TECHNICAL REPORT

APPROVED: 25 July 2016

Relevance of a new scientific publication (Bøhn et al., 2016) for previous environmental risk assessment conclusions on the cultivation of *Bt*-maize events MON810 and Bt11

European Food Safety Authority

Abstract

Following a request from the European Commission, the European Food Safety Authority (EFSA) assessed the scientific publication by Bøhn et al. (2016), including its relevance for the environmental risk assessment of *Bt*-maize events MON810 and Bt11 for cultivation. In their publication, Bøhn et al. (2016) reported that the purified Cry1Ab protein is toxic to the non-target aquatic crustacean *Daphnia magna* (Cladocera: Daphniidae) at concentrations exceeding expected environmental concentrations under field conditions, and thus suggesting cross-order activity of the Cry1Ab protein against *D. magna*. EFSA acknowledges that the study reported in the publication by Bøhn et al. (2016) addresses an objective relevant for the environmental risk assessment of *Bt*-plants expressing the Cry1Ab protein for cultivation, as the data can inform environmental risk assessments by determining the activity spectrum of the Cry1Ab protein, and corroborating or rejecting the risk hypothesis of no harm to *D. magna*. However, owing to limitations associated with the design and reporting of the study, EFSA considers that several uncertainties remain, which do not allow a proper interpretation of the effects observed by Bøhn et al. (2016). In addition, EFSA notes that the observed differences were seen at Cry1Ab protein concentrations above expected environmental concentrations under field conditions, and that the authors did not bring their study results in the context of expected exposure levels in the field. As the evidence reported in Bøhn et al. (2016) is insufficient to indicate the necessity to revise the environmental risk assessment conclusions for maize MON810 and Bt11, EFSA considers that the risk assessment conclusions on maize MON810 and Bt11 for cultivation made by the Panel on Genetically Modified Organisms remain valid and applicable.

Key words: *Bt*-maize, Cry1Ab, daphnids, environmental risk assessment, non-target aquatic organisms

Requestor: The European Commission

Question number: EFSA-Q-2016-00390

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Acknowledgements: EFSA wishes to thank Yann Devos and Fernando Álvarez-Alfageme for the preparatory work on this scientific output, and Konstantinos Paraskevopoulos and Elisabeth Waigmann for the support provided to this scientific output.

Suggested citation: EFSA (European Food Safety Authority), 2016. Relevance of a new scientific publication (Bøhn et al., 2016) for previous environmental risk assessment conclusions on the cultivation of *Bt*-maize events MON810 and Bt11. EFSA supporting publication 2016:EN-1073. 16 pp.

Summary

Following a request of the European Commission, the European Food Safety Authority (EFSA) assessed the scientific publication by Bøhn et al. (2016), including its relevance for the environmental risk assessment (ERA) of *Bt*-maize events MON810 and Bt11 for cultivation. EFSA assessed whether the publication contains new information that would change or invalidate its previous ERA conclusions on non-target (NT) aquatic organisms.

In their publication, Bøhn et al. (2016) reported that: (1) the purified Cry1Ab and Cry2Aa proteins are toxic to the NT aquatic crustacean *Daphnia magna* (Cladocera: Daphniidae) at high concentrations; (2) Cry proteins act in combination, suggesting that 'stacked events' may have stronger effects on non-target organisms (NTOs); and (3) further research is needed to assess potential combinatorial effects of multiple Cry proteins and herbicidal active substances.

The findings reported by Bøhn et al. (2016) are relevant for the ERA of *Bt*-plants expressing the Cry1Ab and/or Cry2Aa protein(s) for cultivation. The GMO Panel issued Scientific Opinions on the cultivation of various *Bt*-plants, of which only *Bt*-maize MON810 and Bt11 express the Cry1Ab protein. Because none of the GM plant applications for market authorisation currently under regulatory review by EFSA or for which the GMO Panel issued a Scientific Opinion cover the cultivation of *Bt*-plants expressing the Cry2Aa protein or both the Cry1Ab and Cry2Aa proteins, EFSA restricted the consideration of the findings reported by Bøhn et al. (2016) to the Cry1Ab protein which is expressed in the single *Bt*-maize events MON810 and Bt11.

