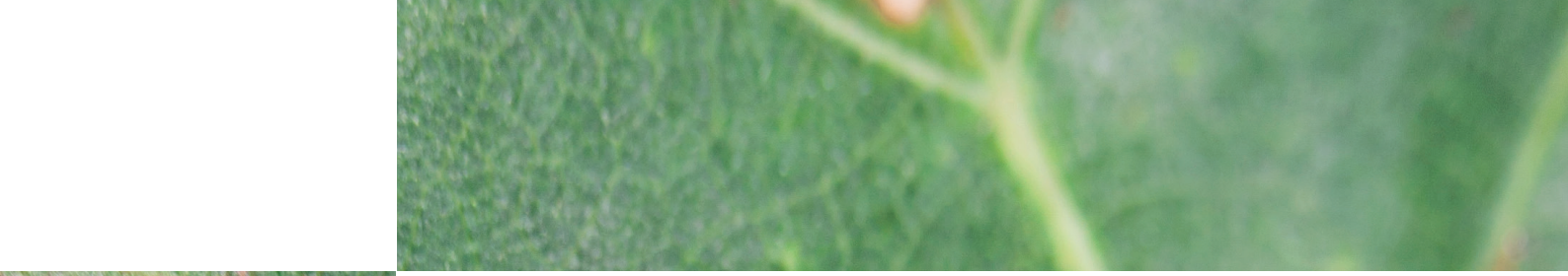

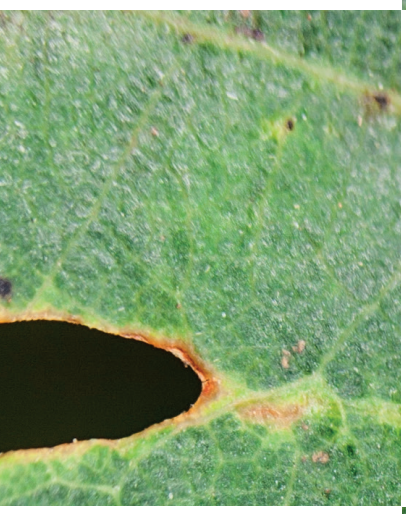

Testing impacts of toxic compounds from transgenic crops on non-target arthropods in tier-1 studies: exposure and response

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Testing impacts of toxic compounds from transgenic crops on non-target arthropods in tier-1 studies: exposure and response

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Preface

"When you know the exposure you know the effect" is an often-heard statement among toxicologists. The underlying argument is that in many cases the variability in toxicological data, especially when exposure is through environmental media, can to a large extent be ascribed to variation in exposure. In a similar spirit, COGEM was faced with a range of toxicity experiments aimed at estimating the environmental risks of Bt toxins to non-target organisms. The data of these experiments proved to be highly variable and in some cases fierce debates arose over the interpretation of the results. Against this background, the present study was commissioned: to shed more light on the reasons why the studies reported in the literature provide such variable results and to develop criteria applicable to solid tier-1 toxicity studies with Bt toxins.

The present report is a significant step in that direction. It concludes that the exposure method cannot be isolated from the test species itself. Drawing on a broad knowledge of entomology, the author argues that every species comes with its own biology, including foraging behaviour and mode of feeding. It is therefore unavoidable that a variety of exposure methods is applied, when each exposure method is optimized with regard to the species of concern. An extensive review of literature data is conducted to support this argument. COGEM will have to consider the biology of the species when evaluating toxicity studies with non-target organisms, implying that such assessments should be done on a case-by-case basis. At the same time, the set of criteria proposed facilitates an easy evaluation of experimental set-ups encountered in risk assessment studies.

Nico M. van Straalen

Chair of the supervising committee

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Summary

To assess potential adverse effects of transgenic crops on non-target organisms (NTOs), a variety of test species is exposed in laboratory studies (tier-1 studies) to toxins that are produced by the transgenic crops. Most commonly it concerns Cry-proteins that originate from genes of Bacillus thuringiensis and have strong toxic effects on a limited number of insect pest species. To study effects on life history traits of a selection of NTOs, different exposure methods are used to mimic worst situations that could negatively impact population densities of such NTOs. Because the receptors for Cry-toxins are situated on the epithelial surface of the midgut, toxic effects can only occur when the Cry toxins are ingested with the food. Because different species have different feeding patterns, different diets and different life histories, the methods of exposure and duration of exposure are key issues in such studies. As these methods are not standardized, the exposure methodology easily leads to debate about the results from different studies. In addition, there is no full consensus about which parameters should be measured to reach meaningful results. In recent years the methodology of toxicity studies with NTOs has continuously been adapted and improved to make results more robust and comparable. Yet there is no golden standard that fits all species and sufficiently reflects natural exposure and the feeding mode of the different NTO species. To obtain clear and more conclusive results in such studies, biologically sound and validated methods are needed for more various NTO species. This report explores available options and limitations of current methods and identifies a number of criteria that should be met to obtain robust and meaningful results.

To evaluate the state of the art in exposure methodology and effect measurements in NTO studies, current methods for different functional and taxonomic NTO groups were critically summarized in this report. From this review, quality criteria were formulated for methodology and completeness of a number of case studies published over the last decades.

In most studies the maximum Cry-concentration as it is maximally expressed in the relevant tissues of the transgenic crop, (the reference), is used to create baseline worst case exposure. Various methods are used to create a 10 times higher exposure dose by adding pure Cry protein to the diet. The most common methods are using pollen with high levels of Cry protein or pure Cry-proteins applied on natural food or in artificial diet. In several cases this reference Cry concentration may deviate considerably from the natural exposure due to the variable and mixed diets and feeding patterns of NTO species. Also where the exposure is indirect e.g. in predators, the choice of relevant exposure concentration can be problematic as there is much uncertainty in uptake patterns. However, in many cases the worst case scenario's used tend to over-dose rather than under-dose the Cry proteins, being conservative in missing effects. To assure that experiments are sensitive, measuring or estimating Cry concentrations in the diet and the uptake by the NTO are important experimental requirements. Also the duration of exposure in the assays should match the maximal exposure duration in the field, which often means a full life cycle. Measuring only mortality, as was done in earlier studies, is too limited to estimate population effects. Measuring various life-table relevant

parameters such as growth, developmental rates, fecundity and behavioural traits strongly enhance the value of the studies.

Technical and methodological principles of tier-1 studies have been described quite extensively in recent years. The evaluation of the case studies shows that guidelines are more and more followed but still technical options and guidelines are not used optimally, in particular where NTOs are less amenable for laboratory studies. This is partly due to the costs involved to overcome experimental constraints. Life time exposure, tracing concentrations and measuring more life-table parameters are key elements. The availability of artificial diets, good rearing methods and methods to measure uptake of Cry are important. For first screening programs it is advised to use a standard set of species, that are easy to handle and for which good protocols are available. The selection of species used and the exposure scenario has to be representative for the natural (field) situation to obtain the most reliable results. Many other potential species may be interesting to assess but feeding modes, uptake and experimental constraints may hinder extensive studies for those species. In particular for species with mixed diets and indirect exposures, it is very important that all experimental aspects are precisely described. Yet the interpretation of results obtained with these more difficult species in tier-1 studies remains questionable. Such results are less reliable in a tier-1 risk assessment

Samenvatting

Om de risico's van genetisch gemodificeerde gewassen op niet-doelwit organismen (NTO's) te beoordelen, worden deze organismen in laboratoriumexperimenten blootgesteld aan de door het gewas geproduceerde toxinen, die in de gemodificeerde plant tot expressie komen. Over het algemeen gaat het om eiwitten die oorspronkelijk afkomstig zijn van de bodembacterie *Bacillus thuringiensis* (Bt-toxines ofwel Cry-eiwitten). Deze Cry-eiwitten zijn sterk toxisch en selectief voor bepaalde organismen, m.n. insecten. Verschillende methoden van blootstelling en verschillende concentraties toxine worden gebruikt om mogelijke effecten op NTO's vast te stellen. Omdat de receptoren voor Cry-toxinen in het darmepitheel liggen, kunnen deze stoffen alleen werken als ze door het organisme worden opgenomen. Omdat iedere NTO zijn eigen manier van voedselopname en zijn eigen dieet heeft, zijn de wijze en duur van blootstelling erg belangrijk. Veel discussie over al dan niet terecht geconstateerde effecten wordt vaak gevoerd naar aanleiding van de manier waarop NTO's in het toxiciteitsonderzoek worden blootgesteld aan Cry-eiwitten. Ook over de relevantie van de gemeten effecten bestaat niet altijd overeenstemming. De methodologie van dergelijke "tier-1" proeven is de laatste jaren voortdurend aangepast om de uitkomsten robuuster en beter vergelijkbaar te maken. In de literatuur is nog geen sprake van een standaardisering die volledig in overeenstemming is met de natuurlijke situatie. Voor het verkrijgen van heldere en eenduidig interpreteerbare resultaten zijn kwaliteitscriteria voor deze proeven nodig. Dit rapport verkent de beschikbare opties en ook de beperkingen en identificeert criteria waaraan voldaan moet worden om robuuste en betekenisvolle resultaten te verkrijgen.

Om een beeld te krijgen van de kwaliteit van de huidige proeven is in dit rapport eerst de problematiek van diverse blootstellingsmethoden in beeld gebracht. Dit gebeurde door beschreven methoden voor diverse functionele NTO-groepen kritisch tegen het licht te houden wat betreft hun doeltreffendheid en robuustheid. Vervolgens werd een aantal recente case studies beoordeeld op een aantal kwaliteitscriteria en op hun volledigheid.

In tier-1 studies met Bt-gewassen wordt meestal gebruik gemaakt van een worst-case scenario. Hierbij wordt een organisme langdurig blootgesteld aan tenminste de concentratie van Cry-toxinen zoals die in het gewas tot expressie komen en aan een 10 maal hogere concentratie. Om dit te bereiken is het noodzakelijk om extra toxine, meestal in pure vorm, toe te voegen aan het op te nemen voedsel. Kunstmatige diëten en het toevoegen van extra Cry-bevattend stuifmeel zijn methodes, die veel worden toegepast bij NTO's uit verschillende taxonomische groepen. De blootstelling dient bij voorkeur gedurende de gehele levenscyclus te worden gerealiseerd en in overeenstemming zijn met hetgeen onder natuurlijke omstandigheden op zou treden. In bepaalde gevallen kan deze blootstelling sterk afwijken van de natuurlijke situatie, maar zolang de blootstelling een worst-case scenario in het veld weerspiegelt hoeft dat geen bezwaar te zijn. Om tot betrouwbare resultaten te komen moet vastgesteld worden dat de toegediende concentratie ook inderdaad wordt opgenomen door het organisme. Opname wordt in slechts 16% van de studies gerapporteerd. Dit kan voorkómen worden door de toxinegehaltes in plantmateriaal, prooisoorten en het uiteindelijke niet-doelwitorganisme terug te meten en nauwkeurig te volgen tijdens de experimenten. Het meten van alleen directe letale toxische effecten via mortaliteitscurven is te beperkt omdat sub-lethale en uitgestelde effecten, zoals larvale ontwikkeling, reproductie en ook gedragseffecten een grote impact kunnen hebben op de omvang en het voortbestaan van

populaties. Een ‘life-table’ benadering waarbij diverse parameters worden gemeten geeft het meeste zicht op potentiële populatie-effecten in het veld. Voor een risicoanalyse kunnen echter ook andere parameters bruikbare informatie geven, bijvoorbeeld het gedrag.

