

Genetically Modified Plants and "Non-Target" Organisms: Analysing the Functioning of the Agro-ecosystem

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Abstract

One of the main concerns for a possible adverse environmental effect due to the cultivation of genetically modified (GM) crops is the threat to the biodiversity in the receiving environments where such plants will be cultivated. In particular, animal biodiversity could be at risk if organisms that come in contact with GM plants and/or their products are harmed. In any ecosystem, including agro-ecosystems, hundreds of species are sustained in food webs, above and below ground, based on cultivated plants as the main primary producers. Therefore, numerous species at higher trophic levels can come in contact with plant metabolites either directly or indirectly. GM crops developed to control insect pests are purposefully hazardous to certain pest species considered to be the target of these new plant varieties (e.g. Cry toxin-expressing GM plants). All other organisms active in the agro-ecosystem and in adjacent habitats are not intended to be harmed by these GM plants and, as a consequence, can operationally be defined as non-target organisms (NTO). In this review the state of the art concerning possible effects of GM crop plants on NTOs is summarised according to the ecological role these organisms play in agro-ecosystems. This criterion is also suggested as a pathway for evaluating possible adverse effects on NTOs when GM crops are being considered for release into a new receiving environment or for the release of new GM events into well-characterised receiving environments. The idea underlining this proposal is that in heavily human-managed ecosystems, such as agro-ecosystems, the preservation of functional biodiversity is an important protection goal in ecological terms, but it is also paramount for the sustainability of agriculture.

Riassunto

Una delle principali preoccupazioni in merito ad un possibile effetto ambientale negativo dovuto alla coltivazione di piante geneticamente modificate (GM) è la minaccia per la biodiversità negli ambienti dove queste piante verranno coltivate. In particolare, la biodiversità animale potrebbe essere a rischio se gli organismi che vengono a contatto con le piante GM e/o con i loro prodotti sono danneggiate. In ogni ecosistema, inclusi gli agro-ecosistemi, centinaia di specie vengono sostenute all'interno di reti alimentari, nel sopra e nel sottosuolo, basate sulle piante coltivate quali principali produttori primari. Pertanto, numerose

specie ai livelli trofici superiori possono venire in contatto con i metaboliti di tali piante direttamente o indirettamente. Le piante GM resistenti agli insetti sono appositamente ottenute per indurre effetti negativi su alcune specie fitofaghe che sono quindi considerate come bersaglio di queste nuove varietà (es. piante GM esprimenti tossine *Cry*). Al contrario, queste piante non hanno lo scopo di interferire con nessuno degli altri organismi attivi nell'agro-ecosistema e negli habitat adiacenti che pertanto possono essere definiti in termini operativi quali organismi non-bersaglio (NTO). In questa review lo stato dell'arte nel campo degli studi degli effetti delle piante GM sugli NTO viene presentato considerando il diverso ruolo ecologico che tali organismi rivestono negli agro-ecosistemi. Questo stesso criterio viene anche suggerito come modalità operativa per valutare i possibili effetti sugli NTO in nuovi ambienti o in presenza di una nuova pianta GM. L'idea che sottende a questo approccio è che in ecosistemi fortemente antropizzati, quali gli agro-ecosistemi, il mantenimento della biodiversità funzionale rappresenta un importante obiettivo di protezione ambientale, ma è anche fondamentale nell'ottica della sostenibilità dell'agricoltura.

1. INTRODUCTION

Genetically modified plants (GMPs) currently in cultivation are principally designed to express characters of herbicide tolerance or insect resistance (IR). In the latter case, commercially available GMPs were produced to express resistance to some Lepidoptera or Coleoptera pest species (e.g. *Ostrinia nubilalis* Hubner, *Helicoverpa zea* (Boddie), *Diabrotica virgifera virgifera* LeConte, *Leptinotarsa decemlineata* Say, *Chilo suppressalis* Walker, etc.), mostly via the expression of modified *cry* genes originally derived from the soil bacterium *Bacillus thuringiensis* Berl. Indeed, pest species represent a small proportion of the organisms that may come into contact with IR crop plants in agro-ecosystems, therefore concerns have been expressed that commercial cultivations of GM crops could affect other possibly sensitive species.

The concept of "non-target" organisms (NTO) has become common in debating the biosafety of GMPs, the specific risk assessment of which is often required by law. For instance, the European Directive 2001/18/EC provides the legal background for NTO testing, requiring the assessment of possible changes in the interactions of GM plants with NTOs prior to their commercial release. The concept of NTO can only be defined in operational terms and in relation to traits newly expressed in plants, as there is no correspondence with any ecological role of these organisms in their ecosystems. In this article, NTOs will be considered as

"all living organisms that are not meant to be affected by newly expressed compounds in GMPs, and that can be potentially exposed, directly or indirectly, to the GM plant and/or its products in the agro-ecosystem where GMPs will be released or in adjacent habitats". However, an additional case is represented by organisms indirectly affected via changes in the cultivation, management and harvesting techniques as a result of the introduced trait in GMPs, for example changes to herbicide application regimes when cultivating herbicide tolerance plants. A few examples of such cases will also be highlighted in this review. For GM plants expressing traits that do not confer resistance to pests, all organisms potentially exposed are therefore considered as non-target. Most of the chosen examples in this paper will refer to arthropods; relevant implications regarding other organisms will be highlighted and the appropriate references will help readers to further reflect on these issues.

Agriculture depends on several ecosystem functions that are essential to soil fertility and agricultural productivity (e.g. microbial decomposition and nutrient cycling, crop pollination by animals, biological control of pests). Each of these ecosystem functions are mediated by several guilds of animal species. Therefore, in any given cropping system, many hundreds of arthropod species, thousands of microbial species, and scores of ecosystem functions can be found (Curtis *et al.*, 2002). It is therefore impossible to estimate the impacts of any new cultivation technique on all potentially exposed species.

To date, the environmental release of GMPs takes place on commercial farms in rural areas. Therefore the concept of the agro-ecosystem (i.e. a specialised, human-managed ecosystem designed for the production of agricultural goods) and its mode of functioning can represent a useful logical criterion to guide the analysis of the large trophic webs that exist therein and where the potential exposure of NTOs to newly produced compounds in GMPs can occur.

Food webs in agro-ecosystems are typically simplified compared to natural habitats, due to the major impact of human activities, the short time span plants remain in the field and the usual uniformity of cultivated plants. Even so, rather complex multi-trophic relationships are established between primary producers and consumers, at least up to the fourth trophic level (e.g. Verkerk, 2004).

Many reviews are already available on the subject of possible effects of GMPs and NTOs seen from different view points. Among the most recent, O'Callaghan *et al.* (2005) gave an overview of the available knowledge concerning the effects of IR GMPs on plant- and soil-dwelling arthropods. Lövei & Arpaia (2005)

concentrated on natural enemies, analysing individual response classes observed in laboratory studies and highlighting several existing knowledge gaps. Romeis *et al.* (2006) summarised the overall effects of Cry toxins on natural enemies using conclusions from laboratory and field studies, as opposed to effects due to chemical pesticides. An updated review by the same group (Romeis *et al.*, 2009) highlighted the lack of direct effects of this group of toxins on many non-target above-ground arthropods and advise that future GM crops should be assessed on a case-by-case basis. Malone & Burgess (2009) examined the existing literature on Hymenoptera pollinators and GMPs, of which some of their results will be commented upon later (Section 3.2.).

The use of meta-analysis to summarise available data has been recently adopted in the reviews of field studies in Bt-expressing maize and cotton (Marvier *et al.*, 2007), functional groups of non-target arthropods (Wolfenbarger *et al.*, 2008), specific effects on honey bees (Duan *et al.*, 2008), and the occurrence of direct and/or indirect effects (Naranjo, 2009). Lövei *et al.* (2009) concentrated their meta-analysis on laboratory studies and natural enemies, performing calculations based on single endpoints measurements and using effect size classes instead of an average effect size. The conclusions of these meta-analysis studies are discussed later (Section 3.1.). Soil communities, including microorganisms, have also been reviewed in recent papers. Widmer (2007) and Fillion (2008) concentrated specifically on microbial communities while soil associated meso- and macro-fauna were also considered by Icoz and Stotzky (2008). Together, these analyses concluded that GMPs will likely induce changes in these communities, but these are expected to be within the range of natural variation (Section 3.3.6).

The present review aims to summarise the specific information available in the literature regarding NTOs and GMPs, particularly those which express insect resistance characters. The review will not try to give opinions about the environmental safety of specific transformation events, but will analyse information and information gaps concerning the main functional guilds active in agro-ecosystems and adjacent habitats. The main biological mechanisms regulating food webs will be reiterated and used to evaluate the possible exposure routes to hazardous compounds that may be expressed in GMPs. The available scientific knowledge will be revisited with the specific aim of “learning lessons” and trying to help researchers, risk assessors and regulators in different parts of the world, including those where specific information is limited, to collect the most relevant data necessary for an effective and scientifically-sound environmental risk assessment.

2. HOW CAN NON-TARGET ORGANISMS BE AFFECTED BY GENETICALLY MODIFIED PLANTS?

The concept of non-target organisms may include a large number of different species. Therefore there are different reasons why the impacts of GMPs on NTOs in agricultural fields and nearby habitats are commonly perceived as possibly serious threats. These possible negative effects can be categorised, according to the functions or values that can be affected, as follows: ecological effects, effects on agriculture, and effects on other anthropocentric values.

Ecological effects relate to threats to biodiversity and ecological functioning in the ecosystems. The protection of biodiversity is deemed important for evolution and for maintaining life sustaining systems of the biosphere, and it is a common concern of humankind (Secretariat of the Convention on Biological Diversity, 1992). Generally, environmental protection goals aim to protect biodiversity through the conservation of natural habitats, wild fauna and flora (e.g Directive 92/43/EEC). Since GMPs will be released in cultivated areas, a possible deterioration of cropping system needs to be considered. The effects on farming activity are related to the biodiversity effects, particularly when “ecosystem services” (Daily, 1997) provided by different guilds can possibly be impaired (e.g. should animal pollination be threatened by the expression of new proteins in crop plants, or natural biological control of pests reduced because toxins accumulate along trophic chains, etc.).

The same biological events can induce, for instance, consequences at different levels. For example, gene flow is a common event in nature and constitutes the base of evolution for every environment, therefore it cannot be considered a negative event *per se*. To evaluate its ecological consequences, it is important to consider the possibility of successful fertile hybrid formation and the successive introgression of a new trait into the population. This latter event is normally foreseeable as long as it confers a selective advantage to the progeny in the specific receiving environment. A possible negative ecological effect, as a consequence of gene flow, could be considered an increase in the invasiveness of a wild plant acquiring a GM trait as a consequence of natural crossing. The corresponding effect on farming activities could be the possible development of an herbicide resistant weed which could affect crop yield due to its newly acquired trait

(Lu, 2008). Finally, other anthropocentric values can be threatened, such as the survival of rare, endangered or charismatic species (e.g. *Danaus plexippus* L., the Monarch butterfly in the USA) or the normal development of economic activities, such as apiculture.

The scientific approach to risk assessment applied in many different fields involves the consideration of two broad categories; hazard characterisation and exposure evaluation. An environmental risk due to release of GMPs indicates the probability of an adverse effect occurring due to the exposure of NTOs to the GM plant and/or its products. In the following chapters, hazard characterisation and evaluation of exposure will be analysed in relation to agro-ecosystems based on the most current scientific literature.

3. A “FUNCTIONAL” APPROACH

As outlined above, the large number of species present in any agro-ecosystem renders impossible any detailed study including all of them. Therefore, it is necessary to make a choice of assessment endpoints that could be considered representative for the specific receiving environment.

Many possible criteria have been proposed, which usually enable the selection of a handful of species based on characteristics such as their abundance in the specific environment, the susceptibility to known stressors, the practicability of conducting laboratory tests, the charismatic value of the species, etc. (e.g. Cowgill and Atkinsons, 2003; Andow and Hilbeck, 2004; Birch *et al.*, 2004; Prasifka *et al.*, 2008; Romeis *et al.*, 2008; Todd *et al.*, 2008). Therefore, it is not surprising that the available body of data does not evenly represent the various animal taxa linked to the agro-ecosystem, and that not every specific guild was studied to the same extent.

