EVENT REPORT

International scientific Workshop "*Non-Target Organisms and Genetically Modified Crops: Assessing the effects of Bt proteins*" (29-30 November 2012, Amsterdam, the Netherlands)[1, 2, 3, 4]

European Food Safety Authority and the Netherlands Commission on Genetic Modification[5]

European Food Safety Authority (EFSA), Parma, Italy and the Netherlands Commission on Genetic Modification (COGEM), Bilthoven, the Netherlands

ABSTRACT

In a continuous strive to improve the environmental risk assessment of genetically modified (GM) insect resistant crops expressing insecticidal *Bacillus thuringiensis* (Bt) proteins, the Netherlands Commission on Genetic Modification (COGEM) and the European Food Safety Authority (EFSA) jointly organised an international scientific workshop on 29 and 30 November 2012 to review the latest scientific insights on Bt proteins, their effects on non-target organisms (NTOs), as well as the assessment of such effects. During the workshop the latest understandings and most recent ideas on the specificity of Bt proteins, the selection of NTOs for assessment and/or testing purposes, the design of lower- and higher-tier studies to assess potential adverse effects of Bt crops on NTOs, and the usefulness of modelling were presented and discussed. The workshop was divided into six successive sessions, each of which focused on a specific set of subjects. The workshop was attended by interested parties from academia, national risk assessment bodies, industry, non-governmental organisations, the European Commission and EFSA. An overview of the presentations given, remarks made and the discussion points put forward during the workshop are presented and summarised in this report.

KEY WORDS

Environmental risk assessment, genetically modified plants, non-target organisms, species selection, study design

1 Question No EFSA-Q-2012-00716.

2 Disclaimer: The views or positions expressed in this event report do not necessarily represent the official position of the Netherlands Commission on Genetic Modification (COGEM) and the European Food Safety Authority (EFSA). COGEM and EFSA assume no responsibility or liability for any errors or inaccuracies that may appear.

3 Scientific Program Committee: The scientific Program Committee of the workshop was composed of Marjan Bovers (COGEM), Willem Jan de Kogel (Wageningen University & Research Centre), Yann Devos (EFSA, GMO Unit) and Nico van Straalen (VU University Amsterdam).

4 Organising Committee: The Organising Committee of the workshop consisted of Marjan Bovers (COGEM), Vanessa Descy (EFSA, CORSER Unit), Yann Devos (EFSA, GMO Unit), Margherita Guidi (EFSA, APDESK Unit), Ruth Mampuys (COGEM), Astrid Schulting (COGEM) and Yustina Olshevska Grigorov (EFSA, GMO Unit).

5 Acknowledgement: COGEM and EFSA wish to thank Marjan Bovers (COGEM), Yann Devos (EFSA, GMO Unit) and Tibor Drost (COGEM) for the preparatory work on this scientific output.

Any enquiries related to this output should be addressed to gmo@efsa.europa.eu and info@cogem.net

Suggested citation: European Food Safety Authority & the Netherlands Commission on Genetic Modification; International scientific Workshop "*Non-Target Organisms and Genetically Modified Crops: Assessing the effects of Bt proteins*" (29-30 November 2012, Amsterdam, the Netherlands). Supporting Publications 2013:EN-484. [30 pp.]. Available online: www.efsa.europa.eu/publications and www.cogem.net

SUMMARY

The Netherlands Commission on Genetic Modification (COGEM) and the European Food Safety Authority (EFSA) jointly organised an international scientific workshop on 29 and 30 November 2012 to review the latest scientific insights on *Bacillus thuringiensis* (Bt) proteins, their effects on non-target organisms and the valued ecosystem services they provide, as well as the assessment of such effects. An overview of the presentations given, remarks made and the discussion points put forward during the workshop are provided in this event report.

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BACKGROUND AS PROVIDED BY EFSA

The European Food Safety Authority (EFSA) was invited by the Netherlands Commission on Genetic Modification (COGEM) to collaborate in the organisation of an international two-day scientific workshop on approaches and challenges to assess the potential adverse effects of plant-produced Bt proteins from genetically modified (GM) crops on non-target arthropods.

The aim of the international scientific workshop was to pursue and contribute to the ongoing discussion on approaches and challenges to assess potential adverse effects of plant-produced Bt proteins from GM crops on non-target arthropods.

TERMS OF REFERENCE AS PROVIDED BY EFSA

To report on the outcomes of the international two-day scientific workshop on approaches and challenges to assess potential adverse effects of plant-produced Bt proteins from GM crops on non-target arthropods that was organised jointly by COGEM and EFSA.

WORKSHOP PRESENTATION & OUTCOMES

1. INTRODUCTION

Genetically modified (GM) crops have been cultivated worldwide since 1996 and in the European Union (EU) since 1998. The rapid adoption of insect-resistant crops expressing insecticidal *Bacillus thuringiensis* (Bt) proteins indicates that they have become a primary tool for managing major lepidopteran and coleopteran target pest species in cotton and maize.

In many jurisdictions, Bt crops undergo an environmental risk assessment as part of the regulatory approval process before market entry to ensure that Bt crops and the Bt proteins they express do not cause harm to non-target organisms (NTOs). In this process, the possible adverse effects that Bt crops and plant-produced Bt proteins may pose to NTOs and the valued ecosystem services they provide (e.g. pest regulation for natural enemies, pollination for bees) are assessed in detail.

The current workshop was part of a series of activities undertaken by the Netherlands Commission on Genetic Modification (COGEM)[6] and the European Food Safety Authority (EFSA)[7] with regard to the assessment of effects on NTOs.

1.1. Aim of the workshop

COGEM and EFSA jointly organised this workshop as part of a continuing effort to improve the environmental risk assessment of Bt crops. Its aim was to review the latest scientific insights into Bt proteins, their effects on NTOs and the ecosystem services they provide, and the assessment of such effects. A major objective of the workshop was to discuss recent scientific developments and how they could be used to further improve and strengthen the NTO risk assessment of Bt crops. Because the details of approaches to be used to assess the potential risk that Bt crops may pose to NTOs are still open to debate, the workshop also sought to identify and describe converging and diverging views and to explore the extent to which more harmonised/integrative approaches for the NTO risk assessment could be developed.

1.2. Participants

The workshop was attended by interested parties from academia, national risk assessment bodies, industry, non-governmental organisations, the European Commission and EFSA. The number of participants was 99, from 25 different nationalities.

2. PROGRAMME

2.1. Opening

The workshop was opened by Prof Bastiaan Zoeteman (chair of COGEM) and Dr Elisabeth Waigmann (head of the EFSA GMO Unit). They welcomed the participants of the workshop and briefly introduced its aim and scope.

2.2. Sessions

The workshop was divided into six successive sessions (Table 1). The first five sessions each focused on a specific set of subjects representing the key elements of the NTO risk assessment, and consisted of several presentations given by distinguished speakers working in different areas related to NTO risk assessment. The topic of the first session was the specificity of Bt proteins, as information on the range of organisms that could potentially be affected forms the basis for NTO risk assessment. The second session focused on the topic of selecting those species which can be used as indicator species to assess potential adverse effects on other species. Laboratory studies were discussed in the third session. Laboratory experiments are a major part of the studies that are carried out to assess effects on

[6] www.cogem.net
[7] www.efsa.europa.eu/

NTOs. Following the tiered testing approach, the results from laboratory experiments are used to determine the need for further testing. Usually, this involves field trials which are used to study potential effects under more realistic, but less controlled conditions. The design of field trials was discussed in the fourth session. In the fifth session, other topics were discussed such as the use of models to address questions which are difficult to answer experimentally and the assessment of potential adverse effects on NTOs in aquatic ecosystems. In addition, the assessment of potential adverse effects of multiple Bt proteins was discussed. The abstracts of the presentations are available at the COGEM website.[8] Each session ended with a discussion during which participants were encouraged to express their views on the subject. In the sixth and final session, the main conclusions and discussion points of the previous five sessions were summarised and their implications for NTO risk assessment of Bt crops were discussed.

Table 1: Overview of the program of the workshop.

