (EFSA, 2007, 2008a, 2009a,b, 2010a, 2011e).

The stability of the inserted DNA and phenotypes was confirmed over several generations.

[12] Technical dossier/Section D2.
[13] Additional information received on 16/01/2012/Annex: Brink and Weaver (2011).
[14] Additional information received on 16/01/2012/Annex: Krauss (2012a).
[15] Technical dossier/Section D3.
[16] Technical dossier/Section D3/Annex 3: Buffington (2004)/Annex 4: Buffington (2005).

Table 1: Ranges in the levels of the Cry34Ab1, Cry35Ab1 and PAT proteins in various parts of maize 59122 (μg/g dry weight) grown in the EU (plants not sprayed with glufosinate-ammonium-based herbicides). Values are combined data from 2003 and 2004 growing seasons. Developmental stages are indicated in parentheses

Plant parts	Cry34Ab1		Cry35Ab1		PAT	
Leaves	< 0.162*–667	(R4)	< 0.162*–307	(R4)	13.0–22.8	(R4)
Roots	16.4–82.1	(V6)	1.12–26.1	(R1)	0.470–1.55	(R4)
Whole plant	49.0–89.0	(R1)	48.7–92.6	(V9)	4.64–16.0	(V9)
Pollen	45.4–146	(R1)	< 0.324*	(R1)	< 0.27*	(R1)
Kernels	23.3–89.3	(R6)	0.59–3.48	(R6)	< 0.068*	(R6)

*These values are the LLOQ ("lower limits of quantification") reported for the corresponding proteins and tissue samples

3.2. Conclusion

The molecular characterisation data establish that maize 59122 contains a single insertion locus. Updated bioinformatic analyses of the ORFs spanning the junction sites within the insert or between the insert and genomic DNA did not raise safety issues. The stability of the inserted DNA and phenotypes was confirmed over several generations. The potential impacts of the Cry34Ab1, Cry35Ab1 and PAT protein levels, quantified in field trials carried out in Europe, are assessed in the Sections on the food/feed safety assessment and environmental risk assessment (see Sections 5 and 6).

4. Comparative analysis

4.1. Evaluation of relevant scientific data

This Scientific Opinion updates the previous EFSA GMO Panel safety evaluation of the food and feed uses, and import and processing of maize 59122 and derived products. Unless specifically indicated, the information provided in this application (EFSA-GMO-NL-2005-23), which is described in the following Sections, has been evaluated previously by the EFSA GMO Panel (EFSA, 2007).

Upon request of the EFSA GMO Panel, the applicant performed a literature search for the period 2007–2011.[17] For the period 2012 to present, EFSA performed a literature search using the databases Scopus (13 February 2013)[18] and ISI Web of Knowledge (25 January 2013)[19], but did not retrieve additional publications or reports dealing with the comparative analysis of maize 59122.

4.1.1. Compositional analysis, agronomic traits and GM phenotype

The information regarding the comparative analysis of agronomic, phenotypic and compositional data in application EFSA-GMO-NL-2005-23 was assessed by the EFSA GMO Panel earlier in the frame of a previous application for the marketing of maize 59122 with a different scope (EFSA, 2007). The information contained agronomic and phenotypic data obtained from field trials performed with maize 59122 and the conventional counterpart in North and South America and in Europe over several seasons (from 2002 to 2004), as well as compositional data on the harvested forage and seed materials.

[17] Additional information received on 16/01/2012/Annex: Ilegems (2012).
[18] Search string: (maize or corn or *zea* or mays) in (Title, abstract, keywords), and (59122 or das59122 or "das-59122" or "herculex-rw") in (Title, abstract, keywords), or (cry34ab1 or cry35ab1 or cry34-ab1 or cry35-ab1) in (Title, abstract, keywords) for all document types.
[19] Topic field: 59122.

While statistically significant differences between maize 59122 (untreated and treated with the target herbicide) and the conventional counterpart were observed for some compositional parameters in the analysis of forage and kernels, none of these differences was consistently observed over years and across locations. In addition, the levels of those parameters were within the literature ranges reported for commercial maize varieties. With regard to agronomic and phenotypic characteristics, the data provided in the frame of the previous application showed statistically significant differences for several parameters in the European field trials during the 2004 growing season. None of these differences were consistently observed over locations and years. The EFSA GMO Panel therefore did not identify any biologically relevant differences in the compositional, agronomic and phenotypic characteristics of maize 59122 compared with its conventional counterpart, except for the newly expressed Cry34Ab1/Cry35Ab1 and PAT proteins (EFSA, 2007).

Subsequently, a compositional analysis of forage and kernels of maize 59122 was published by Herman et al. (2007). For this study maize 59122 was grown, untreated and treated with the target herbicide, together with a conventional counterpart at four locations in North America in 2003 and 2004. The outcomes indicated that the composition of maize 59122 is comparable to that of non-GM maize. The data for one year (2003) was submitted and assessed in the context of a previous application for maize 59122 (EFSA, 2007). Therefore the data from the second year (2004) can be considered as supplementary to what has already been assessed.

4.2. Conclusion

Since the EFSA GMO Panel delivered its earlier opinion on maize 59122, no new information has appeared on the composition or on the agronomic and phenotypic characteristics of maize 59122 that would lead the EFSA GMO Panel to change its previous conclusions.

5. Food/feed safety assessment

5.1. Evaluation of relevant scientific data

This Scientific Opinion updates the previous EFSA GMO Panel safety evaluation of the food and feed uses, and import and processing of maize 59122 and derived products. Unless specifically indicated, the information provided in this application (EFSA-GMO-NL-2005-23), which is described in the following Sections, has been evaluated previously by the EFSA GMO Panel (EFSA, 2007).

Upon request of the EFSA GMO Panel, the applicant performed a literature search for the period 2007–2011.[20] For the period 2012 to present, EFSA performed a literature search using the databases Scopus (13 February 2013)[21] and ISI Web of Knowledge (25 January 2013)[22], but did not retrieve additional publications or reports dealing with the toxicological, allergenicity and nutritional assessment of maize 59122.

5.1.1. Toxicological assessment

The EFSA GMO Panel previously concluded that maize 59122 is unlikely to have an adverse effect on human and animal health, in the context of the proposed uses (EFSA, 2007). In this application (EFSA-GMO-NL-2005-23), updated bioinformatic studies were provided. Analyses of the amino acid sequences of the newly expressed proteins Cry34Ab1, Cry35Ab1 and PAT revealed no similarity to known toxic proteins and thus confirmed the results of the previous studies.

The applicant also provided a reference to 90-day oral feeding study, published by He et al. (2008), not previously considered. Compared with the 90-day feeding study previously assessed in the context of EFSA-GMO-NL-2005-12, diets were prepared containing 50 % and 70 % of maize 59122

[20] Additional information received on 16/01/2012/Annex: Ilegems (2012).
[21] Search string: (maize or corn or *zea* or mays) in (Title, abstract, keywords), and (59122 or das59122 or "das-59122" or "herculex-rw") in (Title, abstract, keywords), or (cry34ab1 or cry35ab1 or cry34-ab1 or cry35-ab1) in (Title, abstract, keywords) for all document types.
[22] Topic field: 59122.

(compared with 35 % used in the study by Malley, 2007). Although a control maize was included its identity was unclear. An additional group of rats was fed a commercial rodent diet. The animals, divided over groups of ten animals/gender/treatment, were measured for feed intake, body weight, mortality, haematology, serum chemistry, and anatomical pathology (including gross and histopathology). It was observed that the high inclusion rate of maize affected various parameters measured in the serum chemistry response of rats fed the maize-containing diets when compared with those fed the standard rodent diet. There were no significant differences between animals fed maize 59122 and those fed the respective control maize. The authors concluded that there were no indications of adverse effects from the consumption of maize 59122 kernels compared with that of diets containing the control.

5.1.2. Allergenicity[23]

The EFSA GMO Panel has previously evaluated the bioinformatic analysis comparing the sequences of Cry34Ab1, Cry35Ab1, and PAT proteins and known allergens and the resistance of these proteins to enzymatic degradation by pepsin (EFSA, 2007).

In this application (EFSA-GMO-NL-2005-23), updated bioinformatic studies were provided.[24] Analyses of the amino acid sequences of the newly expressed Cry34Ab1, Cry35Ab1 and PAT proteins revealed no significant similarities to known allergens, confirming the results of the previous studies.

In the present case, and based on all the available information, the EFSA GMO Panel identified no safety concerns regarding the potential allergenicity of maize 59122. In addition, there is no new scientific information that would invalidate the previous EFSA GMO Panel conclusions on the allergenicity assessment of maize 59122.

5.1.3. Nutritional assessment

The EFSA GMO Panel previously evaluated animal feeding studies in rapidly growing broiler chickens with maize 59122 and the combined events 59122 × 1507 × NK603 in the frame of former applications EFSA-GMO-NL-2005-12 and EFSA-GMO-UK-2005-21, respectively. These feeding studies support the results of the comparative compositional analysis and confirm that maize 59122 is as nutritious as its conventional counterpart (EFSA, 2007, 2009b).

Four additional nutritional feeding studies of interest were identified in a literature search performed by the applicant for the period 2007–2011, including studies on lactating cows (Brouk et al., 2011), laying hens (Jacobs et al., 2008), pigs (Stein et al., 2009) and steers (Huls et al., 2008).[25]

- Brouk et al. (2011) reported a feeding study with lactating dairy cows. Two groups of 15 cows each were fed rations containing both silage (21 %) and kernels (23 %) derived from maize 59122 and from the conventional counterpart during two separate experimental periods (six weeks, measurements made during the last four weeks) according to a crossover design with treatment switchback. While the feeds were analysed for composition (proximates, fibre, lignin, minerals), the animals were measured for feed intake, body weight and condition, milk production, and milk quality (composition and somatic cell count). An improvement in body condition scoring (BCS) was observed between the diet containing maize 59122 and that containing the conventional counterpart, whereas the average total BCS values did not differ between both groups. The authors concluded that their data indicated that the genetic modification in maize 59122 did not alter the nutritional value of maize kernels and silage (Brouk et al., 2011).

- Huls et al. (2008) reported a steer feeding study in which groups of 20 cross-bred steers each were fed rations containing 82 % dry-rolled kernels derived from maize 59122, a conventional counterpart or a non-GM commercial variety, for 109 days. The composition of the feed was

[23] Technical dossier/Section D.7.9.
[24] Technical dossier/Section D.7.9.1/Additional information received on 16/01/2012.
[25] Additional information received on 16/01/2012.

analysed and animal performance various carcass characteristics were measured. No statistically significant differences were identified between the animals fed maize 59122 and those fed the conventional counterpart. The authors concluded that feeding maize 59122 is nutritionally equivalent to commercial non-GM maize kernels when fed to finishing cattle (Huls et al., 2008).

- Jacobs et al. (2008) reported a laying hen feeding study with maize 59122, a conventional counterpart and a commercial maize variety. Groups of 72 hens were used per treatment. Maize kernels were included in the diets at 65 % inclusion level. The composition of the maize and soybean meal used for diet preparation were analysed and animal performance and the quality of the eggs (e.g., albumen, yolk, and shell weight; albumen thickness vs. egg mass) were analysed. No statistically significant differences were noted between dietary treatments with maize 59122 and with the conventional counterpart.

- Stein et al. (2009) reported a growing–finishing pig feeding study with diets containing maize 59122, a conventional counterpart and a commercial maize variety. The treatment groups consisted of 36 pigs each, which were kept in 12 replicate pens with three animals per pen, starting and final weights at approximately 37 and 127 kg, respectively. The inclusion level of maize in the diets ranged from 69 % in starter diets to 82 % in finishing diets. Feeds were analysed for their composition, while during and after the experiment the animals were analysed for performance (weight, feed intake) and various carcass characteristics. No statistically significant differences were observed between the groups fed the maize 59122 and the conventional counterpart. The authors concluded that kernels of maize 59122 have a feeding value that is not different from commercial maize.

In conclusion, the EFSA GMO Panel considers that the data from these four feeding studies support the view that diets formulated with maize 59122 are as nutritious as those formulated with commercial non-GM maize varieties.

5.1.4. Post-market monitoring of GM food/feed

Maize 59122 is intended to have improved agronomic properties. From a nutritional point of view, maize 59122 is similar to conventionally bred varieties. Therefore, maize 59122 will be used as any other maize and only replace a part of the overall maize products within the European market. The risk assessment concluded that no data have emerged to indicate that maize 59122 is any less safe than its non-GM comparators. The opinion of the EFSA GMO Panel is that a post-market monitoring of GM food and GM feed products containing, consisting of or derived from maize 59122 is not necessary, which is in line with its guidelines for the risk assessment of food and feed derived from GM plants (EFSA, 2011b).

5.2. Conclusion

Updates of the bioinformatic studies confirmed the previous findings indicating that there are no similarities between the Cry34Ab1, Cry35Ab1 and PAT proteins, and known toxic proteins and allergens. Various publications on the composition of maize 59122 and its counterparts, as well as a rat feeding study and nutritional feeding studies in target livestock animals with the whole product, have appeared since the previous EFSA GMO Panel Scientific Opinion on maize 59122. The outcomes of these studies revealed that there was no new information that would require changes to the previous EFSA GMO Panel Scientific Opinion on maize 59122, namely that maize 59122 is as safe as its non-GM counterparts, and that the overall allergenicity of the whole plant is not changed and that maize 59122 is unlikely to have any adverse effect on human and animal health in the context of its intended uses.

6. Environmental risk assessment and risk management strategies

6.1. Evaluation of relevant scientific data

The scope of application EFSA-GMO-NL-2005-23 is for food and feed uses, import and processing, and cultivation of maize 59122. Therefore, the environmental risk assessment is concerned with potential direct and indirect environmental effects of the cultivation and the spread of maize 59122 into non-cultivated environments and with the exposure through manure and faeces from animals fed grains produced by maize 59122.

The EFSA GMO Panel considered the following issues in the environmental risk assessment submitted by the applicant: (1) changes in plant fitness due to the genetic modification; (2) potential for gene transfer and its consequences; (3) interactions between the GM plant and target organisms; (4) interactions between the GM plant and non-target organisms; (5) effects on animal and human health; (6) interactions with biogeochemical processes and the abiotic environment; (7) impacts of the specific cultivation, management and harvesting techniques; and (8) risk management strategies (including post-market environmental monitoring).

The NL CA provided to EFSA its report on the environmental risk assessment of maize 59122 on 13 May 2008 in line with Articles 6.3(c) and 18.3(c) of Regulation (EC) No 1829/2003. The report on the environmental risk assessment of the NL CA is provided in Annex H of the EFSA overall opinion, and has been considered throughout this EFSA GMO Panel Scientific Opinion.

Upon request of the EFSA GMO Panel, the applicant performed a literature search for the period 2007–2011.[26] For the period 2012 to present, EFSA performed a literature search using the databases Scopus (13 February 2013)[27] and ISI Web of Knowledge (25 January 2013)[28], and retrieved eight additional publications or reports (Petzold-Maxwell et al., 2012a,b; Rudeen and Gassman, 2012; Siebert et al., 2012; Takács et al., 2012; Zukoff et al., 2012; Devos et al., 2013; Li et al., 2013) relevant to the environmental risk assessment and risk management of maize 59122. These publications have been considered by the EFSA GMO Panel in this Scientific Opinion.

6.2. Environmental risk assessment

As stated in earlier Sections, there are no indications of unintended changes in maize 59122 at the molecular, compositional and agronomic/phenotypic level. The molecular characterisation of the DNA insert and flanking regions of maize 59122 did not indicate unintended changes due to the insertion (see Section 3, above). Moreover, no biologically relevant differences in the composition of key analytes or agronomic and phenotypic characteristics were identified between maize 59122 and its conventional counterpart (EFSA, 2007; Herman et al., 2007; Section 4, above).

6.2.1. Changes in plant fitness due to the genetic modification[29]

A series of field trials with maize 59122 were conducted by the applicant across six locations in Chile in the 2002/2003 growing season[30], five locations in North America in 2003 (three locations in the United States, two locations in Canada)[31], three locations in Bulgaria in 2003[32], and six locations in the EU in 2004 (three locations in Spain, three locations in Bulgaria)[33] to compare the agronomic

[26] Additional information received on 16/01/2012/Annex: Ilegems (2012).
[27] Search string: (maize or corn or *zea* or mays) in (Title, abstract, keywords), and (59122 or das59122 or "das-59122" or "herculex-rw") in (Title, abstract, keywords), or (cry34ab1 or cry35ab1 or cry34-ab1 or cry35-ab1) in (Title, abstract, keywords) for all document types.
[28] Topic field: 59122.
[29] Technical dossier/Sections B2, B3, B4, D4, D9.1 and D9.2.
[30] Technical dossier/Section D4/Annex 37: Essner and Coats (2003).
[31] Technical dossier/Section D4/Annex 38: Buffington (2004).
[32] Technical dossier/Section D4/Annex 3: Buffington (2004).
[33] Technical dossier/Section D4/Annex 4: Buffington (2005).

performance and field characteristics of maize 59122 with its comparators. In an additional field trial in the United States in 2003, yield characteristics were evaluated at four locations.

Information on phenotypic and agronomic characteristics of maize 59122 and its comparators was generated to compare their growth habit, vegetative vigour and reproduction characters. Several endpoints related to growth habit, vegetative growth, reproduction, and yield and grain characteristics were measured.

A randomised complete block design with four replications was used in the field studies. In the field trials performed in Chile, maize 59122 and its comparator received the same conventional herbicide treatments. The US (2003) and EU field trials (2003 and 2004) contained glufosinate-ammonium treated and untreated maize 59122 plants. The comparators in these field trials received the same conventional herbicide treatment as the "glufosinate-ammonium untreated" maize 59122 plants. Agronomic data were collected for glufosinate-ammonium treated maize 59122 in the United States and Canada (2003) and in the EU (2003). In the EU field trials in 2004, agronomic data were collected from glufosinate-ammonium untreated maize 59122.

The breeding tree provided by the applicant confirmed that the near-isogenic lines used in the agronomic and phenotypic field trials had a comparable genetic background to maize 59122.[34]

The EU agronomic and phenotypic field trial data did not show major changes in plant characteristics that indicate altered fitness, persistence and invasiveness of maize 59122 plants. A number of endpoints (i.e., early population count, plant height) showed statistically significant differences in the across-location comparisons between maize 59122 and its near-isogenic line in the 2004 field trials in Spain and Bulgaria. These differences were not consistently observed in each location, and were not considered biologically meaningful with respect to persistence and invasiveness potential. No visually observable response to naturally occurring insects and diseases recorded at maturity provided any indication of altered stress responses of maize 59122 compared with its conventional counterpart.

It is considered very unlikely that the establishment, spread and survival of maize 59122 would be increased owing to the insect resistance and herbicide tolerance traits. These traits can only be regarded as providing a potential selective advantage to maize 59122 under infestation of target pests and/or when glufosinate-ammonium-based herbicides are applied. Moreover, it is considered very unlikely that maize 59122 plants or their progeny will differ from conventional maize varieties in their ability to survive as volunteers until subsequent seasons, or to establish feral populations under European environmental conditions (Section 6.2.2.2, below). Maize is highly domesticated and generally unable to survive in the environment without management intervention (Baker, 1974; Bagavathiannan and Van Acker, 2008). The survival of maize is limited by a combination of low competitiveness, absence of a dormancy phase, and susceptibility to plant pathogens, herbivores and cold climatic conditions (van de Wiel et al., 2011). Maize plants are winter hardy only in European regions with mild winters, and in those situations maize kernels remaining in the field after harvest can germinate, grow, flower, and locally cross-pollinate neighbouring maize plants. The occurrence of maize volunteers was reported in Spain and other European regions (Gruber et al., 2008; Palaudelmàs et al., 2009), but these plants grow weakly and tend to flower asynchronously with the cultivated maize crops in which they occur (Palaudelmàs et al., 2009). While maize 59122 volunteers occurring in cultivated areas will be tolerant to glufosinate-ammonium, they are normally controlled by current agricultural practices, including the use of selective herbicides and/or cultivation techniques (Beckie et al., 2006; Deen et al., 2006). If maize 59122 is rotated with broadleaved crops (such as soybean, oilseed rape, sugar beet, sunflower), potential volunteers can easily be controlled with selective graminicides or glyphosate-based herbicides. The EFSA GMO Panel notes that mechanical weed control such as hoeing is the only solution for weed control if maize 59122 is rotated with another maize crop (either conventional or tolerant to glufosinate-ammonium), as effective herbicides cannot be applied without killing the rotational maize crop itself (Davis et al., 2008). Maize 59122 volunteers are likely to be

[34] Technical dossier/Section D7.1/Figure 46.

controlled by the herbicide programmes applied in glyphosate tolerant crops (Feng et al., 2010; Green and Castle, 2010; Green and Duke, 2011).

Note that the possible impact of maize 59122 volunteers on the efficiency of the insect resistance management plan is considered in Section 6.3.1.1, below.

Despite cultivation for centuries, maize plants do not occur outside cultivated land or in disturbed land in Europe. In addition to the data presented by the applicant, the EFSA GMO Panel is not aware of any scientific report of increased establishment and spread of maize 59122 or any change in survival (including over-wintering), persistence and invasiveness capacity. Because the general characteristics of maize 59122 are unchanged, insect resistance and herbicide tolerance are not likely to provide a selective advantage outside of cultivation in Europe.

As maize 59122 has no altered agronomic and phenotypic characteristics, except for the specific target insect resistance and herbicide tolerance, the EFSA GMO Panel concludes that the likelihood of unintended environmental effects due to the establishment and survival of maize 59122 will be no different from that of conventional maize varieties.

The conclusion of the EFSA GMO Panel is consistent with that of the NL CA. The NL CA concluded that "*there is a negligible risk for 59122 maize to become environmentally persistent or invasive giving rise to any weediness. Maize itself does not possess any traits for weediness. Weediness traits which have been generally described include for example traits as great longevity of seed, ability for seed to germinate in many different environments. The expression of Cry34Ab1, Cry35Ab1 and PAT proteins in 59122 maize does not give rise to any of the described traits for weediness. In addition, in European field trials and trials outside Europe no effect on persistence or invasiveness of 59122 maize was observed. Therefore it can be concluded that the introduced sequences in 59122 maize do not lead to an increased persistence or invasiveness*". In addition, the NL CA considered that "*59122 exhibits no selective advantage or disadvantage as a result of the genetic modification*" (Sections 6.1 and 6.2 of the environmental risk assessment report of the NL CA).

6.2.2. Gene transfer

The EFSA GMO Panel evaluated the potential for horizontal and vertical gene flow of maize 59122, as well as the potential environmental consequences of such gene transfer. A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either through horizontal gene transfer of DNA or through vertical gene flow via the dispersal of pollen and seed.

6.2.2.1. Plant to bacteria gene transfer and its consequences[35]

Bacteria are capable of exchanging genetic material directly between each other and even across species boundaries using mechanisms such as conjugation, transduction or natural transformation. DNA of plants, which may also include DNA derived from GM plants, could hypothetically be acquired by bacteria through horizontal gene transfer. After initial horizontal gene transfer from plants to bacteria, the acquired genes may be further spread to other bacteria.

Current scientific evidence indicates that the transfer of genes derived from GM plants into bacteria and their stable integration either does not occur or, if it has occurred, it has been below the limit of detection in all the studies performed (see Keese, 2008; EFSA, 2009c; Brigulla and Wackernagel, 2010; Ma et al., 2011). The main barriers for horizontal gene transfer from plants to bacteria are the lack of efficient mechanisms of integration of unrelated chromosomal DNA and the limited potential for positive directional selection of the acquired recombinant gene-encoded traits.

[35] Technical dossier/Section D6.

The exposure of bacteria to the recombinant DNA fraction of maize 59122, the factors limiting horizontal gene transfer, and the impact of a hypothetical horizontal gene transfer in receiving environments are described below.

The probability and frequency of horizontal gene transfer of plant DNA (including the recombinant DNA fraction) to exposed bacteria is determined by: (1) the concentration and quality of plant DNA accessible to bacteria in receiving environments; (2) the presence of bacteria with a capacity to develop competence for natural transformation, i.e., to take up extracellular DNA; (3) the ability for genetic recombination by which the plant DNA can be incorporated and thus stabilised in the bacterial genome (including chromosomes or plasmids); (4) the expression and the function of the protein in the bacterial recipient; and (5) the selective advantage provided by the acquired recombinant gene-encoded traits.

(1) The concentration and quality of plant DNA accessible to bacteria in receiving environments—exposure of bacteria to DNA

The release and low-level temporal persistence of gene-sized plant DNA fragments is expected in environments in which crops are grown and in gastrointestinal systems after consumption (EFSA, 2009c).

Genomic DNA is a component of many food and feed products derived from maize. It is well documented that DNA in food and feed becomes substantially degraded during food/feed processing, and in the process of digestion in the human or animal gastrointestinal tracts (Jonas et al., 2001; van den Eede et al., 2004; Ramessar et al., 2007). The DNA is increasingly degraded in the digestive tract, so no full-length genes from plants have been detected in the large intestine or in faeces (EFSA, 2009c, and references therein). In *in vivo* experiments with broilers fed *Bt*-maize, the *cry1Ab* gene was degraded to fragments smaller than 500 bp along the digestive tract (Rossi et al., 2005). Similarly, Chambers et al. (2002) fed chickens with GM maize to explore the *in vivo* fate of the bacterial ampicillin resistance gene bla_{TEM} in bacteria and GM maize. The gene was found in the stomach contents, but not in the lower intestine of animals fed GM maize. In case of Roundup Ready maize (event 39T67), the presence of *epsps* genes arising from feeding on the GM plant material within a field where the crop was grown was reported in soil micro-arthropods, nematodes, macro-arthropods and earthworms (Gulden et al., 2008; Hart et al., 2009). Thus, a low level of exposure of fragments of ingested DNA, including the recombinant fraction of such DNA, to microorganisms in the digestive tract of humans, domesticated animals and other animals, including those inhabiting soil on agricultural fields or neighbouring ecosystems, feeding on maize 59122 or its residues is expected.

Soil bacteria may also be exposed to extracellular DNA released from plant cells into the soil environment throughout and after the growing season (reviewed by Levy-Booth et al., 2007). During active plant growth, free plant DNA may originate from sloughed-off root cap cells (Hawes et al., 1990; de Vries et al., 2003) or necrotic root tissue infected by pathogens (Polverari et al., 2000; Kay et al., 2002). Pollen release at anthesis (de Vries et al., 2003; Webster et al., 2008) and DNA release from decomposing plant residue remaining in agricultural areas after harvest, and which is incorporated into the soil during tillage operations (Widmer et al., 1997; Ceccherini et al., 2003; Stotzky, 2004), can also contribute to the presence of plant DNA in soil later during the growing season. However, the vast majority of plant DNA is expected to be degraded shortly after harvest by plant and microbial DNases in the soil environment. Therefore, plant DNA is only a transient component of the total DNA pool in soil (Levy-Booth et al., 2007; Nielsen et al., 2007; Gulden et al., 2008). Gulden et al. (2008) did not observe accumulation of the epsps gene in the soil environment upon repeated cultivation of Roundup Ready maize (event 39T67). While adsorption to soil particles, particularly clay, can slow down DNA degradation, the vast majority is degraded shortly after harvest. It can therefore be concluded that the concentration of extracellular DNA fragments (including the *cry34Ab1*, *cry35Ab1* and *pat* genes of maize 59122) in gastrointestinal tracts, soil or other environments is relatively low in comparison to those from intact plant material.

(2) The presence of bacteria with a capacity to develop competence for natural transformation, i.e., to take up extracellular DNA

Several bacterial species with the potential to develop competence for natural transformation (take up and recombine with extracellular DNA) belong to the common gut microbial community (Rizzi et al., 2008, 2012; EFSA, 2009c). However, competence development and transformation of such bacteria with genomic DNA of plants has not been observed in the lower gastrointestinal tract, even with optimised model systems providing a selective advantage (Nordgård et al., 2007; EFSA, 2009c; Rizzi et al., 2012). In contrast, some studies have shown that introduced bacteria can be naturally transformed in the oral cavity of humans and animals (Duggan et al., 2000, 2003; Mercer et al., 1999a,b, 2001; Rizzi et al., 2012). Once the recombinant DNA is taken up, it must integrate into the recipient genome to persist during host replication. The likelihood of gene integration is influenced by the gene context (i.e., the surrounding/neighbouring sequences) of the recombinant gene(s) in the plant (EFSA, 2009c).