EFSA acknowledges that *D. magna* represents a member of a taxonomic group not typically tested for terrestrial NTOs, and that early-tier tests with daphnids can inform ERAs by determining the activity spectrum of the Cry1Ab protein, and by corroborating or rejecting the risk hypothesis of no harm to *D. magna*. EFSA is therefore of the opinion that the study reported in the publication by Bøhn et al. (2016) addresses an objective relevant to the NT risk assessment of aquatic organisms in the frame of maize MON810 and Bt11 for cultivation.

Owing to limitations associated with the design and reporting of the study by Bøhn et al. (2016), EFSA considers that several uncertainties remain pertaining to the level of biological activity of the Cry1Ab protein, the suitability of the negative control, and the level of intake of the Cry1Ab protein by *D. magna*. Hence, the study provides insufficient information to allow a proper interpretation of the effects observed.

In addition, EFSA notes that the observed differences were seen at Cry1Ab protein concentrations above expected environmental concentrations under field conditions, and that the authors did not bring their study results in the context of expected exposure levels in the field.

In conclusion, the evidence reported in Bøhn et al. (2016) is insufficient to indicate the necessity to revise the environmental risk assessment conclusions for maize MON810 and Bt11. Therefore, EFSA considers that the GMO Panel risk assessment conclusions on maize MON810 and Bt11 for cultivation remain valid and applicable.

Table of contents

1. Introduction

Following a request of the European Commission, the European Food Safety Authority (EFSA) assessed the scientific publication by Bøhn et al. (2016), including its relevance for the environmental risk assessment (ERA) of *Bt*-maize events MON810 and Bt11 for cultivation. EFSA assessed whether the publication contains new information that would change or invalidate its previous ERA conclusions on non-target (NT) aquatic organisms.

1.1. Background and Terms of Reference as provided by the requestor

EFSA is requested to analyse the publication by Bøhn et al. (2016) and provide the European Commission with a response indicating whether "*the new scientific information contains elements that could lead the GMO Panel to reconsider the outcome of its previous opinions on GM Bt crops*".

2. Data and Methodologies

2.1. Data

In delivering this technical report, EFSA took into account data and findings reported in the publication by Bøhn et al. (2016).

2.2. Methodologies

EFSA took into account the appropriate principles described in the guidelines of the EFSA Panel of Genetically Modified Organisms (referred to hereafter as GMO Panel) for the ERA of GM plants (EFSA, 2010), and relevant scientific publications.

3. Assessment

The EFSA assessment described below is structured into two parts. In the first part of the assessment, the findings reported by Bøhn et al. (2016) are summarised. In the second part, the relevance of the publication for ERA of maize MON810 and Bt11, and its reliability are considered.

3.1. Summary of Bøhn et al. (2016)

Bøhn et al. (2016) assessed the effect of the purified Cry1Ab and Cry2Aa proteins and Roundup® (glyphosate as active herbicidal substance) on the NT aquatic crustacean *Daphnia magna* (Cladocera: Daphniidae). *Daphnia magna* was cultured in medium, and fed algae added to the culture medium. Test substances were dissolved in buffer solutions and added to the culture medium, and lethal and sub-lethal endpoints were measured during the full life-span of the animals (up to 78 days). Test organisms in untreated culture medium served as a control.

No negative effects on mortality, body size, maturation and fecundity were observed when test organisms were exposed to 0.75 mg/L (ppm) of either the Cry1Ab or Cry2Aa protein. A combination of both Cry proteins at 0.75 mg/L plus buffer solutions added to the culture medium resulted in higher mortality. Test organisms exposed to 4.5 mg/L of the Cry1Ab or Cry2Aa proteins, or a combination of both (i.e., 4.5 mg/L of each of the two proteins) added to the culture medium showed significantly higher mortality, lower probability of maturation and lower fecundity (juvenile production), compared to the control.

Exposure to combinations of the Cry proteins (Cry1Ab and Cry2Aa) and Roundup® buffer solution resulted in the stimulation of fecundity in early life-stages compared to the control, but reduced reproductive output at later stages.