Thursday, November 29th	
Welcome / opening: Elisabeth Waigmann & Bastiaan Zoeteman	
Session 1: Specificity of and interactions between Bt proteins (Chair: Nico van Straalen)	
Kees van Frankenhuyzen	Specificity of *Bacillus thuringiensis* pesticidal proteins: what we don't know matters
Discussion	
Session 2: Selection of species for NTO risk assessment (Chair: Salvatore Arpaia)	
Louise Malone	Selecting species for testing non-target impacts of GM Bt crops
Michael Meissle	Use of the EFSA database on European arthropods to support species selection for environmental risk assessment of genetically engineered plants
Discussion	
Session 3: Laboratory studies with NTOs (Chair: Joop van Loon)	
Jörg Romeis	Design of laboratory studies on NTOs to support the risk assessment of Bt crops
Thomas Bøhn	Non-target effects of Bt-transgenic maize in the aquatic ecosystem - the case of *Daphnia magna*
Angelika Hilbeck	How many ways can you feed a ladybeetle larva?
Marcel Dicke	Insect-resistant GM crops and IPM: the chemistry should be right
Discussion	
Friday, November 30th	
Session 4: Field experiments with NTOs (Chair: Yann Devos)	
Stefan Rauschen	Conclusions from 10 years of publicly funded field trials research with genetically engineered Bt-maize in Germany
Ramon Albajes	Assessing the effects of GM crops on non-target arthropods: 14 years of field testing Bt maize in Spain
Discussion	
Session 5: Challenges in NTO risk assessment (Chair: Willem Jan de Kogel)	
Tjalling Jager	Predicting mixture effects: the causality chain from molecule to population
Emma Rosi-Marshall	Examining the effects of Bt maize on adjacent aquatic ecosystems
Joe Perry	Confronting difficult issues in environmental risk assessment - can quantification and modelling help?
Jian Duan	Do laboratory studies of toxicity on non-target invertebrates accurately predict ecological risks of transgenic Bt crops?
Salvatore Arpaia	Non-target organisms and GM crops: shall we stop thinking about it?
Discussion	
Session 6: Workshop lessons on risk assessment, shared views and remaining questions (Moderator: Helmut Gaugitsch)	
Discussion	
Closing: Elisabeth Waigmann & Bastiaan Zoeteman	

[8] http://www.cogem.net/index.cfm/en/news/item/latest-scientific-insights-on-bt-crops-and-non-target-organisms-thoroughly-discussed-at-joint-efsa-cogem-workshop

2.3. Specificity of Bt proteins

Chaired by Prof Nico van Straalen (Department of Animal Ecology, VU University Amsterdam, the Netherlands)

The first session focused on the biological specificity of Bt proteins towards target organisms and NTOs. The target specificity of Bt proteins was discussed using the concepts of activity spectrum (the range of species that are affected within the targeted order, across orders and/or across phyla) and toxicity (the extent to which each target species is affected). Importantly, within-order and across-order activity of Bt proteins was discussed.

2.3.1. Specificity of Bacillus thuringiensis *pesticidal proteins: What we don't know matters*

Dr Kees van Frankenhuyzen (Great Lakes Forestry Centre, Natural Resources Canada, Sault Ste. Marie, Ontario, Canada)

Dr van Frankenhuyzen sketched the context of his presentation by mentioning that 50–60 years of experience of forest protection with Bt sprays show that microbial-produced Bt proteins have an outstanding safety record with regard to non-target effects.

Dr van Frankenhuyzen built the '*Bt toxin specificity database*'[9], which gives an overview of information on the biological specificity and activity of individual Bt proteins. This information was retrieved from the scientific literature and patents over the past 25 years (Van Frankenhuyzen, 2009). This database is mainly restricted to data on spore-free preparations of crystal proteins or protoxins/toxins that have been obtained through the expression of cloned genes or purified from single-gene strains and have been bio-assayed individually. Importantly, the database does not deal with Bt proteins produced by GM crops.

In his presentation, Dr van Frankenhuyzen evaluated the target specificity of Bt proteins by summarising the available information on the activity of Bt proteins. Bt proteins are commonly known to be highly target specific, but some Bt proteins can affect organisms across orders within phyla and even across phyla. Bt proteins are classified according to the sequence identity of the amino acid sequences of full length Bt proteins. A similarity of 40% in the amino acid sequence is labelled with a number (i.e. Cry1, Cry2). Smaller differences between amino acid sequences are indicated with a letter. Fifteen of the 62 families (i.e. Cry1, Cry2) possess proteins that show cross-activity; 27 Bt protein sub-groups (i.e. Cry1Ab, Cry1Ac) are cross-active. Cross-activity is difficult to predict and widespread among Bt protein families. According to Dr van Frankenhuyzen, current data probably underestimate both occurrence of cross-active Bt proteins and the extent of their activity spectra, because few Bt proteins have been tested against organisms from a broad range of taxonomic groups.

Dr van Frankenhuyzen emphasised that many of the entries in the database are based on a single report, and that confirmation and validation of the reports are needed. Although Bt proteins are not as specific as initially reported, in most cases toxicity outside a protein's primary target range is orders of magnitude below its toxicity inside that range. Finally, Dr van Frankenhuyzen recommended that one should expect the unexpected (i.e. occurrence of unknown cross-activity). In some cases, data suggest that cross-activities can occur at toxicity levels that may be ecologically relevant and therefore cross-activity should be taken into account when designing experiments to assess the effect of Bt proteins on NTOs.

[9] van Frankenhuyzen K. & Nystrom C, The *Bacillus thuringiensis* toxin specificity database, http://www.glfc.cfs.nrcan.gc.ca/bacillus/

2.3.2. Remarks and questions

- *Biological relevance of cross-order activity*: A participant asked Dr van Frankenhuyzen to comment on the biological relevance of the activity levels that were reported and asked him to clarify whether Bt proteins which were active only at very high doses are listed as 'active' in the database. Dr van Frankenhuyzen confirmed that these Bt proteins are indeed listed as 'active'. In the analysis of the data, reports of high dose activity would be analysed in more detail and would be discarded if the proper controls were not used.

- *Specificity of crystal vs. plant-produced Bt proteins*: A questioner asked how data on the specificity of Bt crystal proteins relates to the specificity of Bt proteins produced in GM crops. Dr van Frankenhuyzen indicated that the underlying mode of the actions of Bt crystal proteins and Bt proteins in GM crops are most probably the same.

- *Patents as information source*: In response to a question about the interpretation of results on Bt protein specificity from patents, Dr van Frankenhuyzen pointed out that patents often provide little information on how experiments were done. In some cases, however, patents are well documented, as in the case of a patent that reported on Cry3A activity on fire ants (Hymenoptera). In this patent, activated, solubilised and non-activated cloned gene products and purified proteins were used to test Cry3A activity on fire ants. The bioassays even contained information on lethal concentration (LC) values. However, most experiments in patents that are well documented have not been independently reproduced and confirmed, which needs to be taken into account when interpreting the data.

2.4. Selection of species for NTO risk assessment

Chaired by Dr Salvatore Arpaia (Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), UTTRI-BIOTEC, Rotondella, Italy)

The aim of the second session was to discuss criteria and methods for selecting species for NTO risk assessment. Two different tools that could help to select species were presented: the PRONTI (priority ranking of non-target invertebrates) model, which is an approach to select species, and the EFSA database, which gives insight in the occurrence and abundance of arthropods in European fields.

2.4.1. Selecting species for testing non-target impacts of GM Bt crops

Dr Louise Malone (The New Zealand Institute for Plant & Food Research Ltd, Mt Albert Research Centre, Auckland, New Zealand)

To assess potential adverse effects of Bt crops on NTOs, a subset of species is selected and experiments are carried out to analyse whether these species are affected. Dr Malone mentioned five basic selection criteria used to select species. These criteria are: potential susceptibility, potential exposure, ecological function, value to humans (e.g. native, iconic or endangered species) and amenability to testing. The last three criteria vary in importance between countries, but potential susceptibility and exposure are important criteria in all countries.