Ecosystem services denote ecological processes that benefit humankind (Daily, 1997). Several types of ecosystem services ensure agricultural productivity, including soil formation, decomposition of plant residues, pollination, and natural pest control, to name a few. Regardless of GMO introductions, several of these services are already considered to be under pressure and their ability to continue at desired rates is at risk (MA, 2005). The present narrative review of the scientific literature will revise the available knowledge regarding functional groups (i.e. all clusters of biota providing the same ecological function) of organisms active in agro-ecosystems and adjacent habitats (Table 1.) and link each of these groups to ecosystem services and to the main anthropocentric values. The idea behind

this choice is that it can be assumed that a change in biodiversity structure may result in a change in function; therefore, preserving functional biodiversity may guarantee the quality of agro-ecosystems. In fact, increasing biodiversity is known to enhance agro-ecosystem resilience and stability in the presence of redundant species and, more importantly, to increase ecosystem functioning in terms of processes or magnitude of processes in species-poor agro-ecosystems in the short term (Moonen and Bàrberi, 2008). Moreover, not every species or process is equally important for the functioning of the agro-ecosystem and therefore a preliminary problem formulation may successfully use this approach to prioritise which species or functions to be analysed.

The first areas that will be potentially affected by the deployment of GMPs are the cultivated fields and their surroundings. From an applied perspective, we might then essentially concentrate on functions that are important in such environments. Moreover in habitats adjacent to farms, floral and faunal biodiversity may also represent important anthropocentric values to be protected. In the agro-ecosystem, crop plants, weeds and volunteer plants in the field and field margins are included. Adjacent habitats are those where plant parts can be present on a regular basis (e.g. via pollen drift or cultivation residues). This has obviously some limitations, but will likely include the most relevant mechanisms possibly leading to impacts. In relation to agro-ecosystems, the main ways in which a non-target species can be exposed to GMPs or their products are via air, plant and soil. Table 2 summarises the relative exposure mechanisms linked to these elements and the non-target groups possibly involved.

Table 1. Examples of functional group of species and ecosystem services provided to human activities

Functional group	Examples	Examples of ecosystem services or anthropocentric values
Herbivores	<ul style="list-style-type: none"> Sap/cell feeders: e.g. Acarina Tetranychidae, Homoptera: Aphidioidea, Aleurodidae, Cicadellidae; Thysanoptera: Thripidae, etc. Leaf feeders: e.g. Coleoptera: Chrysomelidae, Lepidoptera larvae, etc. 	Secondary pests, quarantine pest species, species of conservation concern, species of charismatic value
Predators	Thysanoptera (e.g. Aeolothripidae); Heteroptera (e.g. Anthocoridae, Miridae, Nabidae); Neuroptera (e.g., Chrysopidae, Hemerobidae); Coleoptera (e.g. Coccinellidae, Carabidae, Staphilinidae); Diptera (e.g., Syrphidae, Cecidomyiidae); Araneae; Acarina (e.g., Phytoseiidae)	Natural control of arthropod pests
Parasitoids	Diptera (e.g. Tachinidae); Hymenoptera Parasitica	Natural control of arthropod pests
Pollinators, pollen feeders	Diptera: Syrphidae; Hymenoptera: Apidae	Crop plant pollination, honey production
Decomposers	Nematoda, Collembola, Acarina, Haplotaxida: Lumbricidae, Isopoda	Soil fertility

Table 2. Possible exposure mechanisms and their consequences for non-target organisms

Exposure channel	Mechanism	Non target groups	Ecosystem services possibly affected
AIR	Pollen flow, seed dispersal	<ul style="list-style-type: none"> • Sexually compatible plants (gene flow); • Herbivores ingesting pollen in field margins and adjacent habitats; • Spermophagous species in adjacent habitats 	<ul style="list-style-type: none"> • Pollination
PLANT	Trophic chain effects	<ul style="list-style-type: none"> • Primary consumers (herbivores) in the field; • Higher order consumers (carnivores) in the field and in adjacent habitats. • These groups are not limited to arthropods, but may include e.g. rodents, birds, mammals, etc. 	<ul style="list-style-type: none"> • Pollination • Natural pest control
SOIL	Horizontal gene transfer, release of plant products	Microorganisms, meso- and macro-fauna	<ul style="list-style-type: none"> • Soil fertility • Natural pest control

3.1. Natural Pest Control

It is estimated that about 95 % of the potential pest arthropod species are maintained below damaging population levels by natural pest control provided by predators and parasitoids (DeBack and Rosen, 1991). This is a typical regulating service provided by numerous animal guilds that prevent most of the herbivores living in agro-ecosystems in becoming pests. The maintenance of this ecosystem service is estimated to have a minimum value of 400 billion USA dollars per year (Costanza *et al.*, 1997). Moreover, natural pest control constitutes a “baseline” value over which any integrated pest management (IPM) programme is built,

since the population dynamics of herbivores and their natural enemies are routinely monitored before any pest management action is considered. It is clear then that preserving this ecosystem service is paramount in order to guarantee the sustainability of agriculture, and to limiting the need for chemical pesticides.

Plants expressing IR characters could introduce novel metabolites into existing food web (e.g. Cry toxins, proteinase inhibitors, lectins), therefore higher order consumers may possibly be adversely affected. The most widely cultivated IR GM crops contain modified versions of genes derived from the soil bacterium *B. thuringiensis* (*Bt*). There is also case of a commercial GM plant expressing a serine protease inhibitor in combination with a Cry toxin in cultivation in China (Malone *et al.*, 2008). These genes enable the production of crystal proteins (Cry) which are selectively toxic to various insect orders. Many different strains of *B. thuringiensis* were isolated from soil samples, plant surfaces, dead insects and stored grains from all over the world. The different strains show a wide range of specificity against different insect orders (Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Phthiraptera/Mallophaga), Acarina, Nematoda and other invertebrates (<http://www.glf.forestry.ca/bacillus/BtSearch.cfm>). The assumed selectivity of Cry toxins is therefore an important characteristic that should limit the negative effects of GM plants to target species. In some cases though, the range of sensitivity to Cry toxins is not completely known.

3.1.1. Predators

Arthropod predators are very active in the natural biological control in agroecosystems; in about 75% of cases generalist predators, either single species or species assemblages, reduce pest numbers significantly (Symondson *et al.*, 2002). To date, 41 predator species (Table 3) have been considered in laboratory studies in relation to IR GMPs. A large part of the available information though, relates primarily to three predatory species, *Chrysoperla carnea* Steph. (Neuroptera: Chrysopidae), *Propylea japonica* Thunberg (Coleoptera: Coccinellidae), and *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae) (Lövei *et al.*, 2009). The limited species spectrum is not the only existing shortcoming in the available data, but also sample size, statistical power and duration of certain laboratory toxicity tests are limiting factors in several papers (Lövei and Arpaia, 2005). By analysing these laboratory studies, Lövei *et al.* (2009) indicated that there are fewer neutral responses, and more positive and negative effects than expected under the assumption of normal distribution of the responses. This would generally translate into the indication of non-neutral effects for both Cry toxins and proteinase inhibitors, the latter giving more significantly negative or positive

effects. Cry3A/Bb generally induced fewer effects in either direction compared to other toxin classes.

Naranjo (2009) also considered laboratory studies on predators but specifically distinguished between direct or prey-mediated effects. When direct-feeding experiments alone were considered, predators showed a significant reduction in their developmental rate when exposed to *Bt* proteins compared with non-*Bt* controls. Conversely, *Bt* toxins had no effect on the survival or reproduction of predators. When experiments only addressing prey-mediated effects were considered, predators showed slightly lower survivorship when provided with low-quality prey exposed to *Bt* toxins, and slightly faster developmental rates when provided with high-quality (i.e. not susceptible to *Bt*) prey exposed to *Bt* toxins. All other predator life history characteristics were unaffected by *Bt* toxins regardless of prey quality.

Whenever a possible hazard is identified in laboratory studies, it is generally advised to further investigate possible environmental impacts in more realistic conditions under more natural exposure conditions in semi-field or field studies. Negative and positive aspects of field studies, as opposed to laboratory tests, are briefly outlined in Section 5. Specific field studies have been conducted on several GM crops. Marvier *et al.* (2007) summarised them by undertaking a meta-analysis of data collected from various field studies of *Bt*-expressing maize and cotton in different parts of the world. They showed that the combined abundance of all non-target invertebrates was significantly lower in *Bt* compared with non-*Bt* crops, but that abundances were significantly higher in *Bt* crops if compared with non-*Bt* crops that had been treated with insecticides.

Wolfenbarger *et al.* (2008) also analysed field studies using arthropod abundance as the measurement endpoint. In this meta-analysis, the authors also included studies with Cry3-expressing GM potatoes, moreover their results were obtained after pooling experimental data according to the functional guilds of the NTO species monitored. The authors showed that in cotton, there were significantly fewer predators in *Bt* crop fields compared to un-sprayed, control (non-*Bt*) fields. This result was not related though to the feeding style within this functional group, but was largely driven by the lower abundance of Nabidae and Coccinellidae found in *Bt* crop fields. Higher numbers of the generalist predator, *C. maculata*, were associated with *Bt* maize but numbers of other common predatory genera were similar in *Bt*

and non-Bt maize. Finally, in the case of the GM potatoes, there were more predators and herbivores in Bt crop fields than in un-sprayed control fields. The analysis of this latter crop though, included only two field studies and significant heterogeneity existed in both of these functional groups.

Cloutier *et al.* (2008) were specifically interested in Bt potato resistant to the Colorado potato beetle and used published studies to perform a specific meta-analysis. The authors found in about 20 % of cases an increased abundance of generalist predators, and suggested that this result could be related to the rather common (42% of cases) higher abundance of sucking herbivores detected on Bt potato. The exposure of predators to Cry toxins expressed in GM plants has been demonstrated experimentally. Many studies are now available which confirm that predators can ingest newly-expressed proteins in GMPs by direct feeding on Bt-expressing plant material (e.g. pollen), and indirectly through the consumption of herbivore prey. Harwood *et al.* (2005) studied the exposure of several groups of non-target organisms to the Cry1Ab toxin from Bt11 maize and reported its observed levels in non-target herbivores and their natural enemies under field conditions. Significant quantities of the Cry1Ab toxin were detected in organisms at higher trophic levels. Obrist *et al.* (2006a) showed that the Cry1Ab toxin from Bt176 maize in some cases accumulated in the food chain at concentrations higher than those in the maize leaves. The Cry1Ab toxin was detected in certain predators (such as *Orius* spp., *Chrysoperla* spp. and *Stethorus* sp.), whilst its presence was negligible in others (e.g., *Hemerobiids*, *Nabis* sp., *Hippodamia* sp., *Demetrias* sp.). This difference might be the result of the different feeding habits of prey species which can ingest variable amounts of toxins. In another study, Obrist *et al.* (2006b) confirmed toxin uptake by larvae of green lacewing, *C. carnea*, via its herbivore prey, *Tetranychus urticae* Koch and *Spodoptera littoralis* Boisduval; the biological activity of the Cry1Ab toxin was maintained after ingestion by both herbivore species. Harwood *et al.* (2007) showed the presence of the Cry1Ab toxin in gut samples of certain predatory coccinellids (e.g., *C. maculata*, *Harmonia axyridis* –Pallas-, *Cycloneda munda* –Say– *Coccinella septempunctata* L.). Álvarez-Alfageme *et al.* (2008) detected Cry1Ab toxin in the coccinellid species *Stethorus punctillum* Weise collected from MON810 maize fields. Generalist predators may in some cases show more complex feeding habits including feeding on plant parts, on numerous herbivore prey species and also intra-guild predation; in some cases with clear preferences among available food sources. These habits make the possible exposure to plant metabolites quite variable and difficult to predict.