(3) The ability for genetic recombination by which the plant DNA can be incorporated and thus stabilised in the bacterial genome (including chromosomes or plasmids)

A successful horizontal transfer would require stable insertion of the transgene sequences into a bacterial genome and a selective advantage conferred on the transformed host. The only known mechanism that facilitates horizontal transfer of non-mobile, chromosomal DNA fragments into bacterial genomes is homologous recombination. Homologous recombination requires the presence of stretches of similar DNA sequences between the recombining DNA molecules and, in addition to substitutive gene replacement, facilitates the insertion of non-homologous DNA sequences if their flanking regions share sequence similarity with bacterial sequences in the recipient.

Maize 59122 contains the coding sequences for the Cry34Ab1, Cry35Ab1 and PAT proteins. The coding genes are codon optimised variants of the sequences of *cry34Ab1* and *cry35Ab1* from *B. thuringiensis* strain PS149B1 and of *pat* from *S. viridochromogenes*. Other DNA fragments with sequence identity refer to the right and left T-DNA borders of 155 bp and 57 bp in length which are located in flanking regions of the expression cassette.

None of the three bacterial species *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*), *B. thuringiensis*, or *S. viridochromogenes* are considered to be prevalent in the gastrointestinal tract of humans or animals. All of them occur in soil and, in addition, *B. thuringiensis* has been frequently isolated from the guts of insects (Jensen et al., 2003).

On a theoretical basis (i.e., without any study providing experimental evidence for horizontal gene transfer in the case of maize 59122 or any other GM plant), it can be assumed that, as an extremely rare event, homologous recombination can occur between the recombinant *cry34Ab1*, *cry35Ab1* or *pat* genes and their natural variants as they may occur in *B. thuringiensis* (for *cry34Ab1* and *cry35Ab1*) and *S. viridochromogenes* (for *pat*) or other not yet characterised soil bacteria with homologous genes. Such recombination events would only replace natural variants (substitutive recombination) and are therefore unlikely to provide any new property connected to a selective advantage for the recipient organisms (EFSA, 2009c). Double homologous recombination of the flanking regions with those on Ti-plasmids of *A. tumefaciens* would result in gene replacement, by which a *cry34Ab1-cry35Ab1-pat* gene construct would substitute genes for crown gall formation (loss of auxin-, cytokinin- and opine-synthesising genes).

In addition to homology-based recombination processes, illegitimate recombination that does not require DNA similarity between the recombining DNA molecules is theoretically possible. However, the transformation rates for illegitimate recombination are considered to be 10^{10}-fold lower than for homologous recombination (Hülter and Wackernagel, 2008; EFSA, 2009c). Illegitimate recombination events have not been detected in studies that have exposed bacteria to high concentrations of GM plant DNA (EFSA, 2009c). Thus, this process, in comparison with homologous recombination, is not

considered to contribute to horizontal gene transfer events. In comparison with the above-described homology-facilitated recombination processes, the contribution of illegitimate recombination is extremely low.

(4) The expression and the function of the protein in the bacterial recipient

The *cry34Ab* and *cry35Ab1* genes of maize 59122 are regulated by promoters from maize and wheat. The *pat* gene is regulated by the promoter of the cauliflower mosaic virus. The expression of these constructs in bacteria is unknown, but generally the expression level of eukaryotic promoters in bacteria is inefficient (Warren et al., 2008).

(5) The selective advantage provided by the acquired recombinant gene-encoded traits

In a worst-case scenario, considering the possibility of expression, an *A. tumefaciens* recipient would become capable of producing Cry34Ab1, Cry35Ab1 and PAT proteins. However, the exposure of bacterial communities to the recombinant genes in maize 59122 must be seen in the context of the natural occurrence and level of exposure to alternative sources of similar genes to which bacterial communities are continually exposed. As mentioned above, *S. viridochromogenes* and *B. thuringiensis* occur in soil and *B. thuringiensis* strains with the capacity to produce Cry34Ab1/Cry35Ab1 proteins are not unusual in terms of their geographic distribution and habitat (Schnepf et al., 2005). The *pat* gene originates from typical soil bacteria and natural variants of this gene have been found in several different species (Omura et al., 1984a,b; Kita et al., 2009). Furthermore, Bartsch and Tebbe (1989) found a large proportion of bacteria with tolerance to phosphinothricin due to mechanisms other than *N*-acetylation, including desamination. Thus, extremely rare transfer events would most likely not signficantly affect the natural background of resistance/tolerance to this compound. Owing to its specific lifestyle as a soil bacterium and plant pathogen, the EFSA GMO Panel considers it unlikely that *A. tumefaciens* would gain selective advantage from such a horizontal gene transfer by double homologous recombination. The loss of gene *5* from the Ti-plasmid will cause a selective disadvantage for *A. tumefaciens* as the tumour induction on plants will be impaired. In addition, further dissemination of the Ti plasmid to bacteria would be limited to the relatives of *Agrobacterium* within the Rhizobiaceae owing to the host range specificity of the Ti plasmid.

The EFSA GMO Panel concludes that the *cry34Ab1*, *cry35Ab1* or *pat* genes from maize 59122 may, on a theoretical basis, be transferred by double homologous recombination to *A. tumefaciens*. Owing to the natural occurrence of sequence similar *cry34Ab1*, *cry35Ab1* and *pat* in the environment, a low level of gene transfer to *A. tumefaciens* is not regarded as conferring a novel selective advantage. Considering its intended uses as food and feed and for cultivation, and the above assessment, the EFSA GMO Panel has therefore not identified any concern associated with horizontal gene transfer from maize 59122 to bacteria.

The NL CA concluded that "*the potential ecological impact due to gene transfer from 59122 to other maize or the soil micro flora is negligible*" (Sections 6.3 of the environmental risk assessment report of the NL CA).

6.2.2.2. Plant to plant gene transfer and its consequences[36]

Maize is a cross-pollinating plant, relying on wind for the dispersal of its pollen. While maize pollen can be collected by honeybees and other insects, these pollinating insects play a minor role in the cross-pollination of maize plants (Eastham and Sweet, 2002; Malone and Burgess, 2009).

Compared with other wind-pollinated species, the pollen grains of maize are relatively large (an average diameter of 90 μm) and heavy (0.25 μg) (Raynor et al., 1972; Di-Giovanni et al., 1995). Owing to their characteristics, maize pollen grains settle to the ground rapidly (Aylor et al., 2003) and have usually a short flight range (Jarosz et al., 2005). Approximately 95–99 % of the released pollen is

[36] Technical dossier/Section D9.3.

deposited within about 50 m from the source. However, vertical wind movements or gusts during pollen shedding can lift pollen up high in the atmosphere and distribute it over significant distances up to several kilometres (Jarosz et al., 2005; Astini et al., 2009; Vogler et al., 2009; Hofmann et al., 2010). The concentrations of viable pollen decrease considerably with height (Aylor et al., 2006) and distance (Jarosz et al., 2005) from the source. Very low levels of cross-pollination can occur over distances up to several kilometres under suitable climatic conditions (Bannert and Stamp, 2007; Delage et al., 2007; Langhof et al., 2010; Kawashima et al., 2011), but most cross-pollination events occur within 40 m of the pollen source (reviewed by Eastham and Sweet, 2002; Devos et al., 2005, 2009b; van de Wiel and Lotz, 2006; Hüsken et al., 2007; Langhof and Rühl, 2008; Sanvido et al., 2008; Ricroch et al., 2009; van de Wiel et al., 2009; Czarnak-Klos and Rodríguez-Cerezo, 2010; Riesgo et al., 2010).

Maize pollen is susceptible to desiccation, and water loss in pollen grains during dispersal reduce their ability to germinate on the stigma (Aylor, 2004). In addition, the water content of maize pollen affects its flight dynamics (Aylor, 2002, 2003; Aylor et al., 2003). During drying, the shape of maize pollen changes from a prolate spheroid to a crinkled, prismatic solid, and its density increases by approximately 16 %, and its settling speed decreases by approximately 34 %. These physical changes impact the potential transport distances of pollen. In general, the lightest pollen will travel the longest distances, but it will be the least viable (Aylor, 2002).

The EFSA GMO Panel does not consider pollen dispersal and consequent cross-pollination as environmental hazards in themselves, and is primarily concerned with assessing the environmental consequences of transgene flow on ecosystems by considering the spread and fitness of hybrid and backcross progeny, as well as exposure to non-target organisms (Section 6.2.4, below).

Theoretically, seeds originating from the cross-pollination of certain sexually compatible wild relatives can mediate the potential spread and establishment of hybrid and backcross progeny (Wilkinson et al., 2003; Morales and Traveset, 2008; Devos et al., 2009a). However, in the EU, there are no sexually cross-compatible wild relatives with which maize can hybridise and form backcross progeny (Eastham and Sweet, 2002; OECD, 2003). The only recipient plants that can be cross-fertilised by maize are other cultivated maize varieties and types (Devos et al., 2005, 2009b; van de Wiel and Lotz, 2006; Hüsken et al., 2007; Sanvido et al., 2008; Bitocchi et al., 2009; Ricroch et al., 2009; Czarnak-Klos and Rodríguez-Cerezo, 2010). As the molecular analysis and food/feed safety evaluation did not raise safety concerns (Sections 3 to 5, above; EFSA, 2007), the EFSA GMO Panel does not consider cross-pollination in maize an environmental risk, but an agricultural management and coexistence issue that is not within its remit.

Seed-mediated establishment of maize and its survival outside cultivation is rare in spite of extensive cultivation in many countries and accidental seed dispersal. Maize plants have lost their ability to release seeds from the cob, so most seed dispersal is the result of harvesting and the post-harvest activities of farmers. The occurrence of some GM maize plants outside cropped areas has been reported in Korea and is attributed to seed spillage during import, transport, storage, handling and processing (Kim et al., 2006; Lee et al., 2009; Park et al., 2010). However, survival of maize outside cultivation in Europe is limited by a combination of low competitiveness, the absence of a dormancy phase, and susceptibility to plant pathogens, herbivores and cold climatic conditions. Furthermore, as these general characteristics are unchanged in maize 59122, it is considered very unlikely that it or its progeny will differ from conventional maize varieties in their ability to establish feral populations under European environmental conditions. The insect resistance and herbicide tolerance traits are not likely to provide selective advantages outside cultivation or other areas where glufosinate-ammonium-based herbicides could be applied in Europe. Therefore, as for any other maize varieties (Raybould et al., 2012), maize 59122 plants are not likely to establish feral populations under European environmental conditions. The contribution of occasional feral GM maize plants to the pollen flow into agricultural fields will be extremely small, compared with that from the crop. Moreover, field observations performed on maize volunteers after GM maize cultivation in Spain revealed that maize volunteers had a low vigour, rarely had cobs and produced pollen that cross-pollinated adjacent plants

only at low levels (Palaudelmàs et al., 2009; Section 6.2.1, above). In comparison with GM maize volunteers, the vigour of occasional feral GM maize plants will be reduced because of the less suitable habitat outside agricultural fields.

Because maize 59122 has no altered agronomic and phenotypic characteristics, except for the specific target insect resistance and herbicide tolerance (Section 6.2.1, above), the EFSA GMO Panel concludes that the likelihood of unintended environmental effects as a consequence of spread of genes from maize 59122 is considered to be extremely low.

The conclusion of the EFSA GMO Panel is consistent with that of the NL CA on maize 59122. The NL considered that "*maize has no sexually compatible wild or weedy species, therefore out crossing of 59122 maize can only occur to other cultivated maize. If this would occur, the potential environmental impact of this maize would be comparable to that of 59122 maize*". The NL CA concluded that "*the potential ecological impact due to gene transfer from 59122 to other maize or to the soil micro flora is therefore negligible*" (Section 6.3 of the environmental risk assessment report of the NL CA).

6.2.3. Interactions of the GM plant with target organisms[37]

The potential of maize 59122 to cause adverse effects through direct or indirect interactions between the GM plant and target organisms was evaluated by EFSA GMO Panel, and this evaluation is described below.

6.2.3.1. Adverse effects due to resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests

There is a concern that the widespread, repeated, and exclusive use of *Bt*-maize that expresses the same *Bt*-protein by individual farmers as the sole pest management option against WCR will create significant selection pressure and increase the risk of the target insect pest evolving resistance (Siegfried et al., 1998). Resistance is defined as the occurrence of a phenotype of an individual of the target insect pest that can survive on the *Bt*-plant and produce viable offspring (Andow, 2008). Susceptibility of target insect pests to plant-produced *Bt*-proteins is viewed in some jurisdictions as a common good that should be preserved (Glaser and Matten, 2003; Bourguet et al., 2005; Gassmann and Hutchison, 2012) owing to the benefits of *Bt*-crops (Qaim, 2009; Carpenter, 2010; Hutchison et al., 2010; Areal et al., 2012; Lu et al., 2012; Wan et al., 2012; Haegele and Below, 2013; Shi et al., 2013) and the broader use of sprayable *Bt*-formulations. Resistance evolution in target insect pests is not considered a direct environmental harm, but the consequences of the establishment of resistant populations may lead to altered pest management practices. Therefore, farmers may have to revert to the currently used pest management tools; e.g., insecticide use, which might have a higher environmental impact, use of biocontrol programmes on a larger scale, or alteration of the cultivation/farming system (i.e., rotate maize with other crops) (Andow, 2008).

Because resistance to chemical insecticides is known to evolve in insect pests (Whalon et al., 2013), the potential evolution of insect resistance to *Bt*-proteins constitutively expressed in *Bt*-crops is considered a relevant environmental and agronomic concern by the scientific community (e.g., Tabashnik et al., 2008a,b, 2009; BEETLE report, 2009). Owing to its behavioural and genetic plasticity, WCR has evolved resistance to broadcast cyclodiene insecticides used for larval control in the 1950s and early 1960s (Ball and Weekman, 1962) and broadcast insecticides used for beetle management in the United States (Meinke et al., 1998; Wright et al., 2000; Siegfried et al., 2004). Moreover, in a growing portion of maize growing areas of the United States, a crop rotation-resistant WCR variant has evolved whereby females have adapted their egg-laying behaviour to lay eggs in crops other than maize, leading to damage in first-year maize in spite of crop rotation (Levine and Oloumi-Sadeghi, 1996; Levine et al., 2002; Onstad, 2008; Miller et al., 2009; Gray et al., 2009). Such a crop rotation-resistant WCR variant has not yet evolved in the EU. Gassmann et al. (2011) reported the first instance of field-selected resistance to a *Diabrotica*-active *Bt*-maize in WCR in Iowa (United

[37] Technical dossier/Section D9.4.

States). They found significantly higher survival of WCR larvae on maize MON 88017 from fields suffering severe WCR feeding damage than from control fields.

In artificial laboratory and greenhouse selection experiments, a decreasing susceptibility of WCR to Cry34Ab1/Cry35Ab1 has been demonstrated for maize 59122. This increased tolerance to Cry34Ab1/Cry35Ab1 in two WCR colonies reared on maize 59122 seedlings was observed over 11 generations of selection (Lefko et al., 2008). An increased WCR survivorship from the first generation to the ninth generation of 15- and 59-fold was found for the two WCR colonies that were selected to survive on maize 59122. While tolerance levels increased over the generations of selection, they fluctuated considerably for the initial four to six generations, but tolerance remained stable after five to seven generations with a survival that was at least ten times greater than that observed during the first generation of selection. After one and six generations of selection, the mean population fitness (number of adults divided by number of hatched eggs) was approximately 0.03 and 1.00, respectively. After ten and 11 generations of selection on maize 59122 with no random mating, the estimated h^2 values declined from 0.29 to 0.11, suggesting that resistance in the two WCR colonies was not fixed under realistic exposure (Lefko et al., 2008).

The expression of *Bt*-proteins in maize 59122 is low to moderate and some susceptible WCR individuals survive on *Bt*-maize. Therefore, only relatively low resistance ratios are expected. The reported values are overall at least an order of magnitude lower than those found for some Cry resistant Lepidoptera (Tabashnik et al., 2009; Siegfried and Hellmich, 2012), but survival can be substantially enhanced on *Bt*-maize (Gassmann et al., 2012). Lefko et al. (2008) did not evaluate their selected populations in the field, but assessed damage to maize 59122 from two selected WCR populations under greenhouse conditions. Damage caused by WCR was shown to increase gradually with repeated generations of selection on *Bt*-maize relative to that caused by WCR from the first generation of selection, but overall damage remained low.

Field-selected resistance of WCR to plant-produced *Bt*-proteins is documented only for Cry3Bb1 in some US maize growing areas in Iowa (Tabashnik 2008; Tabashnik et al. 2008a; Gassmann et al., 2011, 2012). Multiple and increased performance failures of maize MON 88017 were also reported in Illinois, Minnesota, Nebraska and South Dakota (United States) (Gray, 2011a,b,c; US EPA, 2011a; Porter et al., 2012). Given that resistance has evolved in all nine of the artificial laboratory and greenhouse selection experiments conducted with Cry3Bb1-expressing maize within just a few generations (Meihls et al., 2008; Meihls, 2010; Oswald et al., 2011), it is not surprising that resistance evolved under field conditions after three to seven generations of selection (Gassmann et al., 2011, 2012). Cry3Bb1-expressing maize was first to market and has been the dominant *Diabrotica*-active *Bt*-maize (Monsanto, 2009). The first approval for commercial cultivation for maize 59122 in the United States was in 2005, resulting in a lower market share compared with Cry3Bb1-expressing maize. As an increased tolerance to Cry34Ab1/Cry35Ab1 (Lefko et al., 2008) also evolved relatively quickly under laboratory and/or greenhouse settings, field-selected resistance to maize 59122 is possible too and therefore vigilance should be exercised. Tabashnik and Gould (2012) attributed the lack of reported field-selected resistance to Cry34Ab1/Cry35Ab1 so far to the lower exposure of WCR populations to these *Bt*-proteins, rather than an inherently lower risk of evolving resistance compared with Cry3Bb1. According to Tabashnik and Gould (2012), the similar estimated h^2 values for WCR resistance to Cry3Bb1 and Cry34Ab1/Cry35Ab1 suggest that the risk of resistance evolution is similar for both plant-produced *Bt*-proteins. However, owing to the specific protein structures of Cry34Ab1/Cry35Ab1 (which differ from that of three-domain-like *Bt*-proteins), the Cry34Ab1/Cry35Ab1 specific binding sites on WCR midgut membrane and the enhanced specific binding of Cry35Ab1 by Cry34Ab1, there is also the possibility of a different mechanism of action for Cry34Ab1/Cry35Ab1 (Li et al., 2013), which may result in an inherently lower risk of evolving resistance compared with Cry3Bb1.

Based on annual resistance monitoring data for Cry34Ab1/Cry35Ab1 generated by the applicant in the United States, the US EPA (2010b) suspected decreased susceptibility in WCR populations sampled in

2008 (Table 2).[38] The applicant indicated that the susceptibility of WCR in 2008, 2009 and 2010, as measured by the ratio of the highest to lowest LC_{50} and EC_{50} values (representing the least susceptible WCR population to the most susceptible WCR population), was similar to those in previous years. An increase in this ratio would occur if a population shows reduced susceptibility to the Cry34Ab1/Cry35Ab1 proteins. Conversely, the maintenance of the same amount of variation suggests that natural variability is responsible for the range in susceptibility observed. Additional WCR samples taken in Iowa in 2009 exhibited an EC_{50} value similar to that of the susceptible laboratory strain. In addition, only one report of unexpected field damage on maize 59122 caused by WCR that might indicate reduced WCR susceptibility was received by the applicant during 2008–2010. A follow-up investigation did not lead to resistance being suspected in the problem field. Overall, WCR susceptibility to Cry34Ab1/Cry35Ab1 in 2008, 2009 and 2010 had not significantly decreased since monitoring was initiated in 2004.

Table 2: Range of estimated susceptibility of WCR populations to Cry34Ab1/Cry35Ab1 as reported by Lepping et al. (2011).[39] WCR populations were collected in Minnesota, Nebraska, Iowa and Illinois (United States)

WCR population	Sampling year	LC_{50} (µg Cry34Ab1/Cry35Ab1 cm^2)	EC_{50} (µg Cry34Ab1/Cry35Ab1 cm^2)
Field	2004–2005	1.5–9.4 (4- to 7-day bioassays)	0.9–2.4
	2006	1. -7.3 (4- to 6-day bioassays)	0.8–2.3
	2007	3.0–11.5 (4-day bioassays)	0.96–3.7
	2008	6.2–15.4 (4-day bioassays)	2.0–7.9
	2009	45.9–93.3 (4-day bioassays)	1.9–5.7
	2010	6.7–33.7 (5-day bioassays)	2.4–7.6
Laboratory	2010	14.9 (95 % CI: 9.6–24.2)	2.9 (95 % CI: 2.4–3.4)

CI, confidence interval

Based on the available data, the EFSA GMO Panel concludes that WCR has the ability to evolve resistance to the Cry34Ab1/Cry35Ab1 proteins, especially if maize 59122 is used repeatedly and exclusively, and the WCR infestation levels are high. The possible resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests is identified by the EFSA GMO Panel as a concern associated with the cultivation of maize 59122, as resistance evolution may lead to altered pest control practices that may cause adverse environmental effects. The EFSA GMO Panel therefore recommends that appropriate risk management strategies are implemented to delay and monitor resistance evolution. Risk mitigation measures and post-market environmental monitoring are discussed below in Section 6.3.

The conclusion of the EFSA GMO Panel is consistent with that of the NL CA. According to the NL CA, "*resistance to Bt toxins in corn root worm during (commercial) cultivation cannot be excluded*" (Section 6.4 of the environmental risk assessment report of the NL CA).

[38] Additional information received on 02/10/2012/Request 2/page 13/Annex: Storer and Owens (2009)//16/01/2012/Annex: Lepping et al. (2011) and references therein (e.g., Storer et al., 2010).

[39] Additional information received on 02/10/2012/Request 2/page 13/Annex: Storer and Owens (2009)//16/01/2012/Annex: Lepping et al. (2011).

6.2.3.2. Adverse effects on target organisms due to the expression of the PAT protein

Potential effects on target organisms due to the expression of the PAT protein were considered an issue neither by the EFSA GMO Panel nor by the NL CA and Member States, because the protein does not interact with any specific target organisms.

6.2.4. Interactions of the GM plant with non-target organisms[40]

The potential of maize 59122 to have direct or indirect adverse effects on non-target organisms and the ecosystem services they provide, such as pollination, biological control or decomposition (Sanvido et al., 2009; Arpaia, 2010), was evaluated by the EFSA GMO Panel. This evaluation covers the assessment of potential adverse environmental effects on non-target organisms due to intended and unintended changes in the GM plant (e.g., Hjältén et al., 2007; Desneux et al., 2010; Garcia-Alonso, 2010; Raybould et al., 2010; Arpaia et al., 2011). Intended changes in the GM plant are those that fulfil the original objectives of the genetic modification, whereas unintended changes are defined as consistent differences between the GM plant and its appropriate comparator, which go beyond the primary intended changes of introducing the transgene(s) (EFSA, 2010b,c). These changes may have consequences for the environment, and it is the potential adverse nature of these consequences that requires assessment. The EFSA GMO Panel follows two distinct yet complementary approaches for the risk assessment of potential adverse effects on non-target organisms (EFSA, 2010b,c).

The evaluation of potential adverse effects on non-target organisms due to intended and unintended changes in maize 59122 is described below.

6.2.4.1. Adverse effects on non-target organisms due to unintended changes in maize 59122 (weight-of-evidence approach using event-specific *in planta* data)

The molecular characterisation of the DNA insert and flanking regions of maize 59122 did not indicate unintended changes due to the insertion (Section 3, above). Moreover, no biologically relevant differences in the composition of key analytes or agronomic and phenotypic characteristics were identified between maize 59122 and its conventional counterpart (EFSA, 2007; Herman et al., 2007; CERA, 2013; Section 4, above).

In order to reliably conclude on potential adverse effects on non-target organisms due to unintended changes in maize 59122, the EFSA GMO Panel reviewed all *event*-specific studies on main functional groups of non-target organisms such as (1) herbivores, (2) natural enemies, (3) pollinators and (4) decomposers.

(1) *Herbivores*: The applicant provided laboratory studies in which larvae of *Danaus plexippus*, monarch butterfly (Lepidoptera: Danaidae), *Vanessa cardui*, painted lady (Lepidoptera: Nymphalidae) and *Pieris rapae*, cabbage white (Lepidoptera: Pieridae) were exposed to maize 59122 pollen, in order to assess whether this pollen has adverse effects on these lepidopteran herbivores.

- *D. plexippus*: Treatments of the laboratory study included seven doses of maize 59122 pollen or that of the near-isogenic line (50, 100, 200, 400, 800, 1600 and 3200 pollen grains/cm^2) applied evenly over entire leaves of milkweed.[41] No significant differences between the monarch butterfly larvae that ingested maize 59122 pollen and those that ingested the pollen from the near-isogenic line were observed in terms of survival, weight gain, developmental life stage and consumption after 216 hours.

- *V. cardui* and *P. rapae*: Maize 59122 pollen was incorporated into an artificial diet at 10 % of the final dry weight, which corresponds to 2 ng Cry34Ab1 mg^{-1} diet.[42] This concentration

[40] Technical dossier/Section D9.5.
[41] Additional information received on 16/01/2012/Annex: Sears and Rempel (2003).
[42] Additional information received on 08/01/2009/Request 2/pages 5–7.

in the diet corresponds to 10 × the expected environmental concentration of Cry34Ab1 (assuming 171 pollen grains/cm^2 in-field (Pleasants et al., 2001), a pollen grain mass of 250 ng, 71 ng Cry34Ab1 mg^{-1} pollen and 14.5 mg/cm^2 leaf). No differences in mortality of *V. cardui* and *P. rapae* larvae exposed to maize 59122 pollen for seven days was observed between the *Bt*- and non-*Bt*-maize pollen treatments and the no-pollen control diet. In addition, no statistically significant differences in larval weight between *V. cardui* larvae fed *Bt*- or non-*Bt*-maize were noted, but the larval weight of *P. rapae* larvae fed maize 59122 pollen was statistically significantly lower than that for larvae fed non-*Bt*-maize pollen. These latter results are not considered as biologically relevant, as the difference in weight was small and the weight ranges overlapped considerably between all treatments.

Field trials conducted in the United States in 2005–2007, in Hungary in 2006–2008 and in Spain in 2005–2007 indicated no effects of maize 59122 on abundantly occurring herbivore populations such as aphids, lepidopteran pests, plant- and leafhoppers, and thrips.[43]

(2) *Natural enemies*: The applicant performed two laboratory studies to evaluate the response of the predatory coccinellids *Coleomegilla maculata* and *Coccinella septempunctata* (Coleoptera: Coccinellidae) to maize 59122 pollen. *C. maculata* is a non-indigenous (American) species occurring in maize fields and represents potentially exposed predators to maize 59122 under field conditions. It was selected by the applicant as a surrogate species, as it is amenable to laboratory rearing and testing. *C. septempunctata* is a widely spread predatory coccinellid species found in high abundance in maize fields across Europe (Meissle et al., 2012).

- *C. maculata*: In an *in planta* study, larvae were exposed to a diet consisting of 50 % maize 59122 pollen and 50 % ground corn earworm eggs.[44] Neonate larvae were placed in individual bioassay wells and allowed to feed *ad libitum* throughout the 14-day larval growth period. There was no significant difference in mortality, development, or adult weight between *C. maculata* larvae fed a control diet, which did not contain active protein, and larvae fed treatments containing maize 59122 pollen. Following a request of the Belgian Biosafety Advisory Council (in the frame of the initial environmental risk assessment evaluation of application EFSA-GMO-UK-2006-30), the applicant clarified that the maize pollen–egg mixture was ground completely to avoid selective feeding on lepidopteran eggs and to ensure sufficient exposure and ingestion of maize 59122 pollen.