The PRONTI model was developed in New Zealand. Using the information about arthropod species contained in the Eco Invertebase, the PRONTI model enables the generation of prioritised lists of species (Todd et al. 2008). A Bt pine tree case study was used to study the effect of varying the emphasis placed on different criteria on the selection of species to be used for further analysis. Dr Malone presented the results of this study.

One of the assumptions on which the approach to select species relies is that the results obtained with the selected species can be extrapolated to other species. It is often assumed that taxonomic relatedness to the target pest is a good indicator of potential susceptibility. Malone presented the outcome of laboratory tests with a series of lepidopteran species of varying degrees of genetic relatedness and showed that lepidopteran responses to a mixture of Bt toxins do not necessarily follow

predictions based on taxonomy. The significance of these laboratory results was assessed by examining the impacts of the same Bt toxin preparation on the field abundance of several lepidopteran species when applied to pine trees.

Dr Malone emphasised that NTO risk assessment should not only consider mortality of species, but should also take the resilience of species into account.

2.4.2. Use of the EFSA database on European arthropods to support species selection for environmental risk assessment of genetically engineered plants

Dr Michael Meissle (Agroscope Reckenholz-Tänikon Research Station ART, Zurich, Switzerland)

Dr Meissle introduced the EFSA database on European arthropods and its potential use in species selection for the environmental risk assessment (ERA) of GM crops. The database, which includes relevant data retrieved from the scientific literature via a systematic literature search, provides a detailed overview of the arthropod fauna in arable crops (maize, oilseed rape, potato, soybean and sugar fodder beet) across Europe (Meissle et al., 2012). It is a SQL-queryable database and is available on the EFSA website. [10] Almost 3000 arthropod species are known from European crop fields. The most collected taxa for each functional group have been identified. For each species the database contains information on functional group, taxonomy, and number of records. Limitations of the database are: (1) geographical gaps due to the unavailability of literature, (2) some sampling methods collect species that are not necessarily connected with the crop (e.g. light traps), and (3) the database represents collection effort and does not necessarily reflect the situation in the agro-ecosystem.

Dr Meissle illustrated the applicability of the EFSA database using GM maize expressing one Bt protein with activity against *Diabrotica* spp. (Coleoptera: Chrysomelidae), as a case study. The database was used to produce a list of non-target beetles which are abundant in maize fields and which could be exposed to the plant-produced Bt protein. This list of species combined with their potential sensitivity to the Bt protein offers a useful tool to select species for NTO testing, although the testability of the species is also important when making the final selection.

Dr Meissle concluded that the database can be used to identify species known to occur in particular crops and regions. He pointed out that the EFSA arthropod database can facilitate the identification of ecologically and agronomically relevant species in Europe and may be used to identify the most appropriate species for non-target testing. It can therefore support the ERA of GM crops.

2.4.3. Remarks and questions

- *Species relatedness and susceptibility to Bt proteins*: A questioner raised the issue of how representative a selected species is for all other NTOs. The questioner added that, according to Dr van Frankenhuyzen's presentation, taxonomic relatedness might not be a useful indicator of representativeness. Dr Malone answered that they observed a *Pseudocoremia* species which was not susceptible in laboratory experiments, whereas other *Pseudocoremia* species were susceptible to the Bt protein. Dr Meissle said that target taxa have to be separated from taxa for which no effects have been reported. For example, if a ladybird survives a high dose of Bt proteins, it might be considered representative for all ladybirds. If a field dose has an effect on a ladybird species, one should examine in more depth whether other ladybird species are also affected.

- *Model or local species*: A participant raised the following dilemma. One species is found in the field and exposed to Bt proteins, but is not rearable in the laboratory, while another species can be reared in the laboratory, but is not found in the field. What is the best species to choose? For example, *Folsomia candida* represents a functional group, but does not occur abundantly in the field.

[10] Meissle M, Álvarez-Alfageme F, Malone LA & Romeis J (2012). Establishing a database of bio-ecological information on non-target arthropod species to support the environmental risk assessment of genetically modified crops in the EU. Supporting Publications 2012:EN-334, EFSA, [170 pp], http://www.efsa.europa.eu/en/supporting/doc/334e.pdf

Dr Malone answered that it is good to have standardised species and standardised tests, and that evidence from local species is also important, even if such species are difficult to test.

- *Valued species*: One of the participants expressed reservations about the concept of valued species. For example, herbivores are generally seen as pests, yet not all herbivores are pests and they may also have an important ecological function. Although the ecological functions provided by herbivores may not be directly relevant to humans in terms of ecosystem services, herbivores serve as an important food source for other species, and thus contribute to sustaining the food web.

2.5. Laboratory studies with NTOs

Chaired by Prof Joop van Loon (Laboratory of Entomology, Wageningen University, the Netherlands)

The third session of the workshop focused on the criteria and methods for laboratory studies with NTOs. A list of criteria for designing laboratory studies was elaborated upon. In addition, case studies on *Daphnia magna* and *Adalia bipunctata* were used to discuss the selection of measurement endpoints (e.g. lethal versus sublethal effects) and the design of NTO feeding protocols. The possibility of using GM crops in integrated pest management (IPM) was also discussed.

2.5.1. Design of laboratory studies on NTOs to support the risk assessment of Bt crops

Dr Jörg Romeis (Agroscope Reckenholz-Tänikon Research Station ART, Zurich, Switzerland)

In his presentation, Dr Romeis listed and discussed the main points to consider when designing a laboratory study to assess effects on non-target arthropods (Romeis et al., 2008, 2011, 2013). The following nine criteria were put forward:

(1) The test substances used should be well characterised and described (i.e. source, purity, stability and homogeneity of the protein).

(2) The biochemical and functional equivalence of the test substance to the GM crop produced protein should be confirmed (Raybould et al., 2012).

(3) The bioactivity of the test substances should be demonstrated.

(4) The test organisms should be exposed to relative high substance concentrations compared with the concentrations in the field.

(5) The exposure of the test organisms to the test substance should be demonstrated.

(6) The endpoints should be chosen in such a way that they indicate adverse effects on non-target organisms.

(7) The number of replicates should be sufficiently high to measure the size of the effect with enough statistical power.

(8) Negative control treatments should be incorporated in the experiments to assess the quality of the test system, the organisms and the conditions of the experiment.

(9) Positive controls should be incorporated to confirm ingestion of the test substance and to show that the test system is able to detect treatment effects (Li et al., 2011).

Dr Romeis recommended that when a laboratory study reports adverse effects following exposure to a test substance, the following checks should be made:

(1) Are there are indications that the test system was not reliable?

(2) Was the right negative control used?

(3) Can other confounding factors be excluded (e.g. fungal infection of plant material)?

(4) Was a susceptible prey/host used (in tritrophic studies), because an effect may be due to reduced quality of the prey (Lawo et al., 2010).

When a laboratory study reports no adverse effect following exposure to the test substance, he recommended that one should check whether:

(1) The test organisms were exposed to and ingested biologically active test substance.

(2) The test substance was ingested at estimated environmental concentration (EEC) or higher.

(3) The test system was able to detect adverse effects.

Dr Romeis emphasised the importance of a proper study design, as this enhances data quality, increases confidence in the results of the laboratory studies and enables the acceptance of data across regulatory jurisdictions, and reduces data requirements for GM crops that pose a low risk.

2.5.2. Non-target effects of Bt transgenic maize in the aquatic ecosystem: The case of **Daphnia magna**

Prof Thomas Bøhn (GenØk – Centre for Biosafety, Tromsø, Norway; Faculty of Health Sciences, University of Tromsø, Norway)

Prof Bøhn presented the results of experiments assessing the effect of Bt maize (MON810) on *Daphnia magna*, a well-known test organism in toxicology (Bøhn et al., 2008, 2010). *D. magna* is a crustacean that inhabits ponds and lakes in most regions of the world. It is a common inhabitant of ponds in agricultural landscapes and will, like many other zooplankton and benthic arthropods, be exposed to pollen and detritus from drainage water from agricultural fields. In life-cycle experiments over periods up to 42 days *D. magna* exposed to ground grains of maize MON810 showed reduced fitness compared with *D. magna* exposed to ground grains of non-GM maize. In addition, female maturation time in *D. magna* exposed to Bt maize was reduced compared with the control organisms. It was not possible (in these studies) to single out the causes for the observed weak toxicity following exposure to the Bt maize feed. Prof Bøhn mentioned as possible causes a toxic effect of the Bt protein or the down/up regulation of genes. Prof Bøhn also reviewed the debate on experimental procedures that took place in the scientific literature following the publication of the Bøhn et al. (2008, 2010) studies (Ricroch et al., 2010; Bøhn et al., 2012).