3.1.1.1. Details about some taxonomic groups

Lacewings (*Neuroptera: Chrysopidae*)

As indicated above, the number of studies conducted on the green lacewing *C. carnea* has greatly outnumbered that of any other predatory species. More than ten years ago a series of publications (Hilbeck *et al.*, 1998a; Hilbeck *et al.*, 1998b; Hilbeck *et al.*, 1999) reported significantly prolonged larval development and increased mortality when immature *C. carnea* were fed lepidopteran larvae reared on Cry1Ab expressing maize in laboratory. These findings have triggered numerous other studies on the same species. No acute adverse effects were reported when *C. carnea* larvae were fed non-susceptible *T. urticae* containing large amounts of biologically active Cry1Ab toxin (Dutton *et al.*, 2002). Romeis *et al.* (2004) indicated that possible indirect effects may occur to this species due to poor prey quality when lepidopteran larvae are used as prey. Rodrigo-Simón *et al.* (2006) reported that Cry1Ab toxin does not specifically bind *in vitro* to brush border membrane vesicles from the midgut of *C. carnea* larvae, which is considered as a prerequisite for toxicity. Andow *et al.* (2006a) summarised the body of evidence available regarding this predator species, stating that studies agree that no acute toxicity of Cry1Ab to the larvae was demonstrated, when administered either in an artificial diet or in water. However, the laboratory experiments completed on this species do not completely exclude the possibility of chronic effects, as suggested by Hilbeck *et al.* (1998b).

Under field conditions, the continuous exposure of *C. carnea* to diets exclusively based on lepidopteran larvae is considered unlikely, since a variety of prey species is normally available (Dutton *et al.*, 2003). No differences in the abundance of lacewings in *Bt*-expressing and near isogenic control plots were observed in several field studies (e.g. Pilcher *et al.*, 1997; Wold *et al.*, 2001; Bourguet *et al.*, 2002). In the meta-analysis conducted by Wolfenbarger *et al.* (2008), the weighted mean effect size based on field abundance for this species was essentially zero in both cotton and maize studies where no insecticides were used.

Ladybirds (*Coleoptera: Coccinellidae*)

Dhillon and Sharma (2009) studied the effects of Cry1Ab and Cry1Ac proteins on the predatory coccinellid *Cheilomenes sexmaculatus* –L.- under direct and indirect exposure conditions. Direct exposure of *C. sexmaculatus* larvae to *Bt* proteins at high concentrations resulted in reduced larval and adult emergence, as compared to controls. However, there were no adverse effects of the Cry toxins when the larvae were reared on *Aphis craccivora* Koch previously fed different concentrations of Cry1Ab or Cry1Ac in an artificial diet. The tritrophic

experiments of Álvarez-Alfageme *et al.* (2008) demonstrated that when *S. punctillum* were fed *T. urticae* reared on transgenic *Bt*-expressing maize (events Bt176 and MON810), there was no effect on the survival of neonate ladybird larvae through to adulthood. Larval development was similar between treatments even though the fourth instar lasted longer for *Bt*-exposed larvae. Adult *S. punctillum* emerging from the feeding trials were further monitored for a measure of their relative fecundity. The results showed that the *Bt* maize of either event had no significant effect on the mean cumulative ladybird fecundity.

In other papers (e.g. Kalushkow & Hodek, 2005; Ahmad *et al.*, 2006) no significantly negative effects on survival, growth and development of coccinellids were detected using two different Cry3 type of toxins. Zhang *et al.* (2006a) reported a decrease in body mass when *Propylaea japonica* Thunberg young larvae were fed for 72 h with 24 h old *Spodoptera litura* Fabricius larvae reared on *Bt* cotton cultivars GK-12 and NuCOTN 33B (expressing the fused Cry1Ab/Ac toxin and Cry1Ac toxin, respectively), compared to those fed with control-reared *S. litura*. Significantly fewer *P. japonica* larvae molted into second-instar when fed with *S. litura* reared on one of the *Bt* cotton lines used, compared to those fed prey from control plants. In laboratory feeding experiments using transgenic *Bt* cotton plants expressing the fused Cry1Ab/Ac toxin or Cry1Ac toxin, Zhang *et al.* (2006b) found that there were no distinct differences in pre-imaginal mortality between predators reared on aphids feeding on *Bt* cotton or control plants. The pre-imaginal stages of the ladybird beetles also developed faster when reared on prey fed *Bt* cotton cultivars than those fed control prey. However, there was a trend of more adult malformations when the predator was fed with prey from one of two *Bt* cotton cultivars than on control prey. Conversely, there were no significant differences in the pre-ovipositing period or in fecundity. Ladybird beetles preying on *Bt*-reared aphids matured faster and mated more frequently than those fed on aphids which were not exposed to *Bt* plants. When detailed analyses are performed, it is not always observed that all the measurement endpoints unequivocally indicate either the lack or presence of an effect. For instance, Bai *et al.* (2005) quantified and compared 18 predator response parameters of the effects of transgenic cry1Ab rice pollen on the fitness of the coccinellid *P. japonica* on two different *Bt* varieties. Among the considered response parameters, two developmental parameters were significantly negative with respect to the control, whilst two other parameters (mortality and one developmental) were significantly positive. All the other data indicated a neutral effect of the diet.

Cry3 toxins are engineered in crop plants to confer resistance to Coleoptera,

therefore coccinellids that belong to the same order could theoretically be affected. Lövei *et al.* (2009) summarised all the published results from laboratory studies on *C. maculata* DeGeer, one of the two most commonly studied ladybird species in biosafety research with GMOs, and discovered that none of the 101 biological parameters investigated gave significantly negative results. A recent paper by Schmidt *et al.* (2009) reported on laboratory toxicity tests with microbially-produced trypsin-activated Cry1Ab or Cry3Bb proteins fed to different larval stages (L1-L4) of the coccinellid *Adalia bipunctata* L. *Bt* proteins were sprayed on *Ephestia* sp. eggs, which were then offered as food in a no-choice test to first instars of the study ladybirds. The authors report that *A. bipunctata* larvae fed lepidopteran-active Cry1Ab toxin exhibited significantly higher mortality levels than the control group. However, in experiments with the coleopteran-active Cry3Bb toxin, a higher concentration of the toxin (compared to Cry1Ab) was necessary to induce significant mortality levels. No differences were detected with respect to both development time of larvae and body mass of newly-emerged adults. The authors suggest that the increased mortality of larvae in the toxin feeding trials was caused directly by the activated *Bt* toxins and raise questions regarding their postulated specificity and mode of action in *A. bipunctata*. The higher toxicity on Coleoptera of a Lepidoptera-specific Cry1Ab in comparison to Coleoptera-specific Cry3Bb is a new outcome that needs to be confirmed based on more quantitative data of food intake and actual toxin concentrations in the diet.

Coccinellids have been the subject of several field studies with GMPs. No adverse effects of various *Bt* maize events were detected on a range of coccinellid species (e.g., Pilcher *et al.*, 1997; Jasinski *et al.*, 2003; de la Poza *et al.*, 2005; Lundgren and Wiedenmann, 2005; Eckert *et al.*, 2006). Wold *et al.* (2001) did not find adverse effects on *A. bipunctata* in field studies, but reported a lower abundance of *C. maculata* in GM sweet corn expressing Cry1Ab toxin in experimental fields. An important consideration in terms of environmental risk assessment is that it is unlikely for many coccinellid species to be exposed to biologically relevant amounts of Cry toxins from GMPs. This is primarily because aphids, as one of the preferred preys for ladybirds, are known to contain no (e.g. Head *et al.*, 2001; Raps *et al.*, 2001) or limited amounts (Zhang *et al.*, 2006b; Burgio *et al.*, 2007) of Cry toxins. Moreover, the Cry1Ab toxin content in commercialised GM maize pollen (which represents the most likely source for possible toxin ingestion for coccinellids) is usually limited in the most common events. For instance, Cry1Ab toxin in MON810 maize pollen ranges between 1-97 g/g fresh weight (Nguyen and Jehle, 2007). The exposure route used by Schmidt *et al.* (2009) may constitute

a useful model for laboratory studies, but any significant exposure through egg-feeding in the field can be considered very unlikely in field conditions.

Ground beetles (*Coleoptera: Carabidae*)

Because of their complex food webs and ground-dwelling habits, carabids may be exposed to transgenic *Bt*-endotoxins through multiple pathways, including root exudates (Saxena *et al.*, 2002), soil-dwelling prey, ingestion of plant material, and pollen feeding. A relatively large number of ground beetle species have been subjected to specific studies with GMPs (Table 3). Peterson *et al.* (2009) studied the uptake of Cry toxins in carabids using several GMPs, including stacked events. The authors detected different amounts of toxins across predator species and attributed these differences to changes in the non-target food web as well as possibly to differential rates of *Bt*-endotoxin decay in the events studied. Álvarez-Alfageme *et al.* (2009) revealed that there was a great decline in the detection of Cry1Ab toxin through the trophic chain during tritrophic laboratory studies with *Poecilus cupreus* L. Meissle *et al.* (2005) reported a significant increase in the mortality of the generalist carabid predator *P. cupreus* L. fed *S. littoralis* larvae raised on *Bt* maize (event MON810). By speculating on the lack of specific receptors in *P. cupreus*, the authors assume the reported effects to be due to the nutritional quality of the prey and not to the direct toxicity of the Cry1Ab toxin, though they could not exclude direct toxic effects. The tritrophic experiments of Álvarez-Alfageme *et al.* (2009) also used *P. cupreus* larvae fed with *S. littoralis* caterpillars themselves previously fed either Bt176 or control maize. The results demonstrated that indirect exposure to Cry1Ab toxin had no effect on the developmental time of both larvae and pupae of *P. cupreus*. Similarly, no differences were observed on mortality and adult fresh weight. Riddick *et al.* (1998) reported a reduced abundance of *Lebia grandis* Hentz., a predator of the target pest *L. decemlineata*, in experimental fields with Coleoptera-resistant Cry3A-expressing potato. De la Poza *et al.* (2005) noted that the abundance of Carabidae varied from year to year and between locations during a three-year monitoring programme in Spain, but no clear relationships with *Bt* maize was discerned.

Ground beetles were also one of the groups of non-target insects considered in the British farm-scale trials with genetically modified herbicide tolerant (GMHT) crops. The counts of all of these species did not differ between conventional and GMHT treatments in any of three crops studied (beet, maize and spring oilseed rape; Haughton *et al.*, 2003). Dominance in spring oilseed rape was the only significant treatment effect observed (i.e. the abundance of carabids was greater

under GMHT cropping). However, counts of Carabids that feed on weed seeds were reduced in GMHT crops (Brooks *et al.*, 2003), indicating a possible indirect effect due to the decrease of weed populations.

True bugs (*Heteroptera*)

Predatory heteropterans are common and important members of the natural enemy complex of a variety of row crops (Schaefer and Panizzi, 2000). Moreover in a field study performed in Switzerland this group of insects showed the highest correlation with the estimate of overall biodiversity in an agricultural landscape (Duelli and Obrist, 1998). The Cry3Bb1 protein at a concentration ten times higher than expected in *Bt* maize had no adverse effect on the survival and development of *Orius insidiosus* Say nymphs in experiments performed by Duan *et al.* (2008). Rauschen *et al.* (2009) examined the impact of *Bt* maize on the mirid *Trigonotylus caelestialium* (Kirkaldy) in field experiments and concluded that there was no evidence for a negative impact of MON88017 maize on the abundance and body weight of this species, despite its considerable exposure to Cry3Bb1. Torres and Ruberson (2008) assessed the effects of Cry1Ac-expressing cotton on several predatory heteropterans in caged plants experiments. They concluded that the toxin was conveyed to predators via their prey (different species were used), and that there were no effects on the life history traits of the predators (i.e. pre-imaginal development, body weight, reproduction and survivorship). For instance, *Podisus maculiventris* Say exhibited similar life history characteristics (developmental time, survival, longevity, and fecundity) when preying upon *Bt* or non-*Bt* cotton fed *Spodoptera exigua* (Hubner) larvae (Torres and Ruberson, 2008). Arpaia *et al.* (2007) reported the results of a three-year study of several predatory guilds in experimental plots of Cry3Bb-expressing aubergine and their near isogenic lines. Mirids were abundant in these experimental fields and no differences between treatments were detectable. Some species (e.g. *Macrolophus caliginosus* Wagner, *Dyciphus errans* –Wolff–) were significantly associated with GM aubergine areas on a few of the sampling dates.