De principes voor goede blootstellingsprotocollen in het lab zijn in diverse recente artikelen beschreven voor een aantal NTO-soorten. De praktijk laat echter zien dat maar weinig studies optimaal gebruik maken van de beschikbare kennis. De experimentele drempels en kosten die verbonden zijn aan het opzetten van degelijke en diepgaande experimenten met robuuste en natuurgetrouwe blootstelling of het ontwikkelen van een kunstmatig dieet, dat voldoende recht doet aan de natuurlijke voeding, zijn hoog. Ook het volgen van blootstelling- en opname-concentraties over langere periode en het meten van meerdere effectparameters stuit op vergelijkbare bezwaren. Echter in alle gevallen dient de proefopzet beargumenteerd te worden en moeten onzekerheden in de resultaten bediscussieerd worden om de betrouwbaarheid van de NTO test in de risicobeoordeling te waarborgen. Het verdient zeker aanbeveling om naast mortaliteit ook sub-lethale effecten te meten zoals life-table parameters en gedragsparameters, die populatie-dynamische consequenties hebben. Omdat het aantal te testen soorten en potentieel te meten effecten groot is, zou het testen van de potentieel meest gevoelige soorten, waarvoor goede protocollen (en diëten) beschikbaar zijn, prioriteit moeten hebben. Experimenten met soorten waarbij de blootstelling indirect is, die een gemengd dieet hebben of een zwakke link met het gewas hebben, zullen al gauw resultaten geven die moeilijk te interpreteren zijn. Deze kunnen wetenschappelijk wel interessant zijn om onverwachte effecten op te sporen, maar zijn voor de tier-1 risicobeoordeling minder bruikbaar.

1. Introduction

Insect resistant crops are developed on a large scale by incorporating *Bacillus thuringiensis* (Bt) genes in the crop genome. When expressed, the genes produce Cry proteins that are toxic to a specific range of lepidopteran or coleopteran species that attack the crops. The currently used Bt-genes incorporated in transgenic insect-resistant crops produce toxins that primarily affect the target species. However, each variant of the many known Cry proteins is acting in different ways and apart from the target insects other non-target species may be exposed and may be sensitive to these toxins as well, in particular when they are taxonomically related to the target species. But also unrelated organisms can be sensitive and cross-toxicity even exists between orders of insects (van Frankenhuizen 2013).

These often unpredictable potential effects on non-target organisms (NTOs) is one of the reasons that an environmental risk analysis (ERA) has to be done before transgenic insect resistant crops are approved for commercial production. Within an ERA a so-called tiered approach is used to investigate potential effects of Cry proteins on NTOs in particular on non-target arthropods (NTOs). The tiered approach starts at the laboratory level (tier-1) and is extended to semi-field (tier-2) or field level (tier-3) when significant effects are found at the lower level. When no effects are observed in tier-1 worst-case scenario studies, there is little reason to proceed with higher tier studies.

Tier-1 studies are meant to act as a first screen of a limited number of non-target species for potential hazards that may affect their abundance and the ecosystem services they provide in agro-ecosystems. Worst-case scenarios and strict experimental guidelines are used to make sure that no obvious effects are missed. Such a tier-1 worst-case approach has been shown to be consistent with, or to be more conservative than, the effects of Bt-crops in the field for NTOs (Duan *et al*. 2010). To create worst-case scenarios in tier-1 experiments three elements are essential:

1. Species selection: the species should be potentially affected

2. Exposure: exposure in tier-1 studies should reflect worst-case exposure in the field

3. Measurement endpoints: these should be ecologically relevant and potentially affecting population densities

These elements should reflect conditions that are similar or worse than those occurring in the field (the natural or reference situation). Experiments should be transparent, accurate and statistically robust to generate useful and clear results. In case of doubt when a negative effect is identified, further tests are recommended at higher tier levels. For evaluation and use of experimental results in risk assessment and for the approval of experimental designs there is a need for clear-cut criteria. As each crop has its own characteristic properties that determine how arthropod fauna is exposed to Cry proteins, the criteria should be robust but flexible to fit the specific crop conditions.

Methodology for testing potential negative effects of genetically modified (GM) crops on non-target species has been developed in the last two decades, starting with methods that were similar to those for pesticides but steadily becoming more targeted to the more complex situation for GM-crops and NTOs. Copying the procedures from pesticide testing for testing Bt-crop effect on NTOs has been criticized already many years ago and since

then laboratory protocols have been adapted to suit the GM-NTO context (Duan 2007). A key issue that makes pesticide effects different from GM-crops, is the way NTOs are exposed to the Bt-toxin expressed in the plant and are taken up by the NTOs. In Bt crops NTOs can be exposed during a full lifetime and toxins can only be hazardous when they are ingested by NTOs either by direct feeding on plant parts or by feeding on organisms that acquired the toxins at lower trophic levels (often the plant feeding species). This chronic exposure in which ingestion plays a key role is very different from the usual short-term exposure to pesticides by direct contact. For this reason the exposure method is a key issue when developing and improving tier-1 studies for GM crops on NTOs.

A further consequence of long-term exposure in the field is that it is more likely that all developmental stages of the organisms may be exposed by which apart from direct mortality, also life-history traits can be affected and sub-lethal effects may play a more significant role in the ultimate population effects. Based on the issues discussed above and what was already published, Charleston and Dicke (2009) suggested alternative laboratory trials and protocols in which exposure is thoroughly treated and sub-lethal effects are measured in a way that is more close to what may actually happen in the field. At that time also other studies used and described advanced exposure methods and laboratory protocols to test GM-crops expressing Cry toxins. Since then several other studies have focused on improving tier-1 methodology suggesting better and standardized procedures for species selection (Romeis 2013), exposure protocols, measuring life history parameters, including positive and negative controls, creating statistical power and discussing relevance of chosen endpoints and analysis and interpretation of results in relation to risk (Romeis et al 2011, Li et al. 2014).

The international scientific community has established a set of criteria and procedures for tier-1 laboratory tests that are applied worldwide (Romeis 2011), which provides useful information for risk assessors and managers. Although the current testing methods have been well described, from problem definition up to experimental data analysis (Wolt et al 2010, Romeis 2011), the issue of exposure is underrated and there is a need to have a set of practical criteria to design, execute and evaluate tier-1 experiments for specific functional NTO groups associated with a particular GM crop. For this reason a set of experimental criteria (elaborated from Romeis et al. 2011) is used to evaluate recent tier-1 experiments for a number of species and crops and to formulate more case specific criteria and experimental options for a number of situations in maize, potato and oil seed rape.

The aim of this report is to summarize the state of the art for exposure and response methodology in tier-1 effect studies of worst-case scenarios used in NTO testing, and to evaluate recent studies for the application of up-to-date methods and constraints that occur in such studies with regard to methods, analysis and interpretation.

2. Criteria for NTO tier-1 studies

Baseline experimental guidelines and quality criteria have been published for insects and other NTOs by Romeis (2011) and others that reflect the state of the art in tier-1 studies and consensus current research.

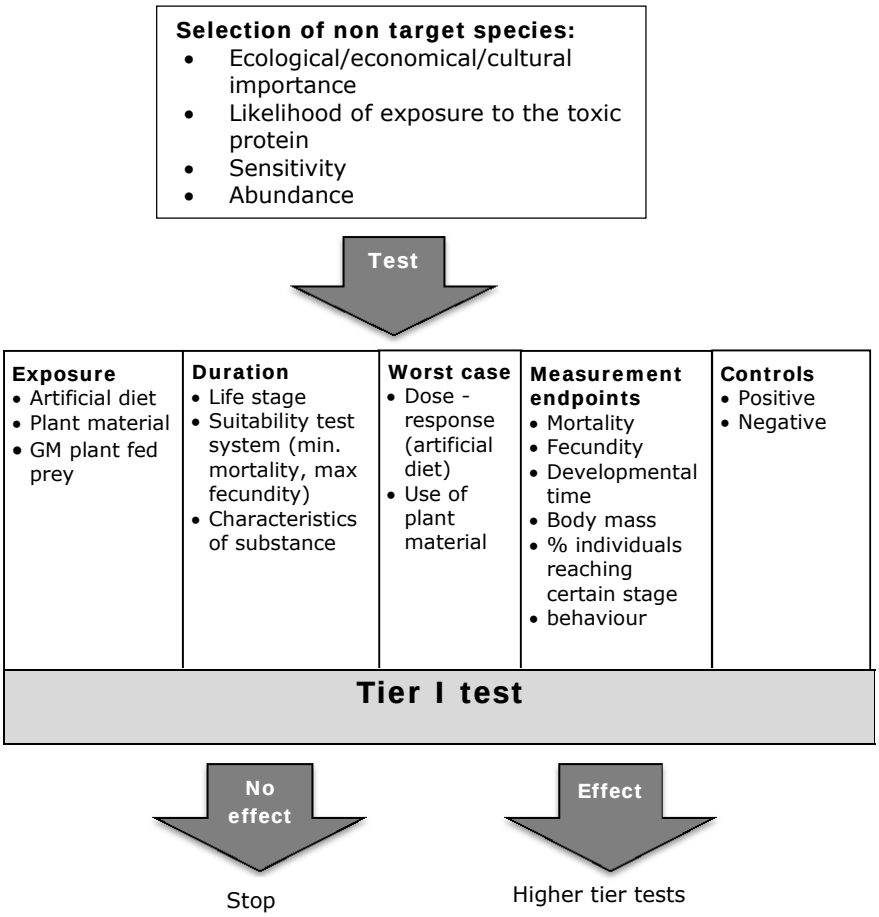
Briefly, these criteria include:

- The Cry protein should be well characterized
- The functional activity of the Cry protein should be equivalent to that in the transgenic crop
- The bioactivity of the substance should be demonstrated
- The test organism should be exposed to a relatively high concentration compared to that in the field
- The uptake of the Cry protein by the organism should be demonstrated and quantified
- The response parameters should be chosen as an indicator for potential adverse effects
- The number of replicates should be sufficiently high to measure effects with enough statistical power
- Positive controls should be incorporated to show that the test system is able to detect treatment effects

One may wonder if these published criteria are sufficiently clear and applicable to all functional groups or whether additional and more specific criteria are needed that cover current and new developments in the near future. At least discussions about species selection, exposure methods and most relevant measurement endpoints continue among researchers and risk assessors. This report focuses on exposure methods and response measurements in tier-1 studies.

In experiments, exposure methods and response parameters should match the ecology and feeding mode of the test species to provide information that can be translated to field situations. It is a key element in environmental risk analysis whereby policy goals, scope, assessment endpoints and methodology are described and developed into an explicitly stated problem and an approach for analysis (Wolt et al. 2009).
Based on the characteristics of the species, appropriate methods should be applied to test the response in worst- case exposure and uptake scenarios.

A background for a number of topics that are relevant for designing tier-1 studies is schematically presented below. This report focuses on different aspects indicated in the diagram below.