Prof Bøhn then discussed other issues related to the cultivation of Bt crops and herbicide tolerant crops. First, pests may evolve resistance to the Bt proteins produced by certain Bt crops. For example, the maize stalk borer (*Busseola fusca*), which is the main lepidopteran pest in South Africa, has evolved resistance to Cry1Ab in certain regions, resulting in the application of pesticides on maize MON810. Second, the cultivation of herbicide tolerant crops –for example, Roundup Ready crops which are resistant to the herbicide glyphosate, the active substance in Roundup – and the cultivation of stacked events is becoming more widespread. Chronic exposure to glyphosate and Roundup leads to adverse effects on *D. magna* at concentrations accepted in the US (Cuhra et al., 2013). Finally, Prof Bøhn argued that Roundup Ready (glyphosate tolerant) soybean contains high levels of glyphosate and its degradation product aminomethylphosphonic acid (AMPA) and therefore recommended that this must be included in the risk assessment of GM herbicide tolerant crops.

2.5.3. How many ways can you feed a ladybird larva?

Dr Angelika Hilbeck (Institute of Integrative Biology, Swiss Federal Institute of Technology, Switzerland)

Dr Hilbeck presented the results of her experiments that indicate adverse effects (significantly higher juvenile mortality) of Cry1Ab and Cry3Bb1 on the coccinellid beetle *A. bipunctata* (Hilbeck et al., 2012), and compared those with other experiments that did not observe adverse effects (Álvarez-Alfageme et al., 2011). Cry1Ab is active against lepidopteran pests, while Cry3Bb1 is known to target coleopteran pests. According to Dr Hilbeck, the different outcomes of laboratory studies reported in the literature in which *A. bipunctata* larvae were exposed to Cry1Ab (Schmidt et al. 2009; Álvarez-Alfageme et al., 2011) can be explained by the different feeding protocols. In an experiment comparing both the continuous and the 24-hr-exposure/recovery feeding protocols, the group of Dr Hilbeck stated that the exposure/recovery protocol is significantly less sensitive than the continuous exposure protocol. She concluded that: (1) the mortality rate of juvenile *A. bipunctata* caused by Cry1Ab is low to medium, but statistically significant, (2) this mortality rate increases over longer exposure times (i.e. 9–10 days), and (3) the mortality caused by Cry1Ab can (but must not) be ecologically significant.

In addition, Dr Hilbeck discussed the finding that Cry1Ab can have adverse effects on green lacewing larvae (*Chrysoperla carnea*) (Hilbeck et al., 1998, 1999; Hilbeck & Schmidt, 2006). Green lacewing larvae have distinct, strictly piercing-sucking mouthparts. The prime testing protocol for regulatory approval studies using green lacewings is exposure to coated meal moth eggs. Uptake of Cry1Ab by green lacewings fed on this diet has never been demonstrated. According to Dr Hilbeck, a lack of uptake might explain the lack of adverse effects observed in other studies on green lacewings. The study by Hilbeck et al. (1999) was followed by reports from other research groups that showed different results owing to the use of different exposure protocols. In 2007, the US Environmental Protection Agency (US EPA) questioned the ingestion of Bt proteins by lacewing larvae when exposed to coated moth eggs and now it recommends the use of minute pirate bugs (*Orius* spp.) (US EPA, 2010). Dr Hilbeck emphasised that results from two studies on several Cry proteins indicate that *Orius* spp. is not affected (Zwahlen et al., 2000; González-Zamora et al., 2007).

The presented examples were shown to illustrate that the choice of exposure methods in ERA on NTOs is important and that the quality of the exposure methods chosen should be thoroughly demonstrated.

2.5.4. Insect-resistant GM crops and IPM: The chemistry should be right

Prof Marcel Dicke (Laboratory of Entomology, Wageningen University, the Netherlands)

At the end of the first day, Prof Dicke reviewed the use of integrated pest management (IPM) as a durable insect pest management strategy. In addition, he discussed the possibilities for using GM crops as a part of IPM. IPM is based on the integration of methods that cause the least disruption of ecosystems. Although IPM relies on many solutions (i.e. biological control and partially resistant crops), very limited attention is given to transgenic crops. Prof Dicke presented a case on Bt cotton in which the number of natural enemies increased due to a reduced number of pesticide applications and consequently no secondary pests occurred (Lu et al., 2012). In this case and in similar cases, the inclusion of transgenic insect-resistant crops in new IPM programmes may provide a first step to replace current control strategies based on chemical pest control.

According to Prof Dicke, the major topics regarding non-target effects that have been discussed for many decades are: (1) selection of species (Scholte & Dicke, 2005), (2) selection of tests, and (3) selection of parameters. He argued that when selecting parameters, it is important to consider not only lethal effects, but also sublethal effects. Parameters that could be studied are population growth rate, development rate, oviposition rate, number of offspring, longevity, intrinsic rate of increase, and life table responses (Charleston & Dicke, 2008).

2.5.5. Remarks and questions

- *Relevance of laboratory results*: It was mentioned that effects observed in the laboratory might not be biologically relevant in the field. Therefore, some participants argued the biological relevance of observed effects should always be assessed.

- *Selection of study duration and test material*: It was argued that the study presented by Prof Bøhn does not meet the OECD criteria. The OECD guidelines on laboratory experiments with *D. magna* state that the test should be stopped after 21 days. In the life-cycle experiments presented by Prof Bøhn, the tests lasted for up to 42 days. Prof Bøhn mentioned that mortality declined slowly and fairly evenly to day 42. Performing a life-cycle experiment with *D. magna* for 42 days generates more data. Another participant mentioned that maize grain is not the normal food for *D. magna* and that it appears to cause nutritional distress. Prof Bøhn clarified having selected grains as test substance because grains are used as food or feed for humans and animals. Prof Bøhn added that when one is interested in environmental exposure the use of leaves would be preferable.

- *GMO vs pesticide assessment protocols*: Dr Hilbeck stressed that the ERA of GM crops follows the pesticide risk assessment protocols, which is why the feeding procedures used include methods like spraying meal moth eggs with Bt proteins and putting plant powder in soil for earthworms. According to Dr Hilbeck, the test duration of the pesticide protocols has to be adapted because the effects of GM crops may develop only after days.

- *Sublethal effects*: A participant questioned why Dr Hilbeck only looked at mortality to compare the sensitivity of the exposure/recovery and the continuous exposure protocol. The inclusion of other endpoints such as weight and development time/rate, which are more sensitive measures of toxicity, would have increased confidence in the dataset. Dr Hilbeck replied that the aim was to reveal differences caused by the experimental setup. Mortality was studied because that was the parameter of discussion. Prof Dicke mentioned a study with caterpillars in which no effect on mortality was observed, but the resulting adults did not reproduce, demonstrating a sublethal effect. It turned out that the seeds of the plants used to rear the caterpillars had been coated with a systemic insecticide (fipronil). This anecdote demonstrates that a population of NTOs could be severely affected even when no effect on mortality is observed, thus highlighting the importance of assessing sublethal effects.

- *Benefits*: One of the participants pointed out that the benefits of Bt crops should also be taken into account in the ERA.

2.6. Field experiments with NTOs

Chaired by Dr Yann Devos (European Food Safety Authority (EFSA), GMO Unit, Parma, Italy)

The second day of the workshop started with a session on criteria and methods for field experiments assessing possible effects on NTOs. Two case studies illustrated 10 years of field testing with Bt maize in Germany and 14 years of field trials in Spain.