3.1.2. Parasitoids

Parasitoids can also be exposed to metabolites expressed in GMPs via one or more trophic levels (e.g. direct feeding on GM plant material, mainly nectar or exudates, or by their host organisms feeding on GMP tissues). When reviewing the available body of data, invertebrate parasitoids appeared to be more sensitive than predators to diets containing Cry toxins or proteinase inhibitors under laboratory conditions (Lövei *et al.*, 2009); in fact 25 out of 31 response classes evaluated in this analysis were significantly non-random. The meta-analysis conducted by Naranjo

(2009) confirmed the higher sensitivity of parasitoids to Cry toxins. In addition, by evaluating tri-trophic and bitrophic experiments separately, this author was able to emphasise that host quality is highly relevant in determining such results. In fact, mostly negative effects occurred when parasitoids were feeding on hosts sensitive to the toxins expressed in GMPs. The intimate relationships of these natural enemies with their hosts renders them sensitive to any change in host quality (Godfray, 1994). However, indirect effects do not fully explain the published results, and other possible mechanisms need to be considered. Ramirez-Romero *et al.* (2007) observed that exposure to Cry1Ab toxin via hosts fed *Bt* maize tissue sub-lethally affected the parasitoid wasp *Cotesia marginiventris* (Cresson). In experiments where the performance of this parasitoid developing on aphids fed MON810 maize was compared with those on aphids fed control maize (which were similar in size), negative effects were detected with respect to the wasp developmental times, adult size, and fecundity. Conversely, these negative effects were not observed when toxin-containing artificial diet was used in tritrophic experiments; the authors were thus able to demonstrate the importance of the plant as a medium in causing negative effects at the third trophic level.

In some cases, an enhanced performance of parasitoids was observed when their hosts were obtained from Cry-expressing plants. For instance, Schuler *et al.* (2003) showed that the number of emerging *Cotesia plutellae* (Kurdjumov) adults was higher on Cry1Ac-expressing oilseed rape compared to control plants. Faria *et al.* (2007) detected a positive effect of six varieties of *Bt* maize (including events Bt11, MON810 and Bt176) on the performance of the maize leaf aphid *Rhopalosiphum maidis* (Fitch) in growth chamber experiments, which in turn enhanced the performance of the parasitic wasp *C. marginiventris* that also utilised the aphid honeydew. In these experiments, GM maize lines were generally significantly more susceptible to aphids than their near-isogenic controls. The analysis of plant sap in selected maize lines revealed significantly higher amino acid levels in *Bt* maize, which might partially explain the observed increased aphid performance. Larger colony densities of aphids on *Bt* plants resulted in an increased production of honeydew that is normally used as food by beneficial insects. In fact, *C. marginiventris* females lived longer and parasitised more pest caterpillars in the presence of aphid-infested *Bt* maize than in the presence of aphid-infested near isogenic maize.

Reports from field studies have highlighted a diminished number of parasitoids (reviewed in Wolfenbarger *et al.*, 2008) in areas with *Bt*-expressing maize compared to respective untreated controls. The author of this review showed that

this pattern was entirely explained by the reduced abundance of *Macrocentris grandii* Goidanich, an exotic specialist parasitoid of the European corn borer, and therefore the absence of the natural host was thought to be the cause of the differences found in maize fields. However, these specific indirect effects need to be carefully considered in an ecological context, e.g. abundance of alternative hosts for the parasitoids in the field and adjacent areas, abundance and diversity of parasitoids for the pest and non-pest species linked to the crop etc. (Arpaia *et al.*, 2006a), in order to evaluate the possible repercussions on this functional guild.

3.2. Pollination

Many crop plants, including crop species that have been genetically modified to date, are dependent upon pollinators for optimal reproduction. In some cases, crop yield is strictly dependent upon pollinators activity in the field (e.g. watermelon, squash, kiwifruit) or in the greenhouse (e.g. tomatoes, strawberries), whilst in other cases, the effective presence of pollinators allows for greater and more stable yields (e.g. oilseed rape, soya bean, cotton; Klein *et al.*, 2007). Some of these crops are also important for honey production in many countries (Crane and Walker, 1986; Free, 1993). The successful establishment of mutualistic interactions between plants and pollinators is dependent upon a series of successive steps that require a finely tuned combination between plant attractants and insect senses. Flowers announce their rewards (most commonly pollen and nectar) to pollinators using colour, scent, size, or shape, making it easier for visitors to recognise them. From an insect perspective, smell and vision are therefore essential for efficiently finding food sources. Pollination ecology plays a fundamental role in protecting biodiversity, and natural selection has resulted in efficient pollinating species to out-compete other species for the same food source.

A recent review on the possible effects of GMPs on pollinators (Malone and Burgess, 2009) concluded that none of the commercially available GM crops expressing herbicide tolerance or insect resistance traits have deleterious impacts on pollinators. The only *Bt* toxin known to be specifically active against Hymenoptera (the order which includes most of the important pollinating insects) is Cry5, which functions with a similar mechanism to Cry1 toxins (Garcia-Robles *et al.*, 2001). No Cry5-expressing GMPs are currently commercially available. Feeding studies performed in controlled conditions with honeybees fed either with *Bt* pollen or mixtures of honey or sugar syrup containing purified Cry1Ab toxin have indicated no direct adverse effects on larvae and adult survival (Ramirez-Romero *et al.*, 2005, 2008; Rose *et al.*, 2007). These findings concur with earlier studies conducted with Cry3B under semi-field conditions (Arpaia, 1996). Based on a meta-analysis of 25

independent laboratory studies assessing direct effects on honeybee survival of Cry toxins from currently commercialised *Bt* crops, Duan *et al.* (2008) concluded that Cry toxins do not negatively affect the survival of either honeybee larvae or adults in laboratory settings. Nevertheless, the same authors considered that in field settings, honeybees might face additional stresses, which could theoretically affect their susceptibility to Cry toxins and generate indirect effects.

Some studies focused on the development of the hypopharyngeal gland in honeybees. Hypopharyngeal glands are considered an important indicator of bee life history, as they are used in nurse bees to prepare brood food (jelly) for the larvae. In this respect, Babendreier *et al.* (2005) fed young adult bees for 10 days with *Bt* maize pollen expressing Cry1Ab toxin (event MON810) or with purified Cry1Ab toxin in sugar solutions. No significant differences in diameter, weight and development of hypopharyngeal glands were found in bees fed either *Bt* pollen or *Bt*-containing sugar solutions, compared to their respective controls. In a field study where colonies foraged on Cry1Ab-expressing maize (event Bt11) and were fed *Bt* pollen cakes for 28 days, Rose *et al.* (2007) did not observe adverse effects on bee weight, foraging activity, and colony performance. Similarly, in a flight cage study in controlled conditions, no significant differences were reported in honeybee mortality, syrup consumption and olfactory learning performance when honeybee colonies were exposed to different syrups containing Cry1Ab protoxin (Ramirez-Romero *et al.*, 2005). In a later paper, Ramirez-Romero *et al.* (2008) state that the toxin did not cause lethal effects on honey bees; however, feeding behaviour was affected when exposed to very high concentrations of Cry1Ab (5000 ppb), with honey bees taking longer to imbibe the contaminated syrup. The authors state though, that these negative effects are unlikely in natural conditions, since exposure to such large amounts of toxin is not foreseeable.

Other insect resistant traits are possibly more hazardous to bees. Serine proteinase inhibitors may affect honeybees and bumblebees at high concentrations while cysteine-based proteinase inhibitors do not seem to have similar effect (Malone and Burgess, 2009). The exception is aprotinin, a serine proteinase inhibitor which affects honeybees but not bumblebees (Burgess *et al.*, 1996; Malone *et al.*, 2000). In the case of herbicide tolerant GM plants, possible negative effects may arise due to changes in cultivation practices which lead to altered weed composition. Reduced flowering in the agro-ecosystem may indirectly lead to a lower local abundance of pollinators (e.g. Haughton *et al.*, 2003). An analysis of the environmental impact of GM plants, if based only on acute toxic response, might prove insufficient to draw general conclusions about their safety for pollinators. In analysing the case study

of *Bt* cotton in Brazil, Arpaia *et al.* (2006b) suggested that other possible adverse-effect scenarios should be duly considered at both the organism and colony level. Amongst these, any possible impaired colony development via the lowering of queen fecundity or any modification of foraging behaviour due to an impaired ability to find food may have important consequences on overall pollination activity in the agro-ecosystem.

The exposure of pollinator hymenoptera to toxins expressed in GM plants is a direct consequence of their feeding habits, since these organisms occupy the second trophic level in food chains. Constitutive promoters in transgene constructs generally lead to limited protein expression in pollen (Potenza *et al.*, 2004). In some events though, significant amounts of Cry toxin is expressed in pollen (e.g. Koziel *et al.*, 1993; Mattila *et al.*, 2005; Yao *et al.*, 2006). However, the presence of newly-expressed proteins in GM plant nectar is considered negligible since it has no cellular content (Malone and Burgess, 2009). The GMP species will also modulate the possible exposure of bees to toxins; for instance, maize does not represent a major pollen source for bees. Babendreier *et al.* (2004) reported that fully grown worker bee larvae contain between 1720 and 2310 maize pollen grains in their gut before defecation, corresponding to 1.52-2.04 mg of pollen consumed per larva. Adult honeybees consume significant quantities of pollen while pollen consumption by larvae is minimal, especially during early growth stages, and therefore larvae are less exposed to GM plant-produced toxins. By directly feeding larvae with pollen, Babendreier *et al.* (2004) calculated the contribution of Cry protein to be less than 5 % in relation to the total amount of protein necessary for complete larval development. Moreover, due to the low concentration of Cry1Ab in pollen, honeybees will only be exposed to low concentrations of proteins. The presence of pollen in honey reserves in the hives can prolong the exposure of the colony to toxins that are expressed in pollen grains and therefore the time of flowering does not fully represent the extent of exposure these social bees may be faced to. Pierre *et al.* (2003) observed the diversity and density of the foraging insect population on GM HT OILSEED RAPE genotypes to be similar to that on the near isogenic controls. Moreover, the foraging behaviour strategy was similar between treatments.

3.3. Biological Components of Soil Fertility

Soil fertility can be defined as the characteristics (biological, physical, chemical, etc.) enabling a soil to provide nutritive elements that support plant growth, and its production in agricultural fields. While it is known that physical, chemical and biological components of soil fertility strongly interact, in this context mainly

the latter will be considered. The availability of nutrients is strongly dependent on effective microbial functioning in the soil. Communities of soil-dwelling invertebrates are also involved in nutrient cycling and decomposition of organic matter that have both an ecological and practical significance for agriculture (Moore *et al.*, 1988). Species diversity in soils is extensive (e.g. Curtis *et al.*, 2002), as soil-dwelling organisms can account for most of the whole agro-ecosystem diversity. Plants themselves, through the release of the exudates in the soil, exert a major influence in selecting communities of micro-, meso- and macro-organisms in the rhizosphere. Brussaard *et al.* (2007) suggest that soil biodiversity can be assessed, managed and conserved, showing examples of successful and unsuccessful practices which have been used in various regions of the world to manage soil biodiversity.

In the context of this review, it is recognised that soil fertility is another important ecosystem service guaranteed to farmers through the establishment of intricate food webs (both grazing and detritus food webs are quite active in agricultural soils). Potential adverse environmental impacts due to the cultivation of GMPs may occur should soil organisms be negatively impacted and/or changes in microbe-mediated functions in the soil occur (Icoz and Stotzky, 2008). GMPs can expose non-target soil-dwelling organisms to proteins via root exudation and cultivation residues. The persistence of toxins in the soil can be quite variable, and is particularly dependent upon soil type; for instance, Cry toxins are known to bind to clay, suggesting that there is potential for their long term persistence and consequently long exposure to non-target organisms (Icoz and Stotzky, 2008). Commonly, experiments have shown GMPs to exert changes in the local populations of soil organisms. Generally speaking though, these differences have been considered to be in line with those effects induced by changing crop species or even varieties within a single species (EFSA, 2009). It is therefore important that both statistical significance and biological relevance are considered when assessing possible specific environmental impacts.

