Effects of insect-resistant transgenic crops on non-target arthropods: first step in pre-market risk assessment studies

A literature-based study, proposing an ecologically
based first step to select non-target organisms

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1 SUMMARY

Although EU legislation requires risk-assessment studies to be done before field trials can be approved, it has not been specified which laboratory studies should be carried out, and which non-target species should be selected for those tests. These issues, with emphasis on the latter, are addressed in the current study.

Based on the scientific literature, ecological food webs have been constructed for three crops. These food webs represent the most relevant arthropod taxa/species and their interactions per crop. Based on these food webs, a total of 18 non-target arthropod species have been selected. It is proposed that for each transgenic crop, 4-6 non-target arthropods should be selected from this list for laboratory tests. To facilitate and guide this selection procedure, a key is presented, in which selection of non-target species for laboratory tests is based on a combination of their prevalence (literature research), ecological and/or economic importance in the crop system (using the ecological food-webs), and probability of exposure to the genetically inserted trait. In the current study we limited ourselves to arthropods of three crops (potato, maize and oilseed rape), with transgenic traits aimed to reduce insect damage (*Bt*-crops), in the geographical area (close to) the Benelux. This approach can be extended to other crops or to other regions of the EU. Suggestions for proceeding in these directions are made.

Based on the approach developed here, a scientifically-based selection of non-target organisms to be tested in the first step of a risk assessment has been made. This is a major step forward in developing a sound and meaningful risk assessment procedure.

2 INTRODUCTION

2.1 Deliberate releases of transgenic organisms

From 1991 until the 17th of Dec. 2004, a total of 1946 SNIFs (Summary Notification Information Format: notification on deliberate releases of transgenic organisms) were notified in the EU for other purposes than placing on the market. Of these, 24.6% were accounted for by the north-western European countries (Benelux and UK): 121 (Belgium) 142 (Netherlands), Luxemburg (0) and United Kingdom (216) (http://gmoinfo.jrc.it/). The majority of these 1946 transgenic organisms were plants, belonging to more than 70 different species and/or varieties. Among the top four of plants in the EU that were released for field trials were maize (*Zea mays*) (529 releases), oilseed rape (*Brassica napus*) (368 releases), and potato (*Solanum tuberosum*) (232 releases). Apart from field trials, genetically modified foods are being sold commercially in the EU. While in the USA almost 30 varieties of crops have been granted permission for commercial planting and use, in the EU only 4 commercial food crops have been approved (oilseed rape, maize, soybean, and chicory). These applications had been submitted under the previous Directive 90/220/EEC. Since regulation 1829/2003 came into place, the number of application requests has increased considerably, specifically those regarding import permits for products containing genetically modified food and feed.

2.2 Regulations

Unlike regulation in the USA, where transgenic insect-resistant plants are not considered different from conventional plant protectants, and where risk-assessment studies are carried out using ‘standard non-target arthropods’ like lady beetles, green lacewings and honey-bees, the EU considers transgenic plants as new entities, which need adjusted risk-assessment. All releases of GMO’s in the environment in the EU have to be authorized under the Deliberate Release Directive 2001/18/EC, which acts through a ‘step-by-step’ progression and uses data from earlier experiments to inform decisions about safety of future field trials (EC, 2001; EC 2005). According to Regulation (EC) No 1829/2003 (Regulation 1829), that provides a harmonized procedure for the scientific assessment and authorization of genetically modified organisms (GMOs) and genetically modified (GM) food and feed, a consent for placing on the market of products, which contain or consist of genetically modified organisms, can only be approved when it is ensured that the potential adverse effects on human health and the environment are accurately assessed on a case-by-case basis (EC, 2004). This assessment takes into account the commitments to environmental protection under legislation such as the EU’s Habitats Directive and the Biodiversity Conventions. For this purpose, a risk analysis and evaluation is carried out in accordance with Annex II of this Directive. This risk analysis is in general based on a risk-assessment of the characteristics of the genetically modified organism and of its intended applications.

2.3 Problem definition

There are concerns about the potential uptake or exposure to the inserted sequences by organisms (Wolfenbarger and Phifer, 2000). If this happens, this may have ecological consequences, leading to effects on the environment. In a bad case scenario, ecological effects may negatively affect the cropping system itself and/or its surrounding (semi)natural areas. For instance, laboratory studies have shown that *Bt*-