2.1 Exposure methods and modes of ingestion

A wide array of exposure methods have been used in tier-1 studies to test the sensitivity of NTOs. The methods developed try to match the natural feeding mode of the NTO in a GM crop environment. As the natural feeding mode is highly diverse among organisms, exposure methods have to be adapted to the species studied.
For comparison and interpretation of different studies, however, some standardisation is preferred.

For tier- 1- studies an exposure scenario should be described that is likely to occur in the field and should include the pathways and coincidence of the test organisms with the GM-crop-expressed toxins. This scenario should be reflected in the tier-1 study, by either feeding the NTO an artificial diet contaminated with the Cry proteins in question, plant-tissues of Bt crops or by other trophic connections. There should be a clear link between natural and lab exposure (this concerns mainly the ingested toxin concentration. Describing the relation between lab and field exposure will make interpretation of studies more transparent. As is commonly accepted in NTO tier-1 studies (Romeis 2011), the tier-1 is based on the maximum gene expression (i.e. highest Cry protein levels in a crop during the growing season). Expression data of Cry-toxin levels within a Bt- crop is available in regulatory dossiers. Expression data are given from multiple sites over the season, because it is known that expression in the field can vary between related Bt-varieties and can change over the growing season as was shown for Bt cotton by Dong et al (2007) and Ullah et al. (2014).

The reference dose in a tier-1 study should reflect the maximum field expression published and a 10 times higher dose should also be tested (worst case scenario). When significant effects are found, follow-up or higher tier) studies using with other doses should be used. For organisms not directly feeding on plant parts such as leafs, stems, roots or pollen or for those plant parts that are only a small part of the normal diet, it may be argued that concentrations of Cry-protein other than maximum expression levels in plant tissue should be used for reference exposure. A good example of how such exposures could be calculated is given by Carstens et al. (2012) for aquatic organisms that are indirectly exposed. In this study, careful calculations are made about how plant material is diluted in the environment and in the food web before it is taken up by the NTO of interest.

An ***exposure checklist*** should consider the following performance criteria:

- Is it clear how, when and along which routes the test species is assumed to be (maximally) exposed to the toxins produced by the GM crop?
- The exposure should reflect the exposure level and duration of the same or similar organisms under natural conditions and a 10 times higher concentration (worst case scenario).
- The concentration of toxins in the exposure medium (usually food ingested by the test organisms) should be measured or estimated in a reliable way
- It should be shown that the toxin is active during the exposure study
- It should be taken into account if the test organism has a mixed diet of toxin-containing and toxin-free items

- A dose-effect relation should be established including a zero-exposure (control), a reference exposure and a 10x higher dose.
- The uptake of toxins should be verified by estimating the uptake of toxin-containing food or the ingested toxin concentration in the organism.

2.2.1 Feeding mode and exposure in different functional groups

As the feeding modes of species in the agro-ecosystems are diverse, and consequently the exposure to toxins by ingestion varies widely, a case by case approach is often necessary to find the right exposure methods in tier-1 studies. Both feeding mode and exposure depend on the functional niche of species in the agro-ecosystem.

As agro-ecosystems can offer habitats for quite a number of (non-target) species that are potentially exposed, exposure studies are necessarily limited to a small number of species that are considered to be representative for the functional groups in the agro-ecosystems. These tests often include common and mostly beneficial species or closely related species (surrogate species) that are suitable for experimentation because of breeding methods, artificial diets and available bioassays (Banks et al. 2014).

Below we review these aspects for each functional group and identify weaknesses and strengths for the different exposure methods. Direct or indirect exposure routes, exposure duration, the use of natural or artificial diets as well following uptake and ingestion are discussed in more detail in the case studies.

Plant feeders

A large group of herbivorous arthropods may be associated with the GM crops feeding either on the Bt crop itself or on plant species that grow as weeds in the crop or in field margins. The latter are exposed mainly through Bt-pollen that disperses from the GM crop.

Herbivorous crop-associated non-target species are usually seen as harmful, so maintenance of population abundance is usually not regarded as a reasonable aim. Exceptions can be imagined e.g. when species of conservation value with a wider distribution are also strongly attracted to the Bt crop.
Exposure of herbivorous species in tier-1 studies can usually easily be achieved by providing them the preferred plant parts in excess to maximise uptake. For this plant-feeding group no surrogate species were found in literature.

It can be imagined that, apart from the use of the main target species (e.g. corn rootworm or maize stem borer) that feed on plant tissue, non-target species with different feeding modes and a known sensitivity for each Bt crop could serve as a reference or positive control in experiments with different exposure protocols.

Plant-feeding species of conservation value that do not live on the crop can still be exposed when living on weeds or wild plants in field margins. The only exposure route identified in such a case is by wind-dispersed pollen that is deposited on leaves and is

ingested by non-crop associated pollen feeders or chewing plant feeders such as caterpillars or phytophagous beetles. Data on pollen deposition from some Bt crops is available so levels of worst-case exposure can be estimated (see e.g. Perry et al. 2012).

Several non-target herbivorous species have been used for tier-1 studies, such as:

Leaf feeding chrysomelid beetles such as *Leptinotarsa* , *Oulema* and *Gastrophysa* that were studied by Huber en Langenbruch (2008), Meissle en Romeis (2009).

Leaf feeding caterpillars (Lepidoptera) can be given pure protein in artificial diet when available (e.g. *Helicoverpa* and *Ostrinia*, (Head et al. 2001) or can be exposed to Cry containing leaves. They can also be fed on their own host plant with additional defined amounts of pollen as was done with *Danaus plexippus* (Matilla (2005) and for *Inachis* en *Aglais* with maize pollen on nettle leaves (Felke 2003).

Leaf and seed feeding species such as weevils (Curculionidae); tests so far have been done only with artificial diet including pure Cry proteins (Head et al. 2001).

Parenchym feeding spider mites such as *Tetranychus urticae* (Li & Romeis 2010) are usually exposed to leaves or additional pollen.

Phloem feeding (plant sucking) species of aphids (Aphididae) or leafhoppers (Cicadellidae), which are normally live in colonies on whole plants (Lundgren and Wiedenmann 2005). For some species artificial diets are available. Due to the fact that Cry proteins are expressed at higher levels in leaf tissue rather than in phloem, uptake and accumulation of Cry proteins by aphids from the plant is very limited (Burgio et al 2011). As a consequence, the concentrations of Cry protein to which aphid-feeding predators are exposed are much lower (20-40 times) than the concentrations in the plant itself.

Seeds are not only consumed by phytophagous species such as weevils but also by omnivorous species that east seeds in addition to insect prey. This is rather common in some carabids. In addition to seeds also pollen is accepted by many carabid species (Mullin et al 2005 and Ahmad 2006)

Pollen and nectar feeding species

Arthropods that feed on pollen or nectar form a diverse group including honey bees, wild bees, parasitoids, hover flies, predatory bugs, and several families of beetles and predatory mites that visit flowers to forage on nectar and pollen.
Also adult lacewings consume considerable amounts of pollen (Li et al 2010). Many species feed only during a restricted period on nectar or pollen of a particular crop and visit other plant species outside that period. It is mainly the adult that has a direct uptake, but larvae form bees and bumblebees are fed by nectar and pollen, so they are indirectly exposed to Cry proteins when the adults forage on Bt-crops. Honey bees are most widely studied as a model (or surrogate) non-target species for Bt crops. However, most experiments thus far indicate that survival and body weight of these species is not affected by to the currently used Cry proteins. In most experiments the arthropods are exposed by feeding them pollen or a sugar solution with pure Cry protein. Experiments so far have been performed with bees, parasitoids and butterflies. Cry protein levels in

pollen have been extensively studied and are part of the regulatory dossiers of the Bt plants.

Cry protein levels in nectar are less frequently measured. A recent study of Paula et al (2014) is a good example of a study where nectar is used for exposure of adult butterflies to Cry protein. Various tier-1 studies using nectar exposure are also found in the literature for honey bees (Maggi 1999, Richards 2011, and Hendriksma 2011, 2012). But in a recent study, Wang et al (2015) state that to their knowledge plant nectar tends to be free of Cry proteins. Also, honeydew produced by aphids is likely to be low in Cry protein levels as the aphids that produce honeydew are phloem feeders with little Cry protein uptake.

Predators

Predatory arthropods are considered as beneficial to the cropping system because they may regulate insect pests. These typical NTOs need to be protected against negative impacts of cropping practices. A diverse group of species (often laboratory strains) have been studied for side-effects of pesticides and more recently also on a wider scale for effects of Bt crops.

Carabidae - ground beetles

Ground beetles represent a highly diverse group of species that are common in arable crops. Most of these species are carnivorous, but many have a range of different prey or even have a mixed carnivore/herbivore diet. Feeding them with only herbivores that have been fed of the with Bt plant material creates a worst-case scenario in itself, but most studies aim at ingestion of a concentration equal or higher than the concentration in the crop. For some species, laboratory methods have been developed including diets to which pure Cry protein can be added. However, usually a mixed diet is provided with natural prey as well as additional Cry protein sources.

For example bioassays are available for *Pterostichus cupreus* fed on maize-fed caterpillars (Meissle 2005) see also Mullin et al 2005, Stacey et al 2006, as well as bioassays for *P. melanarius* and *Harpalus affinis*, and other carabid species.

Staphylinidae - rove beetles

Rove beetles include several beneficial species that feed on small insect eggs, larvae and adults. *Aleochara bilineata* is one of the most common and widespread species that is used in pesticide-effect and Bt tier-1 studies. Only part of these short-winged beetle species is carnivorous while many other species in the group are also detritivorous (but rarely included in tier-1 studies). In a recent example, exposure of the rove beetle *Atheta coraria* to Bt-maize fed spider mites was tested (Carcia et al. 2010). Prey species such as mites and aphids, however, ingest very little Cry protein (Burgio 2011). Hence using such food does not create a worst-case scenario.

Anthocoridae - predatory bugs e.g. flower bugs
Proposed species for tier-1 studies are *Orius laevigatus* or other *Orius* species feeding on

many small prey. They are indirectly exposed by eating Bt-crop feeding arthropods such as mites, thrips, leafhopper larvae and aphids, but they are also directly and more importantly exposed by feeding on pollen. Larvae of these bugs (nymphs) have a diet similar to the adults. Their ecological importance in various crops including maize, makes them a favourite study object for tier-1 studies, though sensitivity to Cry protein has not been shown. They are quite easy to rear and high exposure levels in tier-1 tests can be created by providing Cry protein containing pollen and/or additional pure toxin. See for example Duan et al. (2005).

Coccinellidae

The ladybird beetles (both adults and larvae) are main predators of aphids (which take up little Cry protein from the plants) and mites (more Cry protein uptake); both groups may ingest pollen when available.
Examples are *Coccinella -7-punctata* (Pracar et al 2010, Alvarez-Alfageme 2011, 2012) directly exposed to proteins in diet, and *Stethorus punctulum* which is a predator of mites (Li & Romeis 2010).

Syrphid larvae - hoverflies
While adult syrphid flies feed on pollen and nectar, larvae of some species are important predators of plant-feeding aphids. Since aphids accumulate very little Cry toxins from the Bt crop, the adult flies are more interesting for tier-1 studies as pollen and nectar usually have higher concentrations of Cry toxin and in this type of food, Cry toxin levels can easily be manipulated. Population effects may be translated into the number of predatory larvae in the field in the next generation. Also delayed effects can be studied in the performance of larvae as progeny of exposed adults.