- *C. septempunctata*: Exposing *C. septempunctata* larvae to a 1:3 ratio of maize 59122 pollen and moth eggs (m:m) had no adverse effects on time to adult emergence, adult weight and mortality of larvae, compared with the non-*Bt*-maize pollen:moth egg-containing control treatment.[45] The applicant provided evidence to confirm that the maize pollen–egg mixture was ground completely to avoid selective feeding on lepidopteran eggs, and to ensure sufficient exposure and ingestion of maize 59122 pollen.[46]

Field trials performed in the United States in the growing seasons 2004–2005[47] and 2005–2007[48], in Spain in the growing seasons 2005–2007[49] and in Hungary in the growing seasons 2006–2008[50] delivered comprehensive data for carabids, coccinellids, staphylinids, nabids, *Orius* and

[43] Additional information received on 20/12/2007/Annexes 1–3: Higgins and Hong (2007)//27/01/2010/Annexes 8–9: Pascual and Hong (2009)//16/01/2012/Annexes: Higgins and Hong (2008a,b), Higgins et al. (2009) and Pascual (2010).
[44] Additional information received on 16/01/2012/Annex: Higgins and Binning (2002).
[45] Additional information received on 27/01/2010/Request 1/pages 1–3/Annex 2: Califf and Ostrem (2009)/Annex 3: Hong (2009).
[46] Additional information received on 11/02/2013/Point 4/pages 7–9/Figure 1/Annex: Boeckman (2012).
[47] Additional information received on 16/01/2012/Annex: Higgins and Dively (2006).
[48] Additional information received on 16/01/2012/Annex: Higgins et al. (2009).
[49] Additional information received on 20/12/2007/Annexes 1 and 3: Higgins and Hong (2007)//27/01/2010/Annex 9: Pascual and Hong (2008)//16/01/2012/Annex: Higgins and Hong (2008a).
[50] Additional information received on 20/12/2007/Annex 2: Higgins and Hong (2007)//27/01/2010/Annex 8: Pascual and Hong (2008)//16/01/2012/Annexes: Higgins and Hong (2008b) and Pascual (2010).

Chrysopa species, and confirm the absence of adverse effects of maize 59122 on any of these predatory species.

(3) *Pollinators*: A 26-day laboratory study with three- to five-day-old honeybee larvae (*Apis mellifera*; Hymenoptera: Apidae) was provided by the applicant.[51] Honeybee was selected as representative of potentially exposed pollinators in maize fields (Meissle et al., 2012). Individual larvae were fed 2 mg pollen collected from either maize 59122 or the near-isogenic counterpart. Larval survival was evaluated six and 12 days after treatment, and adult emergence was evaluated 26 days after treatment. No statistical differences in larval mortality between those fed maize 59122 pollen and non-*Bt*-maize pollen were observed. Honeybee development and survival were not affected by exposure to maize 59122 pollen.

(4) *Decomposers*: A 28-day laboratory study with juvenile *Folsomia candida* (Collembola: Isotomidae) was conducted to determine the chronic effects of whole maize 59122 plant material on their survival and reproduction.[52] The collembolan *F. candida* was selected as representative of potentially exposed decomposers in the soil. The test diet consisted of a homogeneous mixture of dry yeast (95.8 %) and lyophilised maize (4.2 %). Maize 59122 plant material had no adverse effect on survival and reproduction of *F. candida* as compared with those exposed to the control diets (yeast only or 4.2 % non-*Bt*-maize with yeast).

The applicant also provided a higher-tier study which was performed in the United States during the growing season 2004–2005, and in which the soil-litter community was monitored.[53] No negative impact of maize 59122 was observed on field densities of abundantly occurring Collembolla and soil mites.

Based on the evidence provided by the applicant on maize 59122, the EFSA GMO Panel concludes that there are no indications of adverse effects on non-target organisms due to unintended changes in maize 59122. Therefore, the EFSA GMO Panel considers *trait*-specific information appropriate to assess whether maize 59122 poses a risk to non-target organisms. The assessment of potential adverse effects on non-target organisms due to the expression of the Cry34Ab1/Cry35Ab1 and PAT proteins is described in Sections 6.2.4.2 and 6.2.4.3, respectively.

6.2.4.2. Adverse effects on non-target organisms due to the expression of the Cry34Ab1/Cry35Ab1 proteins (tiered approach)

Equivalence of microbe-produced Cry34Ab1/Cry35Ab1 proteins to those expressed in maize 59122

The equivalence of the microbe-produced Cry34Ab1/Cry35Ab1 proteins used in some of the lower-tier studies (Tier 1a) provided by the applicant with those expressed in maize 59122 was shown and previously evaluated by the EFSA GMO Panel (see EFSA, 2007).[54] Therefore, the EFSA GMO Panel concludes that the outcomes of lower-tier studies with non-target organisms fed a diet containing microbe-produced Cry34Ab1/Cry35Ab1 proteins can be used to inform the environmental risk assessment of maize 59122.

Effects on non-target terrestrial (plant- and ground-dwelling) arthropods

It has been reported that up to 1000 non-target arthropod species can occur in maize fields in the EU (Knecht et al., 2010; Meissle et al., 2012). Therefore, several non-target arthropods are likely to be exposed to plant-produced *Bt*-proteins when cultivated. These non-target arthropods can be exposed to *Bt*-proteins when feeding on plant material (including pollen) or honeydew excreted from sap-sucking species, and/or when feeding on prey/host organisms which have previously been feeding on *Bt*-maize

[51] Technical dossier/section D.1/Annex 26: Maggi (2001).
[52] Additional information received on 16/01/2012/Annex: Teixeira (2006b).
[53] Additional information received on 16/01/2012/Annex: Higgins and Dively (2006).
[54] Technical dossier/Section D7.8.1/Annex 9: Schafer et al. (2003)/see also Gao and Herman (2000) and Gao et al. (2000).

(Andow et al., 2006; Romeis et al., 2006, 2008a,b; Lundgren, 2009). These species however are only at risk if the *Bt*-proteins show toxicity at a realistic level of exposure (e.g., Head et al., 2001; Dutton et al., 2002; Harwood et al., 2005; Vojtech et al., 2005; Obrist et al., 2005, 2006a,b,c; Torres et al., 2006; Raybould, 2007; Torres and Ruberson, 2008; Meissle and Romeis, 2009a,b; Romeis and Meissle, 2011). Because not all of the exposed species can be tested from a practical viewpoint, the toxicity of *Bt*-proteins is tested generally on a representative subset of species using a tiered approach (Garcia-Alonso et al., 2006; Rose, 2007; Romeis et al., 2006, 2008a). In the case of maize 59122 and in line with the EFSA GMO Panel guidance document on the environmental risk assessment of GM plants (EFSA, 2010c), the applicant selected a representative subset of non-target arthropod species for testing purposes based on the ecological relevance of the species, the likely exposure of the species to maize 59122 under field conditions, species susceptibility to the Cry34Ab1/Cry35Ab1 proteins, and testability. Although the applicants' rationale as to why specific non-target organisms were chosen for testing purposes is comprehensive, the representativeness of some of the selected species as representative of a valued group of non-target organisms (e.g., *C. maculata*, *Nasonia vitripennis*), including their likely exposure to maize 59122 under field conditions, is questionable.

The applicant conducted and reviewed a series of lower-tier studies (dietary bioassays) on several non-target arthropod species representative of different functional groups, including herbivores, natural enemies (predators and parasitoids), pollinators and decomposers.[55] The EFSA GMO Panel notes that some of the lower-tier studies conducted by the applicant do not adhere to the general principles of good laboratory study design (see Rose, 2007; Romeis et al., 2011 for recommendations for the design of laboratory studies on non-target arthropods), and therefore cannot be used to support the risk assessment. These lower-tier studies, limitations in the experiments and the remaining scientific uncertainties are described in more detail below.

Herbivores: Herbivores (prey/host organisms) are an important food source for other species of the food web, can ingest plant-produced *Bt*-proteins when feeding on *Bt*-plants, and can transfer *Bt*-proteins to higher trophic levels. The level at which different herbivores ingest *Bt*-proteins depends on the site and time of protein expression in the plant, the mode of feeding of the herbivore, and the amount of plant material they ingest.

- *Herbivore species from the target taxon (Coleoptera: Chrysomelidae)*: The activity of the Cry34Ab1/Cry35Ab1 proteins is likely to be broader than the target pest species (corn rootworms) or other putative chrysomelid targets such as the cereal leaf beetle, *Oulema melanopus*, and include other non-target Chrysomelidae. The applicant tested the green dock leaf beetle, *Gastrophysa viridula*, and a lower mean adult body weight was observed when larvae were exposed to 600–800 maize 59122 pollen grains/cm^2 compared with those fed non-*Bt*-maize pollen, indicating that the Cry34Ab1/Cry35Ab1 proteins have insecticidal activity against Chrysomelidae other than corn rootworms.[56]

  Non-target Chrysomelids are regularly found in maize fields and can be exposed to the Cry34Ab1/Cry35Ab1 proteins based on their herbivorous feeding habits, as shown for the genera *Chaetocnema*, *Longitarsus*, *Oulema* and *Phyllotreta* (Kiss et al., 2002, 2004; Daly and Buntin, 2005; Eckert et al., 2005; Harwood et al., 2005; Obrist et al., 2006b; Knecht et al., 2010; Rauschen et al., 2010a). Based on a literature review and targeted interrogations of an EU fauna arthropod database (see Knecht et al., 2010; Meissle et al., 2012[57]), the applicant listed 32 different non-target chrysomelid species that may occur in and around maize fields.[58] The EFSA GMO Panel notes that host plants for most of the listed chrysomelid species do not belong to the Gramineae family. Only two non-target chrysomelid species, *O. melanopus* and *Phyllotreta vittula*, which are considered pests, were found to use maize as host plant. This conclusion is consistent with the findings reported by Rauschen et al. (2010a) for Germany and

[55] Additional information received on 08/01/2009, 27/01/2010 and 16/01/2012.
[56] Additional information received on 16/01/2012/Request 1.1/pages 11–19/Annex: Székács and Kong (2011).
[57] http://www.efsa.europa.eu/en/supporting/pub/334e.htm
[58] Additional information received on 16/01/2012/Request 1/pages 11–19.

Kiss et al. (2002, 2004) for Hungary. Rauschen et al. (2010a) 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* spp. (see also Kiss et al., 2002, 2004 for Hungary). Because leaf beetles that use maize as host plant can induce visible damage to maize plants, a reduction in their densities is not regarded as an environmental concern (Rauschen et al., 2010a). Therefore, the EFSA GMO Panel focused its assessment on potential adverse effects of maize 59122 on non-target (non-pest) chrysomelid species found in and around maize fields.

Non-target adult chrysomelids, which may occasionally feed on maize 59122 plants, are not expected to be affected by maize 59122 due to the low activity of Cry34Ab1/Cry35Ab1 on adults. Furthermore, the only protected chrysomelid species (*Macroplea pubipennis*) considered to be at risk across the EU (under Directive 92/43/EEC on conservation of natural habitats and of wild fauna and flora) does not occur in maize fields owing to its aquatic lifestyle. The only remaining potential risk to non-target chrysomelid species from maize 59122 is therefore the ingestion of harmful amounts of pollen deposited on their host plants in and around maize fields. This potential risk is the highest for neonates and younger (early) instars, as older (later) instars and adults are inherently less susceptible to *Bt*-proteins.

To be in a position to evaluate the potential risk to larvae of non-target chrysomelids from exposure to maize 59122 pollen, the EFSA GMO Panel requested the applicant to provide: (1) toxicity data for a representative chrysomelid species; and (2) an exposure analysis.

(1) In the Tier 1b study with the chrysomelid species *G. viridula*, larvae were exposed to three different pollen concentrations: 50–100, 300–400 or 600–800 maize 59122 pollen grains/cm^2.[59] These concentrations were selected to approximate mean in-field pollen deposition rates (e.g., mean in-field rates of 171 pollen grains/cm^2 reported by Pleasants et al. (2001) and 250–500 pollen grains/cm^2 reported by Gathmann et al. (2006), and at least 10 × the reported pollen deposition rate at the field margin (e.g., means ranged from 63.1 pollen grains/cm^2 at the edge of the field (0 m) to 8 pollen grains/cm^2 at 4–5 m from the edge of the field (Pleasants et al., 2001)). A prospective power analysis indicated that 60 larvae per group were sufficient to detect a 20 % mortality increase with a power of at least 70.4 %, and a 20 % weight change with a power of at least 86.0 %. No statistically significant differences in mortality of *G. viridula* larvae were observed between the larvae fed maize 59122 pollen or non-*Bt*-maize pollen at concentrations of 50–100, 300–400 or 600–800 pollen grains/cm^2. In addition, no reduction in mean body weight was found at levels of 50–100 pollen grains/cm^2 and 300–400 pollen grains/cm^2. However, at 600–800 maize 59122 pollen grains/cm^2, the mean adult body weight was lower than that of beetles fed pollen from near-isoline maize plants as larvae. The applicant argued that the difference was < 10 % and was noted only at the highest pollen deposition rate, which is the upper range of reported in-field values, and is therefore unlikely to be biologically relevant.

Because *G. viridula* is taxonomically related to the target pest, the adverse effect on mean adult body weight observed at concentrations of 600–800 maize 59122 pollen grains/cm^2 is not unexpected. It should be noted that the bioactivity of the Cry34Ab1 protein in the thawed pollen, which was used as test substance in the above described Tier 1b study, was not quantified. Therefore, it cannot be ruled out that storage conditions affected the bioactivity of the Cry34Ab1 protein in maize 59122 pollen. Maize 59122 pollen was lyophilised within approximately three months of harvest (pollen was stored frozen (≤ –10 °C) prior to and following lyophilisation). The lyophilised pollen was shipped frozen (on dry ice), and was stored in a freezer maintained at –20 ± 8 °C prior to use. The total length of time the maize 59122 pollen was stored frozen was approximately 12 months.[60] The reported effect on mean adult body weight confirms that the Cry34Ab1 protein in the thawed maize 59122 pollen

[59] Additional information received on 16/01/2012/Request 1.1/pages 11–19/Annex: Székács and Kong (2011).
[60] Additional information received on 02/10/2012/Request 1.1/page 2.

retained some level of bioactivity. ELISA data, provided upon request of the EFSA GMO Panel, confirm that the concentration of Cry34Ab1 protein in the retained subsample of thawed maize 59122 pollen after storage was 160 ng/mg (dry weight)[61], but give no indication of the exact level of bioactivity of the Cry34Ab1 protein in thawed pollen. Therefore, an underestimation of the hazard cannot be excluded.

(2) Upon request of the EFSA GMO Panel, the applicant provided a theoretical exposure assessment, in which pollen deposition rates, protein concentrations and stability in pollen, cropping area, characteristics of the host plant and life history characteristics geographic range, habitat preference and feeding behaviour of non-target chrysomelids, and temporal and spatial overlap of larvae with pollen shed were taken into consideration. Several factors will limit the potential for harm to arise. (i) High pollen concentrations are unlikely to be observed under field conditions. Mean maize pollen densities reported range from 150 to 500 pollen grains/cm^2 in-field and are less than 100 pollen grains/cm^2 in field margins (Wraight et al., 2000; Pleasants et al., 2001; Dively et al., 2004; Kawashima et al., 2004; Lang et al., 2004; Gathmann et al., 2006; Schuppener et al., 2012).[62] (ii) The expression of the Cry35Ab1 protein is very low in pollen (Table 1), which significantly decreases the potential toxicity of maize 59122 pollen compared with other plant tissues in which both *Bt*-proteins are fully expressed.[63] Activity of the Cry34Ab1 is significantly potentiated in the presence of the Cry35Ab1 protein (Herman et al., 2002b). (iii) Non-target (non-pest) chrysomelid species found in maize in Europe are low in abundance due to their preference for other habitats or host plants (Knecht et al., 2010; Meissle et al., 2012). Approximately 70 % of chrysomelids (larvae) in Central Europe are endophagous, rhizophagous or phytosaprophagous on plants other than maize, and will therefore not be exposed to maize 59122 pollen (Huber and Langenbruch, 2008). (iv) Rainfall events or heavy dew may wash pollen from the maize or host plant leaves (Pleasants et al., 2001), or may result in lysis and bursting of the pollen grains (Li et al., 2010). (v) The duration of pollen shed for cultivated maize can be variable, resulting in a different overlap with sensitive larvae occurring in or nearby maize fields (e.g., Oberhauser et al., 2001).

Although the susceptibility of the larvae of most non-target chrysomelid species to the Cry34Ab1/Cry35Ab1 proteins is not known and data on some aspects of exposure, particularly plant–insect phenology, host plant characteristics, pollen consumption and subsequent mortality in field conditions, are rare within Europe, the EFSA GMO Panel concludes that the risk of maize 59122 to non-target (non-pest) chrysomelid species in the field is low due to their low occurrence and abundance in maize fields and because of the low likelihood of encountering harmful amounts of pollen from maize 59122 in and around maize fields. Non-target adult chrysomelids, which may occasionally feed on maize 59122 plants, are not expected to be affected due to the low activity of the Cry34Ab1/Cry35Ab1 proteins on adults. Furthermore, the only protected chrysomelid species (*Macroplea pubipennis*) considered to be at risk across the EU (under Directive 92/43/EEC on conservation of natural habitats and of wild fauna and flora) does not occur in maize fields.

- *Herbivore species from taxa other than the target taxon (non-chrysomelids)*: The applicant provided several lower-tier studies in which the activity spectrum of a mixture of the Cry34Ab1/Cry35Ab1 proteins was assessed on a range of herbivores that either are taxonomically related to the Chrysomelidae family, or that are likely to be exposed to the plant-produced *Bt*-proteins owing to their herbivorous feeding habits. These lower-tier studies, limitations in the experiments and the remaining scientific uncertainties are described in more detail below.

61 Additional information received on 02/10/2012/Request 1.1/page 2/Appendix 2.
62 Additional information received on 16/01/2012/Request 1/pages 18–19/Annex: Székács and Kong (2011).
63 Technical dossier/Section D1/Annex 25: Poletika (2003).

(1) In a Tier 1a study, seven coleopteran storage pests belonging to the families of Bostrichidae, Curculionidae, Dermestidae, Laemophloeidae, Silvanidae and Tenebrionidae, as well as one lepidopteran storage pest, were screened for susceptibility to the Cry34Ab1/Cry35Ab1 proteins.[64] The Cry34Ab1/Cry35Ab1 proteins at 0.9 or 1 % of the diet were tested against larvae of *Rhyzopertha dominica*, lesser grain borer (Coleoptera: Bostrichidae); *Sitophilus oryzae*, rice weevil (Coleoptera: Curculionidae); *Trogoderma variabile*, warehouse beetle (Coleoptera: Dermestidae); *Cryptolestes pusillus*, flat grain beetle (Coleoptera: Laemophloeidae); *Oryzaephilus surinamensis*, sawtoothed grain beetle (Coleoptera: Silvanidae); *Tenebrio molitor*, yellow mealworm and *Tribolium castaneum*, red flour beetle (Coleoptera: Tenebrionidae); and *Sitotroga cerealella*, Angoumois grain moth (Lepidoptera: Gelechiidae). No overall reproducible and statistically significant differences in weight, mortality and/or development time were observed between control and treated coleopteran larvae. No delay in pupation by survivors was observed for the lepidopteran storage pest *S. cerealella*, but larval mortality was significantly higher. In their publication, the authors indicated that significant problems with the *S. cerealella* bioassays occurred, and that no method or diet was judged satisfactory for routine screening of compounds with this insect species. The authors also indicated that the apparent activity of Cry34Ab1/Cry35Ab1 against *S. cerealella* warrants additional investigation to confirm the finding. *S. cerealella* is not considered a pest of field crops but it is primarily associated with stored foods (Oppert et al., 2010).

(2) In another Tier 1a study, three lepidopterans, *Ostrinia nubilalis*, European corn borer (Lepidoptera: Crambidae), *Agrotis ipsilon*, black cutworm, and *Helicoverpa zea*, corn earworm (Lepidoptera: Noctuidae), and the corn leaf aphid *Rhopalosiphum maidis* (Hemiptera: Aphididae) were exposed to artificial diets containing Cry34Ab1/Cry35Ab1 mixtures.[65] Owing to the low replicate numbers and the high variability in mortality, ranging from 0 to 80 % to a single test substance concentration in the experiments with the corn leaf aphid, no conclusions on the potential activity of Cry34Ab1/Cry35Ab1 to aphids can be drawn from this study. Results for the lepidopteran species suggest that their survival was not affected by the maximum dose applied (400 µg Cry34Ab1/Cry35Ab1 cm^{-2}). However, the larvae of the European corn borer exhibited a low level of growth inhibition at high Cry34Ab1/Cry35Ab1 concentrations. The applicant considered that the observed effect on the European corn borer was most likely due to impurities (salt and buffer constituents) in the lyophilised purified *Bt*-protein powders used as test material in the experiment.[66] However, no additional evidence on the purity of the used *Bt*-protein batch was provided by the applicant to substantiate its statement.

(3) In a lower-tier study designed to investigate the potential for synergism between Cry34Ab1/Cry35Ab1 and Cry1F proteins on the European corn borer and SCR, slight growth inhibition was observed in European corn borer larvae exposed to diets containing the Cry34Ab1/Cry35Ab1 proteins at 5 µg/cm^2 each.[67] A single set of concentrations was used for each *Bt*-protein applied alone and in a mixture. The applicant considered that the observed effect on the European corn borer was most likely due to impurities (salt and buffer constituents) in the lyophilised purified *Bt*-protein powders used as test material in the experiment.[68] However, no additional evidence on the purity of the used *Bt*-protein batch was provided by the applicant to substantiate its statement.

64 Additional information received on 08/01/2009/Request 2/page 7.
65 Technical dossier/Section D1/Annex 2: Herman (2000).
66 Additional information received on 11/02/2013/Point 1/pages 2–3.
67 Additional information received on 11/02/2013/Point 1/pages 2–3/Annex: Herman and Storer (2004).
68 Additional information received on 11/02/2013/Point 1/pages 2–3.

(4) Tier 1b studies with the lepidopteran species *D. plexippus*[69], *V. cardui*[70] and *P. rapae*[71] larvae fed maize 59122 pollen showed that these herbivores are not adversely affected by maize 59122 pollen (see Section 6.2.4.1, above). In addition, there was no feeding inhibition or effect on the mortality of European corn borer larvae feeding on maize 59122 leaf tissues.[72]

The applicant also reported on a series of higher-tier studies in which the potential impact of maize 59122 on several phytophagous arthropod species in the United States and EU (Hungary and Spain) was assessed. No negative impact of maize 59122 was observed on field densities of abundantly occurring aphids, lepidopteran pests, plant- and leafhoppers, and thrips.[73] The results of these higher-tier studies confirm the conclusions of the lower-tier studies, and indicate that the Cry34Ab1/Cry35Ab1 proteins have little or no activity on species other than chrysomelids.

The apparent activity of Cry34Ab1/Cry35Ab1 at high concentrations against the lepidopteran species (e.g., *O. nubilalis* and *S. cerealella*) was not expected based on the known spectrum of activity (Coleoptera only) of these binary proteins. The EFSA GMO Panel considers that there are indications of a potential hazard to Lepidoptera owing to cross-order activity at high Cry34Ab1/Cry35Ab1 protein concentrations. However, for those non-target lepidopterans that are pests of maize, some reduction in their densities is not regarded as an environmental concern. Tier 1b studies with the non-target lepidopteran species *D. plexippus*, *V. cardui* and *P. rapae* larvae fed maize 59122 pollen have shown that these herbivores are not adversely affected by maize 59122 pollen. Moreover, a theoretical exposure assessment indicated that the amount of pollen from maize 59122 found in and around maize fields is unlikely to adversely affect a significant proportion of non-target lepidopteran larvae. Based on the submitted toxicity data and a theoretical exposure assessment, no risk to non-target Lepidoptera is expected from exposure to maize 59122 pollen in the field.

Natural enemies (predators and parasitoids): Predators and parasitoids are likely to be exposed to plant-produced *Bt*-proteins when feeding on phytophagous arthropods that contain *Bt*-proteins or *Bt*-containing maize plant material. Several species of spiders, ground beetles, ladybirds, rove beetles, predatory bugs, and larvae of *Chrysoperla* spp. are known to be predators of herbivores (including pest insects) found in maize. Furthermore, parasitic wasps attack a variety of herbivores occurring in maize ecosystems. In addition, many predators are facultative herbivores feeding on pollen, nectar and plant juices, while parasitoids primarily feed from (extra-)floral nectaries. Adults of the green lacewing *Chrysoperla carnea* (Neuroptera: Chrysopidae) are not predacious, but are prevalent pollen consumers in maize fields. Despite the broader feeding habits of some species, the species for which the primary valued function is pest regulation are addressed below.

The applicant conducted lower-tier studies with the following predatory coleopterans *Poecilus cupreus*, *Hippodamia convergens*, *C. maculata* and *C. septempunctata*; the neuropteran *C. carnea*; and the hemipteran *Orius lavigatus*. In addition, the applicant provided a lower-tier study with the hymenopteran parasitic wasp *N. vitripennis*. These lower-tier studies, limitations in the experiments and the remaining scientific uncertainties are described in more detail below.

- *Coleoptera: Carabidae (ground beetles)*: Meissle et al. (2012) reported that *P. cupreus* is one of the most collected carabid species in maize fields in Europe, is likely to be exposed to the plant-produced Cry34Ab1/Cry35Ab1 proteins through prey it consumes, and is amenable to testing. In a Tier 1a study, larvae of the focal species *P. cupreus* were fed blowfly (*Calliphora vomitoria*) pupae injected with a purified protein solution containing Cry34Ab1/Cry35Ab1 at concentrations of 1000 ng Cry34Ab1 mg^{-1} and 333 ng Cry35Ab1 mg^{-1}.[74] This dose represents 10 × the average

[69] Additional information received on 16/01/2012/Annex: Sears and Rempel (2003).
[70] Additional information received on 08/01/2009/Request 2/pages 5–7.
[71] Additional information received on 08/01/2009/Request 2/pages 5–7.
[72] Additional information received on 11/02/2013/Point 1/pages 2–3/Annex: Pascual (2005).
[73] Additional information received on 20/12/2007/Annexes 1–3: Higgins and Hong (2007)//27/01/2010/Annexes 8–9: Pascual and Hong (2009)//16/01/2012/Annexes: Higgins and Hong (2008a,b), Higgins et al. (2009) and Pascual (2010).
[74] Technical dossier/Section D1/page 20/Annex 22: Vinall (2005).

expression level in maize 59122 leaves at time of harvest. Survival of the ground beetle larvae was monitored throughout pupation and adult emergence. There was no statistically significant difference in preimaginal mortality, mean development time, or mean adult weight of beetles fed Cry34Ab1/Cry35Ab1 proteins, relative to the negative controls. Although the Cry34Ab1/Cry35Ab1 concentrations in thawed blowfly pupae used a food source for *P. cupreus* reduced to 350 ng/mg for Cry34Ab1 and 203 ng/mg for Cry35Ab1, they were shown by the applicant still to exceed the worst-case expected environmental concentrations through: (1) direct feeding on decomposing plant tissue; or (2) exposure to soil-bound protein entering the soil via root sloughing or plant tissue decomposition.[75] Like many other ground beetles, *P. cupreus* is a generalist predator that occasionally consumes vegetal matter.

(1) The worst-case expected environmental concentration for ground beetles exposed to Cry34Ab1/Cry35Ab1 through direct feeding on decomposing plant tissue from maize 59122 was set by the applicant as 100 % of the R6 whole plant tissue concentration, i.e., 200 ng Cry34Ab1 mg^{-1} and 47.5 ng Cry35Ab1 mg^{-1} (dry weight).

(2) For ground beetles exposed to soil-bound protein entering the soil via root sloughing or plant tissue decomposition the worst-case expected environmental concentration was calculated to be 20 ng Cry34Ab1 mg^{-1} soil and 4.75 ng Cry35Ab1 mg^{-1} soil.

The EFSA GMO Panel notes that the above-calculated worst-case expected environmental concentrations are very conservative, as they assume that there is no degradation of the Cry34Ab1/Cry35Ab1 proteins in plant tissues or in soil, and that the proteins are 100 % bioavailable, which is unlikely under environmental conditions. Based on the submitted toxicity data and estimated worst-case expected environmental concentrations, no hazard to *P. cupreus* and no risk to carabids are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122 in the field.

- *Coleoptera: Coccinellidae (ladybirds)*: The lower-tier study on the surrogate species *H. convergens* did not reveal adverse effects on survival and behaviour (e.g., signs of lethargy or immobility) of adult beetles at a concentration of 280 µg Cry34Ab1/Cry35Ab1 mL^{-1}, representing 10 × the expected environmental concentration in maize 59122 pollen, after 11 days of exposure.[76]

Further data on coccinellids have been provided by the applicant. Two lower-tier studies were conducted with the surrogate species *C. maculata*.