This section will mainly consider the available information relating to GMPs and soil arthropods, and then will briefly summarise the state of the art and the open issues related to the possible effects on soil microorganisms; while for more detailed information on effects on microorganisms, interested readers are recommended to consult more specific literature.

3.3.1. Earthworms

Earthworms play an important role in decomposing plant litter, and are responsible

for numerous physical changes that affect the biological properties and processes in soil (e.g., through the maintenance of soil structure) and are considered important organisms in the regulation of nutrient cycling processes (Icoz and Stotzky, 2008). Laboratory studies performed on some earthworm species, such as *Aporrectodea caliginosa* Savigny (Vercesi *et al.*, 2006; Schrader *et al.*, 2008), *Eisenia foetida* Savigny (Clark and Coats, 2006) and *Lumbricus terrestris* L. (Saxena and Stotzky, 2001a; Zwahlen *et al.*, 2003; Lang *et al.*, 2006; Schrader *et al.*, 2008) did not reveal significant adverse effects on earthworm survival, growth and reproduction upon ingestion of Cry1Ab toxin.

No adverse effects on mortality or weight were observed on *L. terrestris* exposed to soil incorporating plant material from *Bt*-expressing maize after 40 or 45 days, respectively, compared to non-*Bt* maize (Saxena and Stotzky, 2001a). Zwahlen *et al.* (2003) investigated *L. terrestris* mortality and growth in laboratory and field experiments by exposing juveniles and adults to Bt11 maize (expressing a Cry1Ab toxin) for up to 200 days. Even though earthworms were not lethally affected by the exposure to *Bt* maize, sub-lethal long-term effects were observed in the laboratory study: the growth of adults, expressed as mean fresh weight, was similar for 160 days, but significantly declined thereafter in *Bt*-exposed earthworms. However, in the field no adverse effects of *Bt* maize were found (Zwahlen *et al.*, 2003). Laboratory studies by Clark and Coats (2006), in which *E. foetida* was fed leaf material from either *Bt* maize (events Bt11 and MON810) or the near isogenic counterpart in a soil system did not reveal adverse effects on survival or reproduction after 4 weeks. Vercesi *et al.* (2006) studied the effects of MON810 maize on important life-history traits (survival, reproduction and growth) of *A. caliginosa* under various experimental conditions. The authors measured the growth of juveniles until maturity, as well as cocoon production and hatchability. Finely-ground leaves of MON810 maize added to soil had no adverse effects on these life-history traits in *A. caliginosa*, even when exposed to very high concentrations. In addition, the growth of juveniles was unaffected when they were kept in pots with *Bt*-expressing maize plants for 4 weeks. A statistically significant negative effect of high concentration of *Bt* maize residues was only observed on cocoon hatchability. However, since earthworms were exposed to very high concentration of fresh *Bt*-expressing plant material in the experiment, the authors questioned whether the negative effect would have any ecological significance under field conditions. In recent experiments performed by Schrader *et al.* (2008), *A. caliginosa* and *L. terrestris* both survived incubation in microcosms for 5

weeks, irrespective of whether they grew in the presence of leaves and roots of MON810 or control maize. The content of Cry1Ab toxin detected in the plant residues strongly declined over time.

Saxena and Stotzky (2001a) demonstrated the uptake of Cry1Ab toxin by earthworms via detection in casts and guts. The ingestion of Cry1Ab toxin by earthworms was later confirmed through the detection of the protein in their gut and faeces (Zwahlen *et al.*, 2003). Field surveys indicated no adverse effects on earthworms during the cultivation of *Bt* maize expressing the Cry1Ab toxin (e.g., Zwahlen *et al.*, 2003; Krogh *et al.*, 2007). No significant differences were reported in the population density or total biomass of *Lumbricidae* between soils with *Bt* (events MON810 and Bt176) and non-*Bt* maize at 5 sites during 4 years of maize field cultivation, though the site and the year had a significant influence on both measurement endpoints (Lang *et al.*, 2006).

3.3.2. Nematodes

The composition of nematode communities is considered an useful bioindicator of soil health because it correlates well with nitrogen cycling and decomposition, two critical ecological processes in soil (Neher, 2001). Nematode abundance and diversity has been investigated in several experiments with GMPs; in experimental conditions, these values varied substantially when GMPs were compared to near isogenic controls. The extent and the direction of this variability however, is not unequivocal.

Cry toxins have been proposed to be hazardous to the nematode species *Caenorhabditis elegans* (Maupas) following laboratory studies by Höss *et al.* (2008). The authors studied the potential toxic effects of Cry1Ab toxin on *C. elegans*, either by exposing the species to rhizosphere and bulk soil from experimental fields cultivated with *Bt* maize (event MON810) or to different solutions of Cry1Ab toxin expressed in *Escherichia coli*. Nematode reproduction and growth were significantly reduced in rhizosphere and bulk soil of *Bt* maize as compared with soil from isogenic maize, and were significantly correlated with the Cry1Ab toxin concentrations in the soil samples. However, according to toxicity tests with the pure Cry1Ab protein, it was determined that the concentrations of Cry1Ab toxin measured in the *Bt* maize soil samples were not sufficiently high to produce direct toxic effects on *C. elegans*. Therefore the authors suggest that the adverse effects detected on *C. elegans* reproduction and growth could be indirect effects. In addition, the possibility of interactions of the newly-expressed toxin with other compounds produced by the GM plant should also be considered

as a possible mechanisms to explain these results. Saxena and Stotzky (2001a) found no significant differences in the number of nematodes in the rhizosphere of *Bt* and non-*Bt* maize (event NK4640Bt) in growth chamber studies. In a glasshouse study, Griffiths *et al.* (2006) reported significantly higher nematode populations of *Acrobeloides* spp. and *Pratylenchus* spp. when grown in the presence of *Bt* maize (event MON810) than with non-*Bt* maize. Further, in a later glasshouse study involving 8 different varieties of maize *Bt*, Griffiths *et al.* (2007b) went on to report that nematode abundance varied mainly between maize varieties, rather than between *Bt* and non-*Bt* maize, and commented that differences in previously published studies on soil nematodes in *Bt* maize were smaller than varietal effects. Field experiments confirmed that changes to nematode communities due to *Bt* maize (event MON810) were small and transient, and smaller than those induced by seasonal, soil type, tillage, crop type or varietal effects (Griffiths *et al.*, 2007a). Effects of *Bt* maize (events MON810 and Bt176) on the plant pest *Pratylenchus* spp. and the bacteriovirus *C. elegans*, have also been studied in field trials in Germany (Lang *et al.*, 2006). No adverse effects of GM maize were observed with respect to *Pratylenchus* spp. population density, whilst conversely *C. elegans* growth, number of eggs and reproduction rate were negatively affected. Further studies by Höss *et al.* (2008) confirmed those findings.

Maize does not represent the only GM crop studied for possible effects on nematodes. Manachini *et al.* (2004) studied nematode species assemblage in experimental fields with oilseed rape expressing Cry1Ac toxin. The authors found a statistically significant shift in community structure, and a direct correlation between GM oilseed rape and the abundance of fungal feeding nematodes, while phytophagous nematodes were significantly associated with near isogenic areas. These results were in agreement with similar surveys conducted in Northern Italy (Manachini and Lozzia, 2002) where nematodes feeding on fungi were more abundant in *Bt* maize fields whereas nematodes feeding on bacteria were more abundant in non-*Bt* maize fields. A possible explanation for the presence of different trophic groups between GMPs and control areas could be linked to differences in plant composition that are known to occur in some GMPs (e.g. lignin in maize, Saxena & Stotzky 2001b) which could in turn have effects on the detritus food webs in the soil.

3.3.3. Isopods

The woodlouse (*Porcellio scaber* Latreille) is considered a model decomposer organism, and has been used in laboratory feeding studies for detecting potential adverse impacts of Cry1Ab toxin and Cry1Ab-expressing maize. Assimilation of

the Cry1Ab toxin by *P. scaber* following intentional exposure was demonstrated by the detection of the toxin in the faeces after consuming *Bt*-expressing plant material (Wandeler *et al.*, 2002; Pont and Nentwig, 2005). The latter authors observed that part of the *Bt* toxin taken up by primary decomposers is not digested and therefore is released in its active form into the soils. Under field conditions in autumn and winter, the toxin remains active and available to soil organisms until the next field season. No adverse effects on *P. scaber* consumption, survival and growth were observed when the species was fed plant material from maize expressing Cry1Ab toxin (Escher *et al.*, 2000). The survival and growth of two other common isopod species, *Trachelipus rathkii* (Brandt) and *Armadillidium nasatum* Budde-Lund, were not adversely affected after exposure to the purified Cry1Ab toxin or leaves of different *Bt* maize events under laboratory conditions for 8 weeks (Clark *et al.*, 2006). In other experiments however, some differences in mortality, weight gain and consumption by isopods and in the digestibility of plant material were detected. In these studies (Wandeler *et al.*, 2002; Clark *et al.*, 2006), the differences in the composition and nutritional quality of maize varieties used possibly contributed to determine the differences between treatments, and it is therefore difficult to draw conclusions about the effective consequences from the transgene into the test system.

3.3.4. Collembola

Collembola are an important group of organisms involved in the breakdown and recycling of crop residues, and they are normally abundant in agricultural fields (Hopkins, 2006). They often live in the root zone of plants and can therefore be exposed to root exudates and residues (Icoz and Stotzky, 2008). As such, Collembola are considered good indicator species of soil fertility and health, and have been used to detect the potential impact of Cry toxins. A study adding 4 purified *Bt* insecticidal proteins (Cry1Ab, Cry1Ac, Cry2A, and Cry3A) at concentrations of 200 mg/g to the diet of *Folsomia candida* Willem and *Xenylla grisea* Axelson for 3 weeks did not detect any impacts on the survival or reproduction of the study species when compared with a control diet (Sims and Martin, 1997). No deleterious effects on *F. candida* survival and reproduction were observed when fed leaves of *Bt* maize expressing the Cry1Ab toxin compared with those fed leaves of the isolines (Clark and Coats, 2006). Bakonyi *et al.* (2006) showed that *Bt* maize (event MON810) was less preferred as a food by *F. candida* than was near-isogenic control maize, while this preference was not observed for *Heteromurus nitidus* (Templeton) and *Sinella coeca* (Schott). During these experiments, *F. candida* defecated 30 % less around *Bt* maize in choice tests, but did not show a preference to remain on either of the two

plant material types offered (*Bt* and non-*Bt* maize). When starved individuals were tested in the same choice experiments, they equally consumed both diets. In addition to the presence of the Cry1Ab toxin, available diets also differed in C/N ratios in respective plant material. Heckmann *et al.* (2006) reported that the growth and reproduction of *Protaphorura armata* (Tullberg) reared on roots of *Bt* maize expressing Cry1Ab toxin were not significantly different from those reared on roots of non-*Bt* maize for 4 weeks. *P. armata* performed significantly better on a diet of yeast amended with purified Cry1Ab toxin than on root tissue of *Bt*- and non-*Bt*-expressing maize. In field studies, no significant differences in the population density of collembolans were found in soils cultivated with *Bt*- and non-*Bt*-expressing maize (Lang *et al.*, 2006). Concentrations of Cry toxins in plant material in soils in the field are usually low and estimated to be less than 30 µg/g of fresh weight, suggesting that these concentrations should not pose a relevant threat to Collembola (Sims and Martin, 1997).