Chrysopidae- lacewings

Lacewings are important predators in many crops where they feed on a variety of different prey species including aphids and caterpillars. They also feed on pollen and nectar when available. Screening of effects on lacewings by Cry proteins has been routinely performed by applying the proteins on meal moth eggs as food for lacewing larvae. However, this method has been criticized by Hilbeck (2012) and others as ingestion of Cry protein by this exposure route is poor. Recently artificial diets and pollen have been recognized as better exposure methods (Li et al. 2014b).

Arachnida - spiders

Spiders are a widespread and important group of NTOs that occur in all cropping systems. Depending on their size and feeding style (hunting or web-building) they consume a lot of many different prey species including herbivorous pests, but also other beneficial insects. They can also consume pollen that attach to their webs. Because of this complexity, it is hard to derive a reference exposure concentration from the field situation. However, a worst case scenario could be created by exclusively feeding the spiders on a Bt crop-fed herbivore that contains substantial amounts of Cry protein. As pollen are accepted by many species, (Peterson 2010) this can be a useful source to increase exposure levels. As spiders are taxonomically unrelated to most target organisms and hence are assumed likely to be insensitive to Cry proteins, only a few tier-1 studies have been performed (Ludy and Lang 2006, Meissle and Romeis 2009), generally showing no effect of currently used Cry toxins.

Decomposers and soil organisms

Cry proteins from Bt crops enter the soil ecosystem via root exudates, crops residues, pollen and seed. Though the proteins are gradually broken down, they can also persist for a long period depending on the crop and cropping system. The fate and effects of Bt-crops have been reviewed some years ago by Icoz et al. (2008).

Soil organisms such as mites (Acari), springtails (Collembola) and woodlice (Isopoda) but also earthworms and nematodes are exposed to Cry proteins where Bt crops are grown and they have been subjected to several effect studies. For example a model species such as the springtail *Folsomia candida* and the common woodlice (*Porcellio scaber*) have been tested in tier-1 studies (see e.g. Bai et al 2011, Sims and Martin 1997, Pont and Nentwig 2005) mostly by feeding them with Cry- and non-Cry protein containing leafs or adding pure Cry proteins to the diet. Determining ecologically relevant exposure levels is not easy due to the Cry protein breakdown and little information exists about uptake patterns by soil inhabiting species.

2.2 Concentration and uptake of Cry-proteins

Before tier-1 experiments are performed, it is important that the expression of Cry proteins in various plant tissues is known in order to establish the basic (near field) concentration to be tested in the tier-1 experiment. Cry protein-expression is variable depending on crop stage and external conditions. Principally, in the experiments should make use of data available from in the regulatory dossiers of the Bt crops studied. The standard exposure or other (higher or lower) exposure rates should be derived from this reference concentration.

- Depending on the feeding mode and diet of the NTO species under laboratory settings, (which may differ from the field situation it ideally represents), the exposure should at least be as high as that estimated from the field to create a natural full exposure scenario. As in most other toxicity studies a 10 times higher exposure is applied (worst case scenario). To generate more information in case of lower sensitivity, higher concentrations can be used to create full dose response curves.
- During the different stages of the NTOs in the experiments it would be useful to assess or use indirect estimates of the concentration of Cry protein in the diet and in the studied organisms in order to be informed about uptake, breakdown or accumulation of the toxins in the organisms.
  There is often a discrepancy between the natural feeding modes and experimental constraints to create near natural uptake and to mimic worst-case scenarios. To achieve a higher Cry protein exposure than in the reference in most cases pure protein is added to the diet. While laboratory conditions may often have adverse effects on any life history parameters anyway, artificial diets with high Cry protein levels may create additional stress. As long as this stress factor is similar all treatments and the control, this may be tolerated (see 2.4). A problem sometimes encountered is the effect on the food quality for the next trophic level. In such cases, effects can be due to general food quality rather than the toxic effects of Cry proteins itself. Studies focussing on uptake under laboratory or field circumstances are scarce.

- Uptake rates from plants vary from species to species and transfer rates changes through the food chain. For example it was shown by Burgio et al (2011) that uptake of Cry1Ac from Bt -oil seed rape by the NTO aphid *Myzus persicae* was low; concentrations in the aphid were 30 times lower (5-7 µg/kg) than in the plant (160-230 µg/kg). When aphid predators should are exposed to concentrations that occur in the plant this would create a false picture.

Exposure methods have been described in detail for several NTOs in different functional groups (Duan et al. 2007,Charleston & Dicke 2009, Paula 2014, Li et al 2014b and others). However, there is no standard available that applies to all species. Due to the species- specific biological traits, a case by case approach is needed.
Common exposure methods can be categorized in either artificial diets, natural diets or a combination. Sometimes pure protein is added when higher concentrations are required. When pure protein is used in addition to the natural or artificial diet, it should be well characterized, and its activity in the plant should be known (Romeis 2011).

2.3 Response parameters (stage and life history traits)

Response parameters in tier-1 studies are based on life history parameters such as mortality/survival, developmental time, age-dependent fecundity, as well as on other parameters including physiological state, weight and behavioural effects. Each of those parameters may have its merits for indication of a hazard. The relevance of different parameters, however, may differ from species to species in terms of population effects. Therefore it is very useful when researchers give arguments why a given parameter is assumed to be important for *population maintenance* or *ecosystem services*. For example, parameters that are necessary for life-tables such as age-dependent fecundity and the estimated intrinsic rate of population increase are easy to be interpreted for relevance. Also a parameter such as searching capacity for a predator obviously links to their relevance in biocontrol, and therefore their ecological function.

Functional groups to which the NTO belongs can provide a lead to which parameters are most useful to be measured. For the test on effects to NTOs at a higher trophic level it is suggested by Charleston and Dicke (2008) to use all life-table parameters (r_m and r_i) needed to estimate population growth. r_m and r_i estimates are widely accepted as indications for population fitness, even where other parameters such as dispersal power and natural mortality (which are much harder to retrieve from laboratory experiments) are important for population maintenance. These and other parameters should be clearly defined and their potential impact on the population impact of the NTO substantiated.

It is important to realize that any experimental set-up can be criticised for the parameters not taken into account. In particular delayed effects or effects that are hard to study in the lab can be important for population maintenance. The life-table approach is likely to fulfil the basic criteria to estimate effects on population fitness. Any additional parameters characteristic for the studied species may provide further useful information for the risk assessor.

A checklist for response measurement in exposure studies should include the following:

- The response parameters should be clearly defined including the units in which they are expressed as well as their spatial and temporal dimensions
- The response parameters should be ecologically relevant and contributing to a life-table approach to estimate population dynamical effects.
- There should be a causal (direct or indirect) relationship between the measured response and the mode of action of the toxins

Lethal effects

Parameters that express lethal effects (mortality) are commonly used in studies on NTOs in a way comparable to most other toxicity studies such as for pesticides. Mortality is mostly expressed as the percentage of individuals found dead after a defined period of exposure, as a function of the dose. The dose range should include both lethal and non-lethal (including control) exposures. By using curve-fitting techniques, an estimate may be obtained for the median lethal dose, either expressed as a true dose (LD_{50}) or as a concentration in the exposure medium causing 50% mortality.

In laboratory trials the age or the age distribution of the organisms should be taken into account. At the beginning of the experiments, the age distribution of the tested organisms should be the same in all treatments.
LD_{50} is often used in pesticide trials but is less suitable for Bt toxin effects. This parameter is meant to estimate the dose required to kill 50% of all individuals in the trials. For pesticides this is mostly measured shortly (one day) after the exposure assuming that the effects are instantaneous and the exposure short lasting. For Cry proteins, the exposure is typically long-lasting. Potential mortality effects interfere with natural mortality and the moment of final measurement of mortality for LD_{50} is arbitrary. Preferably, the age- dependent mortality rates should be included in a life-table approach. Still it should be realized that mortality measured under controlled conditions in the lab can be very different from the field where many more factors are affecting the survival. The assumption that those factors act independent of the Cry protein effects can be challenged when sub-lethal effects such as development or behavioural effects make the organism more vulnerable for e.g. adverse conditions and predation. In this way sub-lethal effects may affect survival in the field.

Sub-lethal effects

For risk assessment, sub-lethal effects may be equally important to estimate potential effects on populations of NTOs. Charleston and Dicke (2009) already argued that for ecological relevance complete life-tables would be most useful. For such life-tables sub-lethal effects are crucial. Most relevant parameters in life-table approaches are (age-dependent) fecundity and larval development. Total fecundity obviously affects the number of progeny from one generation to the next and hence the contribution to population maintenance. When eggs are produced at a lower rate or larval development is slowed down due to Cry protein exposure, population growth is retarded. When there is only one generation per year, these effects can lead to asynchrony with the environment, or change the average fitness which is expected to be negative rather than positive. When there are more generations per year such retarding effects can lead to lower populations in winter. In life-table analysis these effects become apparent in lower maximal or intrinsic rates of increase. These rates are clearly defined and are useful in comparative studies. Therefore survival, development and fecundity can be considered as a basic set of parameters that can be included in a life-table approach for studying transgenic Bt crop effects on NTOs.

Additional non-lethal parameters that provide useful information are body weight or size (affecting fitness of F1 generation) and behavioural traits such as mobility (as an indicator for dispersal and searching capacity) or handling time in predators or parasitoids). Also cold tolerance or drought tolerance could be useful aspects as

overwintering is crucial for many organisms. Measuring such additional parameters also helps to estimate ecological impacts.

2.4 Dose - response curves and accumulation

The dose–response relationship describes the adverse effects on an organism caused by different levels of exposure (or dose) of the Cry toxin after a certain exposure time. Effects of different exposure levels can be tested at individual level of individuals or directly on population level (effects on numbers of individuals of a certain stage).

The most meaningful dose-response curves include levels of exposure that generate different effect levels that could indicate potential effects on populations. Obviously a zero exposure, a natural reference exposure and a worst case scenario (10 x higher concentration) should be included

Dose response relationships generally depend on the exposure time and exposure route (in this case various forms of dietary intake). Variation in the way organisms are exposed may lead to different conclusions about the effects of the stressor under consideration. This experimental constraint is inherent to the complexity of the often unknown biological processes operating between the external exposure and the adverse cellular or tissue response. During extended exposure times, accumulation occurs depending on the uptake and breakdown of the toxins. The breakdown and excretion of toxins may be so strong that accumulation does not occur and concentrations of the toxin in the NTO may drop after exposure is stopped. This was for instance shown for the money spider Phylloneta impressa (Meissle and Romeis 2012). In other cases, accumulation may occur after longer exposure times. Therefore generalization of dose response curves to related species can be misleading when exposure times and accumulation vary.

Worst case scenarios are based on the idea that the organism is continuously and exclusively feeding on the plant tissue or prey that contains a known concentration of toxins that are maximally expressed by the transgenes involved. In the field, the Cry gene expression tends to fluctuate during the season (Dong and Li 2007), and average concentration tend to be overestimated. But for tier-1 studies, the maximum expression (concentration in the relevant tissue) is normally used as the natural reference concentration. To realize maximum accumulation and potential activity in the NTO, exposure times should cover the full life cycle of the test organism.