(1) In a seven-day Tier 1a study, larvae were exposed to diets containing purified Cry34Ab1/Cry35Ab1 proteins mixed with artificial diet at 10 × the concentration of the expected field maize 59122 pollen concentration.[77] The Cry34Ab1/Cry35Ab1 protein concentration in maize 59122 pollen is a relevant way of estimating realistic exposure for *C. maculata*, as both larvae and adults commonly feed on pollen in addition to being predacious, and exposure to maize 59122 pollen could be significant during the period of pollen shed. The mixture of Cry341Ab/Cry35Ab1 proteins was administered in a concentration of 901 µg/g of artificial diet. The Cry34Ab1 protein was incorporated at 900 ppm and Cry35Ab1 protein at 1 ppm; this ratio is based on Cry34Ab1 and Cry35Ab1 expression in maize 59122 pollen. The stability of Cry34Ab1/Cry35Ab1 in the artificial diet was not tested. A retrospective power analysis requested by the NL CA demonstrated that this study had sufficient statistical power (80 %) to detect an effect size ranging between 30 % and 50 % or less; the experiment was able to detect a minimum of 25 % increase in mortality at a level of 95 % power and a 25 % decrease in larval weight at a level of 99 %

[75] Additional information received on 02/10/2012/Request 1.5/pages 10–12.
[76] Technical dossier/Section D1/page 19/Annex 20: Bryan et al. (2000).
[77] Technical dossier/Section D1/pages 18–19/Annex 21: Higgins (2003).

power.[78] Because toxins specific to coccinellids are lacking, the applicant indicated that no positive control (toxic/reference) substance could be used in the experiment. The EFSA GMO Panel does not agree with this statement and notes that positive controls including potassium arsenate and the protease inhibitor E-64 could have been used (Álvarez-Alfageme et al., 2012). No adverse effects on survival were observed after seven days of feeding. However, larval weight was reduced significantly in *C. maculata* larvae that fed on the Cry34Ab1/Cry35Ab1-containing diet compared with larvae fed control diets, pointing to a significant growth inhibition (80 % growth reduction). The EFSA GMO Panel notes that a reduction in larval weight can have a pronounced effect on the life expectation and reproduction ability of insects, and thus on population dynamics. Because the *Bt*-proteins (active or heat-inactivated) were dissolved in de-ionised water, the observed effects cannot be attributed to impurities in the *Bt*-formulations.

(2) In line with the tiered approach, the findings from the Tier 1a study triggered further testing under more realistic conditions of exposure (Romeis et al., 2008a). In a Tier 1b study, *C. maculata* larvae were fed diets containing a 1:1 weight mixture of corn earworm eggs and maize 59122 pollen.[79] A retrospective power analysis requested by the NL CA demonstrated that this study had sufficient statistical power (80 %) to detect an effect size ranging between 30 % and 50 % or less; the experiment was able to detect a minimum of a 30 % increase in mortality at a level of 89 % power, and a 25 % decrease in adult weight at a level of 99 % power.[80] The bioactivity of Cry34Ab1 in maize 59122 pollen was not quantified. Following a request from the Belgian Biosafety Advisory Council (in the frame of the initial environmental risk assessment evaluation of application EFSA-GMO-UK-2006-30), the applicant clarified that the maize pollen–egg mixture was ground completely to avoid selective feeding on lepidopteran eggs, and to ensure sufficient exposure and ingestion of maize 59122 pollen. No adverse effects were observed on mortality, development, or adult weight of *C. maculata* larvae fed a diet consisting of 50 % maize 59122 pollen and 50 % ground corn earworm eggs at 1.5 × the expected exposure rate of inbred pollen (see Section 6.2.4.1, above).

Submitted data show that Cry34Ab1/Cry35Ab1 proteins may be toxic to *C. maculata* at dose levels that exceed field exposure (see Tier 1a study, above). However, as adverse effects were not seen at field dose levels (see Tier 1b study, above) when *C. maculata* larvae were fed a mixture of natural prey and pollen, the EFSA GMO Panel considers that there is reasonable certainty that maize 59122 will not adversely affect *C. maculata* (see also US EPA, 2010b).

The EFSA GMO Panel noted the possible sublethal effects on the surrogate species *C. maculata* at Cry34Ab1/Cry35Ab1 dose levels that exceed field exposure, as well as limitations in the experiments. To resolve the remaining scientific uncertainties, the EFSA GMO Panel and other risk assessment bodies (e.g., the Dutch Commission on Genetic Modification and the Belgian Biosafety Advisory Council) requested additional data on a representative European coccinellid species. In response, the applicant provided two types of lower-tier studies (including tritrophic experiments) with the focal species *C. septempunctata*. In addition, the applicant determined the worst-case expected environmental concentration for mite-eating predatory coccinellids such as the focal species *Stethorus punctillum*.

(1) In a Tier 1b study, larvae of *C. septempunctata* were fed a diet containing maize 59122 pollen and moth eggs at a 1:3 ratio by weight (discussed above, under Section 6.2.4.1).[81] The applicant provided evidence to confirm that the maize pollen–egg mixture was ground completely to avoid selective feeding on lepidopteran eggs, and to ensure sufficient exposure

[78] Additional information received on 20/12/2007/Request 1/pages 1–4.
[79] Additional information received on 16/01/2012/Annex: Higgins (2003).
[80] Additional information received on 20/12/2007/Request 1/pages 1–4.
[81] Additional information received on 27/01/2010/Request 1/pages 1–3/Annex 2: Califf and Ostrem (2009)/Annex 3: Hong (2009).

and ingestion of maize 59122 pollen.[82] The bioactivity of the Cry34Ab1 protein in the maize 59122 pollen was not quantified. No statistically significant differences were identified in any of the lifecycle parameters (such as growth and development, as measured by days to adult emergence, adult weight or mortality) recorded for *C. septempunctata* when fed a diet containing maize 59122 pollen.

(2) In tritrophic studies, *C. septempunctata* larvae were fed among others a diet consisting of maize 59122 pollen only, or a mixture of maize 59122 pollen and aphids (*R. padi*) reared on maize 59122.[83] Results from these tritrophic studies indicated no statistically significant differences between larval development, adult weight and survival of *C. septempunctata* between the pollen only, pollen–aphid, and respective non-*Bt*-control treatments (Takács et al., 2010). Maize 59122 pollen and aphids that have been feeding on maize 59122 constitute relevant routes of exposure, as coccinellids such as *C. septempunctata* largely feed on aphids and occasionally on maize pollen. Although *C. septempunctata* larvae and adults largely feed on aphids, exposure of *C. septempunctata* larvae to Cry34Ab1/Cry35Ab1 via aphids reared on maize 59122 is negligible, because only low concentrations of the Cry34Ab1 protein (Cry34Ab1 is the protein responsible for the majority of the insecticidal properties of maize 59122 and is more likely to pass to the phloem owing to its three-fold smaller molecular weight compared with Cry35Ab1) were detected in aphids feeding on maize 59122 (less than 1 ng/mg). The Cry34Ab1 protein content in aphids feeding on maize 59122 was approximately 21 ng/mL (equivalent to 0.13 ng/mg (fresh weight)). Adjusting for the worst-case extraction efficiency observed (19 %) in aphids, the Cry34Ab1 protein concentration in aphids collected from maize 59122 was approximately 0.68 ng/mg, based on fresh weight calculations. This value is approximately 118 × less than the Cry34Ab1 protein content in maize 59122 leaves on which the aphids fed (approximately 80 ng/mg (fresh weight)).[84] In these tritrophic studies, maize 59122 pollen is therefore to be considered the main realistic route of exposure to Cry34Ab1, as maize 59122 pollen contains 73 ng Cry34Ab1 mg^{-1} (dry weight) and coccinellids such as *C. septempunctata* occasionally feed on maize pollen. The bioactivity of Cry34Ab1 in the maize 59122 pollen was not quantified.

In another tritrophic study in which aphids reared on maize 59122 were used as the sole food source for *C. septempunctata*, no significant differences were observed during the test period in either fecundity or fertility of *C. septempunctata* across the *Bt*- and non-*Bt*-based treatments (Takács et al., 2012). Aphids reared on maize 59122 were the sole food source for *C. septempunctata*, alhough they do not represent a significant route of exposure to Cry34Ab1/Cry35Ab1. Because exposure to Cry34Ab1 via aphids is negligible, the study does not allow any conclusions to be drawn about the potential adverse effects of Cry34Ab1/Cry35Ab1 on the fecundity or fertility of *C. septempunctata*.

(3) In contrast to aphids, spider mites reared on maize 59122 are expected to constitute worst-case exposure. Spider mites have been shown to contain *Bt*-protein concentrations that are similar to those measured in the leaves on which they have fed (Romeis et al., 2011, and references therein). Furthermore, bioassays demonstrated that *Bt*-proteins contained in spider mites after feeding on *Bt*-maize retain their biological activity (Romeis et al., 2011, and references therein). The worst-case expected environmental concentration for mite-eating predatory coccinellids such as the focal species *S. punctillum* is 74 ng/mg fresh weight spider mite. The applicant demonstrated that the two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae), fed maize 59122 for two days had Cry34Ab1/Cry35Ab1 concentrations below the limit of detection of the assay for spider mites (i.e., 74 ng/mg fresh weight). The maize 59122 leaves on which the spider mites fed had *Bt*-protein concentrations of 43 ng/mg for Cry34Ab1 and 28 ng/mg for Cry35Ab1. The

[82] Additional information received on 11/02/2013/Point 4/pages 7–9/Figure 1/Annex: Boeckman (2012).
[83] Additional information received on 27/01/2010/Request 2/page 4/Annex 5: Hungarian Academy of Sciences Plant Protection Institute (undated).
[84] Additional information received on 27/01/2010/Request 2/pages 3–4/Annex 4: Anderson et al. (undated).

Cry34Ab1/Cry35Ab1 proteins in the spider mites were not observed at concentrations 1.7 × higher than those found in maize 59122 leaves.[85] These data suggest little likelihood of bioaccumulation of the Cry34Ab1/Cry35Ab1 proteins in spider mites feeding on maize 59122, but confirm that spider mites would constitute a relevant route of exposure to certain predators.

Based on the submitted toxicity data and estimated worst-case expected environmental concentrations, no hazard to *C. septempunctata* and no risk to coccinellids are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122.

- *Diptera: Syrphidae (hoverflies)*: The Belgian Biosafety Advisory Council (in the frame of the initial environmental risk assessment evaluation of application EFSA-GMO-UK-2006-30) requested a toxicity study on a dipteran species, as there is some evolutionary relatedness between the larger Cry35Ab1 protein (44 kDa) and the 42 kDa and 51 kDa dipteran-active toxins from *Lysinibacillus sphaericus* strain 2362 (Baumann et al., 1988; Charles et al., 1997; US EPA, 2010b). Cry35Ab1 has 26 % and 29 % sequence similarity (amino acid level) with the *L. sphaericus* binA (42 kDa) and binB (51 kDa) polypeptides, respectively, which are non-related to most other three-domain-like Cry proteins. Bioinformatic analyses performed by the applicant confirmed that Cry35Ab1 shows 23–24 % identity and 42–43 % similarity to the P42 (BinA) component from *L. sphaericus*.[86] The Bin-toxins are placed in a *Bt*-toxin family consisting of the *L. sphaericus* Bin-toxins, a Cry49 also from *L. sphaericus* and a putative protein from *Chlorobium phaeobacteriodes*. The *L. sphaericus* proteins are active against certain groups of mosquitoes. The Bin-toxins are also found as crystals in the bacterium.

  Hoverflies are the most collected predatory Diptera in maize in Europe, with the main species *Episyrphus balteatus* and *Eupeodes corollae* (Meissle et al., 2012). Although there is limited sequence similarity between Cry35Ab1 and dipteran-active binary (Bin) toxins from *L. sphaericus*, it is unlikely that: (1) a hazard will be realised; and (2) a risk to syrphids will arise.

  (1) *Hazard*: In most cases for which cross-order activity has been shown, toxicity outside the protein's primary target range was orders of magnitude below its toxicity inside that range (van Frankenhuyzen, 2008). Although the authors did not report results in their publication, Ellis et al. (2002) indicated that mosquito screening assays of the type used to identify mosquitocidal *L. sphaericus* and *B. thuringiensis* strains did not identify a similar level of activity for the *B. thuringiensis* binary Cry34Ab1/Cry35Ab1 proteins. Moreover, Cry34Ab1 is the protein responsible for the majority of the insecticidal properties of maize 59122, and both proteins are needed to attain full membrane permeabilisation and insecticidal activity (Ellis et al., 2002; Masson et al., 2004). It is unlikely that Cry35Ab1 is active against dipterans, as the conditions in the gut of dipterans and coleopterans differ substantially (acidic in copleopterans and alkalic in dipterans). The different pH conditions in the gut might influence the tertiary structure of the Cry35Ab1 protein and thereby the binding to midgut receptors in Diptera. In addition, the activity is dependent on specific receptors; analyses indicate that a few sequence differences may be responsible for the specificity of the Bin-toxins.

  Although laboratory methods to assess toxicity of plant protection products to syrphids exist and *E. balteatus* is commercially supplied, no lower-tier study with *E. balteatus* was provided by the applicant. Instead, the applicant performed a 48-hour lower-tier study with the southern house mosquito, *Culex quinquefasciatus* (Diptera: Culicidae).[87] A prospective power analysis indicated that 48 larvae per treatment were sufficient to detect a 20 % mortality increase with a power of at least 80 %. No statistically significant difference in

[85] Additional information received on 08/01/2009/Request 1/pages 2–3.
[86] Additional information received on 11/02/2013/Point 2/page 4/Annex: Krauss (2011).
[87] Additional information received on 11/02/2013/Point 2/page 4/Annex: Fisher et al. (2012).

mortality of *C. quinquefasciatus* larvae exposed to 82 µg Cry34Ab1/mL and 2.5 µg Cry35Ab1/mL (equivalent to the concentration of Cry34Ab1/Cry35Ab1 proteins in maize 59122 pollen) were observed compared with the control treatment. There was 100 % mortality among larvae treated with spheratax, the positive control (toxic/reference) substance, which indicates that the larvae were adequately exposed to the treatments. This study confirms that the Cry34Ab1/Cry35Ab1 proteins are not active against the surrogate dipteran species, *C. quinquefasciatus*, at environmentally relevant concentrations.

(2) *Exposure*: Predatory hoverfly larvae are mostly aphidophagous, and the Cry34Ab1 protein content in aphids feeding on maize 59122 was shown to be negligible (less than 1 ng/mg fresh weight). In addition, adults are pollen and nectar feeders. Evidence provided by the applicant demonstrated that the Cry35Ab1 protein is hardly expressed in maize 59122 pollen. Therefore, exposure to the plant-produced Cry35Ab1 protein of these predatory syrphids is negligible.

Despite the limited sequence similarity between Cry35Ab1 and dipteran-active Bin-toxins from *L. sphaericus*, no hazard to *C. quinquefasciatus* and no risk to syrphids are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 and maize 59122.

- *Hemiptera: Anthocoridae (true bugs)*: The EFSA GMO Panel and other risk assessment bodies (i.e., US EPA (2010b) and the Belgian Biosafety Advisory Council (in the frame of the intial environmental risk assessment evaluation of application EFSA-GMO-UK-2006-30)) requested an additional lower-tier study on a predatory heteropteran species such as bugs of the genus *Orius*. *Orius* spp. are commonly found in maize and are considered important predators that feed on life stage of several pests. Additionally, *Orius* spp. can feed on maize pollen, sap and honeydew (Meissle et al., 2012). In response, the applicant provided Tier 1a data with *O. laevigatus*.[88] *O. laevigatus* nymphs were fed a diet medium containing Cry34Ab1/Cry35Ab1 at two different concentrations. Cry34Ab1/Cry35Ab1 proteins were mixed with an artificial, cooked, meat-based diet at rates of either 82 µg Cry34Ab1 g^{-1} + 2.5 µg Cry35Ab1 g^{-1} diet (dosing solution 1), to represent the average highest measured concentrations of the proteins in maize 59122 pollen, or 820 µg Cry34Ab1 g^{-1} + 25 µg Cry35Ab1 g^{-1} diet (dosing solution 2), to represent 10 × the average highest measured concentrations of the proteins in maize 59122 pollen. No adverse effects on survival of *O. laevigatus* nymphs were observed at treatments rates up to and including 820 µg Cry34Ab1 g^{-1} + 25 µg Cry35Ab1 g^{-1} diet. The time required for *O. laevigatus* nymphs fed the 820 µg Cry34Ab1 g^{-1} + 25 µg Cry35Ab1 g^{-1} diet to develop into adults was marginally shorter (15–19 hours) than that of those fed the non-*Bt*-diet. The EFSA GMO Panel considers this statistically significant difference in development rate as not biologically relevant, because the development time of nymphs is variable, even when the same food source is used. The observed difference in development rate is most likely owing to small differences in the age of nymphs across the test groups. In the experiment, four- to five-day-old nymphs (probably late second instars) of *O. laevigatus* were used at the start of the experiment. These nymphs may have shown small differences in age and nutritional status, which in turn affected their development. No statistical significant difference in development rate was observed between the *Bt*- and non-*Bt*-treatment for the dosing solution 1. There was 75 % mortality among nymphs treated with teflubenzuron, the positive control (toxic/reference) substance, which indicates that the nymphs were adequately exposed to the treatments. Based on the submitted toxicity data, no risk to *O. laevigatus* is expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122 under field conditions.

- *Hymenoptera: Pteromalidae (parasitic wasps)*: Survival and behaviour (e.g., signs of lethargy or immobility) of the surrogate species *N. vitripennis*, a parasitoid of Diptera, were not adversely affected when exposed to a diet containing 280 µg Cry34Ab1/Cry35Ab1 mL^{-1} (combined total of 160 ppm Cry34Ab1 and 120 ppm Cry35Ab1), which is approximately equivalent to 10 × the

[88] Additional information received on 16/01/2012/Request 2/pages 20–27/Annex: Vinall (2011).

maximum protein concentration present in maize 59122 pollen, for 12 days.[89] A retrospective power analysis requested by the NL CA demonstrated that this study had sufficient statistical power (80 %) to detect an effect size ranging between 30 % and 50 %, or less; the experiment was able to detect a minimum of 30 % increase in mortality at a level of 98 % power.[90] Parasitic Hymenoptera do not feed directly on maize plant tissues, including pollen; therefore, minimal exposure of parasitic wasp to plant-produced *Bt*-proteins is expected. In addition, *N. vitripennis* is not related to maize ecosystems, but the applicant has used this species as a surrogate species for hymenopteran parasitoids. The use of more appropriate species that are abundant in maize (Meissle et al., 2012), or at least in cropping systems, like certain ichneumonids, braconids, mymarids or scelionids, has been suggested (Rose, 2007; US EPA, 2010b; Albajes et al., 2012). Based on the submitted toxicity data, no hazard to *N. vitripennis* is expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins.

- *Neuroptera: Chrysopidae (net-winged insects)*: Lacewings have been collected frequently from maize fields in Europe, with the main species *C. carnea* (Meissle et al., 2012). In a lower-tier study, green lacewing larvae were fed a diet containing Cry34Ab1/Cry35Ab1 at a rate of 280 µg/mL (combined total of 160 ppm Cry34Ab1 and 120 ppm Cry35Ab1), which is approximately equivalent to 10 × the maximum protein concentration in plant tissue, for ten days.[91] Compared with the negative control, at day 10, there was no significant increase in green lacewing larval mortality. The control group mortality (28 %) exceeded the allowable rate specified in the test protocol for plant protection products of less than 20 % control mortality, indicating that the test method was not sufficiently robust to provide reliable results (Romeis et al.., 2011). It is also questionable whether green lacewings ingested the Cry34Ab1/Cry35Ab1 proteins coated moth eggs. On account of the feeding mode of *C. carnea* larvae, which have piercing–sucking mouthparts and therefore do not consume the external surface of eggs, exposure to Cry34Ab1/Cry35Ab1 when feeding on the treated lepidopteran eggs is likely to be low. In addition, no positive control (toxic/reference) substance was used to determine whether or not the test substance was actually ingested. Because of the highlighted limitations in the experiment, the study does not allow any conclusions to be drawn about potential adverse effects of Cry34Ab1/Cry35Ab1 proteins on *C. carnea*. Nonetheless, it is not expected that *C. carnea* will be adversely affected by maize 59122, as it is unlikely that they will be exposed to elevated levels of plant-produced Cry34Ab1/Cry35Ab1 proteins in the field. To resolve the remaining scientific uncertainties described above, the EFSA GMO Panel and other risk assessment bodies (such as US EPA (2010b) and the Belgian Biosafety Advisory Council (in the frame of the intial environmental risk assessment evaluation of application EFSA-GMO-UK-2006-30)) recommended the applicant to perform an additional lower-tier study with a predatory heteropteran species such as bugs of the genus *Orius* (see above).

The applicant also reported on a series of higher-tier studies in which the potential impact of maize 59122 on several predatory arthropod species in the United States[92] and EU (Hungary and Spain)[93] was assessed. No negative impact of maize 59122 was observed on field densities of abundantly occurring coleopteran species including coccinellids, and staphylinids; hemipteran nabids and *Orius* species; and neuropteran *Chrysopa* species.

In addition, based on a field study performed in Hungary with maize 59122, Balog et al. (2011) reported that the overall assemblage of rove beetles is not significantly affected by plant-produced Cry34Ab1/Cry35Ab1 through their diet. However, the EFSA GMO Panel considers that no conclusions about potential adverse effects of maize 59122 and the Cry34Ab1/Cry35Ab1 proteins it expresses on non-target organisms and the ecosystem services they provide can be drawn from the

89 Technical dossier/Section D1/page 20/Annex 24: Porch and Krueger (2001).
90 Additional information received on 20/12/2007/Request 1/pages 1–4.
91 Technical dossier/Section D1/page 20/Annex 23: Sindermann et al. (2001).
92 Additional information received on 16/01/2012/Annex: Higgins and Dively (2006) and Higgins et al. (2009).
93 Additional information received on 20/12/2007/Annexes 1–3: Higgins and Hong (2007)//27/01/2010/Annexes 8–9: Pascual and Hong (2009)//16/01/2012/Annexes: Higgins and Hong (2008a,b) and Pascual (2010).

Balog et al. (2011) publication, owing to the limitations in the experiment. The results of higher-tier studies confirm the conclusions of the lower-tier studies, indicating that the Cry34Ab1/Cry35Ab1 proteins have little or no activity on species other than chrysomelids.

Pollinators: Honeybees can be exposed to plant-produced *Bt*-proteins, as they collect, store and consume maize pollen, mainly when alternative pollen sources are scarce. In most cases, however, the proportion of maize pollen as a total of all pollen collected and fed to larvae during summer will be low. It is therefore unlikely that maize pollen would regularly comprise more than 50 % of the honeybee diet.

A Tier 1a study in which five-day-old honeybee larvae were fed diets containing 5.6 µg Cry34Ab1/Cry35Ab1 (representing 100 × the protein concentration in 2 mg maize 59122 pollen) did not reveal adverse effects on larval survival or development.[94] There was 92.5 % mortality among larvae treated with potassium arsenate, the positive control (toxic/reference) substance. Exposing honeybee larvae to 2 mg maize 59122 pollen (representing exposure to 0.056 µg Cry34Ab1/Cry35Ab1) did not affect development and survival (see Section 6.2.4.1). The exact level of bioactivity of the Cry34Ab1/Cry35Ab1 proteins in the pollen used in the Tier 1b study remains unclear, as no data on the bioactivity of the Cry34Ab1/Cry35Ab1 proteins in thawed pollen were provided. However, based on the submitted toxicity data, no hazard to *A. mellifera* is expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122 pollen.

Decomposers: Decomposers can be exposed to plant-produced *Bt*-proteins remaining in plant residues, dead arthropod bodies, or faeces. Decomposers (including soil-inhabiting ones) in maize in Europe are dominated by springtails (Collembola) and soil mites (Acarina), followed by flies and midges (Diptera), and beetles (Coleoptera) (Meissle et al., 2012). Data on springtails and mites are addressed in the Section on non-target soil arthropods.

- *Coleoptera (beetles)*: Based on the submitted toxicity data for the coleopterans *C. septempunctata* and *P. cupreus*, as well as the estimated worst-case expected environmental concentrations through direct feeding on decomposing plant tissue, or exposure to soil-bound protein entering the soil via root sloughing or plant tissue decomposition (see above), no risk to Coleoptera is expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122 in the field.

- *Diptera (flies and midges)*: Saprophytic dipteran larvae in the soil can be exposed to plant-produced Cry34Ab1/Cry35Ab1 proteins via senescent maize 59122 plant material. Despite the limited sequence similarity between Cry35Ab1 and dipteran-active Bin-toxins from *L. sphaericus*, no hazard to *C. quinquefasciatus* and no risk to saprophytic Diptera are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 and maize 59122 (see also scientific rationale followed for predatory hoverflies, above).

Conclusion on effects on non-target terrestrial (plant- and ground-dwelling) arthropods: The EFSA GMO Panel is not aware of identified significant adverse effects of the Cry34Ab1/Cry35Ab1 proteins on non-target terrestrial arthropods. The available data show that the expression of the Cry34Ab1/Cry35Ab1 proteins in maize 59122 has no toxic effect on non-target terrestrial (plant- and ground-dwelling) arthropods outside the coleopteran family of Chrysomelidae, and that the insecticidal activity of the Cry34Ab1/Cry35Ab1 proteins is mostly limited to arthropod species of the Chrysomelidae family (leaf beetles). The risk of maize 59122 to non-target (non-pest) chrysomelid species in the field is low due to their low occurrence and abundance in maize fields and because of the low likelihood of encountering harmful amounts of pollen from maize 59122 in and around maize fields. Non-target adult chrysomelids, which may occasionally feed on maize 59122 plants, are not expected to be affected due to the low activity of the Cry34Ab1/Cry35Ab1 proteins on adults. Furthermore, the only protected chrysomelid species (*M. pubipennis*) considered to be at risk across

[94] Technical dossier/Section D.1/Annex 26: Maggi (2001).

the EU (under Directive 92/43/EEC on conservation of natural habitats and of wild fauna and flora) does not occur in maize fields.

The apparent activity of Cry34Ab1/Cry35Ab1 at high concentrations against the lepidopteran species (e.g., *O. nubilalis* and *S. cerealella*) was not expected based on the known spectrum of activity (Coleoptera only) of these binary proteins. The EFSA GMO Panel considers that there are indications of a potential hazard to Lepidoptera owing to cross-order activity at high Cry34Ab1/Cry35Ab1 protein concentrations. However, based on the submitted toxicity data and a theoretical exposure assessment, no risk to non-target Lepidoptera is expected from exposure to maize 59122 pollen in the field.

Despite the limited sequence similarity between Cry35Ab1 and dipteran-active Bin-toxins from *L. sphaericus*, no hazard to *C. quinquefasciatus* and no risk to Diptera are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 and maize 59122.

The EFSA GMO Panel concludes that there is no evidence to indicate that the cultivation of maize 59122 is likely to cause adverse effects on non-target terrestrial arthropods due to the expression of the Cry34Ab1/Cry35Ab1 proteins.

In its evaluation, the NL CA took into account that "*no adverse effects on C. maculata in field tests were observed and also considered the fact that ladybird beetles do not feed directly on the plant, but on aphids which are usually not present in the maize ecosystem*". The NL CA concluded that "*adverse effects on ladybird beetles are unlikely*", but expressed "*reservations about the conclusion of the applicant that no negative effects were found in the laboratory study on C. maculata*". Therefore, the NL CA was of the opinion that "*the applicant should pay extra attention to the monitoring of C. maculata in 59122 maize*" (Section 6.5 of the environmental risk assessment report of the NL CA).

The EFSA GMO Panel agrees that the submitted data show that Cry34Ab1/Cry35Ab1 proteins may be toxic to *C. maculata* at dose levels that exceed field exposure. However, adverse effects were not seen at field dose levels when *C. maculata* larvae were fed a mixture of natural prey and pollen. Because *C. maculata* is not indigenous to Europe, the EFSA GMO Panel requested additional data on a representative European coccinellid species. In response, the applicant provided lower-tier studies (including tritrophic experiments) with the focal species *C. septempunctata*. Based on the additional toxicity data and estimated worst-case expected environmental concentrations, no hazard to *C. septempunctata* and no risk to coccinellids are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122.

Effects on non-target soil arthropods

Non-target arthropods occurring in the soil ecosystem can be exposed to plant-produced *Bt*-proteins introduced into the soil via physical damage to plant tissues, via decomposition of shed root cells during plant growth, via decomposing plant residues remaining in fields after harvest, which might be incorporated into the soil during tillage operations, and possibly via root exudates (reviewed by Icoz et al., 2008). Collembolans and Acarina are important in the breakdown and recycling of crop residues, and are key indicator species of soil functionality and quality. As these micro-arthropods can be exposed to the Cry34Ab1/Cry35Ab1 protein in the maize 59122 field environment, they and the ecosystem services they provide could potentially be adversely affected by the cultivation of maize 59122 and therefore require assessment.