Based on the observations made, the authors concluded that: (1) the Cry1Ab and Cry2Aa proteins are toxic to *D. magna* at high concentrations; (2) Cry proteins act in combination, suggesting that 'stacked events' may have stronger effects on non-target organisms (NTOs); and (3) further research is needed to assess potential combinatorial effects of multiple Cry proteins and herbicidal active substances.

3.2. Quality appraisal of Bøhn et al. (2016)

In line with EFSA (2015), the appraisal of study quality, outlined below, comprises an evaluation of both its relevance (appropriateness/usefulness) and reliability (accuracy). Relevance considers the extent to which data and/or tests are appropriate for a particular hazard identification or risk characterisation (EFSA, 2010), whereas reliability refers to the inherent quality and validity of the results.

3.2.1. Relevance for ERA of *Bt*-plants

Not all information on GM plants available in the scientific literature is equally relevant to ERA, i.e., provides information on risks to environmental entities of concern (Sanvido et al., 2012; Garcia-Alonso and Raybould, 2014; Devos et al., 2015; EFSA, 2016) and determinants of exposure that place these entities at risk (Raybould, 2006, 2007, 2010; Gray, 2012; Wolt et al., 2012; Layton et al., 2015; Devos et al., 2016). It is therefore important to assess the relevance of a study in contributing to the knowledge that informs the ERA, and thus the risk hypotheses addressed, taking account of both hazard and exposure.

A typical risk hypothesis addressed during the ERA of GM plants for cultivation is that the novel traits intentionally introduced into the GM plant do not adversely affect NTOs, including those occurring in aquatic environments, at field concentrations. Potential harmful effects on NTOs are evaluated within different tiers that progress from laboratory studies representing highly controlled, worst-case exposure conditions (Tier 1) to bioassays with more realistic exposure to the insecticidal protein (Tier 2) and (semi-)field studies carried out under less controlled conditions (Garcia-Alonso et al., 2006; Romeis et al., 2008; EFSA, 2010).

The relevance of the pathway to harm, indicating how the deployment of GM plants would lead to harm, considered by Bøhn et al. (2016), and the representativeness of *D. magna* as test organism are considered below.

Pathway to harm

By-products from GM plants (e.g., pollen, detritus) can be transported in water courses to downstream water bodies where NT aquatic arthropods can be exposed to transgene product(s) through consumption (Axelsson et al., 2010, 2011; Rosi-Marshall et al., 2007; Chambers et al., 2010; Tank et al., 2010; Dijkhuis et al., 2015).

The likelihood of environmental harm to be realised from GM plants expressing the Cry1Ab and/or Cry2Aa protein(s) depends, among others, on the level of exposure to the GM plant. Exposure and potential impact are expected to be the highest under cultivation conditions, but substantially less under import/processing conditions (EFSA, 2010; Devos et al., 2012; Roberts et al., 2013).

Bt-plants for cultivation

The findings reported by Bøhn et al. (2016) are relevant for *Bt*-plants expressing the Cry1Ab and/or Cry2Aa protein(s) for cultivation. The GMO Panel issued Scientific Opinions on the cultivation of various *Bt*-plants (Bt11, 1507, MON810, MON88017 and 59122), of which only *Bt*-maize MON810 and Bt11 express the Cry1Ab protein. At present, none of the GM plant applications for market authorisation currently under regulatory review by EFSA or for which the GMO Panel issued a Scientific Opinion cover the cultivation of *Bt*-plants expressing the Cry2Aa protein or both the Cry1Ab and Cry2Aa proteins. EFSA therefore restricts the consideration of the findings reported by Bøhn et al. (2016) to the cultivation of the single *Bt*-maize events MON810 and Bt11.

The potential for combinatorial effects between the Cry1Ab and Cry2Aa proteins is not considered further here.

Bt-plants for import/processing

The GMO Panel does not consider interactions of *Bt*-maize plants with NTOs a relevant issue under import/processing conditions, as it is unlikely that environmental harm will be realised under these conditions. Due to the extremely low levels of exposure of NTOs to plant material from occasional feral *Bt*-maize plants arising from seed import spills, no plausible pathway to harm for NT aquatic

organisms could be identified in the context of GM maize applications for import/processing. Therefore, the findings reported by Bøhn et al. (2016) are not considered relevant for *Bt*-maize for import/processing.