To create lower doses than the natural reference, a mixed GM/non GM diet may be used. For higher doses the natural of artificial diet should be complemented by additional pure toxins. The ingestion should be measured in some way to verify that higher exposure doses indeed lead to higher uptake.

Dose-response curves should preferably cover a range of concentrations and related uptake levels that a test species is likely to be exposed to under natural conditions in current and future Bt crops to be suitable for predicting effects and to establish critical effect levels that can be used for risk assessment.

Response curves can also be used as a reference or positive control in other comparable studies. In some cases they may serve as predictive tool in higher tier studies where exposure varies according to crops, varieties and conditions with different Cry exposure levels.

2.5 Negative controls, treatments and positive controls

Negative control

In the negative control the treatment conditions should be equal to the other treatments apart from the presence of the Cry protein to be studied. For assays using Bt- plant material to feed the test NTO or to feed the prey of the test species, plant material from the isogenic non-GM counterpart serves as the most obvious negative control.

Providing artificial food media with or without the presence of Cry protein is also a good option.
In some cases, equivalence can be disturbed when the presence of the Bt toxin is associated with other properties in the diet for example when plant properties are influenced by the presence of Cry protein or when Cry proteins ingested by plant feeding species affect their value as prey for predators. In such cases effects of general food quality can be confounded with effects of the Cry itself (e.g. Lawo et al 2010).

Treatments

In the most simple case it is preferred to study the presence (different doses) of Cry protein as the only relevant factor. If food quality is changed due to the presence of the Cry protein one may prefer to provide pure toxin to the test organisms instead of the toxin in the food, to be able to assess the effect of the toxin only. However, one may argue that the combination with natural food is ecologically more relevant as this is how the Bt-crop features works out in the field. So far, very few studies were found in which the effect of the Cry protein was studied in combination with a naturally linked factor (such as a pesticide) or by studying the effects under different environmental conditions (such as temperature). When comparing different studies, experimental conditions should be described.

Positive controls

In case of Cry protein effects studies, positive controls are used in order to proof that the test organism actually ingests the substances offered in the diet and that toxins present in the diet can potentially have effect i.e. confirming the sensitivity of the test.

There are several options:

1. The most obvious proof for exposure and uptake of Cry protein is to measure /monitor the presence and concentrations of active Cry proteins in the diet and in the exposed organisms themselves. For example by taking samples from the exposed organisms at certain intervals during the experiment. In a more qualitative way, one may measure Cry protein levels in the excrements (frass) or in eggs laid by the exposed organisms (and the control).

2. In some cases colour dyes or other labelled substances have been used to prove or quantify the uptake of the Cry containing diet, assuming that this is related to the

uptake of Cry protein itself.

3. Experiments may also include positive controls with a toxic substance that is functional equivalent to Bt, that is orally ingested and known to have effect on the selected end point.
Adding a positive control is also essential when using an artificial diet supplemented with the pure Cry toxin, as the positive control can proof the validity of the testing system.
Commonly used toxic positive controls include potassium arsenate (PA) and a proteinase inhibitor (E-64) Sometimes also insecticides such as teflubenzuron and imidacloprid have been used. (Li et al. 2014, Romeis et al. 2011). Most species are sensitive to those substances. In principle any defined and tested substance can be used that has a known and quantified effect on the effect parameter measured and for which the ingestion is equivalent to the ingestion of the Cry protein used in the experiment.

4. Experiments may include (known) sensitive species with feeding modes equivalent to the test species, so that they undergo exactly the same treatment to confirms the reliability of the assay. Sometimes the target species or a sensitive taxonomically related species is used as positive control. In most experiments however feeding modes of sensitive target and non-target species will be different and other positive controls should be used.

In dose-response studies care should be taken that the uptake of food is dose independent. In some cases higher doses may inhibit food uptake.

3. Criteria-based evaluation of recent tier-1 studies in Bt crops and improvement suggestions for different NTOs

Tier-1 toxicity studies have been published for different Bt-crops including an increasing number of non-target species and the application of new approaches for exposure and effect measurement. To see how current studies follow the experimental guidelines and criteria as presented in this report, a selection of studies from the last 10 years is evaluated to see where methodology could be improved. The experimental set-up of these studies with regard to exposure methods and effect measurements is summarized in Table 1. In the sections below, the methodology used is discussed for the different taxonomic and functional NTO groups.

3.1 Butterflies and moths

Lepidoptera-targeted Cry-1Ab proteins are frequently tested for potential effects on non-target species of butterflies and moths where caterpillars feed on plant tissue and adults feed on nectar. Exposure in this group may occur in a) non-target caterpillars that feed obligatory or occasionally on parts of GM-crop, b) caterpillars of species that are associated with weeds in the crop or live on plants in the near environment and feed on pollen that disperses from the GM crop and c) adults that consume nectar from flowers of the GM crop. For these three categories, crop plant-material, added pollen, or nectar have been used in bioassays to mimic natural Cry levels from the GM plant or higher exposure levels by adding artificial pure Cry protein.
The studies by Felke (2003) and Paula (2014) may serve as representative for tier-1 studies in this group.

Charleston and Dicke (2009) described experimental protocols only for nectar-feeding adult NTOs and suggested that fecundity, emergence and survival of larvae were key parameters to allow a life-table approach for risk assessment. Adult feeding and Cry uptake can be relatively well controlled by providing sucrose solutions with different levels of Cry. For caterpillar exposure, protein containing plant tissue of the transgenic crop can be used or leaves from a non-crop host in combination with additional Cry containing pollen that is ingested.

For nectar-feeding adult NTOs flower-visiting behaviour and the concentration of Cry toxins in the nectar should be leading variables to design exposure regimes in toxicity experiments. The study of Paula (2014) is a good example of how this can be achieved. The nymphalid butterfly, *Chlosyne lacinia* (Bordered Patch) is a frequent flower visitor in (transgenic) cotton crops. Fecundity and subsequent effects on development, survival and (unexposed) offspring are ecologically relevant endpoints to be assessed. By using a relevant range of Cry concentrations in nectar, dose-response curves are relatively easy to establish also for non-lethal effects (Paula 2014).

For pollen-exposed caterpillars feeding on non-GM crop leaves, protocols are more difficult and not elaborated by Charleston and Dicke (2009). Pollen exposure in the field may be variable due to the fact that pollen shed often takes place in a short time-window that may or may not be synchronous with the caterpillar feeding. Pollen amounts deposited on the caterpillar's host plant should be measured to establish a reference exposure concentration to be used in laboratory studies (Felke 2003). Another aspect that should be addressed is that the actual ingestion of pollen applied to the leaf should

be measured in the test caterpillar as they may avoid eating leaf parts that have too much pollen on them.
From the paper of Felke (2003) it can be concluded that when feeding rate decreases when pollen are applied, resulting effects can be attributed to underfeeding rather than to Cry effects. This may in particular be true for higher pollen dosages. When Cry-containing pollen reduce feeding and Cry-free pollen does not, this might be a relevant ecological effect, not of Cry toxicity but of reduced food quality.

Fig 1. Tortoise shell caterpillar (*Inachis io*) on nettles in field margins. Deposition to Cry proteins on nettles is measured during pollen shed to estimate potential exposure levels.

Table 1 Evaluation of exposure methods and response measurements in recent tier-1 studies on for non target in insects in Bt-crops

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Test organism	**Functional group**	**Cry protein**	**Exposure route**	**Life stage**	**Exposure Duration**	**Dose range**	**Endpoint**	**Dose response**	**Protein accumulation**	**Positive control**	**Negative control**	**Sample size**	**Conclusion**	**Reference**	**Notes**
Apis mellifera	Pollinator, honey bee	Cry1Ab	GM maize pollen	adults	12 days	3ppb and 5000ppb	mortality, learning behaviour	two doses	not measured	imidacloprid	uncontaminated syrup	50	Cry1Ab may affect foraging efficiency.	(Ramirez-Romero et al. 2008)	Foraging behaviour is crucial for bees, important to measure behaviour next to mortality.
Apis mellifera	Pollinator, honey bee	Single Bt CP4 EPSPS, and stacked Bt maize (Cry1A.105,Cry3Bb1, Cry2Ab2,)	semi artificial diet	larvae	5 days	1-5 mg pollen	Survival, body mass,	Not tested	not measured	Toxic *Heliconia rostrata* pollen	Pollen of non-Bt maize	20 or 40	No effect	(Hendriksma et al. 2011)	The exact amount of Cry proteins were not measured.
Apis mellifera	Pollinator, honey bee	Single Bt Cry1Ab, and stacked Bt maize (Cry1A.105,Cry3Bb1, Cry2Ab2,)	semi artificial diet	larvae	5 days	4-5 concentrations with 10x gradient	Survival, pupal body mass,	Yes. But no typical dose-response curved obtained for the positive control GNA lectin.	not measured	GNA lectin	Buffer mix and BSA	62-110	Neither single nor stacked Cry proteins affect on honey bee larvae	(Hendriksma et al. 2012)	LD_{50} value was based on 2 dosage points. Would be better to test a range of concentrations between d*100 and d*1000
Chrysoperla rufilabris	predator, lacewing	Cry1Ac, Cry2Ab and Cry1F	larvae of Cry-resistant cabbage looper or armyworm fed on Bt maize fed on Bt plants	larvae	2 generations	leaf Bt maize	Mortality, developmental time, fecundity, egg hatching rate	No tested	not measured	No	leaf non-Bt maize	90	no hazard effect	(Tian et al. 2013)	Bt resistant preys may have special mechanism to degrade Cry proteins. Using these preys may result in low exposure to Cry protein, and not represent the worst case scenario in the field.