2.6.1. Conclusions from 10 years of publicly funded field trials research with GM Bt maize in Germany

Dr Stefan Rauschen (formerly at RWTH Aachen University, Department of Plant Physiology (Bio3), Aachen, Germany)

Dr Rauschen presented the results and conclusions of three field trials in Germany over a period of 10 years. The field trials with the Bt maize events MON810, MON88017 and MON89034 × MON88017 lasted three years each and the outcomes of each trial led to the further refinement of trial designs and assessment methods for the next trial. In the trials, the Bt maize lines were compared with their near-isogenic line, both untreated and treated with insecticide.

Dr Rauschen concluded that:

(1) The assessed Bt maize events do not harm the communities of NTOs typical for maize, including butterflies in adjacent habitats (Gathmann et al., 2006; Rauschen et al., 2010a; Schuppener et al., 2012).

(2) Current practices (e.g. insecticides, management practices) have much stronger negative impacts and need to be taken into account (Eckert et al., 2006; Rauschen et al., 2008).

(3) Conventionally bred maize varieties can differ substantially in their impact on NTOs (Rauschen et al., 2009).

(4) Assessing the impact on NTOs with low densities requires many replications and many sampling years/experimental locations (Rauschen et al., 2010b).

Because it is difficult to assess effects on NTOs in field experiments, Dr Rauschen advocated robust laboratory studies to assess possible adverse effects on NTOs. In specific cases, such as butterflies, Dr Rauschen suggested using modelling approaches and extrapolations from the results of other NTO tests.

2.6.2. Assessing the effects of GM crops on non-target arthropods: 14 years of field testing Bt maize in Spain

Dr Ramon Albajes (Universitat de Lleida, Centre UdL-IRTA and AGROTECNIO Centre, Spain)

Dr Albajes presented the results of 14 years of field trials in Spain. More than 100 maize varieties containing the event MON810 are currently authorised in Spain. In 2012, almost 100,000 ha were planted with Bt maize. Most of the field trials conducted in Spain over the last 14 years have measured the potential impact of Bt (Cry1Ab) maize on the density or activity of arthropods by means of visual counting, pitfall or yellow sticky traps. The results of the field trials show that: (1) non-target lepidopteran pests are unequally affected by Bt varieties, (2) there were occasional higher densities of staphylinids in non-Bt plots (de la Poza et al., 2005; Albajes et al., 2012), and (3) there were consistent higher densities of homopterans (i.e. aphids, leafhoppers) (Lumbierres et al., 2004; Albajes et al., 2011) and generalist predators (e.g. *Orius* spp.) in Bt plots.

Dr Albajes concluded that in general no negative effects of Bt crops (Bt176 or MON810) on NTOs were observed. Occasionally, effects on the tritrophic system composed of maize plant–homopterans–generalist predators were detected. Also some non-target lepidopteran pests are less susceptible to Cry1Ab because, among other things, they are able to excrete the toxin (Pérez-Hedo, et al., 2012). According to Dr Albajes, Cry1Ab maize has no adverse effects on NTOs and no further NTO tests have to be conducted on Cry1Ab maize. Finally, Dr Albajes concluded that the design and analysis of field trials should be reviewed to improve detection capacities and costs.

2.6.3. Remarks and questions

- *Triggers*: A participant asked what the triggers are for deciding whether a field trial is needed or not. Dr Rauschen said it depends on the people you ask and on the specific case and recommended using a stepwise process (the tiered approach). If you want to test a hypothesis and a field trial is the only option, then you should do a field trial. Dr Albajes agreed with the stepwise process to test potential adverse effects of toxic traits. Some impacts of toxic traits are not based on properties of the toxin; in that case, it could be useful to perform a few very simple standard field trials. Such an approach could mask potential effects of non-toxic traits, which makes field trials necessary to assess the effect of GM crops with non-toxic traits on NTOs.

- *Effect size*: Both speakers were asked to give their opinion on the general question: which effect should a field trial be able to detect? For example, one can aim for field trials that have the power to detect effect sizes of 50% or 100%. Dr Albajes replied that to detect small differences you need many trials and many replicates, which is expensive. Rare organisms can be useful as indicator species, but are very expensive to study because many replicates are needed to generate reliable data. Dr Rauschen said that it is important to account for variability when designing and conducting field trials. For example, the weather conditions have a large impact on the number of NTOs that are caught in a field trial. During rainy and windy weather fewer individuals are found. In addition, he stressed the importance of specifying protection goals and defining damage thresholds.

- *Power analysis*: A participant mentioned that the power analyses in the presented field experiments were done retrospectively and asked how challenging it is to do these analyses prospectively. Dr Albajes answered that power analyses are a controversial issue and statisticians may sometimes be too strict in their approach. For example, the abundance of many organisms (e.g. aphids, mites) does not follow a Poisson distribution, although Poisson distributions are the norm according to statisticians. Probably the type of transformation (and power analysis) that is needed depends on the organism. He added that it could be helpful to have a debate on whether transformations should be species-specific or whether a standard transformation should be used for every species.

2.7. Challenges in NTO risk assessment

Chaired by Dr Willem Jan de Kogel (Biointeractions & Plant Health, Plant Research International, the Netherlands)

The aim of the fifth session was to explore the challenges of NTO risk assessment. The issue of predicting synergistic effects caused by interaction between multiple stress factors (i.e. stacking of Bt proteins in GM crops) was elaborated in the light of toxicology and mathematical modelling. A case study on aquatic ecosystems in the US illustrated the issue of possible exposure and adverse effects to organisms adjacent to the field were Bt crops are grown. In addition, the advantages of mathematical modelling for ERA were discussed. Furthermore, using data from meta-analyses the relation between the outcome of laboratory and field studies was reflected upon. Finally, a list of further challenges (i.e. statistical power of experiments and region-specific databases to increase knowledge of agro-ecosystems) was discussed.

2.7.1. Predicting mixture effects: The causality chain from molecule to population

Dr Tjalling Jager (Department of Theoretical Biology, VU University Amsterdam, the Netherlands)

Dr Jager introduced the causality chain from molecule to effect on populations and the challenge of assessing mixture effects. The causality chain refers to the sequence from the external exposure of an organism to a toxin, via internal exposure, molecular targets and life-history traits to population dynamics. Dr Jager emphasised that multiple stress factors occur simultaneously in the field. Most toxicity tests assess the effect of a single trait at one time point under one set of conditions. Such tests are of little use for making educated predictions of the impacts on protection goals in the field, which require a more mechanistic approach. Most of the mechanistic work focuses on the molecular level, but it is difficult to use mechanistic work to make predictions about life-history traits. Dr Jager argued that the causal link between the molecular and the individual level should consider effects on metabolism: how food is used to fuel life-history traits (Kooijman, 2001). This becomes even more important in the event of mixture toxicity, because two stressors may not interact at the molecular level, but at the level of metabolic pathways (Jager et al., 2010). For example, a compound increases maintenance costs at the metabolic level via the molecular interaction with its receptor, while another compound increases growth costs via the interaction with its molecular receptor. Therefore, both compounds interact on the level of metabolic pathways.

Dr Jager drew the following conclusions:

(1) Besides toxicity testing and molecular mechanisms, the successive steps in the causality chain also include the external and internal exposure of the toxin in an organism (toxicodynamics), the effects on metabolic processes and population dynamics. A lot of interdisciplinary research is needed to understand all the steps of the causality chain.

(2) Each step in the causality chain requires mechanistic models. However, effects change with time, environment, etc. Multiple stress factors are present in the field at the same time, but their presence in time and space is dynamic. Standardisation of experiments is therefore not a solution.

(3) Interactions may occur anywhere in the chain. Strong synergism is rare and excluding or predicting synergism is very difficult. If mechanistic knowledge on the causality chain is increased, it might be easier to predict interactions. However, much needs to be done to fill in these knowledge gaps.