The applicant provided two lower-tier studies with the focal species *F. candida*. *F. candida* is a common and widespread collembolan, and has been used as a standard test organism for more than 40 years to estimate the effects of environmental pollutants and GM plants on non-target soil organisms. *F. candida* was selected by the applicant as a representative of valued non-target soil arthropod species. Collembolans are key decomposers in the soil and also serve as food for polyphagous predators.

- *Tier 1a*: Juvenile Collembola were fed diets consisting of purified Cry34Ab1/Cry35Ab1 proteins mixed with dry granulated brewer's yeast at a single treatment level of 12.7 mg Cry34Ab1/Cry35Ab1 kg^{-1}.[95] Fresh diet was provided to test organisms every third day. On days 0 and 28, mortality and observations of sublethal effects on surviving individuals were recorded. No adverse effects on the survival and reproduction of *F. candida* exposed to Cry34Ab1/Cry35Ab1 proteins at 10 × concentrations found in senescent maize 59122 plant tissue were observed. The primary route of collembolan exposure to Cry34Ab1/Cry35Ab1 proteins in the field is from decaying root tissue, in which the Cry34Ab1/Cry35Ab1 proteins are expressed at a range of 3–66 μg/g. The stability and bioactivity of the test substance were confirmed for the study period. During the test no proof was given that the test insects actually ingested the *Bt*-proteins when mixed with brewer's yeast, but a 28-day reference test with a diet including thiodicarb was performed initially to demonstrate that the study design is able to detect toxic effects.

- *Tier 1b*: A 28-day study was conducted to determine the chronic effects of whole maize 59122 plant material on survival and reproduction of *F. candida*.[96] The test diet consisted of a homogeneous mixture of dry yeast (95.8 %) and lyophilised maize (4.2 %). Maize 59122 plant material had no adverse effect on survival and reproduction of *F. candida* compared with those exposed to the control diets (yeast only or 4.2 % non-*Bt*-maize with yeast). The no observed effect concentration (NOEC) for this study was empirically estimated by the applicant to be > 4.2 % maize 59122, which represents 10 × the estimated amount of whole plant material in maize field soil. Because the test diet mostly consisted of yeast, this experiment does not represent worst-case exposure (Clark and Coats, 2006). In addition, even if the mixture was finely ground, *F. candida* may have fed selectively on the yeast, which is a nutritionally superior food source for collembolans compared with lyophilised plant material. There was 17 %, 40 % and 77 % mortality among juveniles treated with thiodicarb, the positive control (toxic/reference) substance, at concentrations of 1.0, 10 and 100 mg/kg respectively, which indicates that *F. candida* was adequately exposed to the treatments. However, the toxic reference did not include maize powder, and therefore the exposure to collembolans was not fully equivalent to the test diet with maize 59122.

Based on the submitted toxicity data, no hazard to *F. candida* and no risk to Collembola are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122 plant material.

The applicant also provided a higher-tier study which was performed in the United States during the growing season 2004–2005, and in which the soil litter community was monitored.[97] No negative impact of maize 59122 was observed on field densities of abundantly occurring Collembolla and soil mites. The results of this higher-tier study confirm the conclusions of the lower-tier studies, indicating that the Cry34Ab1/Cry35Ab1 proteins have little or no activity on species other than chrysomelids.

The EFSA GMO Panel concludes that there is no evidence to indicate that the cultivation of maize 59122 is likely to cause adverse effects on non-target soil arthropods such as springtails and mites due to the expression of the Cry34Ab1/Cry35Ab1 proteins.

The NL CA concluded that "*based on the specificity of the Cry proteins, the demonstrated lack of effect on nontarget organisms, the rapid degradation of the proteins in soil and the fact that the proteins are naturally present in the soil environment, it is concluded that 59122 maize has no significant ecological impact on the soil ecosystem*" (Section 6.7 of the environmental risk assessment report of the NL CA).

[95] Technical dossier/Section D.1/Annex 28: Teixeira (2001).
[96] Additional information received on 16/01/2012/Annex: Teixeira (2006b).
[97] Additional information received on 16/01/2012/Annex: Higgins and Dively (2006).

Effects on non-target aquatic arthropods

By-products from GM plants (e.g., pollen, detritus) can be transported in water courses to downstream water bodies where non-target aquatic arthropods can be exposed to transgene product(s) through consumption (Axelsson et al., 2010, 2011). In the case of *Bt*-maize, Rosi-Marshall et al. (2007) reported that by-products of *Bt*-maize enter headwater streams in the United States and claimed, on the basis of experimental data obtained under lower-tier conditions, that this would reduce growth and increase mortality of some non-target aquatic arthropods, especially trichopteran species (see also Chambers et al., 2010; Tank et al., 2010).

A 48-hour lower-tier study was performed on *Daphnia magna*, a freshwater invertebrate.[98] The test material consisted of the purified Cry34Ab1/Cry35Ab1 proteins added to water at a target concentration of 100 mg Cry34Ab1/Cry35Ab1 L^{-1}. No treatment mortality or behavioural changes were reported between the dosed and control replicates during the 48-hour exposure period. Based on the submitted data, no hazard to daphnids is expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 or maize 59122.

Exposure of non-target organisms to *Bt*-proteins in aquatic ecosystems is likely to be very low (Douville et al., 2005, 2007; Wolt and Peterson, 2010; Carstens et al., 2012). Considering the probability of short-term exposure and acute effects to sensitive species, Wolt and Peterson (2010) indicated no concern in 99 % of cases, with limited opportunity for chronic effects, due to the rapid degradation of *Bt*-proteins. Exposure estimates indicated that shredders such as caddisflies (Cummins et al., 1989) are the functional group most likely to be exposed to *Bt*-proteins in aquatic systems (Carstens et al., 2012). 50 % of filtering trichopterans collected by Rosi-Marshall et al. (2007) from water streams during peak pollen shed had maize pollen grains in their guts and detritivorous trichopterans were located in accumulations of decomposing maize litter in the streams after harvest.

In response to a request of the EFSA GMO Panel, the applicant provided a theoretical exposure assessment in line with that reported by Carstens et al. (2012).[99] For non-target aquatic organisms, there are two routes through which they may be exposed to the Cry34Ab1/Cry35Ab1 proteins from 59122 maize plant material: (1) exposure to freely soluble protein (e.g., proteins that leach out of maize plant tissues and are deposited into an adjacent water body); or (2) exposure to proteins via direct feeding on deposited plant material (e.g., aerially deposited pollen, crop dust, or intact plant material) (Carstens et al., 2012). The worst-case expected environmental concentrations calculated for the two exposure routes and presented in the application are described below.

(1) According to the US EPA standard pond model (1-ha pond with 2 m depth draining a 10-ha watershed planted with maize located $\geq$ 1 m from the edge of the maize field), which is highly conservative, the concentration of freely soluble Cry34Ab1/Cry35Ab1 proteins in an aquatic environment is less than 3 mg/L. Under a more realistic scenario, the concentration of freely soluble Cry34Ab1/Cry35Ab1 proteins in an aquatic environment would be much less than 3 ppm and the duration of exposure short, owing to the rapid degradation of any freely soluble protein. In addition, it is unlikely that all of the plant biomass from a 10-ha maize field would be deposited simultaneously in a 1-ha pond.

(2) Particle feeders and shredders could be exposed to *Bt*-proteins in an aquatic environment if particulate organic matter (e.g., pollen, crop dust, decomposed plant material) or intact plant material (senescent or green plant tissue), respectively, is deposited into an adjacent water body. As discussed by Carstens et al. (2012), there is limited data available in the scientific literature to quantify the amount of particulate organic matter or intact plant material that moves from a maize field into water courses, and there are many factors that affect movement (wind, topography, distance, etc.). Nevertheless, based on conservative assumptions, the worst-case expected

[98] Technical dossier/Section D1/Annex 29: Marino and Yaroch (2001).
[99] Additional information received on 02/10/2012/Request 1.4/pages 8–10.

environmental concentrations calculated was 81.6 ng Cry34Ab1 mg^{-1} and 2.49 ng Cry35Ab1 mg^{-1} pollen (dry weight) for particle feeders, and 200 ng Cry34Ab1 mg-1 and 47.5 ng Cry35Ab1 mg senescent plant material (dry weight) for shredders. In both cases, it was assumed that all *Bt*-protein remains bioactive and does not degrade, which is unlikely under realistic environmental conditions. Moreover, several other mitigation factors described by Carstens et al. (2012) would likely decrease the duration and concentration of exposure of particle feeders or shredders to plant-produced Cry34Ab1/Cry35Ab1 proteins in an aquatic environment.

Based on the submitted toxicity data and estimated worst-case expected environmental concentrations, no hazard to *D. magna* and no risk to non-target aquatic arthropods are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122 plant material. No substantial aquatic exposure to the Cry34Ab1/Cry35Ab1 proteins from maize 59122 plant material is expected.

The EFSA GMO Panel concludes that there is no evidence to indicate that the cultivation of maize 59122 is likely to cause adverse effects on non-target aquatic arthropods due to the expression of the Cry34Ab1/Cry35Ab1 proteins.

The conclusion of the EFSA GMO Panel is consistent with the evaluation carried out by the NL CA on maize 59122, which concluded that "*potential effects of maize 59122 on non-target organisms as a result of the genetic modification are considered to be negligible*" (Section 6.5 of the environmental risk assessment report of the NL CA).

Effects on non-target organisms that are not arthropods

The potential of maize 59122 to have direct or indirect adverse effects on non-target organisms that are not arthropods, as well as the ecosystem services they provide is described below, with a focus on earthworms, mammals, birds and fish. Potential adverse effects on soil microorganisms are considered in Section 6.2.6.2, below.

Annelida (earthworms and enchytraeid worms) play an important role in decomposing plant litter, and are responsible for numerous physical changes that affect the biological properties and processes in soil (e.g., structure, quality, functionality). They are considered important organisms in the regulation of nutrient cycling processes (Didden, 1993; Curry and Schmidt, 2006). Annelida can be exposed to *Bt*-proteins, as *Bt*-proteins can enter the soil by root exudates (Saxena et al., 2002, 2004), plant material (Webster et al., 2008), and by plant residues (Stotzky, 2004). If Annelida populations would be adversely affected by the cultivation of GM crops, this may have negative consequences on the ecosystem services they provide.

Two lower-tier studies with the earthworm species *Eisenia fetida* were provided by the applicant.

- *Tier 1a*: *E. fetida* adults exposed to pure Cry34Ab1/Cry35Ab1 proteins gave no indications of adverse impacts to this earthworm species following short-term exposure to high doses of the *Bt*-proteins.[100] The 14-day LC_{50} for earthworms exposed to Cry34Ab1/Cry35Ab1 in an artificial soil substrate was determined to be greater than 76 mg/kg dry soil (the highest concentration tested), or greater than 20 × the expected field concentration. Earthworm mortality and changes in average body weights were not statistically different among the controls and protein-amended soils. The LC_{50} value for earthworms exposed to chloroacetamide, the positive control (toxic/reference) substance, was approximately 19.4 mg/kg dry soil, and indicates that the earthworms were adequately exposed to the treatments. The Cry34Ab1/Cry35Ab1 protein concentration was not monitored throughout the test period; it therefore remains unclear for which period of time the earthworms were exposed to biologically active Cry34Ab1/Cry35Ab1 proteins.

[100] Technical dossier/Section D1/Annex 27: Bryan et al. (2000).

- *Tier 1b*: A 14-day laboratory study was conducted to determine the effects of whole maize 59122 plant material on the survival and weight change of the earthworm *E. fetida*.[101] Earthworms were exposed to nominal concentration of 4.2 % lyophilised maize 59122 plant material, or 4.2 % lyophilised non-*Bt*-maize plant material. The tested concentration in this study (4.2 % maize tissue in soil) is approximately 10 × the worst-case expected environmental concentration. No mortality was observed at the tested concentration during 14-day exposure, and no significant difference was found in mean percentage weight change among earthworms exposed to any of the treatments tested. There was 0, 30 and 100 % mortality among earthworms treated with carbendazim, the positive control (toxic/reference) substance, at concentrations of 2.5, 5 and 10 mg/kg respectively, which indicates that *E. fetida* was adequately exposed to the treatments.

Based on the submitted toxicity data and estimated worst-case expected environmental concentrations, no hazard to *E. fetida* and no risk to Annelida are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122 plant material.

The lack of toxicity of the Cry34Ab1/Cry35Ab1 proteins to birds[102] and mammals[103] was confirmed in toxicity studies, and the nutritional quality of maize 59122 tested with fish[104] in a nutritional equivalence study (Sections 4 and 5, above; EFSA, 2007 for further details).

The EFSA GMO Panel concludes that there is no evidence to indicate that the cultivation of maize 59122 is likely to cause adverse effects on non-target organisms that are not arthropods, owing to the expression of the Cry34Ab1/Cry35Ab1 proteins.

The conclusion of the EFSA GMO Panel is consistent with the evaluation carried out by the NL CA on maize 59122. The NL CA concluded that "*potential effects of maize 59122 on non-target organisms as a result of the genetic modification are considered to be negligible*" (Section 6.5 of the environmental risk assessment report of the NL CA).

6.2.4.3. Adverse effects on non-target organisms due to the expression of the PAT protein

Based on the mode of action of the PAT protein and the history of safe use of maize 59122 and other glufosinate-ammonium tolerant crops, the EFSA GMO Panel concludes it is unlikely that the expression of this protein in glufosinate-ammonium tolerant crops will cause direct adverse effects on non-target organisms (CERA, 2011, and references therein).

The conclusion of the EFSA GMO Panel on the absence of adverse effects of maize 59122 on non-target organisms due to the expression of the PAT protein is consistent with the evaluation carried out by the NL CA on maize 59122. The NL CA considered that "*PAT has high substrate specificity to the active ingredient of glufosinate-ammonium (L-PPT), and such a substrate does not occur within the maize plant or within the animal and human diets. The PAT protein is assessed several times before. Effects of the PAT protein on non-target organisms were considered to be unlikely*" (Section 6.5 of the environmental risk assessment of the NL CA).

6.2.4.4. Adverse effects on non-target organisms due to interactions between the Cry34Ab1, Cry35Ab1 and PAT proteins

The activity of the Cry34Ab1, Cry35Ab1 and PAT proteins expressed in maize 59122 is not likely to be affected by potential interactions among these proteins, as their modes of action differ. The data submitted by the applicant and the review of published literature did not indicate any interactions in the expression of the proteins or their biological activity compared with GM crops expressing similar single proteins.

[101] Additional information received on 16/01/2012/Annex: Teixeira (2006a).
[102] Technical dossier/Section D7.8.1/Annex 15: Delaney and Smith (2004).
[103] Technical dossier/Section D7.8.1/Annexes 11–13: Brooks and DeWildt (2000)/Annex 14: Malley (2004)/Annex 36: Thomas et al. (2006).
[104] Technical dossier/Section D1/Annex 30: Marino and Yaroch (2002).

The conclusions of the EFSA GMO Panel are consistent with those of the NL CA which concluded that "*an interaction between the Cry proteins and the PAT protein is not expected, since the proteins have different mechanisms of action and they are located in different cell components. Potential effects of 59122 maize on non-target organisms as a result of the genetic modification are considered to be negligible*" (Section 6.5 of the environmental risk assessment report of the NL CA).

6.2.5. Effects on human and animal health[105]

The molecular analysis and the food and feed safety assessment of maize 59122 did not raise safety concerns for human and animal health (Sections 3–5, above; EFSA, 2007). In its previous Scientific Opinion on maize 59122 (EFSA, 2007), the EFSA GMO Panel concluded that "*maize 59122 is as safe as its non genetically modified counterparts with respect to potential effects on human and animal health or the environment*", and that "*maize 59122 is unlikely to have any adverse effect on human and animal health or on the environment in the context of its intended uses*".

6.2.6. Interactions with biogeochemical processes and the abiotic environment[106]

The newly expressed proteins in maize 59122 can be introduced into the soil via physical damage to plant tissues, via decomposition of shed root cells during plant growth, via decomposing plant residues remaining in fields after harvest, which might be incorporated into the soil during tillage operations (Stotzky, 2004), and via root exudation (e.g., Saxena et al., 2002, 2004; Icoz and Stotzky, 2007; Icoz et al., 2008), resulting in exposure of non-target soil organisms to the Cry34Ab1/Cry35Ab1 proteins. Indirect exposure through manure and faeces from animals fed maize 59122 was also considered, although most of the Cry34Ab1/Cry35Ab1 proteins would be degraded by enzymatic activity in the intestinal tract and subsequently by microbial processes in the manure.

6.2.6.1. Fate of the Cry34Ab1/Cry35Ab1 proteins in soil

Proteins can be a major source of energy, carbon and nitrogen for soil microorganisms. They are readily degradable by widely abundant extracellular microbial proteases (Jan et al., 2009) and there is no indication that *Bt*-proteins would generally behave differently compared with other proteins (reviewed by Icoz and Stotzky, 2008). The degradation rate of *Bt*-proteins in soil depends upon multiple factors, such as soil management, soil texture, pH value, temperature and humidity.

While the fate of some plant-produced *Bt*-proteins such as Cry1Ab and Cry3Bb1 in soils has been extensively studied and reported in the scientific literature, knowledge on the fate of the Cry34Ab1/Cry35Ab1 proteins in soils is not equally documented. Results from studies with other than Cry34Ab1/Cry35Ab1 *Bt*-proteins indicate that they degrade rapidly in decaying plant residues and should readily be degradable in soil, where they occur—if detected—at extremely low concentrations (< 1.0 ng/g soil). While there is certain potential of proteins including *Bt*-proteins to be sorbed by soil surface active particles, which can decrease degradation rates and thus increase their transient environmental persistence (Icoz and Stotzky, 2008), there is no indication of accumulation of *Bt*-proteins in soils upon the subsequent cultivation of *Bt*-crops in the soil (Gruber et al., 2012). In soils from fields with *Bt*-crops, concentrations of *Bt*-proteins found in soil were very low, several orders of magnitude below concentrations to expect unintended adverse effects on non-target organisms (Baumgarte and Tebbe, 2005; Gruber et al., 2012). However, studies on the fate of *Bt*-proteins in soil suggest that degradation rates differ depending on the specific *Bt*-protein. For example, it was found that, under comparable environmental conditions, plant-produced Cry3Bb1 was less stable than Cry1Ab (Miethling-Graff et al., 2010). To the knowledge of the EFSA GMO Panel, there is no suitable information to assess whether the persistence of Cry34Ab1/Cry35Ab1 proteins in soil differ or not from that from better characterised Cry1Ab or Cry3Bb1 proteins. In addition, sequence comparisons with other known *Bt*-proteins failed to reveal homology between Cry34Ab1 or Cry35Ab1 proteins with other previously described three-domain-like *Bt*-proteins (OECD, 2007). No conclusions

[105] Technical dossier/Sections D9.6 and D9.7.
[106] Technical dossier/Sections D9.8 and D9.10.

can be drawn from the two available studies on the fate of Cry34Ab1/Cry35Ab1 proteins owing to limitations in the experiments.

- Herman et al. (2002a) conducted a laboratory study in which microbe-produced Cry34Ab1/Cry35Ab1 proteins were added to soil to obtain a concentration of 5 mg of each *Bt*-protein per gram of soil (which would correspond to the amount present in approximately 500 g of root material, considering 10 µg/g root fresh weight). According to the EFSA GMO Panel, the concentrations of *Bt*-proteins applied are unrealistically high, and therefore it considers this study as insufficient evidence to draw conclusions on the degradation rates of the *Bt*-proteins in soil in which maize 59122 has been cultivated. Degradation rates of lower *Bt*-protein concentrations can be highly different from those of very high concentrations owing to sorption processes.

- In a study provided by the applicant, the authors analysed Cry34Ab1/Cry35Ab1 protein concentrations in samples from three different soils after three years of continuous cultivation of maize 59122, using ELISA.[107] The authors did not detect any of the *Bt*-proteins in any of the soil samples. A comparison of the sensitivity of the applied detection method with detection thresholds reported in the peer-reviewed literature indicated that the thresholds applied by Dunville et al. (2010) were 45 to 180 × less sensitive.[108] Furthermore, the study report does not include information on how the extraction efficiency for *Bt*-proteins from soils was established, nor does it include the analyses of a positive control, so that it cannot be excluded that the negative results were caused by inefficient soil extraction and/or failure, e.g., inhibition, of the applied detection method (ELISA). Additional data provided by the applicant confirmed that the detection method used was specific and able to detect the *Bt*-proteins in the soil samples, but not sufficiently sensitive.[109]

The EFSA GMO Panel is not aware of any further published information on the fate of Cry34Ab1/Cry35Ab1 proteins in soil.

Based on general knowledge of the degradation of plant-produced *Bt*-proteins in soils and the overall concentrations of Cry34Ab1/Cry35Ab1 proteins in maize 59122, the EFSA GMO Panel concludes it is unlikely that the Cry34Ab1/Cry35Ab1 proteins will reach soil concentrations that would affect non-target organisms, in context of the intended uses of maize 59122. Although no risk was identified in the short term, scientific uncertainties pertaining to the specific potential of Cry34Ab1/Cry35Ab1 to accumulate and persist in soil during subsequent years of cultivation of maize 59122 remain, owing to the lack of experimental evidence. Therefore, the EFSA GMO Panel recommends that the remaining scientific uncertainties can be resolved with data acquired during post-market environmental monitoring.

The NL CA concluded that "*based on the specificity of the Cry proteins, the demonstrated lack of effect on nontarget organisms, the rapid degradation of the proteins in soil and the fact that the proteins are naturally present in the soil environment, it is concluded that 59122 maize has no significant ecological impact on the soil ecosystem*" (Section 6.7 of the environmental risk assessment report of the NL CA).

6.2.6.2. Adverse effects on soil microorganisms due to the expression of the Cry34Ab1/Cry35Ab1 proteins

A small-scale field study with maize 59122 plants did not reveal significant differences in soil microorganisms between soil samples taken near maize 59122 and non-*Bt*-maize.[110] *Bt*-proteins do not act as antimicrobials but rather as insecticides with a narrow host specificity. During the relatively

[107] Additional information received on 16/01/2012/Request 3/pages 31–33/Annex: Dunville et al. (2010)//02/10/2012/Request 3/pages 14–16.
[108] Additional information received on 23/01/2013/Point 3.2/pages 5–7/Table 1.
[109] Additional information received on 23/01/2013/Point 3.2/pages 5–6/Annexes: Shan and Embrey (2009a,b).
[110] Additional information received on 16/01/2012/Appendix 2/Annex: Urtz (2004).

long period of scientific exploration of *Bt*-proteins and their potential biotechnological or agricultural applications, there has been no scientific report to the EFSA GMO Panel's knowledge that these proteins would exhibit adverse effects on bacteria or other microorganisms or interfere with microbial activities (Icoz et al., 2008; Yanni et al., 2010; Barriuso et al., 2012; Fließbach et al., 2012; Prischl et al., 2012). Therefore, there is no indication of a hazard from maize 59122 or its Cry34Ab1/Cry35Ab1 proteins to soil microorganisms and the ecosystem services they provide, including their contribution to biogeochemical processes. Where effects of GM crops on microbial communities have been reported, these effects were in general considered spatially and temporally limited, and small compared with those induced by differences in geography, temperature, seasonality, plant variety, soil type and changes in soil management (Sessitsch et al., 2004; Fang et al., 2005, 2007; Griffiths et al., 2005, 2006; Lilley et al., 2006; Filion, 2008; Icoz and Stotzky, 2008).

Various studies have reported decreases in the decomposition rate of *Bt*-maize (e.g., Saxena and Stotzky, 2001b; Flores et al., 2005; Poerschmann et al., 2005; Fang et al., 2007; Raubuch et al., 2007). These differences in decomposition rate have been shown to result from increased lignin contents in certain maize varieties, and not from an inhibition of soil microorganisms by the plant-produced *Bt*-protein (Griffiths et al., 2007a,b; Hönemann et al., 2008; Lehman et al., 2008a, 2010; Tarkalson et al., 2008; Zurbrügg et al., 2010). Altered lignin content in maize varieties is not an effect attributed to the insertion of the transgene, but from the genetic background of the maize varieties under consideration (Fernie et al., 2006; Griffiths et al., 2007a,b; Lehman et al., 2008b, 2010; Poerschmann et al., 2008; Zurbrügg et al., 2010; Yanni et al., 2011).

Effects of crops on soil microbial communities, which are especially expected in the rhizosphere or on decaying plant material, depend more on the plant species, variety or age than whether they are genetically modified. Rearrangements in structural diversity and population abundance of non-target soil organisms occur frequently in the agricultural environment. They are typically associated with several sources of variation, caused by natural variability (e.g., soil heterogeneity, weather conditions) and agricultural practices (e.g., soil tillage, crop rotation, irrigation measures) and are thus not necessarily an indication of environmental harm. The EFSA GMO Panel concludes that potential effects on soil microorganisms and microbial communities, as well as the ecosystem services they provide, due to the cultivation of maize 59122, if they occur, will be transient and minor, and are likely to be smaller or within the range currently caused by other agronomic and environmental factors.

The NL CA concluded that "*based on the specificity of the Cry proteins, the demonstrated lack of effect on nontarget organisms, the rapid degradation of the proteins in soil and the fact that the proteins are naturally present in the soil environment, it is concluded that 59122 maize has no significant ecological impact on the soil ecosystem*" (Section 6.7 of the environmental risk assessment report of the NL CA).

6.2.6.3. Adverse effects on biogeochemical processes and the abiotic environment due to the expression of the PAT protein

Based on the mode of action of the PAT protein and the history of safe use of maize 59122 and other glufosinate-ammonium tolerant crops, the EFSA GMO Panel concludes it is unlikely that the expression of this protein in glufosinate-ammonium tolerant crops will cause direct adverse effects on biogeochemical processes and the abiotic environment (CERA, 2011, and references therein).

The conclusion of the EFSA GMO Panel is consistent with that of the NL CA who reported that "*the potential effect of the PAT protein on biogeochemical processes has been assessed several times before. Potential effects of the PAT protein were found to be negligible*". The NL CA concluded that "*based on the specificity of the PAT protein, the demonstrated lack of effect on nontarget organisms, the rapid degradation of the proteins in soil and the fact that the proteins are naturally present in the soil environment, it is concluded that 59122 maize has no significant ecological impact on the soil ecosystem*" (Section 6.7 of the environmental risk assessment report of the NL CA).

6.2.7. Impacts of the specific cultivation, management and harvesting techniques[111]

6.2.7.1. Changes in pest management practices

Pest management options for WCR are usually directed towards reducing larval feeding and consist of crop rotation, the use of maize seed coated with systemic insecticides and the application of soil insecticides (applied at planting) (Levine and Oloumi-Sadeghi, 1991; Széll et al., 2005; Boriani et al., 2006; Ma et al., 2009; van Rozen and Ester, 2010; Meissle et al., 2011b). Crop rotation is highly effective in controlling WCR, as females lay their eggs mainly in maize fields and the larvae hatching in the following year do not survive well on other crop roots (Levine and Oloumi-Sadeghi, 1991; Kiss et al., 2005b; Boriani et al., 2006; Meissle et al., 2011b). Foliar broad-spectrum insecticides are sometimes applied to suppress adult populations, especially in continuous maize, in order to decrease egg laying by adult females and hence the number of overwintering eggs and hatching larvae in the following year (Levine and Oloumi-Sadeghi, 1991; Boriani et al., 2006). Foliar insecticides can also be applied to prevent silk clipping by adults in seed and sweet corn production, where high grain quality is essential for marketing (Levine and Oloumi-Sadeghi, 1991; Tuska et al., 2002; Boriani et al., 2006; van Rozen and Ester, 2010; Meissle et al., 2011b). In EU regions where WCR populations have been detected (EC, 2012), but are not yet established, mandatory eradication programmes require the application of insecticides and planting restrictions on maize in buffer zones surrounding new introduction points (FCEC, 2009; Carrasco et al., 2010).

Compared with pest management currently practised against WCR in conventional maize cropping systems, cultivation of *Diabrotica*-active *Bt*-maize can reduce the use of insecticides that are more harmful to the environment, given that fewer or no treatments with soil or foliar broad-spectrum insecticides may be needed (Porter et al., 2012). Therefore, maize 59122 is expected to result in a reduced environmental load from chemical insecticides (Alston et al., 2002; Rice, 2004), and lead to fewer adverse side effects on non-target arthropods in the maize ecosystem, when it replaces chemical insecticides (Marvier et al., 2007; EFSA, 2008b; Wolfenbarger et al., 2008; Naranjo, 2009). As indicated in Section 6.2.3.1, the cultivation of maize 59122 could lead to the evolution of resistance in the target pest and so cultivation practices will need to be adapted accordingly.