3.3.5. Other taxonomic groups

Even though diplopods are not the most important group of decomposers of plant litter in soil, they are widely spread in the agricultural landscape and regularly occur in maize fields (EFSA, 2009). Laboratory studies have been performed on *Allajulus latestriatus* (Curtis) to analyse the effects of *Bt* maize (event Bt11) on mortality, food consumption, weight gain, and faeces production. No significant differences were found when test organisms were fed *Bt* maize compared to the near isogenic control and two commercial varieties used as references. Exposure to very high Cry1Ab concentrations (more than 100 times higher than those detectable in leaves) did not result in a significantly higher mortality (Weber and Nentwig, 2006), Faeces production was significantly increased when animals were maintained on *Bt* maize. Diplopods prefer feeding on partly degraded plant material which usually contains lower Cry1Ab concentrations (Weber and Nentwig, 2006), therefore their exposure in field conditions is estimated to be rather low. A recent paper (Hönemann and Nentwig, 2009) described laboratory feeding studies using GMPs and the enchytraeid *Enchytraeus albidus* Henle. Through their feeding activities, enchytraeid worms support mineralisation processes and improve the structure of soil. As for other soil-dwelling arthropods, enchytraeids can be exposed to the toxins released over time in the soils by GMP exudates or residues. Enchytraeids were observed to feed on diets that contained leaf material of *Bt* maize, though no Cry1Ab toxin was detected in adults after 3 weeks of feeding (Hönemann and Nentwig, 2009). In addition, *E. albidus* survival and reproduction showed no significant differences between the Cry3Bb1-expressing treatment and the untransformed counterpart. Different results were obtained

when Cry1Ab toxin was part of the diets of the enchytraeid species. Significantly more individuals were reported to survive in the treatment with Bt11 maize than with the corresponding near-isogenic control. In contrast, a significantly higher number of offspring were generated in the control treatment, as compared with the Bt11-containing diet. Due to differences in plant composition between the maize varieties, the authors could not tease out how much the presence of *Bt* maize leaf material in their diet contributed to such differences. Generally, enchytraeid worms do not feed on a single food source, but take up all degradable organic matter of adequate size in the field and therefore their exposure to toxins is likely to be reduced in normal field conditions (EFSA, 2009).

3.3.6. Effects on soil microorganisms

Microorganisms are the most abundant organisms in soils and are involved in many fundamental processes (e.g. decomposition of organic matter, mineralisation, decomposition of chemicals, improvement of soil structure, etc.; Gupta and Yeates, 1997). Root exudates released by plants selectively regulate which organisms reside in the respective rhizosphere (e.g. Lynch, 1994). Therefore, qualitative and quantitative changes in their emission could influence the diversity and activity of soil microbiota (Icoz and Stotzky, 2008). Populations of soil microorganisms are known to be affected by many factors in field conditions, therefore isolating the possible effects of a new stressor requires accurate experimental plans, moreover not all soil microorganisms can be easily grown in the laboratory and therefore experiments in controlled conditions can not always be easily performed.

While the majority of the studies summarised by Icoz and Stotzky (2008) indicated that *Bt*-expressing plants cause no or minor changes in microbial communities, in some studies relevant differences in the presence of microorganisms between soils cultivated with *Bt* and non-*Bt* maize were demonstrated. Xue *et al.* (2005) found a lower ratio between gram-positive and gram-negative bacteria in soil with *Bt* maize compared to their controls, while the effect was reversed in *Bt*-expressing potato. Root exudates of *Bt* maize (event Bt176) were shown to reduce pre-symbiotic hyphal growth of the arbuscular mycorrhizal fungus *Glomus mosseae*, as compared with those of another *Bt* maize (event Bt11) and control maize (Turrini *et al.*, 2004). Castaldini *et al.* (2005) also reported consistent differences in rhizosphere heterotrophic bacteria and mycorrhizal colonization (including *G. mosseae*) between *Bt* maize (event Bt176) and its conventional counterpart. According to the authors, the genetic modification in Bt176 maize might have led to changes in plant physiology and composition of root exudates, which in turn may have affected symbiotic and rhizosphere microorganisms. For

instance, microbial activity could have been affected by soluble sugar content (Burns and Dick, 2002); percentage differences in sugar content in the plants used in the study were relatively high and this could have also contributed to the observed changes. From an ecological perspective, Widmer (2007) suggests that effects observed on symbiotic microorganisms will only be disadvantageous for the crop itself, without representing a concern for the ecosystem. A more recent field study (Knox *et al.*, 2008) reported very different results when the production of arbuscular mycorrhizae was studied in GM cotton plants. The experiments included commercial cultivars of cotton expressing genes for insect resistance (Cry1Ac and Cry2Ab), glyphosate tolerance (5-enolpyruvylshikimate-3-phosphate synthase gene), or both, and their conventional parent lines. The development of mycorrhizae in cotton roots increased rapidly in the first three weeks after sowing and the pattern of colonisation was virtually identical among both conventional and GM cultivars of cotton at each assessment.

Reported effects on microbial communities were in general considered spatially and temporally limited, and small compared with those induced by differences in geographic location, temperature, seasonality, plant variety and soil type (Fang *et al.*, 2005, 2007; Griffiths *et al.*, 2005, 2006; Lilley *et al.*, 2006; Fillion, 2008; Icoz and Stotzky, 2008). Factors such as plant growth stage and field heterogeneity produced larger effects on soil microbial community structure than MON810 maize (Baumgarte and Tebbe, 2005; Griffiths *et al.*, 2007b). However, some issues in this area are still to be resolved. For instance, in some cases it is not clear if the methodology used for analysing soil samples has an influence in the appearance of diverging results obtained for similar groups of microorganisms in the literature. Moreover, the relationship between the high biodegradability and adsorption of Cry toxins in the soil and their biological activity should be further clarified. However, a more detailed discussion in this area goes beyond the goal of the present review.

3.4. Measuring the Overall Functionality

It should be apparent to the reader by now that selecting the appropriate assessment endpoint(s) is very important in analysing any non-target effects of GM crops. In line with the suggested “functional approach”, the choice of one or a few species amongst each functional group is a sensible way of studying possible environmental impacts. However, in some cases, the preliminary selection of indicator species for the risk assessment of GMPs may be difficult to the extent that it might not even be considered as the most appropriate approach. In that case, an alternative or complementary strategy could be the estimate of the functionality of a given ecosystem service.

In a field study on the arthropod fauna in Cry1Ac-expressing cotton, Naranjo (2005a) demonstrated that the abundance of some predator species was significantly diminished in plots with GMPs compared to those with near-isogenic untransformed cotton plants. However, in a parallel study conducted in the same cotton fields, the author found that the overall predation rate on the pink bollworm *Pectinophora gossypiella* (Saunders), one of the target species of Cry1Ac cotton, was similar between control and GMP plots (Naranjo, 2005b). Therefore, the detected changes in the guilds of predators did not impair the ecosystem service of natural predation on this pest. In this case, the consideration of only a given predator-prey dynamics might have resulted in the wrong conclusions. In two field studies conducted with GM potato and aubergine (and their respective untransformed controls), Arpaia *et al.* (2009) observed that the predation rates on *L. decemlineata* Say egg masses due to a guild of predatory species were similar in GM and control plots. In the two different experiments, *L. decemlineata* was the potential target for GM aubergine expressing the Cry3Bb toxin, while it was a non-target species for GM potato which were resistant to Lepidoptera by expressing a Cry1Ab toxin. The fact that the eggs were preyed might have limited the predator exposure to the Cry toxins in all cases, since the eggs deposited on leaves are not likely to absorb the toxin from plants. These results enabled the authors to conclude that the ecological function of natural predation on *L. decemlineata* eggs in GM plots was not impaired.

An indirect measure of pollination activity (e.g. by comparatively measuring fruit set between pollinated GM and control crops) might furnish a general indication of the foraging activity of pollinators in this type of experiment. Studies in which the decomposition of *Bt* maize was compared with that of non-*Bt* isogenic lines mostly showed that Cry1Ab-expressing maize did not affect decomposition rates or the mass of carbon remaining over time (e.g. Cortet *et al.*, 2006; Tarkalson *et al.*, 2008). Likewise, litter-bag experiments with *Bt* maize (event Bt11) reported by Zwahlen *et al.* (2007) did not reveal major changes in the decomposition rate of *Bt*-maize residues. Van Toan *et al.* (2008), discussing a possible risk assessment strategy for *Bt* cotton in Viet Nam, state that the complexity of soil ecosystems render studies based on species lists impractical and unreliable. The authors suggest that ecosystem processes such as biomass decomposition, cellulose and lignin breakdown, phosphorous and nutrient uptake should constitute the focus of the biosafety studies.

These examples show that on a case-by-case manner, different assessment endpoints can be effectively chosen in line with the proposed functional

approach and therefore risk assessors should evaluate this possibility considering the combination of plant, newly expressed trait(s), and the receiving environment. An additional criterion, while not strictly based on ecological characteristics, that may help to assess an environmental impact on NTOs is the consideration of anthropocentric values. This criterion will possibly lead to the consideration of other NTOs that could not be selected among the above-mentioned functional groups. Anthropocentric values are related to human goals and require the consideration of organisms characterised as secondary pest species, rare or endangered species, species that generate income, and species of social or cultural value (Birch *et al.*, 2004).

A very well known case of risk assessment of GMPs for a non-target species concerns the Monarch butterfly *D. plexippus* L., a species which can not be ascribed to any functional group of NTOs directly involved in ecosystem services. This lepidopteran species though has an important charismatic value, especially in the USA, due to its fascinating habits, especially its periodical massive migrations across the continent. The report of the hazard presented to *D. plexippus* larvae from the ingestion of Cry1Ab-expressing maize pollen in laboratory experiments (Losey *et al.*, 1999) triggered extensive laboratory and field studies to determine whether Monarch butterfly populations would be at risk under realistic exposure conditions from *Bt*-expressing maize pollen in the USA. Monarch larvae do not directly feed on maize leaves, therefore pollen deposition on their host plants, the milkweed *Asclepias syriaca* L., is the most realistic exposure route for the larvae to Cry toxins expressed in maize. Field studies indicated that the overall proportion of Monarch butterfly populations exposed to toxic levels of *Bt* pollen was small due to the limited spatial distribution of pollen (Pleasant *et al.*, 2001) and the limited temporal overlap between larval development and pollen shed (Oberhauser *et al.*, 2001). Based on the USA data, a risk assessment model estimated that 50 % of the breeding population of the Monarch butterfly was potentially exposed to Cry1Ab-expressing pollen in the USA corn belt (Sears *et al.*, 2001), but that only an additional 0.6-2.5 % mortality would be generated due directly to the cultivation of *Bt*-expressing maize (Dively *et al.*, 2004). Anderson *et al.* (2004) and Prasifka *et al.* (2007) both reported a reduction in feeding and weight gain of the *D. plexippus* larvae under laboratory conditions due to food induced behavioural changes, but how these results would correlate with feeding habits in the field was not elucidated. Under field conditions early instars, the most susceptible to the Cry1Ab toxin, are less exposed to *Bt* pollen drift, as they mainly feed on the upper third of milkweed plants where the lowest

densities of anthers occur (Pleasants *et al.*, 2001; Anderson *et al.*, 2004). In addition, larvae can move to the underside of leaves where they would avoid any contact with anthers (Pleasants *et al.*, 2001; Jesse and Obrycki, 2003).

Maize plants expressing lepidopteran-specific Cry toxins were shown to be hazardous to a range of other Lepidopteran species, some of which are species of conservation concern (e.g. Felke *et al.*, 2002; Lang and Vojtech, 2006). These species could also be exposed to potentially toxic pollen deposited on their host plant in and around maize fields. The possible effects on non-target Lepidoptera has been invoked as a justification for several safeguard clauses that were risen by several European nations (e.g. Austria, France, Greece, Germany, Hungary, Luxembourg) who asked for the ban of MON810 maize cultivation in their territories. The European Food Safety Authority GMO Panel (EFSA, 2009) conducted an exposure assessment based on a simulation model to help quantify the risk assessment. Exposure was modelled for 3 combinations of lepidopteran species and their host plants, all of which occur widely throughout the European Union. These were: the butterflies *Inachis io* L. and *Vanessa atalanta* L. both feeding on the host plant *Urtica dioica* L.; and the pest species *Plutella xylostella* L. (diamondback moth) and its host plant species in the Brassicaceae family. The analysis was based on an 11-parameter deterministic mathematical model, of which 7 parameters were specific to particular geographic regions and 4 parameters were more generic to the particular species/host plant combination. For the majority of areas where *U. dioica* is known to occur, for both the butterfly species considered, the best estimate for mortality was less than one individual in every 1800, whilst sub-lethality was estimated at less than one individual in every 550. When the diamondback moth was considered, for the majority of areas the best estimate for mortality was less than one individual in every 300, whilst sub-lethality was less than one individual in every 100 (EFSA, 2009). A further elaboration of this mathematical model (Perry *et al.*, 2010), which includes more experimental values for two of the estimated parameters and accounts for the possibility of aggregated pollen dispersion, suggests that the probability of sublethal effects might be four times less than previously estimated. In order to obtain these estimates, extrapolations were made about the toxicity of MON810 maize pollen in relation to other *Bt* maize events with different toxin expression in pollen. It has become clear that a case-by-case evaluation will be necessary. This will have to consider the biology of NTOs in relation to the specificity of the receiving environment, and also the specific GMP and its molecular characteristics (including toxin expression in different plant parts).