Plant protection products

Within the EU, the approval of plant protection products (PPPs) is regulated by the Regulation (EC) No 1107/2009 (repealing Directive 91/414/EEC) and the Regulations (EU) No 283/2013 and 284/2013, which establish the data requirements. The use of PPPs, including their environmental impact once on the market, is regulated by the Sustainable Use Directive 2009/128/EC. The ERA includes the investigation of the fate and behaviour of the pesticide active substance in the environmental compartment soil, water body, groundwater, air, and the evaluation of the effects and of the risk to NTOs (i.e. birds and other terrestrial vertebrates; aquatic organisms; bees and non-target arthropods; earthworms, other soil macro-organisms and micro-organisms; other non-target organisms (flora and fauna) and organisms involved in biological methods for sewage treatment). The risk assessment of herbicidal active substances is therefore not in the remit of the GMO legislation.

Representativeness of *D. magna* as test species

Because not all NTOs potentially at risk can be tested from a practical viewpoint in ERAs, a representative subset of species is typically selected for testing. These species are usually selected based on their ecological relevance, their likely exposure to Cry proteins under field conditions, their expected susceptibility to Cry proteins, and their testability (Todd et al., 2008; EFSA, 2010; Devos et al., 2012; Meissle et al., 2012; Barratt et al., 2013; Romeis et al., 2013a, 2014; Carstens et al., 2014; Riedel et al., 2016; van Capelle et al., 2016; Wach et al., 2016).

Testing focuses on species that play a role in ecosystem services (e.g., natural enemies for pest regulation, honeybees for pollination, springtails and earthworms for soil-related processes), or are of conservation concern (e.g., rare and protected species, or species of aesthetic or cultural value). The representativeness of *D. magna* as test species is considered against the aforementioned selection criteria, below.

Ecological relevance

Daphnia magna is considered an important species in aquatic environments. It is a lake dweller occupying lentic habitats (i.e., lakes, shallow ponds rich in organic matter sediment). It has a central position in aquatic food webs as filter feeder of small, suspended particles such as unicellular algae, bacteria and detritus (herbivore involved in the removal of algae and potentially pathogenic microbes), and serves as prey for planktivorous fish and other organisms. Daphnids are also indicators of water quality. Since *D. magna* is a water column species, it is not necessarily representative of benthic or epibenthic communities (Carstens et al., 2012).

Exposure

There are two routes through which NT aquatic organisms may be exposed to Cry proteins from *Bt*-plants: (1) exposure to free protein (e.g., proteins that leach out of maize plant tissues and are deposited into an adjacent water body); and (2) exposure to proteins via direct feeding on deposited plant material (e.g., aerially deposited pollen, crop dust, or intact plant material) (Rosi-Marshall et al., 2007; Tank et al., 2010; Carstens et al., 2012).

Exposure of NTOs to the Cry1Ab protein in aquatic ecosystems is likely to be very low due to its rapid degradation (Douville et al., 2005, 2007; Wolt and Peterson, 2010; Carstens et al., 2012; Strain and Lydy, 2015). Cry1Ab protein concentrations in water bodies are small compared with the amount known to cause adverse effects on sensitive target organisms (Jensen et al., 2010). *D. magna* is therefore unlikely to be exposed to significant levels of the Cry1Ab protein derived from maize MON810 and Bt11 in the water column.

Exposure of *D. magna* to the Cry1Ab protein via intact plant material (i.e., pollen) is also expected to be low, as it is not clear whether daphnids are able to digest maize pollen grains following ingestion. Moreover, the Cry1Ab protein content in maize MON810 and Bt11 pollen is low (Nguyen and Jehle, 2007; EFSA, 2009a,b). Degradation rates of the Cry1Ab protein from decaying plant material in

aquatic environments are comparable to those of non-*Bt*-maize (Griffiths et al., 2009; Swan et al., 2009). In their early-tier study with the European corn borer, Jensen et al. (2010) detected no bioactivity of the Cry1Ab protein in senesced maize tissue exposed to aquatic environments for two weeks, confirming the rapid degradation of the protein.