2.7.2. Examining the effects of Bt maize on adjacent aquatic ecosystems

Dr Emma Rosi-Marshall (Cary Institute of Ecosystem Studies, Millbrook, New York, USA)

Dr Rosi-Marshall presented the results of laboratory and field studies in the US (state of Indiana) on the effects of Bt maize on aquatic NTOs (Rosi-Marshall et al., 2007). In the US, maize is cultivated in large fields where other vegetation is scarce. The maize is planted right up to the edges of ditches. After harvest of the corncob, crop by-products remain on the fields and may enter adjacent streams via wind and water (Jensen et al., 2010). This results in a potential high level of exposure of aquatic organisms to plant-produced Bt proteins.

To assess whether Bt maize poses a risk to aquatic ecosystems, Dr Rosi-Marshall pointed out that one should first establish whether there is a connection between them. The following questions should be asked (Carstens et al., 2012): (1) Does maize and Bt maize enter and persist in agricultural streams? (2) Do laboratory and field studies indicate that stream dwelling organisms are sensitive? (3) Does the widespread planting of Bt maize pose a threat to aquatic ecosystems. Dr Rosi-Marshall demonstrated that agricultural streams receive, store and move crop by-products (Rosi-Marshall et al., 2007; Griffiths et al., 2009). Furthermore, Cry1Ab is detectable in maize by-products found in agricultural streams in the State of Indiana, and dissolved Cry1Ab can be detected in the stream water (Tank et al., 2010). An unknown aspect is whether the detected Cry1Ab in stream ecosystems has any residual insecticidal properties. In laboratory assays, Bt maize reduced aquatic insect growth (e.g. caddisflies) (Chambers et al., 2010). In addition, the question remains whether the observed reduced growth rates have ecological consequences.

Dr Rosi-Marshall concluded that:

(1) Streams are closely linked with their watersheds ('drainage basins' in North America).

(2) The adoption process and management of new agricultural technologies should consider this linkage.

(3) The possible effects on streams should be measured from individuals (e.g. growth rates) to ecosystems.

2.7.3. Confronting difficult issues in environmental risk assessment: Can quantification and modelling help?

Prof Joe Perry (Oaklands Barn, Broome, Norfolk, United Kingdom)

Prof Perry started with a short introduction to the EFSA approach to assess the possible adverse effects of GM crops on NTOs. He mentioned the 4-step approach to species selection:

(1) Identification of NTO functional groups exposed to the GM crop (e.g. herbivores, predators, pollinators).

(2) Categorisation of NTO species from identified functional groups.

(3) Ranking NTO species based on ecological criteria (e.g. exposure, abundance).

(4) Final selection of focal species/functional groups (e.g. testability).

Besides the value of different statistical approaches (as also elaborately discussed in EFSA documents; EFSA, 2010), particularly the difference test and the equivalence test, Prof Perry elaborated on the advantages of using mathematical models in ERA (Perry et al., 2010, 2012). Among the advantages of using models are that they: (1) force transparency, (2) expose inaccuracies/uncertainties indicating where new data needs to be collected to improve the model, (3) may give an order of magnitude estimate, and (4) can help build consensus during risk assessment. Modelling can identify the key parameters of a process and help to expose areas where further data are required. In addition, by its very nature, modelling attempts to quantify uncertain biological processes – the quantification and communication of uncertainty is increasingly recognised as a vital part of risk assessment. Conversely, modelling may help to identify situations where some forms of risk management would be a disproportionate response to the estimated risk.

Prof Perry underlined the importance of discussing controversial issues. By confronting these issues the science can move forward. While modelling can never provide the final answer, it can help risk assessors to make progress in difficult areas and to highlight where further research efforts should be directed.

2.7.4. Do laboratory studies of toxicity on non-target invertebrates accurately predict ecological risks of transgenic Bt crops?

Dr Jian Duan (USDA-ARS, Beneficial Insects Introduction Research Laboratory, Newark, Delaware, USA)

In his presentation, Dr Duan presented the results of meta-analyses using databases containing information from studies that assessed the effects of Bt crops on non-target invertebrates (Marvier et al., 2007; Wolfenbarger et al., 2008; Naranjo, 2009; Duan et al., 2010). A meta-analysis quantitatively combines and analyses the results of multiple experiments by using standardised effect sizes that take into account variability, sample size and the magnitude of differences in comparative studies. Dr Duan concluded that laboratory studies show that:

(1) Life-history performance of 'susceptible' herbivores and some valued herbivores, such as Monarch butterflies, are negatively affected by lepidopteran-active Bt proteins, while pollinators and other non-target herbivores are unaffected.

(2) Natural enemies are largely unaffected when exposed directly to Bt proteins.

(3) Tritrophic exposure of natural enemies must account for prey/host quality effects to accurately assess direct effects of Bt proteins.

In addition, he concluded that field studies show that:

(1) Non-target effects of Bt crops are largely neutral.

(2) Positive or negative effects are probably related to ecological factors such as prey/host availability (trophic interactions).

(3) The negative effects of Bt crops are small in comparison to the use of broader-spectrum insecticides.

He concluded that laboratory studies conservatively predict the direct effects of Bt crops in the field, suggesting that the tier testing system does identify risks in the field. In a meta-analysis study using a random effect model to compare laboratory and field studies on the effect of lepidopteran-active Bt proteins on different functional guilds, it was found for parasitoids that both bitrophic and tritrophic laboratory studies were significantly different from results from the field. The significant negative effect which was observed in the field was not detected in the bitrophic laboratory studies, and was significantly smaller in the tritrophic laboratory studies (Duan et al., 2010). The negative effect observed in the field was largely the result of an adverse effect on *Macrocentrus*, which is a specialist parasitoid of the targeted lepidopteran pest. Adverse effects arising from the lack of the European corn borer host (the target pest) could not be predicted in bitrophic laboratory studies. The tritrophic exposure studies on parasitoids are correlated with, but not necessarily predictive of, field effects.

2.7.5. Non-target organisms and GM crops: Shall we stop thinking about it?

Dr Salvatore Arpaia (Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), UTTRI-BIOTEC, Rotondella, Italy)

Dr Arpaia discussed some challenges that require careful consideration in order to ensure that the ERA meets the highest scientific standards. He made the following points:

(1) There is a relatively limited number of species studied, although this has improved in the last few years.

(2) There is a limited choice of measurement endpoints. In most cases, only acute toxic effects on development and growth are measured, whereas parameters like fecundity, fertility and population growth rate might also be of interest.

(3) There is a need for properly designed experiments with sufficient power to detect adverse effects.

(4) The availability of a European database containing ecological datasets from European and regional programs is fundamental to avoiding future overlap of similar activities.

(5) Experimental protocols for NTO studies should define proxies for long-term effects, as current tests are mainly based on short-term acute toxicity.

(6) The assessment endpoints should be defined in more detail for each of the receiving environments. Currently, the protection goals are defined too generally.

(7) As there is currently only partial knowledge of the agro-ecosystems, region-specific databases containing data on the species assemblages of main functional groups in each region should be developed.

2.7.6. Remarks and questions

- *Trigger for NTO testing*: A participant raised the question of when effects on NTOs should be assessed. Should NTOs always be studied, or only if there is a target species? The questioner added that the Cartagena Protocol only requires the possible effects on NTOs to be assessed if there is a scientifically plausible reason to suspect effects. Dr Jager responded that from a toxicologist point of view 'the dose makes the potion' and so one should always look at the possible effects on NTOs. According to Dr Arpaia, assessing everything does not mean testing everything. The EFSA uses this view to do risk assessment. Sometimes studies are needed, while in other cases the literature is enough or no data are needed at all to perform a proper risk assessment.

- *Multiple stressors and synergism*: Another participant raised the issue of multiple stressors and synergistic behaviour. Dr Jager answered that during his presentation he talked about strong synergistic effects. Although in the field there are many different stressors, the effects due to synergistic behaviour tend to be small and in Dr Jager's experience are no more than ten times as strong.

- *Limited assessment of aquatic organisms*: A participant asked whether there are known cases in which the risk assessment was too limited. Dr Rosi-Marshall replied that the aquatic ecosystem has been underrepresentedin the NTO risk assessment. So far, no effect was observed in the few field experiments that were performed with a limited number of stream organisms. Dr Rosi-Marshall mentioned that stream organisms have not been sufficiently tested and that further research is needed.