6.2.7.2. Changes in weed management practices

Although maize 59122 is tolerant to glufosinate-ammonium-based herbicides, the EFSA GMO Panel did not assess the potential adverse effects associated with the use of such herbicides on maize 59122, as maize 59122 will not be marketed in the EU as a herbicide-tolerant crop.[112]

6.2.8. Conclusion on the environmental risk assessment

As the scope of the current application covers cultivation, the environmental risk assessment considered the environmental impact of full-scale commercialisation of maize 59122.

The EFSA GMO Panel concludes that maize 59122 is unlikely to have any adverse effect on the environment, except for the possible resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests. The possible resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests is identified by the EFSA GMO Panel as a concern associated with the cultivation of maize 59122, as resistance evolution may lead to altered pest control practices that may cause adverse environmental effects. The EFSA GMO Panel therefore recommends that appropriate risk management strategies are implemented to delay and monitor resistance evolution.

The apparent activity of Cry34Ab1/Cry35Ab1 at high concentrations against the lepidopteran species (e.g., *O. nubilalis* and *S. cerealella*) was not expected based on the known spectrum of activity (Coleoptera only) of these binary proteins. The EFSA GMO Panel considers that there are indications of a potential hazard to Lepidoptera owing to cross-order activity at high Cry34Ab1/Cry35Ab1 protein

[111] Technical dossier/Section D9.9.
[112] Communication of applicant to EFSA on 11/03/2010.

concentrations. However, based on the submitted toxicity data and a theoretical exposure assessment, no risk to non-target Lepidoptera is expected from exposure to maize 59122 pollen in the field.

Scientific uncertainties pertaining to the specific potential of Cry34Ab1/Cry35Ab1 to accumulate and persist in soil during subsequent years of cultivation of maize 59122 remain, owing to the lack of experimental evidence. Therefore, the EFSA GMO Panel recommends that the remaining scientific uncertainties can be resolved with data acquired during post-market environmental monitoring.

The conclusions of the EFSA GMO Panel on the environmental safety of maize 59122 are largely consistent with those of the NL CA. The NL CA concluded that "*cultivation of line 59122 poses a negligible risk to human health and the environment, under the condition that specific monitoring for ladybird beetles is incorporated*" (Section 8 of the environmental risk assessment report of the NL CA). Based on the additional toxicity data on a representative European coccinellid species requested by the EFSA GMO Panel and estimated worst-case expected environmental concentrations, no risk to coccinellids are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122.

6.3. Risk management strategies[113]

6.3.1. Risk mitigation measures

According to the EFSA GMO Panel guidelines on the environmental risk assessment of GM plants (EFSA, 2010c) and in line with Annex II of the Directive 2001/18/EC, the risk assessment can identify risks that require management and propose mitigation measures to reduce the levels of risk. Risk mitigation should be proportionate to the levels of risk identified in the environmental risk assessment and the remaining scientific uncertainties.

The EFSA GMO Panel recommends that appropriate risk mitigation measures are implemented to delay resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests. The EFSA GMO Panel evaluation of the efficacy and scientific quality of the risk mitigation measures proposed by the applicant is described below.

6.3.1.1. Risk mitigation measures to delay resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests

Insect resistance management plan proposed by the applicant

In line with the applicants' EU working group on insect resistance management (as referred to by Alcalde et al. (2007)), the applicant proposed to put in place risk mitigation measures to delay the possible resistance evolution in the target insect pests. According to the insect resistance management plan proposed by the applicant, farmers growing more than 5 ha of maize 59122 in the EU shall establish refuge areas with non-Cry34Ab1/Cry35Ab1-expressing maize, corresponding to at least 20 % of the area planted with maize 59122. The applicant's reasoning for implementing the *refugia* only on farms where the area of maize 59122 is greater than 5 ha is based on: (1) the high fragmentation of the European agricultural landscape; (2) the lack of economic feasibility for providing *refugia* on farms with less than 5 ha maize 59122; and (3) the negligible risk of evolution of resistance in areas with maize 59122 smaller than 5 ha (Alcalde et al., 2007).

In addition to maintaining an adequate level of refuge areas with non-Cry34Ab1/Cry35Ab1-expressing maize, the insect resistance management plan proposed by the applicant covers the following elements: (1) monitoring for resistance (Section 6.3.2, below); (2) the implementation of a comprehensive education (training) programme to aid farmers to understand the importance of adhering to insect resistance management requirements, which is key to the success of the high dose/refuge strategy (Section 6.3.2, below); and (3) remedial measures to respond to confirmed

[113] Technical dossier/Section D9.10//Additional information received on 16/01/2012/Request 2/pages 27–30//02/10/2012/ Requests 4–5/pages 16–22/Appendix 5.

resistance (either to mitigate the further evolution of resistance (prevent its spread) or eradicate resistance (if detected timely)) (Glaser and Matten, 2003; Alcalde et al., 2007; MacIntosh, 2010; Head and Greenplace, 2012).

High dose/refuge strategy

The EFSA GMO Panel considers that appropriate insect resistance management strategies are capable of delaying possible evolution of resistance under field conditions (see also Alstad and Andow, 1995; Andow, 2008; Tabashnik et al., 2008a, 2009; Huang et al., 2011). Insect resistance management strategies are designed to minimise the selection pressure associated with *Bt*-crops, in order to prevent or at least delay resistance evolution in the target insect pests and to extend the durability of *Bt*-crops (Bates et al., 2005; Alcalde et al., 2007; Andow, 2008; MacIntosh, 2010; Head and Greenplate, 2012). As currently implemented for several *Bt*-crops in several countries, the insect resistance management plan proposed by the applicant relies on the high dose/refuge strategy (Gould, 1998; Glaser and Matten, 2003; MacIntosh 2010). The high dose/refuge strategy prescribes planting *Bt*-crops that produce a very high concentration of the *Bt*-protein (25 × the amount needed to kill > 99 % of susceptible individuals (LC_{99})), so that nearly all target insect pests that are heterozygous for resistance do not survive on it. In addition, a nearby structured refuge of the non-*Bt*-crop is required where the target insect pest does not encounter the *Bt*-protein (Alstad and Andow, 1995; Gould, 1998; Ives and Andow, 2002). Note that non-*Bt*-crops or refuges are intended to mean areas under a crop that does not express *Bt*-proteins that are active against the target insect pest. Under these conditions, most of the rare resistant individuals surviving on the *Bt*-crop will mate with abundant susceptible individuals emerging from nearby refuges to produce heterozygous offspring that are phenotypically susceptible. If inheritance of resistance is recessive, then the hybrid progeny from such matings will die on the *Bt*-crop.

The success of the high dose/refuge strategy is aided if the following conditions are met: (1) the *Bt*-protein is expressed at appropriate levels in relevant plant parts; (2) initial resistance alleles are rare in the target insect pest population, so that nearly all resistance alleles will be in heterozygote individuals that cannot survive on the *Bt*-crop; (3) random mating occurs between resistant insects emerging in *Bt*-crops and susceptible insects preserved on refuges at sufficient levels; (4) resistance alleles are partially or fully recessive; and (5) fitness costs are associated with the resistance. Whether these conditions of the high dose/refuge strategy are met for WCR and maize 59122 is considered below.

(1) *Bt-protein is expressed at appropriate levels in relevant plant parts*: The predicted duration of susceptibility of target insect pests to the *Bt*-protein is dependent upon many factors (e.g., Tyutyunov et al., 2008), including its dose in the *Bt*-crop (Onstad et al., 2001a). It is generally assumed that the *Bt*-protein concentration in relevant plant parts must be sufficiently high to kill a high proportion of heterozygous resistant genotypes, so that any resistance allele in the target insect pest population remains functionally recessive (Gould, 1998; Andow, 2008). Instances of field-selected resistance reported so far (reviewed by Tabashnik et al., 2009; Huang et al., 2011) support model predictions that target insect pests are at greater risk of evolving resistance if managed by *Bt*-crops that are not high dose (Tabashnik et al., 2004).

The average reduction in adult WCR emergence on maize 59122 in different field studies ranged from 94.2 % to 96.48 % (Storer et al., 2006; Binning et al., 2010; Hibbard et al., 2010a; US EPA, 2010b). Lefko et al. (2008) reported that the F_1 generation of two WCR colonies reared on maize 59122 in a laboratory experiment had mortality rates of 99.6 % and 98.7 % (see also Nowatzki et al., 2008). In all studies reported above, the observed survival was > 100-fold higher than the US EPA standard of 0.01 % for a *Bt*-crop that is truly high dose (Tabashnik and Gould, 2012). These findings confirm that maize 59122 fails to meet the high dose condition and that the expression of *Bt*-proteins in this event is to be considered low to moderate. The ability of heterozygous resistant WCR progeny, resulting from the mating between individuals emerging from the refuge and *Bt*-maize fields, to survive on *Bt*-maize may diminish the efficacy of the high dose/refuge strategy to delay resistance evolution (Gassmann et al., 2011).

Because WCR is not extremely susceptible to Cry34Ab1/Cry35Ab1 proteins and older instars are inherently less susceptible than neonates, larvae can survive the exposure to maize 59122 (Siebert et al., 2012). It has been postulated that larvae surviving on *Bt*-maize may also do so by grazing on the outside of *Bt*-protein-expressing roots, thereby minimising exposure to *Bt*-proteins. Root growing points are more metabolically active, and have a higher content of total soluble Cry34Ab1/Cry35Ab1 proteins compared with older root tissue (Lefko et al., 2008).[114] Moreover, Cry34Ab1/Cry35Ab1 proteins appeared relatively more concentrated in the endodermis and epidermis and less concentrated in the vascular tissues. Results suggest that a repellent factor in roots or root exudates may contribute to the overall efficacy of maize 59122 (Rudeen and Gassman, 2012).

(2) *Initial resistance alleles are rare in the target insect pest population*: The resistance alleles must be sufficiently rare (the frequency should be typically < 0.001, which has been taken as a default value when modelling the evolution of resistance to *Bt*-proteins (Roush, 1994)), so that nearly all resistance alleles are in heterozygote genotypes that are eliminated by the *Bt*-crop (Andow, 2008). Studies in which the frequency of resistance alleles to *Bt*-proteins in populations of WCR are directly estimated have not been published in the scientific literature. In the case of maize 59122, evidence suggests complex inheritance of resistance, owing to the involvement of one or more minor genes (Lefko et al., 2008). Characterisation of the selected WCR colonies by Lefko et al. (2008) suggests that the frequency of a major resistance allele in US populations is low. Annual resistance monitoring with no apparent shifts in WCR susceptibility to Cry34Ab1/Cry35Ab1 (Table 2) provides indirect evidence that the initial resistance allele frequency may be low.[115] However, based on the outcomes of the artificial selection experiments conducted by Lefko et al. (2008), Onstad and Meinke (2010) calculated that the initial resistance allele frequency may range between 0.05 and 0.1 for maize 59122.

(3) *Random mating occurs between resistant insects emerging in Bt-crops and susceptible insects preserved on refuges at sufficient levels*: For the refuge to be effective its placement, configuration and size should ensure that resistant and susceptible insects mate more or less randomly, and that susceptible insects outnumber resistant ones. How much mixing and mating will occur between individuals emerging from the refuge and *Bt*-maize fields is determined by the scale of adult movement. Although adult WCR can move substantial distances (Coats et al., 1986; Toepfer et al., 2006; Carrasco et al., 2009), most movements are quite local, and limited to short-ranged movements within fields or between adjacent fields, especially prior to mating (Naranjo, 1990, 1991, 1994; Storer, 2003; Meinke et al., 2009; Szalai et al., 2011). The range of adult movement measured in maize fields was shown to be less than 30 m per day (Coats et al., 1987; Nowatzki et al., 2003; Spencer et al., 2003, 2005), with an average dispersal rate of approximately 15 m per day (Spencer et al., 2009). The tendency for short-distance dispersal may delay resistance evolution at a landscape level (Caprio and Tabashnik, 1992), but it may contribute to the persistence and intensification of resistance in localised areas (Gassmann et al., 2011).

WCR females are unlikely to disperse before mating, meaning that males are the primary dispersers before mating (Spencer et al., 2003; Marquardt and Krupke, 2009); pre-mating movement of males can be extensive when responding to reproductive females (Meinke et al., 2009). Mating typically occurs within 24–48 hours of female adult emergence within the maize fields they emerged from or nearby. Males normally emerge before females and are capable of mating multiple times (on average twice during their lifespan), although they are less likely to mate as they age, whereas females generally mate only once (Kang and Krupke, 2009a). Given the known ambit of males, planting refuges for maize 59122 adjacent to, or within the *Bt*-maize field, preferentially in large blocks or as row strips of at least four or more rows, is considered

[114] Additional information received on 02/10/2012/Request 4/page 16/Annex: Lefko and Diehn (2008).
[115] Additional information received on 02/10/2012/Request 2/page 13/Annex: Storer and Owens (2009)//16/01/2012/Annex: Lepping et al. (2011).

adequate (US EPA, 2007, 2010a,b) to ensure that males from refuges encounter receptive females on *Bt*-maize in time to mate.

A non-synchronous emergence of WCR from refuges and *Bt*-maize fields could result in non-random (assortative) mating and contribute to resistance. Based on a series of laboratory experiments, Kang and Krupke (2009a) argued that the realised mating activity between susceptible males from refuges and potentially resistant females on *Diabrotica*-active *Bt*-maize may be low, because the mating ability of males declines rapidly and adults in *Bt*-maize may emerge later than those in the refuge. Storer et al. (2006) and Rudeen and Gassmann (2012) reported a seven-day delay in initial emergence of WCR from maize 59122, compared with the near-isogenic line. Further, males have been shown to prefer larger females under laboratory conditions (Kang and Krupke, 2009b), which could result in assortative mating too (Murphy et al., 2011).

(4) *Resistance alleles are partially or fully recessive*: If resistance is completely recessive, then heterozygous offspring resulting from crosses between resistant and susceptible individuals are expected to be susceptible to the *Bt*-protein, thus preventing or slowing resistance evolution (Bates et al., 2005). The longest delays in resistance evolution are expected for resistance traits that are completely recessive. The applicant indicated that maize 59122 has been cultivated in the United States for eight years without an instance of resistance, providing indirect evidence that resistance is probably recessive.[116] Using the data reported by Lefko et al. (2008), Onstad and Meinke (2010) determined that the dominance value (*h*) values (defined by Liu and Tabashnik, 1997) range from 0.5 to 0.75 for maize 59122. The calculations of *h* values point to non-recessive inheritance of resistance under artificial selection experiments, which could accelerate the evolution of resistance in WCR in the field (Onstad and Meinke, 2010; Pan et al., 2011).

(5) *Fitness costs are associated with the resistance*: Fitness costs associated with resistance occur when fitness on the non-*Bt*-crop is lower for resistant insects than the susceptible ones (Gassmann et al., 2009). As the most likely cause of instability of resistance to a *Bt*-protein is the fitness cost associated with resistance (Tabashnik, 1994), such costs could cause declines in resistance when the selection exerted by *Bt*-maize ceases. In refuges where resistant insects are not exposed to the *Bt*-protein, fitness costs would exert control over the frequency of resistance alleles, and delay or reverse resistance by selecting against resistant genotypes, thereby increasing the effectiveness of refuges for delaying resistance (Gould, 1998; Carrière and Tabashnik, 2001; Crowder and Carrière, 2009). Refuges would delay resistance evolution not only by providing susceptible individuals to mate with resistant individuals, but also by selecting against resistance. Gassmann et al. (2009) reported that the magnitude of fitness costs is positively correlated with resistance ratios, with more resistant strains suffering greater fitness costs. Based on reported resistance ratios, only low fitness costs are expected to be associated with resistance to *Bt*-proteins in WCR.

Few studies have analysed fitness costs associated with Cry3Bb1, Cry34Ab1/Cry35Ab1 and mCry3A resistance in WCR (Devos et al., 2013), but available data for Cry3Bb1 suggest that fitness costs associated with Cry3Bb1 resistance are minimal (Meihls et al., 2008, 2012; Gassmann et al., 2012; Oswald et al., 2012). Because most of the data indicate that no or limited fitness costs are associated with resistance to Cry3Bb1, it is prudent to infer that major fitness costs are not necessarily present in field populations and, thus, fitness costs may not help to substantially delay WCR resistance.

While caution must be exercised when extrapolating laboratory and greenhouse results to field conditions, the evidence discussed above indicates that several conditions contributing to the success of the high dose/refuge strategy are not met for maize 59122 and WCR: (1) the *Bt*-proteins are expressed heterogeneously at a low-to-moderate dose in roots; (2) resistance alleles may be present at

[116] Additional information received on 02/10/2012/Request 2/page 13/Annex: Storer and Owens (2009)//16/01/2012/Annex: Lepping et al. (2011).

a higher frequency than initially assumed; (3) WCR may mate in a non-random manner due to short-ranged movements before mating and non-synchronous emergence; (4) resistance traits could have non-recessive inheritance; and (5) fitness costs may not necessarily be associated with resistance evolution. Because the high dose condition is not met, the strategy for managing resistance in WCR would rely solely on a refuge, in order to maintain a susceptible population.

Models developed to estimate the evolution of resistance in WCR populations predicted that a 20 % refuge can delay resistance evolution for *Bt*-maize under certain conditions (Onstad et al., 1999 2001a,b; Storer, 2003; Crowder and Onstad 2005; Crowder et al., 2005, 2006; US EPA, 2007, 2010a,b; Onstad and Meinke, 2010; Pan et al., 2011). In some of these models, a range of efficacy and genetic parameter values were explored; adaptation to low- to moderate-dose *Bt*-crops were simulated by accounting for the fact that many or most of the individuals surviving on *Bt*-crops have susceptible phenotypes; multi-locus models for resistance were considered instead of single-locus, two-allele models for resistance; a spatially explicit model structure was accounted for; and more realistic data on the biology of WCR were integrated. Depending on the underlying model assumptions and parameter values used in these models, which explore more or less conservative scenarios, resistance has been predicted to evolve in three to more than 20 years. With a 20 % block refuge planted every year in the centre of a *Bt*-maize field of 80 ha, Pan et al. (2011) estimated delays in resistance of at least 20 years when the initial resistance allele frequency was 0.001. For an initial resistance allele frequency of 0.01, the resistance allele frequency was likely to exceed 50 % (0.5) in seven years. With the annual relocation of the 20 % block refuge, the resistance allele frequency would exceed 50 % in nine and five years, if the initial resistance allele frequencies were 0.001 and 0.01, respectively. Without adequate risk management strategies, resistance evolved in five and three years for initial resistance allele frequencies of 0.001 and 0.01, respectively (Pan et al., 2011). Similar trends were reported for dominance: as the *h* value increased, the time to 50 % allele frequency decreased. For cases of additive resistance ($h = 0.5$) resistance was predicted to evolve in 7–11 years under 20 % block refuge scenarios (Onstad and Meinke, 2010). However, the EFSA GMO Panel notes that resistance may evolve faster, because some of the parameter values used in the above-mentioned models are not conservative. For instance, the initial resistance allele frequencies in WCR for maize 59122 may be 10–100 × higher than typical empirical estimates of 0.01–0.001 for other target insect pests (Carrière et al., 2010; Onstad and Meinke, 2010). In addition, modelling predictions often assume complete compliance with refuge requirements (Pan et al., 2011; Gassmann, 2012).

If the high dose condition of the high dose/refuge strategy is not achieved, modelling predictions indicate that resistance evolution can be delayed by increasing refuge abundance to compensate for the survival of hybrid progeny on *Bt*-crops (Gould, 1998; Tabashnik et al., 2004). The strategy for managing resistance in WCR would rely solely on a refuge to maintain a susceptible population (Murphy et al., 2010). Tabashnik and Gould (2012) recently advocated increasing refuge abundance by requiring a 50 % refuge of non-*Bt*-maize WCR instead of the current 20 % for the first generation of *Diabrotica*-active *Bt*-maize expressing a single *Bt*-protein against WCR. As the effectiveness of larger refuges may be diminished by the probably uneven dispersal of WCR under certain configurations, the authors recognised the need to fine-tune their recommendation and to account for different spatial configurations of refuges. However, depending on refuge configurations, increased refuge abundance may have economic trade-offs, which may offset incentives to implement refuges and lead to reduced farmer compliance.

It should be noted that maize 59122 itself may act as a (unstructured) refuge, because WCR larvae feeding on maize 59122 roots may not be exposed to *Bt*-proteins uniformly owing to their heterogeneous expression in roots. Hibbard et al. (2010b) demonstrated that many or most of WCR individuals initially surviving on maize MIR604 after one generation of selection in the field had a susceptible phenotype, suggesting that resistant individuals from the *Bt*-maize are mating not only with susceptible individuals from the refuge but also with susceptible individuals that emerged from the *Bt*-maize field. *Bt*-maize that is not truly high dose could thus yield susceptible adults that are available to mate with any WCR potentially carrying resistance alleles, and hence contribute to slow the onset of resistance evolution (Crowder and Onstad, 2005). Moreover, evidence has shown that

several grass species can support the growth of WCR larvae (Clark and Hibbard, 2004; Oyediran et al., 2004; Wilson and Hibbard, 2004; Breintenbach et al., 2005) and may therefore serve as an additional (unstructured) refuge in which such host plants are abundant and appropriately distributed (Chege et al., 2005, 2009; Oyediran et al., 2005).

Volunteers

The extent to which maize 59122 volunteers in subsequent crops (including maize 59122) may affect the rate of resistance evolution is unclear. In the case of maize MON 863, Krupke et al. (2009) argued that the unpredictable and varying levels of the Cry3Bb1 protein in the roots of volunteer plants may facilitate more rapid evolution of resistance in WCR populations; larvae may survive exposure simply because the dose is lower, even without any differential feeding behaviour. It is also possible that due to larval movement (Hibbard et al., 2003, 2004, 2005; Zukoff et al., 2012; Schumann and Vidal, 2012) larvae would be exposed to sublethal doses of the Cry3Bb1 protein at later instar stages by feeding on a combination of volunteer and *Bt*-maize plants (Meihls et al., 2008; Krupke et al., 2009; Murphy et al., 2010). However, there is also the possibility that larvae may exhibit a feeding behaviour that minimises exposure to *Bt*-proteins. How much each of these mechanisms will contribute to the speed of resistance evolution overall is dependent upon the amount and type of *Bt*-maize planted, the number of maize volunteers present and the level of *Bt*-proteins expressed by these plants. The EFSA GMO Panel notes that the early and timely control of volunteer plants in subsequent crops may decrease the potential selection pressure on WCR populations, as these plants would be killed before the larval development of WCR is completed (Olmer and Hibbard, 2008; Marquardt et al., 2012).

Compliance with refuge requirements

Compliance with refuge requirements is a critical factor contributing to the success of insect resistance management plans in delaying the rate at which resistance evolves (Bourguet et al., 2005; Kruger et al., 2009, 2012; Huang et al., 2011; Onstad et al., 2011). In the case of maize MON 863 and MON 88017, failure to fully comply with the refuge requirements and to carry out the operational details of insect resistance management plans may have contributed to the field-selected Cry3Bb1 resistance reported in the United States (Andow et al., 2010; Gassmann et al., 2011). It is therefore important that education (training) programmes form an integral part of insect resistance management plans, as they aid farmers to understand the importance of adhering to insect resistance management requirements and are key to the success of the high dose/refuge strategy (Glaser and Matten, 2003; Bates et al., 2005; Andow, 2008; Head and Greenplate, 2012). The insect resistance management plan proposed by the applicant for maize 59122 proposes education programmes and specific means for communicating insect resistance management requirements (e.g., technical user guides, newsletters, technical bulletins, product brochures, sales meetings, presentations by local experts to farmers, and the requirement to attend education meetings for purchase of the product). Some of these tools will be used by the applicant, in addition to the traditional label that accompanies the *Bt*-crop and which outlines the contents of the product and standard directions for use (Alcalde et al., 2007; MacIntosh, 2010). Besides education programmes, the EFSA GMO Panel notes that compliance can be maximised via farmer contracts, certification tests, audits, rewards for compliance, crop insurance for refuges, databases of non-compliant farmers, sales restrictions, and fines for non-compliance.

Seed blends

Seed blends (also termed seed mixtures or refuge in a bag), composed of a 5–10 % blend of non-*Bt*-maize serving as refuge seed in the *Diabrotica*-active *Bt*-maize seed bag, are approved for commercial cultivation in the United States (US EPA, 2010c, 2011b). Seed blends will result in 100 % compliance and are more convenient for farmers to plant than the usual block and row strip refuges (Onstad et al., 2011). The use of seed blends also distributes refuge plants relatively uniformly within the *Bt*-crop field. Further, when compared with block refuges, WCR emerging from refuge plants emerges more synchronously with those emerging from *Diabrotica*-active *Bt*-maize plants in seed blends (Rudeen and Gassmann, 2012). This increased proximity in both space and time may facilitate random mating

between adults emerging from *Bt*-maize and refuge plants compared with block refuges (Murphy et al., 2011). However, the advantages of seed blends may be offset by the potential for larval movement between roots of *Bt*-maize and refuge plants (Hibbard et al., 2003, 2004, 2005; Zukoff et al., 2012; Schumann and Vidal, 2012), and the exposure of later instars to sublethal doses of the toxin (Goldstein et al., 2010; Murphy et al., 2011; Onstad et al., 2011; Razze and Mason, 2012; Zukoff et al., 2012). For *Bt*-crops that are truly high dose, Mallet and Porter (1992) indicated that the movement of larvae between *Bt*-crop and refuge plants may lower the selective differential between susceptible and resistant genotypes, and increase the effective dominance of resistance by producing more heterozygote individuals (Glaum et al., 2012; Siegfried and Hellmich, 2012; but see Tabashnik, 1994).

Overall, resistance evolved more slowly under seed blend scenarios than for WCR colonies reared fully on *Bt*-maize (Onstad, 2006; Meihls et al., 2008; Binning et al., 2010; Pan et al., 2011; Rudeen and Gassmann, 2012; Zukoff et al., 2012), indicating that the WCR biology seems to lend itself to the seed blend concept (US EPA, 2009). Fully rearing WCR larvae on *Diabrotica*-active *Bt*-maize led to resistance within three generations, while selection for resistance when first instars were fed the near-isogenic maize and third instars were fed *Diabrotica*-active *Bt*-maize led to the evolution of resistance within six generations of selection (Meihls et al., 2008).

Recommendations to revise insect resistance management (including refuge) requirements

According to the insect resistance management plan proposed by the applicant, only farmers growing more than 5 ha of maize 59122 in the EU shall establish refuges with non-Cry34Ab1/Cry35Ab1-expressing maize, corresponding to at least 20 % of the surface planted with maize 59122. In practice, this would mean that *refugia* of non-Cry34Ab1/Cry35Ab1-expressing maize would not be implemented on a considerable proportion of farms in certain EU countries, as the area planted to maize 59122 on these farms would cover less than 5 ha. In most cases, it is likely that sufficiently large areas of non-Cry34Ab1/Cry35Ab1-expressing maize will remain, providing widely distributed mosaics of both non-Cry34Ab1/Cry35Ab1-expressing and maize 59122 at regional scales. However, if maize 59122 was adopted on a larger scale in a region or in a cluster of fields with an aggregate area greater than 5 ha, then the potential for resistance evolution would be likely to increase. Therefore, the EFSA GMO Panel recommends that there should always be *refugia* equivalent to 20 % of the aggregate area, irrespective of individual field and farm size.

Modelling predictions indicated that increasing refuge abundance beyond 20 % may delay resistance evolution if the high dose condition of the high dose/refuge strategy is not achieved (Tabashnik and Gould, 2012). Until resistance monitoring data relevant to maize 59122 become available and point to Cry34Ab1/Cry35Ab1 resistance evolution in WCR, the EFSA GMO Panel considers the implementation of greater than 20 % refuges disproportionate to the level of risk identified at this time. Nonetheless, the EFSA GMO Panel requires that the insect resistance management plan proposed by the applicant should be complemented with additional insect resistance management measures, as the Panel does not accept the 20 % refuge as the sole insect resistance management measure (see below).

To ensure that males from refuges encounter receptive females on *Bt*-maize in time to mate, refuges for maize 59122 should be planted adjacent to, or within, the *Bt*-maize field in large blocks, as row strips of at least four or more rows, or as seed blends (US EPA, 2007, 2010a,b). The model of Storer (2003) showed that refuge strips, refuge blocks and refuge in nearby fields were all similarly effective at delaying resistance to maize 59122. The EFSA GMO Panel considers that refuge options for large fields of *Diabrotica*-active *Bt*-maize such as maize 59122 are the least optimised outside the *Bt*-maize field as separate refuge. Modelling predictions suggest that refuge strips and blocks would be more effective than a separate refuge in varying locations at delaying resistance to *Diabrotica*-active *Bt*-maize (Storer, 2003). If a portion of the refuge were to be planted in the same fields or in-field blocks each year, WCR adaptation would be delayed substantially. Seed blends were shown to produce equal or greater durability than block refuges that were relocated each year (Onstad, 2006; Pan et al., 2011).