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Rhopalosiphum padi	herbivor, aphids	Cry1F	Bt maize	all	18 days	leaf Bt maize	survival rate, alata vivipara production, or host preference	Not tested	Measured, Cry concentration increased in the body of aphids feeding upon Bt maize	No	non-Bt maize	20	No negative effects found, but Cry concentraion accumulated in aphids	(Kim et al. 2012)	
Cotesia marginiventris	parasitoid	Cry1Ab	Spodoptera frugiperda on artificial diet with Cry1Ab protein	all	1 generation	1x, 20x and 200x con. in leaves of Bt maize	survival, developmental time, grother rate	Three concentrations	not measured	No	Spodoptera frugiperda on artificial diet without Cry1Ab protein	12 or 20	Exposure to Cry1Ab protein via Bt-maize tissue affected parasitoid developmental times, adult	(Ramirez-Romero et al. 2007)	
Cotesia marginiventris	parasitoid	Cry1Ab	Spodoptera frugiperda on Bt maize	all	1 generation	leaf Bt maize	parasitism, survival, developmental time, longevity, size, fecundity	No	not measured	No	Spodoptera frugiperda on non-Bt maize	160	size, and fecundity	(Ramirez-Romero et al. 2007)	
Adalia bipunctata	predator, ladybird beetle	Cry1Ab and Cry3Bb	Neonate A. bipunctata fed with *Tetranychus urticae* fed on Bt maize	larvae	L1-L3	Dosage in Bt maize leaves	Mortality, developmental time, dry weight of L3.	No		No	Neonate A. bipunctata fed with *Tetranychus urticae* fed on non-transgenic maize	45	No effect through tritrophic feeding	(Alvarez-Alfageme et al. 2011)	It would be better to chose a better prey of *A. bipunctata.*

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Adalia bipunctata	predator, ladybird beetle	Cry1Ab and Cry3Bb	Neonate A. bipunctata fed with *Tetranychus urticae* fed on Bt maize	larvae	2d or 5d (=L1 and L2)	Dosage in Bt maize leaves	NR	No	Bt protein levels in Bt maize leaves, *T. urticae* and *A. bipunctata* measured with DAS-ELISA	No	Neonate A. bipunctata fed with *Tetranychus urticae* fed on non-transgenic maize	6	Cry protein concentration lowest in A. bipunctata. No accumulation effect	(Alvarez-Alfageme et al. 2011)	
Adalia bipunctata	predator, ladybird beetle	Cry1Ab and Cry3Bb	*A. bipunctata* fed with Sucrose solution with Cry protein	1st day of each instar	1d	45 µg/ml Cry1Ab, 200 µg/ml Cry3Bb1. (10x conc. In spider mites fed on Bt maize)	Larval mortality, pupal mortality, larval developmental time, pupal developmental time, adult dry weight	No	not measured	10,000 µg/ml, GNA, 300 µg/ml or potassium arsenate	Sucrose solution without Cry protein	34-41	No effect via direction injection of pure protein	(Alvarez-Alfageme et al. 2011)	Time fed on sucrose solution (exposure) was only during the first day of each instar, may underestimate the effect of Cry protein.
Adalia bipunctata	predator, ladybird beetle	Cry1Ab and Cry3Bb	*A. bipunctata* fed with eggs of *Ephestia kuehniella* sprayed with Cry proteins	larvae (24h old)	L1 until pupation	5 - 50 µg/ml	mortality, developmental time, adult body mass	5, 25 50 µg/ml	not measured	No	10 and 100 µg/ml of pBD10, the expression factor	120	Cry1Ab caused higher mortality, Cry 3Bb caused marginal higher mortality	(Schmidt et al. 2009)	The exposure method used in this test dose not simulate the real situation. Lack of linkage between the used dosage and the realistic dosage of Cry proteins in the eggs of Lepidopteran

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Atheta coriaria	predator, rove beetle	Cry1Ab	Fed with *Tetranychus urticae* reared on Bt maize	Newly emerged larva and adults	L1-L3 or 4d for adults.	Bt maize expressed with Cry1Ab	survival, sex ration, adult body weight and size, preoviposition period, total fecundity, fertility	No	Fate of Cry1Ab protein through the trophic chain measured with ELISA.No accumulation effect, but decrease through the trophic chain.	No	Rearing food, T. urticae+non-Bt maize	30 for larvae and 10 for adults	No effect of prey reared on Bt maize detected on any life-histry parameters.	**Carcía et al. 2010 Bio. Control 55:225-33.**	
Euselius concordis	predatory mite (develops and reproduces best on pollen)	Cry1Ia12	Feed on Bt suspension and Cry protein	Newly hatched larvae	24h	0.006mg/ml and 0.018mg/ml	Life-table parameter	One dosage of Bt suspension and two dosages of Cry1Ia12	Not measured. Blue clour dye used to confirm ingestion though	No	Solution with dye	30, 38 and 68	No effect found with Bt suspension and Cry1Ia12 at 0.006 mg/m, 0.018 mg/ml Cry1Ia12 reduce fecundity	De Castro et al. 2013 Exp. Appl. Acarol 59:421-33.	Life-table parameters were used as endpoints. No positive control and no quantitative measurement of ingestion of Bt or Cry1I12 protein.
Neoseiulus californicus	Predatory mite (prefer spider mites to pollen)	Cry1Ac	Fed with *Tetranychus urticae* reared on Bollgard® expressing Cry1Ac gene	Whole life	Whole life	NA	Life-table parameter	NA	Not measured	No	Non-transgenic isoline	52	No effect found	De Castro et al. 2013 Exp. Appl. Acarol 59:421-33.	Life-table parameters were used as endpoints
Inachis Io	plant feeding caterpillar	Cry1Ab	Bt maize pollen on leaves fed to caterpillars	larvae from 2th - 4th instar	1d-2d	10, 20, 40, 80, 160 an 320 pollen/leaf	mortality, weight gain	yes	not measured	No	rearing on leaves with non-Bt maize pollen	variable but 5 -30 per treatment	significant effects found in higher dose range	Felke et al 2003	dose was not related to exposure level in the field, no positive control, no intake measured

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Test organism	Functional group	Cry protein	Exposure route	Life stage	Exposure Duration	Dose range	Endpoint	Dose response	Protein accumulation	Positive control	Negative control	Sample size	Conclusion	Reference	Notes
Chlosyne lacinia	plant feeding caterpillar	Cry1Ac	Pure Cry protein in artificial nectar for adults , leaves dipped in Cry solution	Adults and larvae life time long	life time long	100 ug/ml for adults, range of concentration for larvae essay on leaves	larval and adult mortality F0. Adult Fecundity , larval development and mortality of F1	yes, for larvae	protein uptake was measured in frass and egg masses	casein as a control for protein uptake.	Cry free nectar for adults and Cry free leaves	300 individuals	when adults or larvae are exposed life long to sublethal levels, negative effects were found in F1 larval development	Paula 2014, Plos one 9:1-7	dose used 100 times higher than natural level.
Orius albidipennis	Predatory bug	Cry1Ac	Starved O. albidipennis were offered with a drop of Cry protein solution	Nymphs	1-3 times of 2d in cyclus	1 and 10ug/ml	prey consumption developmental time, nymph survival, fecundity and egg hatching	two doses	not measured	No	drop of water	30	No effect on any of the observed parameters	Donzalez-Zamora et al. 2007, Environ. Entomol. 36(5):1246-53.	No positive control, no measurement of Cry protein ingestion either. The exposure method is questionable. No explanation of the chosen dosage and the Cry expression level.
Orius insidiosus	Predatory bug	Cry3Bb1	Nymphs exposed to pollen diet including Cry protein	Nymphs 3d after hatching	up to 13d	930ug/g≈10x highest expected environmental concentration of Cry3Bb1 in Mon863 corn plant tissue	survival and developmental time to adults	no	not measured	protease inhibitor E64 at 53ug/g	pollen diet with buffer solution, and pollen diet only	25	No negative effects detected at the tested dosage	Duan et al. 2008 Environ. Entomol. 37(3):838-44	No dose response tested. Actual ingestion of Cry protein not measured.

Table 1 Evaluation of exposure methods and response measurements in recent tier-1 studies on for non target in insects in Bt-crops.

Test organism	Functional group	Cry protein	Exposure route	Life stage	Exposure Duration	Dose range	Endpoint	Dose response	Protein accumulation	Positive control	Negative control	Sample size	Conclusion	Reference	Notes
Protaphorura armata	spring tail	Cry1Ab	Dried ground root tissue of maize varieties, baker's yeast diet contained Cry protein	adults	4 weeks	Cry concentration in the root tissue	Somatic growth, body surface, population growth rate	No	not measured	No	Dry baker's yeast	10	No negative effect found, recommend species interactions in long-term, multi-species experiments	Heckmann et al. 2006, Environ. Pollution 142:212-6	Sample size low.
Poecilus chalcites (F1-from field collected adults]	ground beetle	Cry3Bb1	published culture diet with pure Cry added, refreshed every 2 days, concentration measures	larvae	continuous during larval development, 4 weeks	930ug/g≈10x highest expected environmental concentration of Cry3Bb1 in Mon863 corn plant tissue	weight and survival			uptake not measured, but check with the same diet was mad with sensitive lady bird larvae and diet with potassium arsenate	diet with only buffer solution(no Cry) and a water control	6 for survival curves, 40-60 beetles weighing per treatment	no effects on survival and weight (growth)	Duan 2006	exposure to 10x concentration in plant tissue which is not their natural food which are animal prey part of which feed on corn
Chrysopa carnea	lacewing	Cry1C	Pollen and artificial media for adults. Artificial diet for larvae	Adults and larvae	lifetime	1, 10 and 100 x tissue concentration in food	Larval development Weight Fecundity	No	No	Potassium arsenate	Protein free artificial medium and pollen	25-45	No effects detected	Li et al 2014b	High quality set up.

Table 1 Evaluation of exposure methods and response measurements in recent tier-1 studies on for non target in insects in Bt-crops.

Test organism	Functional group	Cry protein	Exposure route	Life stage	Exposure Duration	Dose range	Endpoint	Dose response	Protein accumulation	Positive control	Negative control	Sample size	Conclusion	Reference	Notes
Carabidae (6 species)	Ground beetles, collected from field crops	Cry3Bb1 and Cry1Ab (combined with neonicotin!)	Gt pollen and isogenic pollen ad libitum		continuous	Adapted to weight, but not exactly measured.	Survival differences between Cry3 and Cry1		No	no	no	Low, 3-11 beetles/ treatment	No difference between CRy3 and Cry1 survival	Mullin 2005	No negative control, unknown uptake, one unknown exposure, statistically very poor, highly variable
Aphis gossypii	*Herbivore cotton aphid*	Cry1Ac and combined with CpTI	Standardized colonies on cotton plants		Continuous, over 3 generations	Natural level	R0, Rm, generation lifespan, lifetable parameters, fecundity and survival Feeding behaviour, fluctuating asymmetry (developmental stress indicator)		No	No, Cry +CpTi might be considered as a positive control?	Untransformed isoline	5 parameters sets per treatment	Development, survival and Rm fecundity and probing behaviour equal between Cry and control (but almost all different in CpTi) Fluctuating assymetry was different in all 3 treatments	Xiang et 2005	
Rhopalosiphum padi	*Herbivore aphid on maize*	CryF1	Standardized colonies on maize leaves on wet cotton	Adult/ nymphs	Full cycle of 14 days	Natural level (in phloem	Fecundity, survival, wing formation	no	yes	no	Non-Isogenic Non-Bt variety	3 colonies starting with 20 individuals	Positive effect on fecundity, no effect on other parameters	Kim et al 2012	Low # replicates, maize strains not isogenic

3.2 Plant feeding beetles

Cry3-Bb is toxic for leaf beetles (Chrysomelidae) living on the transgenic crops or in nearby other plant species (contaminated with pollen).
Huber and Langenbruch (2008) performed a range of laboratory experiments on five non target leaf beetle species by providing pollen suspensions and Cry3Bb protein solutions with different concentrations on leaf discs as food for the larvae. Effects on survival, development and weight were measured but not in a life-table context. The applied methods appeared useful to show some effects even with small numbers of insects. However there was no reference to field exposure. No information is available for Cry3Bb concentrations in the NTO beetles themselves and part of the effects can be due to reduced feeding instead of to the Cry protein.
Charleston and Dicke (2009) did not provide protocols for leaf-feeding beetles, but a life-history approach similar to predatory beetles could be imagined.. Supplying pure toxins to leaf discs seems adequate for leaf beetles as long as uptake can be estimated and more life-history parameters are measured. For species feeding on plant species other than the crop itself, information is needed on pollen deposition to estimate a reference concentration of pollen or pure toxins to be applied as the standard. No advanced studies in this field were found by us.