3.5. What Else is Non-Target?

Agro-ecosystems and adjacent habitats are the first environments to be exposed to GMPs and their products, and therefore they are correctly considered in risk assessments. Nevertheless, the spatial-temporal dispersion of GM products can influence other trophic chains that in specific cases might become relevant. For example, pollen dispersal might bring other herbivore species in contact to newly-expressed toxins at distances from crop fields; pollen and plant residues may enter water bodies surrounding cultivated fields and therefore enlarge the possible exposure to expressed toxins. Trophic chains do not stop with predatory arthropods, such as spiders, but commonly involve other taxa, for instance, birds. Finally it must be considered that in some conditions non-arthropod herbivores (e.g. snails, rodents, wildlife) may commonly feed on cultivated plants.

The water flea *Daphnia magna* Straus is generally considered a surrogate species for assessing the effects of pollutants on aquatic organisms, and as such, the species is commonly included in eco-toxicological tests performed by the applicant when submitting dossiers for the commercialisation of GM crops or products. It has been reported that the species did not suffer acute toxic effects in laboratory studies with Cry1Ab toxin (Mendelson *et al.*, 2003). However, in contradiction, a laboratory experiment performed by Bøhn *et al.* (2008) revealed that *D. magna* fed with a suspension of MON810 maize flour had a higher mortality and lower proportion of females reaching sexual maturity. Since maize flour is not a common natural diet of flea beetles, and that *D. magna* fed non-Bt maize also experienced delays in development, the experiment could not rule out the possibility that these results might have been caused by nutritional deficiencies related to the maize-based diet. Rosi-Marshall *et al.* (2007) reported that by-products of Cry1Ab-expressing maize entered headwater streams. In laboratory experiments, the authors found reduced growth and increased mortality in aquatic Trichoptera; nevertheless, no effects were found during samplings in natural conditions by Chambers *et al.* (2007). The study by Rosi-Marshall *et al.* (2007) has attracted a lot of controversy especially with respect to its experimental design, but it did highlight a potential hazard of high doses of Cry1Ab toxin for Trichoptera under laboratory conditions. However, due to the low level of Cry toxins in aquatic systems (Douville *et al.*, 2005), and the rapid decomposition of maize leaves (Griffiths *et al.*, 2009), exposure of Trichopterans in aquatic ecosystems is likely to be low in many cases.

In an acute toxicity test, Cry3Bb1 toxin from MON863 maize root extracts was fed to *Chironomus dilutus* Shobanov larvae for 10 days. A significant decrease

in *C. dilutus* survival at nominal concentrations of 30 ng/ml was found; however, no effect on growth among the surviving larvae was observed (Prihoda and Coats, 2008). No direct effects on the snail, *Cantareus asperses* Müller (Stylommatophora: Helicidae), were detected after exposure to purified Cry1Ab toxin for 4 weeks (Kramarz *et al.*, 2007a) or to growing Bt and non-Bt maize for 3 months in microcosm experiments, though the Cry1Ab toxin was detected in snail faeces and thus it represents an additional route of exposure for soil microorganisms (de Vauflleury *et al.*, 2007). In a no-choice feeding experiment at the end of growth (47 weeks of exposure), snails exposed to Cry1Ab toxin in food and soil had a growth coefficient 25 % lower than unexposed snails (Kramarz *et al.*, 2009). After the first period of reproduction (68 weeks), a significant difference remained for body mass growth between the two treatments. Differences in body mass were not significant at the end of exposure (88 weeks).

Indirect effects of GMPs on birds were studied by Gibbons *et al.* (2006) and by Chamberlain *et al.* (2007) based on data from the British farm-scale trials. The study aimed to compare bird abundance between GM herbicide-tolerant and conventional crops. The observed differences were in agreement with likely differences in food availability (Chamberlain *et al.* 2007). Moreover, using farmland birds as a model system, Butler *et al.* (2007) have developed a generic risk assessment framework that accurately predicts the current conservation status of each bird species and population growth rate associated with past changes in UK agriculture. They concluded that replacing equivalent conventional crops in the current UK agricultural landscape with GMHT crops would only have a limited effect on the Farmland Bird Index (a measure of their biodiversity). Forecasts about possible effects at landscape levels can be obtained when spatial and temporal models incorporating agricultural landscaped and their cropping patterns are available (Castellazzi *et al.*, 2007).

4. LACK OF KNOWLEDGE AND FURTHER IMPROVEMENT OF RISK ASSESSMENT PROCEDURES

Even though the scientific literature dealing with non-target organisms and GMPs is continuously increasing, there are still knowledge gaps that will need to be addressed in future studies. Table 3 lists the number of species of natural enemies and pollinators subjected so far to specific studies using GMPs and/or their products. Several new species were studied in the last few years, nevertheless there are some important groups that are still not, or only poorly, represented. In spite of their relevance in some agro-ecosystems, no information on predaceous

Diptera or Odonata are available, and only 2 spider and 1 spider mite species have been studied to date. Also, Coleoptera Staphilinidae are unfortunately lacking in the list of organisms considered so far in biosafety research. This guild constitutes, for instance, the third most abundant group of soil-dwelling arthropods in maize crops in Spain, and it has a species richness and diversity similar to ground beetles and spiders (Farinós *et al.*, 2008). Similarly, obvious knowledge gaps persist for groups of parasitoids of relevant economic importance in several agro-ecosystems (e.g. Eulophidae, Aphelinidae, Tachinidae) and establishing their compatibility with the use of GM crops would be very important for integrated pest management programs. When it comes to pollinators, information is only available concerning honeybees, 3 bumblebee species and the red mason bee. In some areas, solitary bees may have a very important ecological role, especially when crops are cultivated near natural habitats (Arpaia *et al.*, 2006b), therefore important information might be gathered in more specific studies.

Also when information about relevant non-target species is available, risk assessment of non-target organisms can be improved by considering their ecology in natural conditions better. Charleston and Dicke (2008) reviewed some of the dossiers for commercialisation of GMPs submitted to the Dutch National Competent Authority and highlighted several serious shortcomings in the tests conducted on non-target organisms. Firstly, the choice of assessment endpoints was often limited to the detection of acute toxic effects, and sub-lethal effects were ignored. While mortality is obviously a main life history factor to be measured, it is important to consider that sub-lethal effects alone can also drive a population to extinction (Hallam *et al.*, 1993). Therefore, other measurement endpoints such as development, growth, fecundity, fertility, etc. need to be considered to predict any possible environmental effect. Secondly, even when the selected species for testing was considered appropriate, in several cases the life stage selected may not have been entirely appropriate (Charleston and Dicke, 2008). A sound analysis of the possible exposure in field conditions (e.g. which stage is used as a prey in the experiment, which stage of the non-target is exposed, whether bi-trophic or tri-trophic exposure is more likely, etc.) is very important in order to properly design experiments. Last, but not least, a properly designed experiment with the sufficient power to detect any adverse effect is fundamental to collect meaningful information for environmental risk assessment (Andow, 2003; Perry *et al.*, 2009).

Table 3. Number of species of natural enemies and pollinators studied for testing the effects of genetically modified plants and/or their products. Note: species which were only surveyed in field trials as part of species assemblages are not included. The list is complete until July 2009.

Functional group	Order	Family	No. of species
Predators	Heteroptera	Anthocoridae	4
"	"	Nabidae	1
"	"	Geocoridae	2
"	"	Miridae	2
"	"	Reduviidae	1
"	"	Pentatomidae	1
"	Coleoptera	Coccinellidae	9
"	"	Carabidae	17
"	Neuroptera	Chrysopidae	1
"	Araneae	Araneidae	2
"	Acarina	Phytoseidae	1
Parasitoids	Hymenoptera	Braconidae	8
"	"	Ichneumonidae	3
"	"	Eulophidae	1
"	"	Aphelinidae	1
"	"	Encyrtidae	1
"	"	Trichogrammatidae	1
"	"	Apidae	5

Many discussions about possible negative environmental effects due to the cultivation of GMPs identified the need of predicting long-term impacts of these plants (e.g. the possible development of resistant pest strains, build up of populations of secondary pests, etc.). Long-term effects may be difficult to measure as the environment is dynamic and agro-ecosystems undergo substantial change due to varietal improvement, agronomic innovation and climatic shifts. In addition, agro-ecosystems have a relatively short time-scale over which they remain in cultivation. Some of the possible effects, e.g. linked to a chronic exposure to a particular GM plant that results in a delayed response by organisms (or their progeny), might possibly be assessed in confined experimental systems maintained over several generations. In this case the selection of appropriate measurement endpoints based on the biology of the chosen organism is essential. Other effects that may occur at a later stage due to spatial and temporal complexities are not likely to be revealed in highly confined experimental systems. In these cases, research studies together with modelling and monitoring are appropriate tools to investigate possible long-term environmental effects resulting from GMO cultivation (BVL, 2009).

5. ANALYSIS OF RISK ASSESSMENT APPROACHES

Testing of non-target organisms is deemed necessary in risk assessment in order to evaluate possible direct and indirect effects of the environmental release of GMPs. However, there are broad discussions on the potential and flexibility of different approaches and the nature of the conclusions derived from different testing regimes (see EFSA, 2008). Several risk assessment approaches have been proposed for the analysis of the possible impacts on non-target organisms; the two illustrated here represent alternative approaches to which, even with some modifications, many other risk assessment proposals refer to.

The tiered eco-toxicological approach using clearly defined risk hypotheses and the selection of surrogate species is suggested for instance by Romeis *et al.* (2008). This approach was originally developed in pesticide toxicology and is currently applied to insect resistant plants in the USA. In this approach, the potentially toxic product expressed in the GMP is considered the stressor to be characterised. The authors consider necessary to select species which are representative of their genera and/or of particular functional groups (including herbivores, pollinators, predators and parasitoids, decomposers of plant material) to serve as surrogates that can be tested under laboratory and/or field conditions and which represent ecologically and economically important animal taxa likely to

be exposed to the GM crop or its products. The availability of standard laboratory tests is an important requirement, and guides surrogate species selection. As a first step ('tier'), very high doses of the toxin ("worst case" conditions) are used to test the sensitivity of surrogate species to the newly-expressed products in GMPs. Any insensitivity of tested species to high toxin doses in this framework implies that no effect will occur even under field exposure on these species, and therefore no further testing is required. By contrast, any species found to be sensitive are assessed further in more detail and in semi-field and field experiments (higher 'tiers'), in order to verify whether negative effects detected in laboratory conditions will occur in more realistic settings. Field studies are therefore not always considered necessary in this approach. In addition, as tests move from the lab to the field, it is acknowledged that more sources of potential experimental variation are introduced and thus can increase the difficulty in confirming causal relationships in the field. Based on the experience with Cry toxins, tier 1 tests appear to represent useful predictors for results at higher tier tests (Duan *et al.*, 2010) providing that designs include all ecologically-relevant routes of exposure (e.g. including tri-trophic experiments with *Bt* plants). However, studies with soil-dwelling organisms performed in a multi-year project in the EU (Birch *et al.*, 2007) do not seem to be in agreement with this general trend.