Susceptibility

In feeding studies with *D. magna* fed maize MON810 plant material, either ground kernels (Bøhn et al., 2008; 2010) or leaves (Holderbaum et al., 2015), negative effects have been observed, suggesting toxic effects of the Cry1Ab protein on daphnids. However, uncertainty remains on whether these effects have been caused by the Cry1Ab protein, nutritional deficiencies related to the maize-based diet, the genetic/varietal background of the conventional counterpart used as comparator, or other unintended effects (EFSA, 2009a, 2012; Ricroch et al., 2010; Bøhn et al., 2012; Romeis et al., 2013b).

Based on the known spectrum of activity of the Cry1Ab protein and its selectivity to lepidopteran species (Romeis et al., 2013b; van Frankenhuyzen, 2013; De Schrijver et al., 2014), and the phylogenetic distance between *D. magna* and target species (pests of the family Lepidoptera), susceptibility of daphnids to the Cry1Ab protein is not expected at field concentrations. However, EFSA acknowledges that early-tier tests with daphnids contribute to determining the activity spectrum of the Cry1Ab protein, and corroborating or rejecting the risk hypothesis of no harm to *D. magna*.

Testability

Daphnia magna has a long history of use for chemical toxicity testing on NT aquatic organisms where it serves as a surrogate species, and has been used previously for early-tier testing for GM plants to determine the activity spectrum of Cry proteins (Carstens et al., 2012; Romeis et al., 2013a). Standardised guidelines exist for chemical toxicity testing and rearing (US EPA 2000; OECD 2004, 2012; ASTM 2010), allowing consistent detection of adverse effects on ecologically relevant parameters.

Suitable life-stages of the test species are commercially available and can be obtained in sufficient quantity and quality (Carstens et al., 2012; Romeis et al., 2013a).

Conclusion

The study reported by Bøhn et al. (2016) addresses an objective relevant for the ERA of *Bt*-plants expressing the Cry1Ab and/or Cry2Aa protein(s) for cultivation. The GMO Panel issued Scientific Opinions on the cultivation of various *Bt*-plants (Bt11, 1507, MON810, MON88017 and 59122), of which only *Bt*-maize MON810 and Bt11 express the Cry1Ab protein. Because none of the GM plant applications for market authorisation currently under regulatory review by EFSA or for which the GMO Panel issued a Scientific Opinion cover the cultivation of *Bt*-plants expressing the Cry2Aa protein or both the Cry1Ab and Cry2Aa proteins, EFSA restricted the consideration of the findings reported by Bøhn et al. (2016) to the Cry1Ab protein which is expressed in the single *Bt*-maize events MON810 and Bt11.

Based on the limited exposure to significant levels of the Cry1Ab protein via intact plant material and particulate organic matter in the water column, the known spectrum of activity of the Cry1Ab protein and its selectivity to lepidopteran species, and the phylogenetic distance between *D. magna* and target species, EFSA does not consider *D. magna* the most representative NT aquatic organism for testing. However, EFSA acknowledges that *D. magna* represents a member of a taxonomic group not typically tested for terrestrial NTOs, and that early-tier tests with daphnids can inform ERAs by determining the activity spectrum of the Cry1Ab protein, and by corroborating or rejecting the risk hypothesis of no harm to *D. magna*.

Overall, EFSA is of the opinion that the study reported in the publication by Bøhn et al. (2016) addresses an objective relevant to the NT risk assessment of aquatic organisms in the frame of maize MON810 and Bt11 for cultivation.

3.2.2. Reliability

Any study designed to test relevant risk hypotheses should be carried out in such a way that it minimises the probability of erroneous (i.e., false negatives and false positives), or inconclusive results. Adhering to quality standards increases confidence in the results and adds certainty to the conclusions. In the frame of the assessment of potential adverse effects of GM plants on NTOs, the reliability of test systems is optimised if the following conditions are met: (1) the purity of the test substance is well characterised and described; (2) the test substance is biochemically and functionally equivalent to the novel proteins produced in the GM plant; (3) the bioactivity of the test substance, as provided to the test organisms, is established; (4) test organisms are exposed to high concentrations of the test substance relative to predicted exposures in the field; (5) ingestion of the test substance by the test organisms is confirmed; (6) endpoints are measured that are likely to indicate the possibility of adverse effects on the abundance of NTOs or other assessment endpoints; (7) the number of replicates in the study is such that defined effect sizes can be detected with sufficient statistical power; (8) negative control treatments are included to assess the suitability of the test system; and (9) positive control treatments are included, where feasible, to demonstrate that the test system is able to detect treatment effects (as reviewed by EFSA, 2010, 2011; Romeis et al., 2011, 2013b; Raybould et al., 2013; Booij and Qiu, 2015; De Schrijver et al., 2016).