3.3 Aphids, leafhoppers, thrips and spider mites

Phloem-sucking insect species such as aphids and leafhoppers commonly occur in many (transgenic) crops such as maize, cotton, potato and rice. Also parenchym feeding thrips and spider mites occur in almost every crop. As many are considered pests rather than NTOs, development of tier-1 protocols for these groups got little attention thus far. Natural exposure is straightforward by testing growth and reproduction on the Bt and non-Bt varieties. Exposure to higher doses can only be done when the test animals are cultured on an artificial diet which is available for some species. Cry protein levels in the plant and uptake and accumulation patterns in the test organisms can be irregular and excretion can be high. Uptake in these phloem-feeding aphids has been reported to be very limited but thrips and spider mites accumulate significant amounts of Cry (Romeis & Meissle 2010). These plant-feeding groups are not described by Charleston and Dicke (2009).

We did not find any references for tier-1 studies in thrips, aphids or leafhoppers but two relevant exposure studies were found for the aphids *Rhopalosiphum padi* on maize (Kim et al 2012) and *Aphis gossypii* on cotton (Xiang et al. 2005) respectively.
The case of *Rhopalosiphum padi* is an interesting one as this widespread species occurs on a variety of host plants including cotton and soybean. This species is an important host for parasitoids and prey for predators and therefore is a staple food for these natural enemies in several crops. Both adults and nymphs of the aphids are phloem feeders and are assumed to have negligible uptake of Cry proteins. However the authors showed that the aphids accumulated significant amounts of Cry1F proteins when feeding on Bt maize leaves. When this finding is confirmed for other Cry toxins this species may serve as a surrogate species as it is a good representative for non-target herbivore/sucking insects. Due to fast development and colony forming properties, the life-history approach is relatively easy to apply in tier-1 studies.
Xiang et al (2005) studied the effect of transgenic cotton varieties with genes expressing Cry proteins and CPtI cowpea trypsin inhibitors on various life history parameters,

behaviour and growth of the Cotton Aphid *Aphis gossypii*. When both genes were present most life-table parameters were affected as well as feeding behaviour and stress indicators. However, when only one Cry protein was expressed in the plant, no effect could be found for the life-history traits studied. This may be explained by the minimal uptake of Cry. However asymmetric growth and slower development occurred on the Cry-only variety and not in the control which indicates that the aphids were stressed by the presence of Bt genes but maybe as an indirect effect. The study indicates that feeding behaviour and development of aphids may be disturbed by Cry toxins. The study also showed that other parameters than life-table data can provide useful additional (sub-lethal) information. Though the population rate of increase (population fitness) was not significantly affected, the stress imposed on the aphids is likely to reduce their quality as prey for natural enemies. This reduced prey quality effect was also shown for caterpillars consumed by lacewings (Lawo et al. 2010).

3.4 Lady bird beetles

Lady birds are common and widespread predators all over the world and occur in almost every crop where prey is available. Both adults and larvae prey primarily on aphids, but also on white flies, psyllid larvae and many other prey types that they encounter. When hungry they may also feed on nectar and pollen, though this is not their primary food. In NTO trials, they are exposed mostly to the Cry toxins by the diet. Aphids or spider mites grown on the transgenic Bt crop and on non-Bt plants served as controls (Alvarez-Alfageme et al. 2011). Worst-case scenarios are based on the idea that they maximally feed on prey originating from grown the Bt-host or Cry containing pollen. This scenario is quite realistic for the larvae that stay on the host plant from the egg to the pupal stage. Using aphids however, though often their primary food, is not very useful since they accumulate very little Cry. In addition, since lady bird beetles also readily feed on lepidopteran eggs, these can be used for as an exposure medium by spraying them with a defined dose of pure Cry.
In nature the adults will normally have a mixed diet from different host plants as they move readily from one place to another. To create an exposure dose above natural field levels, other food such as lepidopteran eggs or honey / sucrose solutions with added pure Cry protein can be used. However, choosing an ecologically relevant reference dose is rather arbitrary. In practice spider mites also have been used (Schmidt et al 2009) but they accumulate little Cry-proteins, hence exposure levels tend to be relatively low compared to that in the GM plant. Experiments done so far indicate that adult lady birds beetles and larvae themselves do not accumulate much toxin. One may wonder if more toxins are ingested when other more natural prey species such as aphids are used.. Due to the mixed and variable diet of these predators it is difficult to establish a well-funded standard exposure for these non-target insects. Tracing Cry concentrations in the diet as well as in the larvae and adult beetles is needed for consistent results and to make the different studies (with different exposure methods) comparable.

3.5 Ground and rove beetles

Ground beetles (Carabidae) and rove beetles (Staphylinidae) occur widespread and numerous in many crops. A few widespread species are tested in laboratory studies when rearing methods are available. Field exposure for the different species in different

cropping systems can be highly variable as many species have opportunistic feeding strategies switching to the most abundant and rewarding prey types (in case of carnivores) but also to plant material and detritus (in omnivorous species).
Worst-case scenarios assume that the test species eat herbivores feeding on the GM crop e.g. corn rootworms or caterpillars. Artificial diets are based on this assumption and allow for higher Cry exposure by adding more Cry protein to the diet.
For example, survival, development and growth of the ground beetle *Poecilus chalcites* was measured after exposure to Cry3Bb1 in a dose 10 times higher than in plant tissue. Information about the Cry concentration in their real food was lacking and no dose response analysis was made. The positive control in this case was arsenate which –as expected- indeed caused severe mortality. This study typically lacks a good link to the natural exposure and using one exposure concentration and no uptake verification makes its conclusion of no-effect questionable.
Another approach was taken by Mullin (2005) who exposed different field collected carabid species to a diet of Cry1 and Cry3 containing pollen *ad libitum* and followed their survival for several weeks. Uptake was not measured and negative or positive controls were lacking. Regarding the weak experimental setup, using very few animals and high experimental variability, their conclusion that neither Cry1 nor Cry3 causes no mortality is not well substantiated.

A last example of again a different approach is described in the paper of Garcia et al (2010) who studied Cry1Ab impacts on *Atheta coriaria* by feeding this rove beetle for a number of days with spider mites reared on Bt and non-Bt maize. They did not find any effect on a variety of life-history traits measured. Although the set-up of these experiments was sound, no clear information came out about the uptake of Cry, as ELISA showed very little uptake indicating that the prey accumulated very little Cry. As no real dose-response could be established the rove beetle can still be sensitive when exposed in another way. In this study a positive control was lacking.

The case studies above exemplify that the feeding modes and diets in this group prevent a straightforward approach. Also in the protocol study of Charleston and Dicke (2009) no clue is given for sound experiments with ground and rove beetles. Furthermore, the long generation time of predators in this group make them unsuitable for life-table studies. This may lead to the conclusion that there is a lack of good methodology for this common group of beneficial insects.

3.6 Predatory bugs

Several species of predatory bugs are important predators of a variety of pests such as mites, aphids and psyllids. In particular the genus *Orius* (flower bugs) is a valued group for biocontrol and these bugs are considered as non-target species that are frequently included in GM effect studies in crops such a maize and cotton. They have a mixed feeding mode, feeding on insects as well as pollen. They can be easily kept in laboratory cultures and exposure levels of Cry can be manipulated by using artificial diets, pollen and additional pure protein (Duan et al 2005, Tan et al. 2005). No protocols are described by Charleston and Dicke (2009). The studies summarized in Table 1 concentrate on worst-case scenario's using pollen plus added Cry protein. In the field, however, mixed diets are the rule, the diet including prey that accumulates little Cry. Using mites or thrips that are parenchym feeders would be an option when a more natural exposure is aimed for.

3.7 Parasitoids

Tier-1 approaches have been extensively reviewed by Charleston and Dicke (2009). In the field adult parasitoids are exposed to Cry toxins when feeding on the nectar of a flowering GM-Bt crop. They may subsequently lay their eggs in a host that feeds on a GM crop or a non GM crop. Therefore potential life-time exposure depends on the life style of both adults and larvae. When both are exposed, exposure patterns and toxin uptake are complex. Preferably, laboratory trials should match the most relevant field situation to create a worst case scenario. Higher exposure levels can be created by adding pure toxin to honey/ sucrose solutions and uptake by the adult parasitoids can be estimated by the ingestion of the solutions or by measuring concentration in the parasitoids.
Optimal protocols and suggestions for life-table parameter to be measured are described by Charleston and Dicke (2009). Several additional parameters of interest, such as searching behaviour, can be nicely studied in these NTA's.

3.8 Honey bees

In social insects such as honey bees, any factorial population effect should be preferably studied at the colony level. However such studies are expensive and often not feasible. Therefore tier-1 studies for Cry effects are more or less adapted to the EPPO guidelines for pesticide studies and use pollen and sucrose solutions with added Cry proteins. Protocols for this group have been described by Charleston and Dicke (2009). Ramirez-Romero (2011), Hendriksma et al (2011, 2012) For adult mortality and behavioural aspects are studied. For larvae survival and growth are key parameters.
Duan et al. 2008 concluded in their meta-analysis that exposure levels in laboratory studies are well above field levels (due to mixed diets) and most studies concentrate on survival of larvae or workers. Some additional life-table parameters were measured in a tier-1 study by Wang et al. (2015) No effects were found so far, and effects of Cry on enzyme activities in the larvae were found to be absent using an artificial diet (Wang et al 2015). Other parameters that are relevant to colony survival such as fecundity, disease sensitivity or foraging capacity of workers are missing in current studies.

3.9 Hover flies

Although adult hover flies (Diptera, Syrhidae) readily feed on pollen and nectar of crops and syphid larvae are important predators of aphids living on modified crops like Bt-corn and Bt-cotton, we could not find any publication for a tier-1 study on this group to evaluate.

3.10 Lacewings

The larvae of lacewings (Neuroptera, Chrysopidae) are voracious predators of many crop inhabiting pests and adults feed on pollen as well (Li et al 2010). This led to concerns about their vulnerability for Cry proteins. Several tier-1 studies have been performed for this group. Differences in exposure protocols led to much controversy about the experimental results some 10 years ago. The first effects reported by Hilbeck et al (1999) have later been explained by changes in prey quality rather than by Cry protein exposure (Lawo et al. 2010). The latter showed that measuring Cry protein in the prey (caterpillars in their experiments) and uptake of Cry by the predator was essential to

distinquish direct effects of the Cry protein from other factors associated with properties of the Bt crop or from prey-mediated indirect effects. Uptake and digestion of Cry containing pollen has been demonstrated by Li et al (2010) by measuring pollen consumption and digestion in adults both in the laboratory as well as in the field. Tier-1 studies are not easily performed due to the variable response of larvae and adults to different diets. Recently, however, a thorough high-quality analysis (Li et al 2014b) was published for *Chrysoperla sinica* adults and larvae on different diets where Cry concentrations were measured in the exposure diet as well as in the individual insects both using pollen diets for adults and artificial diets for larvae, showing no difference in a several life-table parameters.

3.11 Spiders

Spiders are common beneficial non-target organisms that are common and widespread in almost all crops. Almost all spider species are polyphagous eating a wide variety of prey including plant feeding insects and carnivorous species. Therefore their place in the food web is mostly complex being on secondary and tertiary trophic levels. Like insects they also feed on pollen when available. Hence their exposure to Cry protein in food is hard to estimate. In laboratory trials they are usually fed with one Cry-containing prey item. Charleston and Dicke (2009) did not provide a protocol for spiders in non-target effect studies. The uptake, accumulation and breakdown of Cry proteins was studied for *Phylloneta impressa* by feeding them with corn rootworm, lacewings and spider mites (Meissle and Romeis 2012). Concentrations in the spiders were lower than in their prey and no accumulation occurred. However no data on lethal or sub-lethal effects were provided.