- *Modelling*: A further question was whether modelling is considered important for current risk assessments e.g. by providing new hypotheses or confirming the risk assessment. Dr Jager replied that modelling should play a big role in chemical risk assessment, because it is impossible to test everything under all possible conditions. Some predictions have to be made. For these predictions EC_{50} values from laboratory studies can be used. Other measurement endpoints are important for assessing adverse effects other than just toxicity (e.g. life-history traits). For example, the *D. magna* data presented by Prof Bøhn contain a lot of information (there are multiple endpoints over time). Most of the data from such experiments are usually not included in the analyses. In most cases, only endpoints at the last time point (e.g. reproduction and EC_{50} values) are used in the analyses, but the data that is currently discarded can be used to make predictions (e.g. on time varying exposure) using mathematical models.

2.8. Closing session: Workshop lessons on risk assessment, shared views and remaining questions

Moderated by Dr Helmut Gaugitsch (Land Use & Biosafety, Umweltbundesamt, Austria)

Dr Gaugitsch presented a summary of all five sessions as a starting point to initiate discussion among the workshop participants (see Appendix A). Some points of the summary met general agreement, whereas other points led to a lively discussion among the participants. Additional comments were made as well. The main points discussed and comments raised are presented below.

2.8.1. Session 1: Specificity of Bt proteins

- *Data requirements*: A participant stated that the aim of risk assessment is to make a decision based on what we need to know, while what we do not know can be addressed in general surveillance (which is part of post-market environmental monitoring). The speaker argued that much testing being done is redundant (i.e. on Cry1Ab) and does not necessarily have to be part of the risk assessment. It is important to go beyond describing what we do or do not know and to translate knowledge into recommendations for ERA. In other words, we somehow have to put this knowledge together as tools for applicants. Another participant disagreed with the previous speaker's assertion that there is much redundant data and thus considered there is a need to generate additional data to

complete the ERA. In addition, cross-order activity should be assessed on a case-by-case basis. Initial first-tier testing should be extensive to make field testing unnecessary.

- *Mode of action*: The issue of mode of action was put forward and it was suggested that if effects of Bt toxins on NTOs occur it would then be useful to know which mechanisms underlie these effects.

- *Protoxins, activity and specificity*: It was mentioned that the microbial Bt toxins Dr van Frankenhuyzen talked about are not the active ingredients of Bt crops. A participant argued that protoxins should not be used for Bt crop studies because they have a lower affinity than hydrolysed proteins and so the concentration of Bt proteins in the plant will be underestimated. Another participant disagreed with this viewpoint and pointed out that most of the studies use trypsinised toxin instead of the protoxin. Dr van Frankenhuyzen responded that there are only a few cases where it was reported that proteolytic processing changed the level of activity of the Bt toxin, but the spectrum of activity does not change. In other words, proteolytic processing could change the amount of protein needed for an effect, but usually there is no difference on specificity.

2.8.2. Session 2: Selection of species for NTO risk assessment

- *Data transferability*: One of the participants mentioned that the EFSA database is useful to support data transferability. If one chooses to test the organisms that are widely distributed, then the different areas that have to be tested are reduced. It was stated that the major power of the EFSA database is that it describes the receiving environment very well.

- *Problem formulation*: The problem formulation should drive which species should be tested or not, especially when local or regional species are involved. Whether new data should be generated and hence a specific species be tested will thus depend on the outcomes of the problem formulation.

- *GMO vs. pesticide risk assessment*: It was mentioned that the species that are commonly used for testing pesticides (i.e. *Chrysoperla* and *Daphnia*) are not necessarily relevant for GMO risk assessment.

- *Receiving environment*: A participant mentioned that the receiving environment is essential in the risk assessment. However, risk assessment needs to be practical: therefore, not all organisms from every receiving environment should be tested. It was noted that not all necessary data to support environmental risk assessments for the entire EU can be centralised at EU level, because there is too much variability at the local level. Local managers have therefore to consider whether there are additional risks that have to be assessed.

- *Adjacent habitats*: It was mentioned that the focus of the EFSA database is on organisms in the field and that off-field environments (i.e. adjacent habitats) are not really discussed. Dr Meissle responded that the information on arthropods in field margins of the crops included in the EFSA database was inserted in the database, but that the number of records in the database is too limited to provide useful data on off-field environments. As the database highlights knowledge gaps, it might stimulate research in these areas. Another participant added that EFSA is intending to update the database and keep it up to date. Furthermore, it was also suggested that the database might be useful to pesticide scientists and risk assessors.

2.8.3. Session 3: Laboratory studies with NTOs

- *Equivalence of test proteins*: A participant stressed that Bt proteins should be tested for equivalence: the pure microbial-produced Bt protein used in the laboratory and the Bt proteins produced by the Bt crop should be functionally equivalent.

- *Measurement endpoints in laboratory or field*: Following the discussion on the criteria for measurement endpoints during Session 3, Dr Romeis said that in his view population growth is a measurement endpoint for the field and not for the laboratory, and that other endpoints (growth rate, fecundity and mortality) are more relevant for the laboratory.

- *Study design*: It was mentioned that demonstration of a dose-response effect in the validation of measurement endpoints is needed in the laboratory. Another participant responded that this is only valid if there is a hazard or susceptibility. Yet, another viewpoint was that power analyses should be incorporated into the laboratory study design, while another participant missed time-dependence as an aspect of study design.

- *IPM*: It was suggested that Bt crops can play a role in IPM, as was proposed during the talk by Prof Dicke. Another participant disagreed and mentioned that, although Bt proteins are more benign than pesticides, Bt proteins are produced continuously whereas in IPM the use of pesticides is limited to situations where a certain damage threshold is exceeded. In addition, IPM cannot be tested in laboratory studies.

- *Purpose of tier1a and 1b studies*: A participant raised the issue of tier 1a (microbially produced pure protein) and tier 1b (plant material) (as proposed in the EFSA ERA guidelines; EFSA Guidance, 2010) and asked for further clarification on these tiers. According to one of the participants, tier 1a and tier 1b answer two different relevant questions. Tier 1a focuses on the direct effects and asks the question: how broad is the range of organisms affected? Tier 1b involves other stressors to make sure there are no unintended effects. Doubts were raised about what a tier 1b study on pollen can say about the unintended effects of a plant. Dr Romeis added that tier 1b is meant to identify unintended effects, but that this is inappropriate as these effects should be assessed elsewhere and should not be studied in the laboratory. Dr Romeis mentioned that tier 1b is not highly relevant for testing the toxicity of Bt proteins because worst case exposure is not possible with plant material. In addition, it is difficult to find suitable controls and the baseline is unknown. For *in planta* material, proper controls are needed as many confounding effects can be involved.

2.8.4. Session 4: Field experiments with NTOs

- *Triggers for field trials*: It was noted that more guidance is needed on the decision to move from laboratory tests to field trials. It was clarified that the EFSA guidance does not require routine field experiments. Another participant mentioned that at the moment, field studies seem to have limited value, because effects are almost never reported, but that does not mean that field trials should not be performed. Dr Rauschen stated that a case-by-case approach is needed to ensure that not too few or too many studies are done. Dr Albajes argued that the need to conduct field trials is a controversial issue, which depends on the test hypothesis, and thus requires a case-by-case approach.

- *Risk hypotheses*: It was mentioned that the research questions addressed in laboratory and field are different, because field trials are more complex. In the laboratory, one investigates whether hazardous effects occur, while in the field one investigates whether the Bt crop causes harm to NTOs.

- *Field trial design*: A participant stressed the need for a better link between laboratory and field experiments. The results from laboratory tests could be used to design hypothesis-driven field trials.

- Knowledge of the variability of the system is needed beforehand if trials are to be designed with sufficient statistical power. In addition, it would be useful to draw up a list of organisms that should be tested.