However, the use of maize 59122 seed blends is not being recommended at this time until further data are gathered on the performance and suitability of this potential refuge option.

To optimise and encourage compliance with refuge requirements, stewardship agreements should allow farmers to select appropriate refuge options, based on the size and spatial distribution of maize fields on a local scale. In regions dominated by large maize fields, refuges should ideally be planted as two- or four-row strips within the maize 59122 fields. Alternatively, it can be more convenient for farmers to plant the refuge along one side of the maize 59122 field as a block refuge, or to plant the entire perimeter of the maize 59122 field to the refuge. In regions with mainly small maize fields, farmers with multiple maize fields may choose to plant the refuge as the entirety of one of those fields (separate refuge) and maize 59122 in the others.

Refuges planted as separate fields may be effective only if planted within a designated distance from the *Bt*-maize field. Therefore, a separate refuge should be in the immediate vicinity and separated by no more than 10 m from the longest side of the maize 59122 field. The refuge should be planted in a manner to minimise the average distance between the *Bt*- and non-*Bt*-maize plants and to maximise the common boundary between the *Bt*- and non-*Bt*-maize field.

In cases in which larger fields are planted to maize 59122, the effectiveness of a single block or a single separate refuge may be diminished because of the probably uneven dispersal of WCR with a higher density of insects near the refuge (Gassmann et al., 2012). Therefore, stewardship agreements should specify the upper limit of the maize 59122 surface at which interspersed block or separate refuges should be established.

As the life cycle of WCR extends over two consecutive maize growing seasons, the EFSA GMO Panel considers that separate fields designed to deliver susceptible WCR adults are suitable as refuges only if they have been cropped with non-*Diabrotica*-active *Bt*-maize for at least two successive years in the EU.

To improve the development of optimal refuge requirements on a regional scale in the future, resistance monitoring data could be analysed retrospectively in conjunction with data on the spatial and temporal distribution of maize 59122 and refuges (e.g., Carrière et al., 2012).

To limit non-synchronous emergence of WCR from refuge and maize 59122 fields, the type of maize to be planted as refuge should be of a similar hybrid/variety, as close as possible to the GM maize variety containing event 59122. Refuge maize should therefore be selected based on its equivalent maturity to the GM maize variety containing event 59122, and be planted within the same planting window as the maize 59122 variety. They should also be managed using comparable agronomic (fertilisation, weed and pest management and irrigation) practices. The EFSA GMO Panel considers it acceptable to treat refuges for maize 59122 with seed treatments or soil-applied insecticides to control WCR larvae when WCR infestation levels are high, as this is not expected to adversely affect adult emergence from the refuge (US EPA, 2007, 2010a,b). However, it is not acceptable to treat refuges for adult WCR control as these treatments may diminish the efficacy of the refuge. Foliar applications for adult control are an option only if both refuge and *Bt*-maize fields are treated equally, and only if adult population densities are very high (US EPA, 2007, 2010a,b).

To decrease the potential selection pressure on WCR populations, the early and timely control of maize 59122 volunteer plants in subsequent crops is advisable, as these plants would be killed before the larval development of WCR is completed.

Owing to the remaining scientific uncertainties pertaining to the appropriateness of the high dose/refuge strategy in delaying resistance evolution for *Bt*-crops that are not truly high dose, the EFSA GMO Panel requires that the insect resistance management plan proposed by the applicant should be complemented with additional insect resistance management measures, so that additional and diversified resistance management strategies, reliant on multiple tactics to control WCR, are

implemented. Stewardship agreements should prescribe diversity in cropping and WCR management practices, and recommend: (1) rotating fields to crops that are not hosts of WCR larvae; (2) alternating maize 59122 with other *Bt*-maize events that express one or more different *Bt*-protein(s) active against WCR; and (3) using additional pest management measures, such as insecticides or biological control agents, only when and where necessary in maize 59122 fields.

(1) Crop rotation is a key component of integrated pest management, and is an effective tool to manage WCR in areas in which no crop rotation-resistant WCR variant occurs. If maize 59122 is followed by a different crop in the consecutive spring, then hatched WCR larvae do not find enough food and starve quickly (Levine and Oloumi-Sadeghi, 1991; Kiss et al., 2005b; Boriani et al., 2006; Meissle et al., 2011b). To delay the evolution of resistance to *Bt*-proteins in WCR, or of a crop rotation-resistant WCR variant, sufficient diversity in crop rotations in space and time should be ensured, ideally at a regional scale. Alternating *Bt*-maize with another crop whenever possible is therefore considered useful, especially in fields with a high probability of WCR infestation levels. Gassmann et al. (2011, 2012) found a significant positive correlation between the number of years that maize MON 88017 had been grown in a field and the survival of WCR populations on maize MON 88017 seedlings in laboratory bioassays. Multiple and increased performance failures of maize MON 88017 were mostly reported in fields in which maize MON 88017 was grown for more than three successive years without crop rotation (Gray, 2011a,b). To delay Cry34Ab1/Cry35Ab1 resistance to evolve in WCR, it is advisable not to grow maize 59122 for more than three consecutive years on the same field.

(2) An additional essential component of integrated pest management is the alternation of insecticides with different modes of action. Rotation of *Bt*-maize expressing one or more different *Bt*-protein(s) active against WCR as a WCR management strategy has been neglected in the United States (Porter et al., 2012). In areas with significant WCR infestation levels, *Diabrotica*-active *Bt*-maize expressing the same *Bt*-protein is often planted in the same field year after year (Gassmann et al., 2011; Gray, 2011a,b; US EPA, 2011a). The EFSA GMO Panel considers that the use of a *Diabrotica*-active *Bt*-maize expressing a *Bt*-protein different from the one that performed poorly in the previous year would avoid repeated selection pressure, provided that no cross-resistance occurs. Gassmann et al. (2011, 2012) reported that there was no significant correlation among WCR populations for survival on maize 59122 and MON 88017. Offspring from WCR collected from *Bt*-maize MON 88017 problem fields and control fields had a similar survival on maize seedlings of *Bt*-maize DAS-59122-7 and the near-isogenic line, suggesting a lack of cross-resistance between Cry3Bb1 and Cry34Ab1/Cry35Ab1. Because Cry3Bb1 is a typical three-domain-like *Bt*-protein and has no sequence similarity with the binary Cry34Ab1/Cry35Ab1 proteins (Bravo and Soberón, 2008), it acts on WCR midgut receptors independently of Cry34Ab1/Cry35Ab1 (US EPA, 2010a,b; Gassmann, 2012; Gassmann et al., 2011, 2012). Cry3Bb1 is, however, more similar to mCry3A than Cry34Ab1/Cry35Ab1, and therefore cross-resistance is more likely between Cry3Bb1 and mCry3A (Rausell et al., 2004; Crickmore et al., 2013). Based on the analysis of WCR midgut membrane binding sites, Li et al. (2013) demonstrated the lack of shared binding sites for Cry34Ab1/Cry35Ab1 and Cry3Aa, Cry6Aa or Cry8Ba. These results indicate that midgut receptors involved in the mechanism of action differ between Cry34Ab1/Cry35Ab1 and the other proteins, and therefore suggest a low likelihood of receptor-mediated cross-resistance between Cry34Ab1/Cry35Ab1 and Cry3Aa, Cry6Aa or Cry8Ba.

The pyramiding[117] in the same plant of two or multiple toxins acting independently on WCR midgut receptors is also expected to delay the evolution of resistance to either toxin effectively when most individuals that are resistant to one toxin are killed by the other, and when selection for resistance to one of the toxins does not cause cross-resistance to the other (Roush, 1998; Zhao et al., 2005; Storer et al., 2012). In the absence of cross-resistance, model predictions by Onstad and Meinke (2010) showed that evolution of resistance to a *Bt*-protein in WCR is generally

[117] A pyramided *Bt*-crop combines related traits such as insect resistance against target insect pest species of the same Order.

delayed by pyramided traits in *Diabrotica*-active *Bt*-maize compared with two single traits deployed sequentially. However, in populations in which WCR has begun adapting or has evolved resistance to one of two *Bt*-proteins, the benefit from pyramiding may be diminished or offset, respectively. The efficacy of pyramided *Diabrotica*-active *Bt*-maize will also be diminished if cross-resistance occurs. However, factors facilitating greater larval survival on pyramided *Bt*-maize than the additive effect of the individual proteins have not yet been identified (Hibbard et al., 2011).

(3) The use of insecticides or biological control agents when and where necessary is an additional essential component of integrated pest management. *Diabrotica*-active *Bt*-maize is being used prophylactically in US areas with little or no need for it. Under these conditions, Porter et al. (2012) argued that planting non-*Diabrotica*-active *Bt*-maize can be profitable and should be one of the integrated pest management tools to maintain the sustainability of *Bt*-maize; non-*Diabrotica*-active *Bt*-maize, used in conjunction with soil-applied insecticides or not, would not cause selection for resistance. Treatment of *Diabrotica*-active *Bt*-maize with insecticides targeting WCR should be considered only under special circumstances, and is therefore not a recommended routine management strategy, as it masks the geographic extent and in-field severity of *Bt*-resistance and selects for resistance to the insecticides (Porter et al., 2012). In addition, entomopathogenic nematodes can serve as biological control agents (Toepfer et al., 2008, 2009, 2010; Meissle et al., 2009; Petzold-Maxwell et al., 2012a,b). The EFSA GMO Panel considers that the decision to apply WCR management measures should be based on scouting, past experience and the population density of adult WCR in the preceding year's crop.

The EFSA GMO Panel pinpoints the importance of implementing educational (training) programmes to encourage farmers to establish appropriate refuges and to ensure compliance with the insect resistance management requirements recommended by risk managers.

6.3.1.2. Conclusion on risk mitigation measures

The EFSA GMO Panel evaluated the efficacy and made recommendations on the scientific quality of the insect resistance management plan proposed by the applicant. While caution must be exercised when extrapolating laboratory and greenhouse results to field conditions, evidence indicates that several conditions contributing to the success of the high dose/refuge strategy are not met for maize 59122 and WCR (see above). Scientific uncertainties related to the appropriateness of the proposed strategy in delaying resistance evolution in WCR remain. Therefore, the EFSA GMO Panel does not accept the high dose/refuge strategy as the sole insect resistance management strategy, and requires that the applicant's insect resistance management plan should be complemented with additional resistance management measures. Stewardship agreements should prescribe diversity in cropping and WCR management practices, and recommend: (1) rotating fields to crops that are not hosts of WCR larvae; (2) alternating maize 59122 with other *Bt*-maize events that express one or more different *Bt*-protein(s) active against WCR; and (3) using additional pest management measures, such as insecticides or biological control agents, only when and where necessary in maize 59122 fields. The additional recommendations made by the EFSA GMO Panel to revise the applicant's insect resistance management plan in terms of refuge requirements should also be implemented by the applicant.

The EFSA GMO Panel recommends that resistance and compliance monitoring is conducted to allow the periodic evaluation of the adequacy and efficacy of the revised insect resistance management strategy.

Models developed to estimate the evolution of resistance in WCR populations predicted that a 20 % refuge can delay resistance evolution for maize 59122 under certain conditions. However, the EFSA GMO Panel considers that some of the underlying model assumptions and parameter values used in these models are not sufficiently conservative and mainly represent best-case scenarios. Moreover, each model is subject to scientific uncertainties. Therefore, caution is recommended when predicting the future responses of WCR in specific regions based on other target insect pest species, or on

experiences elsewhere, as resistance evolution is dependent upon many factors. The EFSA GMO Panel recommends that further research is conducted by the applicant to improve future modelling predictions of resistance in WCR populations due to the cultivation of maize 59122.

If appropriate insect resistance management measures are implemented, the EFSA GMO Panel concludes that resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests could be successfully delayed.

The NL CA acknowledged the potential for resistance to the Cry34Ab1/Cry35Ab1 proteins to evolve within the *Diabrotica* spp. population. The NL CA noted that "*the insect resistance management approach proposed by the applicant will only be adequate in case of recessive inheritance of Bt-resistance*", but did not assess the appropriateness of the insect resistance management plan further (Section 7.1 of the environmental risk assessment report of the NL CA).

6.3.2. Post-market environmental monitoring[118]

Directive 2001/18/EC introduces an obligation for applicants to implement a post-market environmental monitoring plan, in order to trace and identify any direct or indirect, immediate, delayed or unanticipated effects on human health or the environment of GMOs as or in products after they have been placed on the market. This plan should be designed according to Annex VII of the Directive. According to Annex VII, the objectives of a post-market environmental monitoring plan are: (1) case-specific monitoring—to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO or its use in the environmental risk assessment are correct (i.e., hypothesis based); and (2) general surveillance—to identify the occurrence of adverse effects of the GMO or its use on human health or the environment which were not anticipated in the environmental risk assessment (Sanvido et al., 2005, 2009, 2011a,b; EFSA, 2006b, 2011c).

6.3.2.1. Case-specific monitoring

The EFSA GMO Panel recommends case-specific monitoring to monitor (1) resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests, and to resolve the remaining scientific uncertainties pertaining to: (2) modelling predictions of resistance in WCR populations owing to the cultivation of maize 59122; and (3) the potential of the Cry34Ab1/Cry35Ab1 proteins to accumulate and persist in soil following subsequent years of continuous maize 59122 cultivation.

In its evaluation report, the NL CA expressed reservations about the conclusions of the applicant that no negative effects were found in one of the two lower-tier studies with the surrogate coccinellid species *C. maculata*. The NL CA noted that "*although laboratory toxicity testing demonstrated a possible adverse effect on the growth of C. maculata larvae; in the field no such effect was observed on ladybird beetles*". Nonetheless, the NL CA was of the opinion that "*the applicant should incorporate specific monitoring for ladybird beetles*" in the frame of case-specific monitoring (Section 7.1 of the environmental risk assessment report of the NL CA). The EFSA GMO Panel agrees that the submitted data show that Cry34Ab1/Cry35Ab1 proteins may be toxic to *C. maculata* at dose levels that exceed field exposure. However, adverse effects were not seen at field dose levels when *C. maculata* larvae were fed a mixture of natural prey and pollen. Because *C. maculata* is not indigenous to Europe, the EFSA GMO Panel requested additional data on a representative European coccinellid species. In response, the applicant provided lower-tier studies (including tritrophic experiments) with the focal species *C. septempunctata*. Based on the additional toxicity data and estimated worst-case expected environmental concentrations, no hazard to *C. septempunctata* and no risk to coccinellids are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122. Therefore, the EFSA GMO Panel concludes that case-specific monitoring of coccinellids is not necessary.

[118] Technical dossier/Section D9.10//Additional information received on 02/10/2012/Request 5/page 22/Appendix 5.

The EFSA GMO Panel evaluation of the efficacy and scientific quality of the case-specific monitoring proposed by the applicant is described below.

Resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests

The applicant proposed to follow a two-pronged approach to detect early warning signs indicating increases in tolerance in the field. This approach consists of: (1) measuring the baseline susceptibility of WCR populations to the Cry34Ab1/Cry35Ab1 proteins and changes in that susceptibility in the EU; and (2) monitoring of unexpected field damage caused by WCR. The EFSA GMO Panel considers these two approaches to be complementary, because monitoring for WCR susceptibility is more likely to detect changes in susceptibility occurring on a broader spatial scale than reports of unexpected field damage that target the detection of localised resistance. The EFSA GMO Panel agrees with this approach and considers it adequate to detect early warning signs of increased tolerance, so that actions to limit the survival of resistant insects and to slow or prevent their spread, should resistance have evolved among field populations, can be taken timely. Acquired data will also contribute to resolving the remaining scientific uncertainties related to the appropriateness of the high dose/refuge strategy in delaying resistance evolution in WCR. In addition, those data may allow the periodic evaluation of the adequacy and efficacy of the revised insect resistance management strategy. To ensure that any resistance is detected timely, resistance monitoring should be performed annually.

(1) *Baseline and monitoring WCR susceptibility*: Resistance monitoring aims to measure the baseline susceptibility of WCR to the *Bt*-proteins and shifts in that susceptibility over time. This baseline susceptibility should represent the natural variability in response to the *Bt*-proteins among WCR populations across their geographic distribution range, preferably prior to the first introductions of maize 59122 (Siegfried et al., 2005). To obtain comparable data and to detect actual shifts in susceptibility at an early stage, a consistent methodology in terms of sampling, laboratory bioassays and toxin standardisation is required (Andow, 2008; Tabashnik et al., 2008a, 2009; Siegfried and Spencer, 2012; Devos et al., 2013).

- The EFSA GMO Panel recommends utilising appropriate sampling strategies to collect individuals in the field. Setting the most adequate and precise susceptibility baselines can be achieved through random sampling. Measuring shifts in that susceptibility can be realised through targeted sampling in areas in which the selection pressure is believed to be highest and which correspond to those areas where WCR populations are known to regularly reach high infestation levels and where maize 59122 uptake is highest ('hotspot areas'). The target pest population needs to be large enough to provide sufficient numbers of healthy individuals for collection. Widely adopted guidelines for sampling corn borers recommend the sampling of 200 larvae, 200 adults, 100 mated females or 100 egg masses per sampled population and set the minimum population size considered to be a valid sample for testing at 50 larvae, 50 adults, 25 mated females or 25 egg masses. Similar guidelines for sampling WCR are under development (Siegfried and Spencer, 2012). The sampling strategy should include fields cropped to maize 59122 and adjacent fields cropped to non-Cry34Ab1/Cry35Ab-expressing maize or conventional maize, annual sampling during each maize growing season, follow-up sampling of the same populations in subsequent seasons and sampling at appropriate times. As resistance is less likely to evolve rapidly in maize-growing areas with a low uptake of maize 59122, sampling in such areas could be at a lower frequency, compared with hotspot areas, and serve to establish susceptibility baselines. Baseline data should be established preferably before the first introductions of maize 59122, but at least during the initial years of its launch prior to high market penetration. Ideally, the same areas should be monitored over time to reduce the natural geographical variation in susceptibility (Farinós et al., 2004, 2011, 2012; Saeglitz et al., 2006; Gaspers et al., 2011). Appropriately designed sampling strategies should account for the abundance, distribution and dispersal behaviour of WCR, and local variability in susceptibility levels.

- Most resistance monitoring studies have used insect diet bioassays to determine LC_{50} and EC_{50} values in individuals derived from field-collected populations exposed to *Bt*-crops, and to compare those with that of susceptible laboratory reference or non-exposed field-derived colonies (Siegfried et al., 2007; Andow 2008; Tabashnik et al., 2008a, 2009; Siegfried and Spencer, 2012). The estimation of LC_{50} and EC_{50} values and the establishment of dose–response relationships require data from several toxin concentrations, and allow the calculation of resistance ratios. An increase in the resistance ratio indicates a decrease in susceptibility, which may be heritable. The dose–response bioassay method has proved adequate for documenting resistance that reached high levels, but is insensitive to small changes in resistance allele frequency, especially in the early stage of resistance evolution when resistance is first appearing and the frequency of resistant individuals is relatively low (Bourguet et al., 2005; Siegfried et al., 2007; Tabashnik et al., 2009; Siegfried and Spencer, 2012).

  Alternatively, susceptibility testing is performed with larvae (F_1 offspring) obtained from field-collected individuals using a diagnostic or discriminating dose of the *Bt*-protein incorporated into an artificial diet (Siegfried et al., 2007; Andow, 2008; Tabashnik et al., 2008a, 2009; Siegfried and Spencer, 2012). Such a dose, when carefully selected, ensures 100 % mortality of fully susceptible WCR populations (LC_{99}), survival of only resistant individuals, and discrimination between resistant and susceptible individuals. Decreased susceptibility and potential field-selected resistance are then demonstrated as the percentage of individuals surviving exposure to a fixed amount of the *Bt*-protein. Ideally, resistant individuals are needed to determine the discriminating dose, but in the absence of resistant individuals, some multiple of the LC_{50} or LC_{99} for susceptible larvae is commonly used (Andow, 2008). The discriminating dose bioassay is a cost-effective method that allows the testing of many individuals at an appropriate dose, and will detect low frequencies of both polygenic and multiple resistance (Bates et al., 2005). However, Bourguet et al. (2005) indicated that the discriminating dose bioassay is more likely to detect dominant resistance alleles, and would be inefficient at detecting recessive alleles in heterozygotes (see also Siegfried and Spencer, 2012). As individuals heterozygous for a recessive resistance allele have a susceptible phenotype, they will not survive the discriminating dose, and therefore reliable detection of allele frequencies below 10 % (0.1) is impractical (Siegfried et al., 2007).

  The F_2 screen was proposed as a method to detect rare and highly recessive resistance alleles in a heterozygous state (Andow and Alstad, 1998, 1999; Andow and Ives, 2002). This methodology involves establishing single-female family lines from a large number of field-collected individuals by inbreeding the offspring of each collected female within family lines. The offspring of these matings (i.e., the F_2 of the collected generation) are then screened at a discriminating dose to detect any homozygous individuals (Zhao et al., 2002). The purpose of the inbreeding process is to allow potentially heterozygous offspring of the field-collected females to mate with each other, generating a significant and easily detectable fraction of homozygous resistant offspring. Through back-calculation of the frequency of family lines containing a resistant allele, the frequency of the resistance allele in the sampled population can be estimated (Siegfried and Hellmich, 2012; Siegfried and Spencer, 2012). The F_2 screen is far more sensitive than a discriminating dose bioassay to detect recessive traits, although it does not allow the detection of polygenic resistance (Zhao et al., 2002). Moreover, it does not require obtaining a resistant WCR colony beforehand. However, given the time and effort required for the F_2 screen, the fact that resistance to Cry34Ab1/Cry35Ab1 is most probably polygenic and that resistance alleles may be more common than initially thought in WCR, the F_2 screen may not offer significant benefits over the dose–response and discriminating dose bioassay.

  The surface treatment of diet is usually utilised in diet bioassays to generate dose–response curves, or to discriminate between resistant and susceptible individuals. Instead of

incorporating the *Bt*-protein uniformly in the diet, it is added to the surface of the diet (e.g., Marçon et al., 1999; Siegfried et al., 2000, 2005; Farinós et al., 2004, 2011, 2012; Blanco et al., 2008; Gaspers et al., 2011). Important advantages of the surface treatment are the lower amount of *Bt*-protein required for each test and the reduction in costs associated with *Bt*-protein preparation (Blanco et al., 2008). However, compared with the surface treatment, the incorporation technique allows a more homogeneous distribution of the *Bt*-protein solution in the diet and thus a consistent exposure of each larva. As larvae may be exposed inconsistently to the *Bt*-protein when directly burrowing and feeding into the diet instead of grazing on the diet surface, this technique may be prone to error. However, a side-by-side comparison between the surface and incorporation treatment led to similar levels of variability in susceptibility, indicating that there are no major differences between both techniques (Saeglitz et al., 2006; Siegfried et al., 2007). Further, Siegfried and Spencer (2012) pinpointed the fact that strict quality control of bioassays using surface treatment through visual inspection is essential to minimise potential inconsistencies in terms of non-uniform treatment and inconsistent exposure of larvae (see also Gaspers et al., 2011). Given the costs associated with *Bt*-protein preparation, its instability and limitations in the amount that can be produced, Siegfried et al. (2007) considered that the advantages of the surface treatment outweigh the possible increased uniformity of exposure that may be associated with incorporating the *Bt*-protein in rearing diets.

The sensitivity of diet-based bioassays used to monitor WCR susceptibility in order to detect WCR resistance has been questioned (Nowatzki et al., 2008; Siegfried and Spencer, 2012). This is because WCR larvae are not that susceptible to Cry3Bb1, Cry34Ab1/Cry35Ab1 and mCry3A, so achieving significant mortality in WCR larvae can be challenging even at the highest toxin doses used. In addition, available WCR baseline susceptibility data for Cry3Bb1 (Siegfried et al., 2005; US EPA, 2011a), Cry34Ab1/Cry35Ab1 (US EPA, 2010b) and mCry3A (US EPA, 2007) have shown that the range of natural variation in baseline susceptibility can be greater than five-fold (Siegfried and Spencer, 2012).[119] Therefore, discerning populations with decreased susceptibility from those with actual resistance to the *Bt*-protein can be challenging. The consequence is that small changes in *Bt*-protein susceptibility, which could significantly affect product performance, could go undetected (Nowatzki et al., 2008). Additional challenges are that artificial diets are prone to microbial contamination, resulting in high rates of control mortality, WCR larvae may survive without feeding in three-day diet bioassays leading to an underestimation of the actual percentage mortality due to exposure to the *Bt*-protein, and only one generation of WCR can be tested in a given year, as WCR is univoltine and its life cycle involves an obligatory egg diapause (US EPA, 2011a; Siegfried and Spencer, 2012).

Alternative methods to the dose–response and discriminating dose bioassays such as the sublethal seedling assay have been developed (Nowatzki et al., 2008) and applied to detect resistance in WCR (Lefko et al., 2008; Gassmann et al., 2011, 2012). Alves et al. (2012) also used the sublethal seedling assay to assess within and among population variation in susceptibility of neonate WCR to maize 59122 from six populations across Europe over two years. The sublethal seedling assay consists of exposing populations of neonate WCR to seedlings from either *Bt*-maize or the near-isogenic line for a fixed duration of time (usually 17 days) and measuring the total larvae recovered and age structure of the larval population. Delays in larval development are detected in the distribution of the different larval instars, which are determined based on head capsule width (Hammack et al., 2003). Higher proportions of later instars recovered on *Bt*-maize roots during the fixed duration of exposure to maize roots indicate higher rates of larval population development and increased tolerance to *Bt*-maize. This method has proved adequate to detect subtle changes in population susceptibility and is more sensitive than the standard diet bioassays that typically rely on

[119] Additional information received on 02/10/2012/Request 2/page 13/Annex: Storer and Owens (2009)//16/01/2012/Annex: Lepping et al. (2011).

mortality or growth inhibition as endpoints (Lefko et al., 2008; Nowatzki et al., 2008). This can be attributed to the more ecologically relevant larval exposure that is similar to that under field conditions, and to the use of the increased sensitivity of a sublethal endpoint (Lefko et al., 2008).

- The susceptibility of the target insect pest has been shown to vary considerably depending upon the source of *Bt*-protein used. Therefore, the same *Bt*-protein source should be used throughout the duration of resistance monitoring (Farinós et al., 2004; Saeglitz et al., 2006; US EPA, 2011a).

The EFSA GMO Panel concludes that the F_2 screen is not proportionate for routine resistance monitoring in the case of maize 59122, and that the dose–response and discriminating dose assays using the same *Bt*-protein source are suitable methods for monitoring WCR susceptibility and therefore should be adopted. The EFSA GMO Panel recognises the limitations of the current diet-based bioassay methods, but considers that results from these assays can assist in monitoring for WCR resistance. The top Cry34Ab1/Cry35Ab1 concentrations used in bioassays should be sufficiently high to cause consistently > 50 % mortality for either field or laboratory WCR populations. Should a more sensitive method than the dose–response and discriminating dose assays be required to detect subtle changes in population susceptibility, the sublethal seedling assay should be used. Resistance monitoring, through targeted field sampling in areas where maize 59122 uptake is the highest and selection pressure is greatest, should reveal changes in susceptibility of these WCR populations.

(2) *Unexpected field damage caused by WCR*: The monitoring of greater-than-expected field damage due to WCR is an important component of the early detection of resistance, as it allows capturing early warning signs indicating increased tolerance in the field and reporting those timely. Greater-than-expected field damage resulting from WCR control failures can easily be observed and reported by farmers, provided that farmers know what level of WCR damage is to be expected under various conditions and what level of WCR control is normally achieved (see Gassmann et al., 2011; Gray, 2011a,b; US EPA, 2011a). Ideally, a comparison of performance of maize 59122 and refuge plants should occur; if damage levels on maize 59122 plants surpass economic thresholds and exceed those observed on refuge plants, then field resistance could be a concern. Such observations may reveal the occurrence of localised tolerance before it spreads, and may serve as a trigger for further investigation. For maize 59122 the EFSA GMO Panel recommends setting the greater-than-expected field damage threshold at 1.0 (node-injury scale; Oleson et al., 2005; US EPA, 2011a). This could serve as a trigger: (i) to instruct farmers to use alternative WCR management options; and (ii) to initiate sampling of WCR adults in the fields of concern for the purpose of further evaluation and laboratory testing to confirm potential resistance. If adult beetles cannot be collected from problem fields during the season, adult sampling should occur in the problem area the following season, irrespective of the pest infestation levels and damage in the problem year (US EPA, 2011a). The majority of WCR adults do not disperse long distances, so the greatest probability of capturing resistant genotypes is in problem fields and, possibly, in adjacent fields. Sampling in neighbouring fields is reasonable during the following year, as adults may have moved from the problem fields to those fields, but only after in-field collection in problem fields has occurred.