3.12 Soil macrofauna (Collembola)

Soil macrofauna include many different arthropod species that can feed on plant debris and pollen that may contain Cry proteins which are not readily broken down. Springtails (Collembola), mites (Acari), and also soil inhabiting larvae of many flies may feed on these plant remains.
However, potential effects of Cry proteins have been studied for very few of these organisms so far. Concentrations of Cry proteins in the plant remains tend to be lower than in the crop but still can be measured.
One of the standard test organisms in the soil macrofauna is the springtail *Folsomia candida* that has been used in some tier 1 studies. Bai et al. (2011) maintained strains of this species on leaf-soil mixtures containing different levels of Cry1Ab containing and Cry1Ab-free leaves to study effect on reproduction and size of individuals. In such a way they were able to vary Cry concentrations in the diet and although uptake could not be precisely measured they found stress-related enzyme activity at higher levels of Cry in the diet. For this group of organisms the methodology needs to be further developed.

Table 2 Case studies classified according to fulfilment of exposure and response criteria
A well described / tested/ defined/ measured **B** weakly described/tested/defined/measured **C** not described/tested/defined/measured

Test organism	Reference	Natural exposure route described	Worst case reflects natural	Correction mixed diet	Toxin in medium /diets estimated	Toxin activity confirmed	Dose response including zero, reference and 10x	Uptake verified and quantified	Parameters clearly defined	Parameters lifetable ready	Parameters linked to mode of action
Apis mellifera	(Ramirez-Romero et al. 2008)	A	C	C	A	C	B	A	A	C	C
Apis mellifera	(Hendriksma et al. 2011)	A	C	C	A	C	B	C	B	C	
Apis mellifera	(Hendriksma et al. 2012)	A	C	C	A	B	A	C	A	C	
Chrysoperla rufilabris	(Tian et al. 2013)	B	B	B	B	C	C	B	B	B	B
Rhopalosiphum padi	(Kim et al. 2012)	A	A	A	A	C	C	A	B	C	
Cotesia marginiventris	(Ramirez-Romero et al. 2007)	A	B	B	A	A	A	C	A	A	C
Adalia bipunctata	(Alvarez-Alfageme et al. 2011)	B	C	C	A	*A*	A	C	A	A	B
Adalia bipunctata	(Schmidt et al. 2009)	B	C	C	B	*C*	B	C	A	B	
Euselius concordis	De Castro et al. 2013	B	B	C	A	C	B	B	A	A	C
Neoseiulus californicus	De Castro et al. 2013	B	B	C	A	C	B	B	A	A	C
Inachis Io	Felke et al 2003	B	C	C	B	C	A	C	B	C	B
Chlosyne lacinia	Paula 2014	B	C	C	A	A	A	A	A	A	A
Orius albidipennis	Donzalez-Zamora et al. 2007	B	C	C	B	C	B	C	B	B	C
Orius insidiosus	Duan et al. 2008	A	B	B	B		C	C	B	C	
Protaphorura armata	Heckmann et al. 2006	B	C	C	B	C	C	C	B	B	C
Poecilus chalcites	Duan 2006	C	B	C	A	A	B	B	A	B	B
Chrysoperla sinica	Li et al. 2014b	A	A	B	A	A	A	A	A	B	B
Carabidae (6 species)	Mullin 2005	C	C	C	B	C	C	B	B	C	C

Test organism	Reference	Natural exposure route described	Worst case reflects natural	Correction mixed diet	Toxin in medium /diets estimated	Toxin activity confirmed	Dose response including zero, reference and 10x	Uptake verified and quantified	Parameters clearly defined	Parameters lifetable ready	Parameters linked to mode of action
Aphis gossypii	Liu 2005	A	A	A	C	C	C	C	A	A	C
Classiified with	N	8	3	2	13	5	6	4	10	6	1
an A	%	42	16	11	69	26	32	21	53	32	5

3.12 Overall conclusions from the case studies

While Table 1 gives a general overview extracted from a number of recent tier-1 studies, Table 2 summarizes our findings about the extent to which the studies match the exposure and response measurement criteria described earlier in this report. From Table 1 and 2 a number of conclusions can be drawn.

In many cases in which the NTO does not feed directly on the plant tissue or pollen it is difficult to establish a reference exposure dose for the worst case scenario. This is especially true when the NTO has a mixed diet or natural exposure time is very variable because the organism is linked only temporarily to the Bt crop for part of their life cycle. In such cases, the organisms are likely to be exposed to much higher doses than would occur in reality. This is not a problem when effects are absent, however, when effects are found this may be an artefact of the set-up and further study is needed. In species with a mixed diet worst-case exposure should be done using a diet that contains the highest Cry concentration. The use of phloem-feeders such as aphids that do not acquire Cry in tier-1 studies for polyphagous natural enemies is rather useless, when other Cry containing prey in the diet or pollen is also provided. In this sense non-target species with mixed diets can be exposed to concentrations that can be either too high or too low. Most of the discussions about the presence or absence of adverse effects are due to the relation between natural uptake from the diet and the experimental exposure.

Determining and establishing defined concentrations of Cry protein in the diet appears to be relatively easy. In case plant tissue is used, the exposure concentration depends on the highest concentration of Cry in the plant tissue or in the pollen that was measured in these plant tissues. When pure protein is added to the diet more options are available to create a 10 times higher dose than the standard (reference). Ranges of more than 3 doses (zero, standard and 10 times) – as recommended in this report- are rarely used. However, the use of those 3 doses will do for a rough effect screening. When no effects are found on any of the parameters measured even at higher and sometimes unrealistic doses, no further tests have to be performed.
Parameters are mostly quite well defined but not always directly useful for a life-table as all necessary (also age-dependent) parameters are rarely measured. Full life-table analyses were only made for short-lived and easy to handle organisms such as aphids. Delayed effects such as F1 development or reproduction are rarely measured. Also behavioural parameters are rarely studied while they can be very relevant. Such complementary data, however, are not included in tier-1 toxicological studies.

When ingestion of amounts of Cry similar or higher than natural exposure is shown in the experiments, and no indirect effects other than from the Cry itself are confounding the results, the outcome of those experiments is clear. In case of low replication causing low statistical power or of short exposure duration, the potential negative effects of the Cry proteins can be overlooked.

4. Conclusions and recommendations

Tier-1 laboratory trials under more or less controlled conditions have been adopted as the first phase of screening for the potential impact of GM-crops on NTOs. The central question in this report was how suitable currently used exposure methods in tier-1 studies are to obtain reliable information about the potential effects of transgenic insect resistant crops on NTOs. An additional question was if and how exposure methods and methodology in general can be standardized and improved to obtain more robust and conclusive results. The rationale in tier-1 laboratory studies is that exposure concentration and rate of uptake of Cry proteins are equivalent to those occurring in the field and that they are preferably also tested at a 10 times higher dose to represent a worst-case scenario.

To simplify exposure protocols, artificial diets became mainstream for the non-target species that thrive on such diets that can be easily manipulated for dose–response studies. That is also the reason that model species and laboratory strains are preferred in many tier-1 studies. However, it should be realized that effects on surrogate species or other single tested species cannot be automatically extrapolated to other species that are relevant for the GM-crop studied.

The danger of using surrogate species and artificial diets is taking attention away from the complexity and diversity of the interactions of NTOs with their food under natural conditions (Duan 2010). This may widen the gap between what is or can be measured in the laboratory and how relevant it is for field situations. As we have noticed in this report, the chosen exposure concentrations tend to be higher than what is actually occurring in field situations due to mixed diets of many or only temporary exposure in species that move between habitats. This may lead to overestimating risks in lab studies rather underestimation. On the other hand many untested species occur in the field and many possible sub-lethal effects are still rarely studied in the laboratory.

It is likely that for each case new non-target species of interest will pop up for which no artificial diet is available and natural food should be used in the trials. To manipulate the dose of Cry toxins, additional Cry-containing feed such as pollen is often easily accepted by arthropods. Also pure Cry can be added to the food to create worst-case scenarios. For herbivores the natural reference concentration in the food and the uptake are relatively easy to estimate, but at higher trophic levels the natural exposure, the uptake and possible accumulation, dilution or breakdown processes are much more complicated. In all cases the measurement of real concentrations in both the diet and the test organisms gives very useful information for the interpretation of results. Such measurements may also replace the positive controls that are often arbitrary.

Guidelines and protocols for tier-1 studies have been published for several non target organisms. For several commonly occurring not target species, advanced exposure methods and useful endpoints have been developed and proven to be useful (Romeis 200). However, for other species such protocols are not available and no consensus exists on which criteria should be fulfilled. The absence of standard protocols may not be a key issue as long as the chosen protocol is well documented, the experiments are accurately analysed, and conclusions are statistically valid.

In order to obtain relevant and conclusive results from tier-1 studies, providing the following information is recommended to be compulsory in the Environmental Risk Analysis (ERA):

Exposure

1. Measurement of toxin activity and exposure concentration are required and should equal the maximum concentration in the tissue of the transgenic plant as well as a 10-times higher concentration.

2. The use of artificial diets to increase Cry concentration 10-times compared to the maximum concentration in the crop requires that it is demonstrated that the performance of the Cry-free control group is equivalent to the performance on the Cry-free plant.

3. Ingestion of the toxic protein by the NTO should be quantified.

Regarding different and newly developed Bt-crops in the future, the aspects that will affect the experimental protocols are the type of Bt included, the concentration in different tissues and their presence in nectar and pollen. In particular nectar and pollen are important as many non-target species use these as feed. This common exposure route is also interesting for tier-1 experiments as concentrations of Cry can be manipulated more easily in tier-1 studies when pollen or nectar is used in combination with pure Cry proteins.

Selection of NTO species

With regard to selection of NTO-species, the following recommendations are made:

5. Due to the diversity in feeding modes and life history traits among different NTO species, a case-by-case approach is warranted.

6. Selection of NTO-species that are ecologically relevant in the transgenic crop must be made based on criteria published by EFSA (2010).

7. Experiments with omnivorous species that have mixed and variable diets and long life cycles are less suitable for tier-1 studies.

Response parameters

With regard to response parameters any variable that relates to population effects can be informative, though it should be realized that some parameters may have more impact on population trends than others. Measuring mortality is often not enough as non-lethal and delayed effects can be equally important.

8. It should be strongly recommended to measure variables that contribute to life-table approaches.

As this report shows, most recent studies scrutinized do not comply with all of these recommendations. When the robustness and field-relevance of experimental results is ascertained, the use of several approaches can be complementary and should be evaluated by competent risk managers. At present the lack of uniformity in experimental protocols, exposure routes, the use of different NTO-species and effect parameters, calls for standardization of tier-1 studies. The diversity and advancement of methodology

supports the development of this research field. Even when standardized protocols can be developed for a single species or a group of related species, case-by-case approaches are likely to remain necessary when unrelated species with different feeding modes and response types have to be studied.

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