The reliability of the laboratory bioassays conducted by Bøhn et al. (2016) with the Cry1Ab protein is considered against the aforementioned study design criteria, below.

Purity, equivalence and biological activity of the test substance

The purified Cry1Ab protein used in the bioassay with *D. magna* was produced in the bacterium *Escherichia coli*. The authors did not state the purity of the protein, and whether this was considered for the calculations of the final concentration in the stock solution. The nominal concentrations of test materials were not analytically confirmed in any of the treatments. Since the protein batches were purchased from a well-established source (Dr Marianne Carey, Department of Biochemistry, Case Western Reserve University, Cleveland, US), high purity of the Cry1Ab protein is likely. However, uncertainty remains on protein purity and the true amount of test substance delivered to the test organisms.

Since the protein batches were purchased from a well-established source (see above), the biochemical equivalence of the purified Cry1Ab protein produced in *E. coli* to that expressed in maize MON810 and Bt11 is likely. However, Bøhn et al. (2016) provides no information on this matter in their publication.

The biological activity of the Cry1Ab protein once dissolved in the buffer solution and in the culture medium was not confirmed by, e.g., using a sensitive insect bioassay. Likewise, the authors did not provide any information regarding the storage conditions of the stock solution after its preparation, which can affect the biological activity of Cry proteins (Nguyen and Jehle, 2009). Therefore, uncertainty remains on the level of insecticidal activity of the test substance during the study period.

There is also no information provided by the authors on what Cry1Ab protein variant (full-length, 'protoxin' form or a protease-treated 'activated' variant) was actually used in the bioassays, which can influence the biological activity of the protein (Saeglitz et al., 2006). A trypsin-treated Cry1Ab protein variant would need to be carefully purified so that any residual protease activity and other impurities caused by the protease treatment do not significantly influence the stability and/or integrity of the tested protein (Nguyen and Jehle, 2009). Therefore, additional uncertainty remains on the nature of the test substance and its possible consequences on the effects observed in the reported study.

Exposure of the test organisms to the test substance relative to predicted exposures in the field

In early-tier bioassays, test organisms are typically exposed to the test substance at the margin hazard dose (MHD) level ($\geq 10\times$ the expected environmental concentration (EEC) using protein expression data gathered in field trials performed in representative receiving environments where the GM plant is grown) (EFSA, 2010; Romeis et al., 2011). In the bioassays performed by Bøhn et al. (2016), *D. magna* was exposed to 0.75 and 4.5 mg/L of the Cry1Ab protein. Considering the expression levels of the Cry1Ab protein in maize MON810 and Bt11 (Nguyen and Jehle, 2007; EFSA, 2009a,b), and EECs under field conditions (see Carstens et al., 2012 and Raybould et al., 2014 for calculations using the US Environmental Protection Agency's standard pond and generic estimated

environmental concentration models and the EU ditch model), EFSA notes that Bøhn et al. (2016) exposed *D. magna* to Cry1Ab protein concentrations above conservative EECs for free Cry1Ab protein and maize MON810/Bt11 pollen and plant debris in the water column.

Confirmation of exposure of the test organisms to the test substance

Ingestion of the test compounds by *D. magna* in the treated groups was neither confirmed, nor quantified (e.g., by enzyme-linked immunosorbent assay (ELISA) test).

Measurement endpoints and test duration

Bøhn et al. (2016) measured both lethal (i.e., mortality) and sub-lethal endpoints. The latter included body size of adults and juveniles (as a measure of growth), maturation (as a measure of development), and cumulative and daily fecundity. The endpoints measured are in accordance with international standards (OECD, 2012), and therefore considered appropriate.