An "ecological approach" to the risk assessment of NTOs was originally proposed by Andow and Hilbeck (2004) and further elaborated in a series of case studies of environmental risk assessment of *Bt*-expressing crops in developing countries (Hilbeck and Andow, 2004; Hilbeck *et al.*, 2006; Andow *et al.*, 2008). The approach stresses the differences between environmental exposure of a chemical product and of a toxic compound expressed in a GMP. The approach requires that effects of the GMP be tested on a few important non-target species specifically selected from the range of environments where the GM crop is likely to be grown. Several additional criteria are considered in a selection matrix, helping to rank the importance of each candidate species in the specific agro-ecosystems. This risk assessment scheme further requires a process relying on risk hypotheses to guide the characterisation of exposure, adverse effects and risk, and a dynamic and adaptive tiered process where field studies are considered an essential component (Andow *et al.*, 2006b). The obligatory integration of field studies is deemed essential for introducing ecological realism, which occurs in the more complex spatial-temporal environment because laboratory trials might not capture any potential significant environment-organism interaction. Another reason for advocating field studies is represented by the possible synergistic effect (e.g. for cry toxin) with the presence of other environmental stressors that

may enhance toxic effects (e.g. Brousseau *et al.*, 1998; Wraight and Ramos, 2005; Lawo *et al.*, 2008) and that are not normally included in laboratory studies.

While these two approaches were mostly considered mutually exclusive, it can be recognised that there are objective merits in both; for instance the growing knowledge about some specific GM events might be handled with some confidence with an eco-toxicological approach, while the ecological approach might better fulfil the Precautionary Principle (Principle 15 of the “Rio Declaration”; General Assembly of the United Nations, 1992) for new available events. As a follow up of the Scientific Colloquium on Environmental Risk Assessment of GMPs (EFSA, 2008), the European Food Safety Authority committed to produce a scientific document, currently in preparation, which incorporates some aspects from both approaches. The implications for risk assessment are manifold and a detailed discussion goes beyond the scope (and the length) of the present review. However some general recommendations can be made, in particular it may be useful to recall here two points that may be critical in properly framing a risk assessment on NTOs. It is very important to determine what type of agriculture is considered as the comparator for the specific receiving environment. The appropriate basis for comparison should be determined during the initial phase of an environmental risk assessment (USA EPA, 1998), and the current agricultural practices should be considered (Arpaia, 2004) especially referring to pest management measures. Additional factors that may influence this determination include the policy goals of the regulatory authority and the potential users of the technology (Andow *et al.*, 2006a).

The use of mathematical models for risk assessment is widespread, however it is not trivial to remember that models are only as good as the data which drive them and thus risk assessors should ensure that dependable empirical data informs any modelling presented. This should be achieved by more cooperation between modellers and data-gatherers to ensure that expensive field trials generate data which not only help inform the environmental risk assessment but also help develop/validate models, which in turn may add confidence to risk characterisation and field trial design (EFSA, 2008).

6. FUTURE TRAITS

According to the latest available official data on the commercialisation of GM crops (James, 2009), the adoption of GM crops expressing multiple characters (stacked events) is increasing relative to other traits; a total of 28.7 million hectares

of stacked biotech crops were planted in 2009 (up from 26.9 million hectares in 2008). Insect resistance currently represents the third diffused category of traits introduced in GMPs (Figure 1) accounting for 15 % of the cultivated GMPs worldwide.

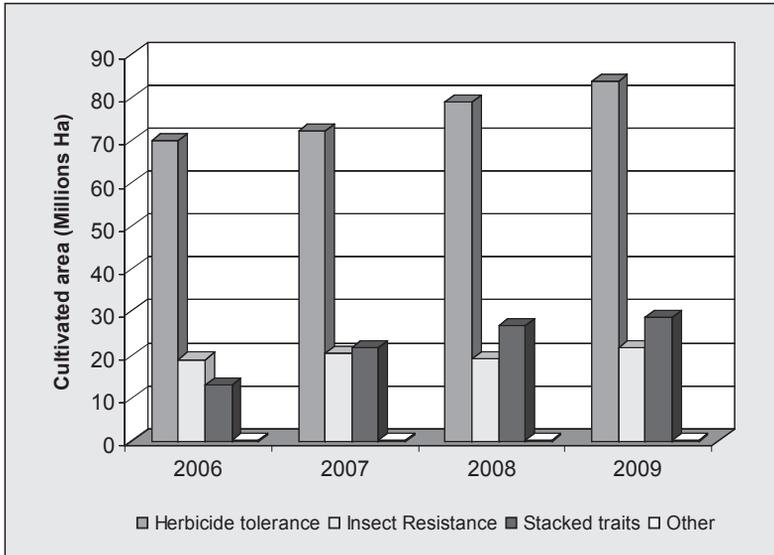


Figure 1. Adoption of Genetically modified plants by trait in recent years. Data source: <http://www.isaaa.org>.

This trend has clear implications for the risk assessment of GMPs on non-target organisms. When dealing with GMPs featuring insect resistance characteristics, the risk hypotheses considered were rather specific (e.g. a Cry toxin might not be as specific as predicted, and therefore hazards on other taxa need to be ascertained). With GMPs expressing other characteristics and producing metabolites whose toxic properties are sometimes not known, the consideration of more generic risk hypotheses (e.g. will the GMP cultivation impair predator activity?) should be included in the initial problem formulation phase. Unanticipated effects during the development of new GMPs might arise due to pleiotropy, positional effects, *in vitro* regeneration, re-arrangement in

metabolic patterns, etc. This is because transgene integration in plants occurs through illegitimate recombination. DNA integration is random, with a preference for generic regions, and gene disruptions, sequence changes and the production of new proteins can occur as a consequence of the recombination event (Rischer and Oksman-Caldentey, 2006). When such changes result in significant phenotypic modifications of the agronomic characteristics, these events will certainly be eliminated by the selection processes of breeders and companies. On the other hand, such changes may remain unnoticed if these metabolic rearrangements do not trigger unfavorable phenotypic changes.

Plant-insect interactions are largely mediated by chemical clues, even though colours, shapes, textures, etc. also play an important role. Insect herbivores and plants have co-evolved in order to optimise, each for its own benefit, the interpretation of chemical clues to successfully occupy their ecological niche. Therefore familiar food webs are ultimately constructed thanks also to the fine tuning, at all trophic levels, of such chemical perception. Under this scenario, it seems logical to hypothesise that the strict observation of an eco-toxicological approach for risk assessment on NTO will unlikely be sufficient, for instance, for GMPs in which some metabolic pathways were purposefully altered (e.g. GMPs for the production of industrial or medicinal products, energy production, phytoremediation, etc.). In the near future GMPs will likely express different metabolites for which there is much less experience in toxicological studies. In order to rule out the possibility of unintended effects linked to the genetic transformation, more general risk hypotheses will have to be considered. It has to be reflected that both the newly-produced metabolites and the GM plant itself might potentially represent an environmental stressor to be introduced in the agro-ecosystem. The author believes that *in planta* tests with NTOs are paramount when working with GM plants, such as pharma plants or plants with post-transcriptional gene silencing mechanisms, with which risk assessors have limited experience and need to predict their possible ecological implications. A further consideration of a comparative analysis of chemical composition of GM and control plants may also help in this assessment, since other groups of metabolites that are only partly considered during food or feed safety assessments become important in regulating plant relationships with arthropods (e.g. nutrients in all plant parts, secondary metabolites involved in plant direct defences, volatiles constituting indirect plant defences, etc.).

7. DEVELOPING COUNTRIES

Agricultural biotechnologies have the potential of meeting food and agricultural needs in developing countries. However, potential environmental

risks and benefits need to be taken into account when making decisions about the use of GMOs, especially in highly bio-diverse countries mostly located in the tropics. For those developing countries which are also important centres of origin for the major crop species, a thorough environmental risk assessment is extremely important before the cultivation of GM plants is considered.

Lövei *et al.* (2009) considered the geographical distribution of the published studies dealing with NTOs in laboratory experiments. A detailed analysis of the database demonstrated that no NTO species endemic to the continents of South America, Africa or Australia have been subject to detailed laboratory studies, as compared to species populating the same agro-ecosystems in the USA, Western Europe and China. Basic biological knowledge is clearly lacking in many cases, even though the situation is far from being uniform in this respect. During two specific workshops held to plan an environmental risk assessment for the adoption of *Bt* maize in Kenya and *Bt* cotton in Brazil, several groups of non-target organisms were considered in order to start a species selection process. Pollinators and pollen feeders were identified, amongst others, as important guilds likely to be exposed to GMPs and therefore included in the analysis. In the case of *Bt* maize in Kenya, the working group agreed that no systematic observations on flower-visiting species associated with maize had been conducted in Kenya and they could only come up with a short list of some pollen-feeders known to be present in significant numbers in maize cultivations (Birch *et al.*, 2004). The outcome of a similar exercise was quite different for *Bt* cotton in Brazil, where a working group could produce a list of several antophylous species including bumblebees, honeybees, stingless bees, solitary bees, orchid bees and several pollen feeders including ladybeetles, boll weevil, predatory mites, lacewings, earwigs (Arpaia *et al.*, 2006b).

Fragile ecosystems tend to be characterised by high rates of species turnover and high fluctuations in population sizes (Nilsson and Grelsson, 1995); features which are often associated with agro-ecosystems in developing countries, particularly those in marginal climates for agriculture (Grant, 1989). It is generally recognised that there is a lack of baseline bio-ecological information and limited local funds and trained personnel for studies to generate such data. Moreover, specific local expertise to perform environmental risk assessment and post-release monitoring programs for GMPs is often lacking in developing countries. These needs are the focus of

several capacity building projects currently operating in these regions. There are great expectations that GM traits addressing the primary agricultural needs of developing countries (e.g. drought tolerance) will become widely available in the future. It is therefore important that the benefit-sharing objective stated in the Convention on Biological Diversity will be pursued in order to ensure that developing countries will benefit from biotechnology without endangering their natural resources and their ecosystems.

8. CONCLUDING REMARKS

The commercialisation of genetically modified plants is probably the innovation that has experienced the fastest rate of adoption ever in agriculture, in both developed and developing countries (James, 2009). However, the uneven adoption of GMP cultivation around the world reveals differences in societal attitudes toward these crops, for instance, in Europe as opposite to the USA (Marshall, 2009). The political debate repeatedly involves governments, regulators, farmers, traders as well as non-governmental organisations, environmentalists and consumers associations. Examples are reported daily by the press and media in many areas of the world.

From a researcher's perspective, it is always to be hoped that the distinction between rigorous scientific reasoning and the socio-economic interests of the various stakeholders is clearly differentiated in ongoing discussions. There are still a number of differing legitimate views and interests in the scientific community in the area of potential environmental risks linked to the use of GMOs, and an attempt has been made by the author to highlight areas of uncertainties where science could furnish further inputs to existing knowledge and to help regulators.

As already stated elsewhere (Andow *et al.*, 2006a), the author reiterates his optimism that "the rapidly accumulating base of empirical knowledge will soon make possible that the likelihood of realizing most benefits of GMPs is increased and the likelihood of environmental harm is reduced." As with any other technology, risks to the environment are possible, and in some cases practical ideas for their management can be proposed (EFSA, 2009). On the other hand, environmental benefits appear achievable with the cultivation of GMPs; for example, the reduction of insecticide use is certainly a remarkable goal that seems realistic in several conditions (e.g. Morse *et al.*, 2005; Kleter *et al.*, 2007; Naranjo 2009). Agriculture has a long record of affecting biodiversity and its functioning at several levels, and a major change, for instance, in pest management practices will certainly induce a shift (either positive or negative) on biodiversity. This review proposes an approach to a scientifically-sound consideration

of the receiving environments where GMPs are to be released; this approach can be useful in summarising relevant information, identifying knowledge gaps and set up regional environmental risk assessment programs.

Plants provide the basis for complex food webs which host hundreds of different organisms; not all of these organisms can realistically become the subject of specific studies. The analysis of the main ecosystem services provided to agriculture can represent the underlying criterion enabling the estimation of the possible environmental impacts of GMP cultivation. In every agro-ecosystem, key functions such as plant pollination, natural pest control and maintaining soil fertility are mediated by NTOs, and thus represent pillars of farming systems. It is therefore suggested that the main functional groups involved in these functions be studied for their response to GMPs at the level of organism, colony and population, and by thoroughly considering both species abundance and diversity. Anthropocentric values should also be considered. The author considers that a scientifically-sound regulatory framework which considers the ecology of the agro-ecosystems in which a GMP is to be authorised for released is the best guarantee that biotechnological innovations, as well as other future environmental challenges, can be managed in a sustainable way without renouncing their benefits.

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