The EFSA GMO Panel concludes that greater-than-expected field damage due to WCR control failures should be reported. The EFSA GMO Panel considers that farmer questionnaires provide a relevant early alert system to report unexpected field damage caused by WCR larvae (see Section 6.3.2.2). Stewardship agreements should specify what level of WCR damage is to be expected under various conditions and what level of WCR control is normally achieved. Additional communication mechanisms should be put in place for the timely reporting of farmer complaints regarding maize 59122 performance. It is critical that responses to farmer complaints about product failure and hence greater-than-expected field damage are made timely, so that

suspected resistance can be declared confirmed resistance and remedial measures be implemented, or be refuted without undue delays.

The EFSA GMO Panel concludes that the overall framework to monitor resistance evolution proposed by the applicant is consistent with those described in the scientific literature, but requests that its recommendations to strengthen it are implemented.

Modelling predictions of resistance in WCR populations

Models developed to estimate the evolution of resistance in WCR populations predicted that a 20 % refuge can delay resistance evolution for maize 59122 under certain conditions. However, the EFSA GMO Panel considers that some of the underlying model assumptions and parameter values used in these models are not sufficiently conservative and mainly represent best-case scenarios. Moreover, each model is subject to scientific uncertainties. Therefore, caution is recommended when predicting the future responses of WCR in specific regions based on other target insect pest species, or on experiences elsewhere, as resistance evolution is dependent upon many factors. The EFSA GMO Panel recommends that further research is conducted by the applicant to improve future modelling predictions of resistance in WCR populations due to the cultivation of maize 59122.

Potential of the Cry34Ab1/Cry35Ab1 proteins to accumulate in soil

Based on general knowledge of the degradation of plant-produced *Bt*-proteins in soils and the overall concentrations of Cry34Ab1/Cry35Ab1 proteins in maize 59122, it is unlikely that the Cry34Ab1/Cry35Ab1 proteins will reach soil concentrations that would affect non-target organisms, in the context of the intended uses of maize 59122. Although no risk was identified in the short term, scientific uncertainties pertaining to the specific potential of Cry34Ab1/Cry35Ab1 to accumulate and persist in soil during subsequent years of cultivation of maize 59122 remain, owing to the lack of experimental evidence. Therefore, the EFSA GMO Panel recommends that the remaining scientific uncertainties can be resolved with data acquired during post-market environmental monitoring.

Data on the fate of the Cry34Ab1/Cry35Ab1 proteins in soil may be gathered either via a laboratory-based study, or by quantifying the concentration of *Bt*-proteins from soil samples, which originate from representative fields previously cultivated with maize 59122 (see below).

In the case of a laboratory study, the fate of the Cry34Ab1/Cry35Ab1 proteins should be studied over a period of up to 20 weeks or until degradation by at least two orders of magnitude. Initial concentrations of the Cry34Ab1/Cry35Ab1 proteins in soils should not be above 1–10 µg/g soil, corresponding to the amount present in 0.1–1 g of root material of maize 59122. The Cry34Ab1/Cry35Ab1 proteins should be extracted from the soils with a buffered solution, which also elutes the particle-adsorbed proteins, or a defined proportion of them. The buffer should stabilise the protein to avoid their disintegration. It is suggested that these analyses be conducted in the context of soil incubations at room temperature or below, and use the Cry1Ab protein as a reference, thus allowing comparison of the fate of the Cry34Ab1/Cry35Ab1 proteins in soil to the as yet best characterised plant-produced *Bt*-protein. It is recommended that these studies be conducted with three native (not sterilised) field soils differing in their texture (one should have a clay content of 30 % or above) and pH (one should have a pH value above 7, one below). Quantifications should be conducted with ELISA using appropriate positive controls to determine their concentration.

Alternatively, the applicant may quantify Cry34Ab1/Cry35Ab1 on agricultural or experimental fields with three different representative soils, as described above, on which maize 59122 has been consecutively cultivated for a minimum period of five years. The field sites should be representative of climatic regions with relevance for maize cultivation in Europe. The soil sampling strategy should include independent replicates to consider the heterogeneity of agricultural fields, and the limit of detection should not be above 10 ng/g soil. Sampling of bulk soil should be conducted starting in the second year on an annual basis before seeding of maize 59122. The extraction should be conducted

with a buffered solution, as described above, to achieve maximum extraction efficiency and stabilise the extracted proteins to be detectable and quantifiable by ELISA.

6.3.2.2. General surveillance

The applicant proposed conducting general surveillance for maize 59122 throughout the period of validity of the authorisation. The general surveillance will take into consideration and be proportionate to the extent of cultivation of maize 59122 in the EU Member States. The applicant proposed to build its general surveillance on four approaches: (1) the use of annual farmer questionnaires; (2) the review of scientific information provided by existing monitoring networks; (3) the monitoring and review of ongoing research and development, as well as scientific literature; and (4) the implementation of industry stewardship programmes, in order to identify potential adverse effects associated with the intended uses of maize 59122. The EFSA GMO Panel evaluation of the efficacy and scientific quality of the general surveillance approach proposed by the applicant is described below.

(1) Farmer questionnaires

The EFSA GMO Panel agrees with the general surveillance approach of the applicant to establish farmer questionnaires as a reporting format that provides relevant information. The questionnaires to farmers exposed to or using GM plants are regarded by the EFSA GMO Panel as an adequate tool to address several aspects of general surveillance (EFSA, 2006b, 2011c). The EFSA GMO Panel is of the opinion that farmer questionnaires enable the reporting of any on-farm observations of effects associated with the cultivation of maize 59122, as this approach uses first-hand observations and relies on farmers' knowledge and experience of their local agricultural environments, comparative crop performance and other factors that may influence events on their land (Schmidt et al., 2008; Wilhelm et al., 2010). Some of the questions link directly to assessment endpoints or give indirect indications of effects on assessment endpoints (EFSA, 2011c).

Farmer questionnaires should be designed to determine whether any unanticipated effects have occurred as a result of cultivation of the GM plant. The farmer/manager/worker should be asked to record any differences they observe between the management of the GM crop and a conventional (non-GM) crop of the same crop type, either on the same farm or cultivated nearby or in previous years (EFSA, 2011c). The applicant and risk managers are advised to consider the new EFSA GMO Panel guidelines on post-market environmental monitoring (EFSA, 2011c) and the specific recommendations on the annual post-market environmental monitoring report of maize MON 810 cultivation in 2009 and 2010 (EFSA, 2011d, 2012) when finalising and/or or evaluating their monitoring plans.

Farmer questionnaires should also be designed to collect information on the implementation of refuges, technology adoption levels and farmer use patterns (such as applied pest management practices). Such information will give indications on whether farmers followed and adhered to the insect resistance management (including refuge) requirements when growing maize 59122, and hence on compliance levels (EFSA, 2011c,d, 2012). Specific questions on the following should be considered: the proportion of non-*Bt*-maize compared with *Bt*-maize such as maize 59122 on the farm; refuge plant configurations; the distance between the refuge and the monitored *Bt*-maize field if the refuge is planted as a separate field adjacent to the *Bt*-maize field; differences in pest management practices of the refuge; and whether the separate refuge has been cropped with non-*Bt*-maize for at least two successive years. The reporting of non-compliance with refuge requirements, especially in areas where the uptake of maize 59122 is high, may serve as a trigger to strengthen education (training) programmes to aid farmers in understanding the importance of adhering to refuge requirements, and to impose penalties for non-compliance (such as the lack of access to the technology for deviations from the refuge requirements).

While the EFSA GMO Panel considers the format and contents of the farmer questionnaire, as provided by the applicant, comprehensive, it proposes the following modifications:

- In addition to the occurrence of (GM) volunteer maize from previous crops (whenever relevant), questions should be added on the possible occurrence and observation of (GM) feral maize plants (if any) in field margins for the consideration of unanticipated effects on the potential persistence and invasiveness of maize 59122;

- In addition to the questions on pest and disease incidences on maize 59122, the farmer questionnaire should specifically request information on the occurrence of possible unexpected field-damaged maize 59122 plants which might be associated with WCR control failures, as this information will complement the case-specific monitoring of the possible evolution of resistance to the Cry34Ab1/Cry35Ab1 proteins in target pests. It is critical that responses to farmer complaints about maize 59122 failure and hence greater-than-expected field damage are taken timely, so that suspected resistance in WCR can be declared confirmed resistance and remedial measures be implemented, or be refuted without undue delays (see above);

- Questions should be added on the proportion of non-Cry34Ab1/Cry35Ab1-expressing maize compared with maize 59122 on the farm, the distance between the refuge area and the monitored maize 59122 field in case the refuge is planted as a separate field adjacent to the *Bt*-maize field, the differences in pest management practices of the refuge, and whether the refuge has been cropped with non-Cry34Ab1/Cry35Ab1-expressing maize for at least two successive years.

In line with the general recommendations on the farmer questionnaire set out in its 2011 Scientific Opinion on post-market environmental monitoring (EFSA, 2011c), the EFSA GMO Panel advises that farmer questionnaires:

- are designed to ensure the appropriate statistical validity and representativeness of the collected data, including the proportion of fields growing maize 59122 in a region and a minimum percentage or number of questionnaires required to achieve statistical power in the data collected;

- are designed to generate data on the agronomic management of maize 59122, as well as data on impacts on farming systems and the farm environment;

- use a field or group of fields growing maize 59122 as the basic unit for monitoring in representative farming regions and for representative cropping systems within the country: the precise fields should be identified, so that their locations can be subsequently retrieved from registers of GM plant sites;

- clearly identify the comparator (e.g., variety, location) and whether it is being grown adjacent to maize 59122, on the same farm or in another location; if no comparators are being grown spatially or temporally close to maize 59122, then the rationale for selecting another comparator (e.g., historical data) should be fully described;

- where appropriate, observe the field/fields in subsequent years for any unusual residual effects;

- provide information on other GM plant events being grown at the same sites and farms;

- are adapted, where needed, to each GM plant monitoring on a case-by-case basis by considering additional data requirements relevant to each species/event, its management and its receiving environments;

- are user friendly but also information rich;

- are constructed to encourage independent and objective responses from farmers, land managers and others involved with maize 59122 or its transgene products;

- are audited to ensure the independence and integrity of all monitoring data.

In addition to the general recommendations on the farmer questionnaire (EFSA, 2011c) and in line with its 2011 Scientific Opinion on the annual post-market environmental monitoring report on maize MON 810 cultivation in 2009 and 2010 (EFSA, 2011d, 2012), the EFSA GMO Panel advises the applicant to take into account the following points:

- The sampling frame should be comprehensive and a stratification should be applied consistently in each country. Adequate sampling should be carried out from the previous stratification exercise;

- Cultivation areas with high uptake of maize 59122, and where maize 59122 has been continuously grown in previous years, should be over-represented in the sampling scheme;

- The number of farmers not participating in the survey and the reasons thereof should be documented;

- Impartial and standardised interviews should be carried out by independent parties and effective quality and auditing procedures should be considered;

- Questions additional to the farmer questionnaire should be considered to better describe the cultivation of *Bt*-maize in the local area and/or the previous years, the receiving environments and the management systems in which maize 59122 is being grown;

- Relevant data as from other sources of information (e.g., official statistics on crop management practices) should/could be considered for validity checking of the questionnaires (e.g., consistency, representativeness);

- The raw data, programmes, logs and output files related to the statistical analysis of the farmer questionnaires should be provided. Confidence intervals for the analysis of the monitoring characteristics should be included in the statistical report;

- Appropriate statistical procedures should be used based on using a distribution for appropriate outcomes;

- The use of a standard default effect size of 5 % is not relevant to all assessment endpoints and, where scientifically justified, different default effect sizes should be considered for some assessment endpoints;

- Data should be pooled and statistically analysed over years. At the end of the ten years of general surveillance, the applicant should conduct a statistical analysis with all pooled data;

- A codification for farmers repeatedly surveyed over years should be set up. These farmers should be monitored in particular;

- The number of years the surveyed farmer has grown maize 59122 and other GM plants should be indicated.

The NL CA concluded that "*general surveillance will take place through a predefined format that will be provided to the growers and other users of 59122 maize. An example of the format is included in Annex VII. This is considered to be sufficient. It is indicated by the applicant that reporting to the EC will take place immediately if any adverse effects arising from 59122 maize will be reported. Other reporting of results of the case-specific and general surveillance will be according to the requirements of the consent*". The NL CA advised to report results on an annual basis (Section 7.2 of the environmental risk assessment report of the NL CA).

(2) Existing monitoring networks

The EFSA GMO Panel supports the consideration of additional information sources for general surveillance (EFSA, 2006b, 2011c). Directive 2001/18/EC proposed making use of established routine surveillance networks, in order to obtain data on environmental impacts in the landscape where GMOs are cultivated from a range of existing monitoring networks which observe changes in biota and production practices from farm up to regional level. EU Member States have various networks in place—some of which have a long history of data collection—that may be helpful in the context of general surveillance of GM plant cultivations. Existing monitoring networks involved in routine surveillance offer recognised expertise in a specific domain and have the tools to capture information on important environmental aspects over a large geographical area. The EFSA GMO Panel recognises that existing monitoring networks fully meeting all the needs of the monitoring of GM plant cultivations can be limited (Bühler, 2006; Mönkemeyer et al., 2006; Schmidtke and Schmidt, 2007; Graef et al., 2008; Smit et al., 2012). The development of harmonised criteria for the systematic identification, specification and analysis of existing surveillance networks across the EU is therefore considered important (EFSA, 2011c).

The EFSA GMO Panel agrees with the proposal of the applicant to describe the generic approaches for using existing monitoring networks. The applicant has also given consideration to the use of any future surveys of conservation goals as defined in the Directive 2004/35/EC on environmental liability (EC, 2004) in farming regions where maize 59122 will be cultivated and intends to investigate their suitability for providing data on potential changes in biota.

Knowing the limitations of existing monitoring networks, it is important to describe the processes and criteria that will be used for selecting and evaluating existing monitoring networks for supplying data related to the unanticipated adverse effects of GM plants in general surveillance. Therefore, the applicant, in consultation with Member States, should:

- consider the protection goals, the assessment endpoints and their indicators that could be monitored through existing monitoring programmes;
- identify the type of existing monitoring networks that would be appropriate to survey the protection goals considered to be at risk in the countries where maize 59122 will be grown;
- describe the generic approach and develop more detailed criteria to evaluate existing monitoring networks and how appropriate networks will be selected;
- identify what changes need to be made to these monitoring networks and describe how these might be implemented, and identify gaps in information that could be filled by additional surveys;
- encourage these networks to adopt the proposed modifications and describe how data from these networks will be integrated and assessed.

In addition, when selecting existing monitoring networks to be part of general surveillance, it is recommended that the applicant considers the following points for assessing the suitability of these existing networks to supply relevant general surveillance data:

- the relevance of protection goals and their indicators monitored through existing monitoring networks;
- the type (e.g., raw data) and quality of the data recorded;
- the statistical power and the effect sizes detected by monitoring networks, where appropriate;

- the ease of access to the data collected by existing monitoring networks (e.g., availability of data via Internet, free access to data or access subject to a fee, protected data of ongoing research projects);

- the track record and past performance of existing monitoring networks;

- the methodology used by existing monitoring networks (e.g., sampling and statistical approach) including:

  (1) the spatial scale of data collection (e.g., local, regional, national, zonal): existing monitoring networks focusing on agricultural areas cultivated with GM plants or with conventional plants such as maize, potato (for which GM plants are also available and grown) should be preferred;

  (2) temporal scale of data collection: appropriate frequency of data collection and reporting (e.g., short-term vs. long-term data sets, regularity of data collection);

  (3) other parameters such as the language of the reports, impartiality.

Furthermore, the EFSA GMO Panel recommends that the applicant describes arrangements with any third parties participating in its general surveillance plan. It is recommended that consideration is given to how arrangements for collecting, collating and analysing data will be made, and descriptions are given of how formal agreements, procedures and communication will be established with the European Commission and Member States or other third parties, although detailed arrangements may not have been agreed at the time of the application.

The EFSA GMO Panel also recommends including in the sources of information that support general surveillance of maize 59122 existing monitoring networks that monitor herbicide usage, botanical diversity on farms and the evolution of weed resistance, so that the scientific requirements for the detection of any unforeseen environmental effects due to altered farm management practices associated with maize 59122 cultivation are met.

(3) Monitoring and review of ongoing research and development, as well as scientific literature

An additional approach to support general surveillance is to review all new scientific, technical and other information pertaining to maize 59122, including information on GM plants with similar traits or characteristics, that has emerged during the reporting period. This will include reviewing results from ongoing research and development studies (i.e., variety registration trials) and all publications including peer-reviewed journal articles, conference proceedings, review papers and any additional studies or other sources of information relevant to the cultivation of the plant/trait combination for which the report is being drafted (EFSA, 2011c). This approach enables an assessment of whether the risk assessment conclusions and risk management recommendations made in the context of the original environmental risk assessment remain valid and applicable in the light of new scientific information.

The EFSA GMO Panel recommends that the applicant:

- to cover all relevant peer-reviewed publications, including peer-reviewed journal articles, conference proceedings, review papers and any additional studies or other sources of information relevant to the cultivation of the plant/trait combination for which the report is being drafted;

- to describe the criteria for selecting and evaluating the scientific reliability of publications;

- to adhere to systematic literature review methodology to select relevant papers (EFSA, 2010d).

(4) Industry stewardship programmes

The EFSA GMO Panel welcomes the applicant's proposal to develop stewardship programmes for the introduction, marketing, management and stewardship of maize 59122, because they aim to facilitate compliance with risk management conditions, to ensure that maize 59122 is cultivated in a way that has similar or less environmental impact compared with conventional crop cultivation, and to ensure the sustainable use of the technology. The EFSA GMO Panel advises that these programmes should be made available well in advance of the time of commercialisation so as to allow risk managers to validate the implementation of proportional risk mitigation measures and detailed monitoring plans.

6.3.2.3. Reporting the results of post-market environmental monitoring

The applicant will submit a report on an annual basis covering case-specific monitoring and general surveillance. In the case of adverse effects altering the conclusions of the environmental risk assessment, the applicant will immediately inform the European Commission and Member States. The EFSA GMO Panel agrees with the proposal made by the applicant on reporting intervals. The EFSA GMO Panel recommends that effective reporting procedures are established with the Competent Authorities of Member States and the European Commission as required under the Council Decision 2002/811/EC on monitoring.

The results of post-market environmental monitoring should be presented in accordance with the standard reporting formats established by the 2009/770/EC Commission Decision on standard reporting formats. In addition, it is recommended that the applicant provide raw data, in order to allow different analyses and interrogation of the data and to allow scientific exchange and co-operation between Member States, the European Commission and EFSA. The EFSA GMO Panel recommends that the applicant describes whether the post-market environmental monitoring reports contain cumulative analyses of data with previous years' results.

6.3.2.4. Conclusion on post-market environmental monitoring

The EFSA GMO Panel gave its opinion and made recommendations on the scientific quality of the post-market environmental monitoring plan proposed by the applicant.

The EFSA GMO Panel agrees with the two-pronged approach proposed by the applicant to detect early warning signs indicating increases in tolerance in WCR in the field. This approach consists of: (1) measuring the baseline susceptibility of WCR populations to the Cry34Ab1/Cry35Ab1 proteins and changes in that susceptibility in the EU; and (2) monitoring of unexpected field damage caused by WCR. The EFSA GMO Panel considers these two approaches complementary, because monitoring for WCR susceptibility is more likely to detect changes in susceptibility occurring at a broader spatial scale than reports of unexpected field damage that target the detection of localised resistance. Acquired data will also contribute to resolving the remaining scientific uncertainties related to the appropriateness of the high dose/refuge strategy in delaying resistance evolution in WCR, and allow the periodic evaluation of the adequacy and efficacy of the revised insect resistance management strategy.

The case-specific monitoring plan proposed by the applicant focuses on monitoring resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests only. To resolve the remaining scientific uncertainties pertaining to the modelling predictions of resistance in WCR populations owing to the cultivation of maize 59122, and the potential of the Cry34Ab1/Cry35Ab1 proteins to accumulate and persist in soil following subsequent years of continuous maize 59122 cultivation, the scope of the case-specific monitoring as proposed by the applicant should be extended to include additional studies to address these issues too.

The EFSA GMO Panel accepts the approach of the applicant to general surveillance: (1) to establish farmer questionnaires as a reporting format for any on-farm observations of effects associated with the cultivation of maize 59122; (2) to use existing monitoring networks that observe changes in biota and

production practices from farm up to regional level to obtain data on environmental impacts in the landscape where maize 59122 is cultivated; (3) to review all new scientific, technical and other information pertaining to maize 59122; and (4) to develop stewardship programmes for the introduction, marketing, management and stewardship of maize 59122. However, it requests that its recommendations to strengthen general surveillance are implemented. The EFSA GMO Panel agrees with the reporting intervals and modalities proposed by the applicant.

In its evaluation report, the NL CA expressed reservations about the conclusions of the applicant that no negative effects were found in one of the two lower-tier studies with the surrogate coccinellid species *C. maculata*. The NL CA noted that "*although laboratory toxicity testing demonstrated a possible adverse effect on the growth of C. maculata larvae; in the field no such effect was observed on ladybird beetles*". Nonetheless, the NL CA was of the opinion that "*the applicant should incorporate specific monitoring for ladybird beetles*" in the frame of case-specific monitoring (Section 7.1 of the environmental risk assessment report of the NL CA). The EFSA GMO Panel agrees that the submitted data show that Cry34Ab1/Cry35Ab1 proteins may be toxic to *C. maculata* at dose levels that exceed field exposure. However, adverse effects were not seen at field dose levels when *C. maculata* larvae were fed a mixture of natural prey and pollen. Because *C. maculata* is not indigenous to Europe, the EFSA GMO Panel requested additional data on a representative European coccinellid species. In response, the applicant provided lower-tier studies (including tritrophic experiments) with the focal species *C. septempunctata*. Based on the additional toxicity data and estimated worst-case expected environmental concentrations, no hazard to *C. septempunctata* and no risk to coccinellids are expected from exposure to plant-produced Cry34Ab1/Cry35Ab1 proteins or maize 59122. Therefore, the EFSA GMO Panel concludes that case-specific monitoring of coccinellids is not necessary.

With regard to general surveillance, the NL CA concluded that "*general surveillance will take place through a predefined format that will be provided to the growers and other users of 59122 maize. An example of the format is included in Annex VII. This is considered to be sufficient. It is indicated by the applicant that reporting to the EC will take place immediately if any adverse effects arising from 59122 maize will be reported. Other reporting of results of the case-specific and general surveillance will be according to the requirements of the consent*". The NL CA advised to report results on an annual basis (Section 7.2 of the environmental risk assessment report of the NL CA).

OVERALL CONCLUSIONS AND RECOMMENDATIONS

The EFSA GMO Panel concludes that maize 59122 is unlikely to have any adverse effect on the environment, except for the possible resistance evolution to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests. The EFSA GMO Panel recommends the implementation of appropriate and diversified insect resistance management strategies and case-specific monitoring to delay and monitor the possible evolution of resistance to the Cry34Ab1/Cry35Ab1 proteins in coleopteran target pests, respectively. In addition, the EFSA GMO Panel recommends revision of the applicant's insect resistance management plan and the proposed post-market environmental monitoring plan.

The remaining non-critical scientific uncertainties pertaining to the modelling predictions of resistance in WCR populations owing to the cultivation of maize 59122, and the potential of the Cry34Ab1/Cry35Ab1 proteins to accumulate and persist in soil following subsequent years of continuous maize 59122 cultivation are to be resolved with data acquired during post-market environmental monitoring.

Although maize 59122 is tolerant to glufosinate-ammonium-based herbicides, the EFSA GMO Panel did not assess the potential adverse effects associated with the use of such herbicides on maize 59122, as maize 59122 will not be marketed in the EU as a herbicide-tolerant crop.

This Scientific Opinion also updates the previous EFSA GMO Panel safety evaluation of the food and feed uses, and import and processing of maize 59122 and derived products.

The EFSA GMO Panel concludes that the information available for maize 59122 addresses the scientific comments raised by Member States and that maize 59122, as described in this application, is as safe as its conventional counterpart and commercial maize varieties with respect to potential adverse effects on human and animal health.

If subjected to appropriate management measures, the cultivation of maize 59122 is unlikely to raise safety concerns for the environment.

DOCUMENTATION PROVIDED TO EFSA

1. Letter from the Competent Authority of the Netherlands (NL CA), received on 21 October 2005, concerning a request for placing on the market of maize 59122 submitted by Pioneer Overseas Corporation in accordance with Regulation (EC) No 1829/2003.

2. Acknowledgement letter, dated 10 November 2005, from EFSA to NL CA.

3. Letter from EFSA, dated 31 March 2006 selecting the NL CA to perform the initial environmental risk assessment (ERA) evaluation of application EFSA-GMO-NL-2005-23.

4. Letter from EFSA to applicant, dated 20 November 2006, requesting additional information under completeness check.

5. Letter from applicant to EFSA, received on 28 November 2006, providing additional information under completeness check.

6. Letter from EFSA to applicant, dated 10 January 2007, requesting additional information under completeness check.

7. Letter from applicant to EFSA, received on 26 January 2007, providing additional information under completeness check.

8. Letter from EFSA to applicant, dated 1 March 2007, requesting additional information under completeness check.

9. Letter from applicant to EFSA, received on 8 March 2007, providing additional information under completeness check.

10. Letter from EFSA to applicant, dated 9 March 2007, delivering the 'Statement of Validity' of application EFSA-GMO-NL-2005-23, maize 59122, submitted by Pioneer Overseas Corporation under Regulation (EC) No 1829/2003.

11. Letter from EFSA/NL CA to applicant, dated 13 March 2007, requesting additional information and stopping the clock.

12. Letter from applicant to EFSA, received on 2 April 2007, providing additional information.

13. Letter from EFSA to applicant, dated 13 April 2007, re-starting the clock.

14. Letter from EFSA/NL CA to applicant, dated 30 July 2007, requesting additional information and stopping the clock.

15. Letter from EFSA/NL CA to applicant, dated 8 October 2007, requesting additional information and maintaining the clock stopped.

16. Letter from applicant to EFSA, received on 20 December 2007, providing additional information.

17. Letter from the NL CA delivering the ERA report of application EFSA-GMO-NL-2005-23 to EFSA, received on 13 May 2008.

18. Letter from EFSA to applicant, dated 14 May 2008, re-starting the clock.

19. Letter from EFSA to applicant, dated 29 May 2008, requesting additional information and stopping the clock.

20. Letter from applicant to EFSA, received on 22 September 2008, providing additional information.

21. Letter from EFSA to applicant, dated 1 October 2008, requesting additional information.

22. Letter from applicant to EFSA, received on 8 January 2009, providing additional information.

23. Letter from EFSA to applicant, dated 13 February 2009, requesting additional information and maintaining the clock stopped.

24. Letter from applicant to EFSA, received on 28 April 2009, providing additional information.

25. Letter from EFSA to applicant, dated 28 May 2009, requesting additional information.

26. Letter from applicant to EFSA, received on 27 January 2010, providing additional information.

27. Letter from applicant to EFSA, received on 11 March 2010, providing additional information.

28. Letter from applicant to EFSA, received on 18 March 2010, providing additional information.

29. Letter from EFSA to applicant, dated 30 April 2010, requesting additional information and maintaining the clock stopped.

30. Letter from applicant to EFSA, received on 15 July 2010, requesting clarifications.

31. Letter from EFSA to applicant, dated 16 September 2010, providing clarifications.

32. Letter from applicant to EFSA, received on 16 January 2012, providing additional information.

33. Letter from EFSA to applicant, dated 29 February 2012, requesting additional information and maintaining the clock stopped.

34. Letter from EFSA to applicant, dated 12 July 2012, requesting clarifications.

35. Letter from applicant to EFSA, received on 1 October 2012, providing clarifications.

36. Letter from applicant to EFSA, received 2 October 2012, providing additional information.

37. Letter from EFSA to applicant, dated 9 January 2013, re-starting the clock.

38. Letter from applicant to EFSA, received on 23 January 2103, providing additional information spontaneously.

39. Letter from applicant to EFSA, received on 11 February 2013, providing additional information spontaneously.

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