The test duration was in tune with the measured endpoints, as the test organisms were exposed during their full life cycle. EFSA notes that the authors extended the test duration recommended by OECD (2012) from 21 days to 78 days. Moreover, the bioassay was terminated upon the death of the last individual in the control group (day 78), instead of when control mortality rose above a predefined threshold (Rose, 2007; Romeis et al., 2011).

Number of replicates

Bøhn et al. (2016) used 10 replicates for the treatment groups, and 20 replicates for the control group. One replicate consisted of a single *D. magna* contained in one glass. The number of replicates used in the bioassay is consistent with the sample size recommended in the OECD guidelines (OECD, 2004, 2012) for *D. magna* testing.

Inclusion of appropriate negative control treatment(s)

The Cry1Ab protein used in the study was "*carefully weighed and dissolved in a small amount of buffer at pH 10.5*", and then added to the 50 ml of culture "*M7*" or "*water*" medium containing a single *Daphnia*. The authors did not specify in which buffer the Cry proteins were dissolved and which volume of buffer solution was added to the culture medium since the Cry1Ab protein concentration in the stock solution was not provided. Furthermore, no information was provided on how the Cry1Ab protein was quantified. The type of method(s) used to determine protein concentration can be critical when interpreting results of insect bioassays (Crespo et al., 2008). Therefore, uncertainty remains on the buffer composition and amounts used.

EFSA considers that insufficient information is reported in Bøhn et al. (2016) to judge the suitability of the negative control. For example, the negative control should have contained the same volume of buffer that was added to the Cry1Ab protein treatments (Romeis et al., 2011; Booij and Qiu, 2015), and ideally, the heat-deactivated Cry protein at the same concentration, as this is useful to distinguish direct toxic effects from non-specific effects of proteins on the nutritional status of *D. magna* (Romeis et al., 2011; Raybould et al., 2014). Although the buffer volumes and its toxic potency are expected to be low, it cannot be ruled out that the reported negative effects were caused by the addition of the buffer instead of the test substance. This issue might be particularly relevant for the highest Cry protein concentration tested, because depending on how the purified protein was prepared, this treatment could have received six times more buffer than the lower concentration. Therefore, uncertainty remains on the interpretation of the effects observed.

Inclusion of positive control treatments

A positive control (toxic/reference) substance is typically used in bioassays assessing NT effects to (indirectly) demonstrate that the test system was able to detect treatment effects, and confirm exposure to Cry proteins, as these have no contact toxicity and must be ingested by a susceptible organism to be effective. Direct dietary intake is thus required to evaluate the toxicity of Cry proteins. The study by Bøhn et al. (2016) did not include a positive control treatment to confirm the intake of the test substance by the test organisms.

Conclusion

Owing to limitations associated with the design and reporting of the study by Bøhn et al. (2016), EFSA considers that several uncertainties remain pertaining to the level of biological activity of the Cry1Ab protein, the suitability of the negative control, and the level of intake of the Cry1Ab protein by *D. magna*. Hence, the study provides insufficient information to allow a proper interpretation of the effects observed.

In addition, EFSA notes that the observed differences were seen at Cry1Ab protein concentrations above EECs under field conditions, and that the authors did not bring their study results in the context of expected exposure levels in the field.

4. Conclusions

Owing to limitations associated with the design and reporting of the study, EFSA considers that several uncertainties remain, which do not allow a proper interpretation of the effects observed by Bøhn et al. (2016). As the evidence reported in Bøhn et al. (2016) is insufficient to indicate the necessity to revise the environmental risk assessment conclusions for maize MON810 and Bt11, EFSA considers that the GMO Panel risk assessment conclusions on maize MON810 and Bt11 for cultivation remain valid and applicable.

Documentation provided to EFSA

1. Letter from the European Commission, dated 1 June 2016, to the EFSA Executive Director requesting scientific assistance on new scientific information (Bøhn et al., 2016) in relation to their risk assessment of genetically modified *Bt*-crops.

2. Acknowledgement letter, dated 1 July 2016, from the EFSA Executive Director to the European Commission.

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