- *Pesticidal control*: A participant recommended including relevant management perspectives and using a pesticidal control, or maybe even a 'biological' control, to place data generated in field studies within a broader perspective. Dr Albajes replied that including an insecticide is a good idea, but only if the use of this insecticide is standard practice in that geographical zone. However,

including a pesticidal control is not necessarily useful, because it is known that pesticides are more harmful than Bt crops.

- *Transferability of data*: One of the participants underlined the danger of over-interpreting data from field trials, saying that caution should be taken against transferring data on NTOs to different crops. Although the effect of Bt maize on NTOs is extensively studied in the field, it does not mean that in other Bt crops the same effect on NTOs will occur since different organisms are present in each crop.

- *Risks and benefits*: A participant argued that IPM could be the way forward and that Bt crops could be used in IPM. Bt crops can be compared to current practice and both risks and benefits can be assessed. Furthermore, GM crops should not be seen as a special case. A considerable amount of information from conventional crops is available.

2.8.5. Session 5: Challenges in NTO risk assessment

- *Protection goals*: A participant noted that there is a lack of clearly defined protection goals. It was mentioned that the protection goals in the EU should be uniform for both pesticide risk assessment and GMO risk assessment, as both assessments deal with the same organisms in the environment and aim to preserve the environment from harm. An additional comment was that applicants are asked to specify which protection goals, assessment endpoints and measurement endpoints they use. This participant considered an agreement is needed between the industry and EFSA on which goals and endpoints should be adopted, and recommended that the industry and EFSA build on the existing, practical approach proposed by applicants.

- *Synergism*: It was stated that antagonism and synergism are not the norm. A participant mentioned that a presentation on Bt protein interaction study design should have been part of this session.

- *Biological relevance*: Another participant stated that statistical significance is a starting point for further analyses of the observed effect. When a statistical significant effect is observed, the biological relevance of the effect needs to be assessed. The concept of 'biological relevance', however, can only be used if it is clearly defined, which is not the case in both the scientific and regulatory discourse.

- *Sampling*: A participant indicated that sampling efforts are greater when studying rare organisms compared with studying functional groups. Strategies and efforts required for both kinds of research objects are quite different and should be discussed further.

- *Modelling*: One of the participants said that mathematical models give a false sense that we have complete knowledge, but in fact they are not robust enough to make predictions. Models are only as good as the data used. Moreover, in models, the sensitivity of insects should be incorporated. Some non-target Lepidoptera, for example, are more sensitive than the pest. Another participant responded that modelling is not a magic bullet, but that it can deliver relevant data. According to this participant, questions are often too broad to be tested in experiments and these questions could be solved by modelling. The goal of modelling is to obtain information from the data that are already available.

2.8.6. Conclusions of the moderator

Based on the reactions of the participants following the summaries of the first five sessions of the workshop, Dr Gaugitsch concluded that there was general agreement on his summary of Session 1 on biological specificity of Bt proteins. Cross-order activities and activities across phyla are a fact. However, the level of knowledge on specificity is preliminary and limited; only a few Bt proteins have been tested intensively.

Dr Gaugitsch also concluded there was broad agreement on his summary of Session 2 on species selection. The receiving environment should be taken into account during ERA, including regional

ecological characteristics, such as the occurrence of local species. Databases (like the EFSA and the New Zealand examples), although different in scope and contents, may be appropriate instruments for facilitating species selection. A few suggestions were made during Session 2, such as striking a balance between regional characteristics that should be taken into account and a practical approach to use databases.

It was concluded that the subject of Session 3, laboratory testing of NTOs, needs further discussion, especially the topics of experimental criteria and methods.

Based on the reactions of the participants to his summary of Session 4 on field testing of NTOs, Dr Gaugitsch concluded that field trials are an important aspect and that more focus should be on the continuum of laboratory and field tests.

Regarding the discussion on Session 5 on the challenges in Bt crop risk assessment, Dr Gaugitsch concluded that there are areas that provide further challenges. Among these are that the risk assessment of Bt crops: (1) has to take into account knowledge from other areas (e.g. pesticide protection goals), and (2) it should include the topics of statistical significance versus biological relevance, and (3) mathematical models.

Dr Gaugitsch thanked the audience for the rich and productive discussion.

2.9. Closing

The workshop was closed by Dr Waigmann (EFSA) and Prof van Straalen (COGEM). They both thanked the speakers and the audience for their contributions and wished them a save trip home.

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APPENDIX

A. SUMMARY OF THE FINAL DISCUSSION SESSION AS PROVIDED BY THE WORKSHOP MODERATOR

Session 1: Specificity of and interactions between Bt proteins

- Mode of action as well as specificity of Bt proteins is an important starting point in NTO risk assessment
- There is a vast range of Bt proteins, most of them have proven specificity cross order activities and activities across phyla are a fact
- Our level of knowledge on this topic is preliminary and limited, only a couple of Bt proteins have been tested intensively
- There is a large range of primary toxicity levels (three to four orders of magnitude) but cross activities can occur at relevant toxicity levels
- We have available methods (such as modelling) for assessing combined effects
- Recommendation: Case-by-case assessment of the Bt protein at question, if necessary by providing data on the basis of additional testing

Session 2: Selection of species for NTO risk assessment

- Selection of species for NTO risk assessment is a crucial step
- Established criteria for that purpose: hazard, exposure, ecological function, value to humans, testability
- Take into account the receiving environment, including regional ecological characteristics such as local species
- Species and ecological function groups may be a good basis for the selection
- Problem formulation as the starting point: what is the type of harm, the ecosystem service, the protection goal we want to assess?
- Databases (NZ and EFSA example), although different in scope and contents may be an appropriate instrument for facilitating this selection
- Different relevant functional groups: herbivores, predators, decomposers, parasitoids, pollinators, species of conversation/cultural concern

Session 3: Laboratory studies with NTOs

- What are the questions we want to answer? What do we do with the data resulting from the lab tests?
- Lab tests play an important role in a tiered approach to NTO risk assessment
- Lab tests are an important first step but need to be followed up, e.g. by field trials

- Scientifically valid test design is needed: test substance (toxin, GM plant material etc.), test organism, appropriate controls (positive, negative), length of the study, number of replicates, statistical design, validation of exposure and ingestion, dose dependence of the effect

- Different types of studies with different types of measurement endpoints: lethal, sub-lethal, behavioural

- Focus of the study? It is population growth rate that matters

- Harmonized test protocols and criteria are necessary

Session 4: Field experiments with NTOs

- What are the questions we want to answer? What do we want to detect? What do we do with the data resulting from the field trials?

- Rationale for field trials can be different from the rationale for lab studies

- The environment is complex, with a lot of interactions

- This situation can be better mirrored by field trials rather than less complex lab studies

- Scientifically valid test design is needed: test substance (toxin, GM plant etc.), test organisms, sampling methods, appropriate controls (positive, negative), length of the study (longer than 2 years?), number of plots, replicates, statistical design and power

- What is the goal of the field trial in the context of integrated pest management (IPM)?

- The results of field trials may be of limited value

- Natural variation should be taken into account

Session 5: Challenges in NTO risk assessment

- Predictions at the population/community level are relevant

- Causality chain from exposure to population effect – a multidisciplinary approach is necessary

- Take into account interactions (synergism, antagonism), which are the norm rather than the exception

- Include assessment of effects on adjacent aquatic ecosystems, case-by-case, with a valid experimental design (take into the account the quantitative aspect of exposure in this case)

- Integrate different statistical test approaches – difference test, equivalence test: each approach is valid, together they serve best for the purpose

- Rationale for a combination of both approaches: identify unintended effects, put results into the context

- Mathematical models can aid in the quantification of uncertainty, can be analysed critically and thereby contribute to transparency

- Meta-analysis on one Bt event confirms that lab and field studies are in agreement

- Two approaches to protection goals: ecosystem services may be linked to species (e.g. rare species), or functional diversity – functional groups in agro-ecosystems (pros and cons for both approaches)

- Biological relevance is still a rather vague concept

- Define protection goals – a task for risk managers and regulators

- Commonly accepted and verified protocols for environmental risk assessment should be developed (including long-term effects)

- Assessing everything does not mean testing everything