toxins were present in several non-target phytophagous arthropods (Dutton *et al.*, 2004) that had fed on Bt-Cry1Ab transgenic maize, as well as in a natural enemy that fed on those non-target organisms (Hilbeck *et al.*, 1998a,b), indicating the potential for adverse effects of insect-resistant transgenic crops on non-target organisms. Although there is consensus among regulatory bodies in the EU to require appropriate pre-market risk-analysis, described above, there is a lack of guidelines on exactly what information should be provided by applicants for deliberate release (field trial) of an insect resistant transgenic plant: This relates to (a) which non-target organisms should be tested, (b) what kind of non-target tests should be carried out, (c) exactly how these tests should be carried out, and how the results of these tests should be interpreted. In a first step to fill in this gap, Dutton *et al.* (2003) have proposed a sequential test procedure for risk-assessment to evaluate the effects of insect resistant plants on entomophagous arthropods, consisting of a three-tiered testing system. This system is based on a case-by-case scenario, rather than the standardized testing procedure typically used in pesticide risk-assessment procedures. They propose that in the first place it should be determined which arthropods play a role in the cropping system of interest. Then, to assess whether a non-target arthropod species in that particular agro ecosystem is at risk, sensitivity tests should be carried out. Also, it should be determined if, and at what level non-targets are exposed to the transgenic product, followed by toxicity tests. This proposed three-tiered system is a variation of the testing approach used for assessing the effects on pesticides on different organisms, and starts with laboratory tests (1st tier), followed by semi-field (2nd tier), and field (3rd tier) tests (Candolfi *et al.*, 2001; Dutton *et al.*, 2003).

2.4 Objective of current study

Recognizing the above described problems, the COGEM assigned a literature desk study called ‘standardization of criteria regarding effects on non-target organisms’ (Annex II), with the goal ‘to define which criteria should be used to assess effects of genetically modified organisms, in order to improve methodologies. Although taking an academic approach to address the selection of non-target arthropods, a pragmatic approach was used subsequently in an attempt to balance theory with ‘the real world’. Agreement on these criteria will lead to a better insight for applicants as to which data is necessary for the risk-assessment and to a more transparent authorization procedure’.

How this goal is approached is in line with the framework proposed by Dutton *et al.* (2003), and focuses on the first part of a tiered system: an appropriate pre-market risk-analysis of insect resistant transgenic plants in the laboratory. The main objective is to develop guidelines to assess which non-target organisms should be selected for the laboratory tests: Rather than testing a standard set of non-target species, as is common practice in risk-assessments of conventional insecticidal pesticides, it may be desirable to test only those non-target arthropods that are considered ‘important’ for each particular crop. The term ‘important’ species is used in relation to species that are known to be 1) of ecological importance (e.g. pollinators, natural enemies of pest-species), 2) of economic importance (e.g. bees that produce honey, important pollinators in fruit containing crops), 3) likely to get exposed (directly or indirectly) to the transgenically produced insecticidal proteins, and 4) so rare or endangered that any loss due to exposure to transgenic insecticidal proteins may further weaken their already critical populations. The current approach differs from Dutton *et al.* (2003) in that we did not limit the non-target organisms to entomophagous arthropods only, but include pollinators and parasitoids, as well as protected Lepidoptera.

Each crop has its own particular ecological food-web, i.e. herbivores (both pest and non-pest), carnivores, and 2nd order carnivores that live in, on, or near the GM plants. The current report is restricted to the three GM crops that have most often been released in the EU for field studies: potato, maize, and oil-seed rape. Although legislation of GM crops is regulated by the EU, we limited our study to the geographical areas of north-western Europe (Benelux, north-western Germany, north eastern France, and UK), with special emphasis on the Netherlands. This was done to develop the methodology of selecting non-target organisms for a restricted food web. We will indicate how the findings of this study can be extended to the EU as a whole.

3 ECOLOGICAL FOOD-WEBS

3.1 Description of the most important pest species and their natural enemies.

The text below is accompanied by Figures 1, 2, and 3. These figures consist of the ecological foodwebs of the three different crops, arranged by ecological groups of the most important pest insects, natural enemies thereof (predators and parasitoids), pollen and/or nectar feeders, and soil organisms, but does not mention 'unimportant' herbivores (those that feed on the plants but do not cause any considerable damage). It should be stressed that these are strong simplifications of actual ecological food webs: in a regular agronomic ecosystem there is an enormous species diversity with hundreds of species (Asteraki *et al.*, 2004), with an overwhelmingly complex network of interactions between these species. As an example of species richness, a recent ecological study on species diversity in a grassland margin area in southern England identified a total of 343 beetle species (Asteraki *et al.*, 2004). In addition to species richness, species compositions are not stable but change between years, and even between seasons (Rott & Godfray, 2000).

Members of these ecological groups can, at various levels and likelihood, become exposed to the GM trait of a crop plant: Pest species and pollen feeders, by feeding from the transgenic plant, are exposed directly to the *Bt* toxins. Their natural enemies are exposed indirectly by feeding on herbivores. Also many soil organisms are likely to get exposed directly to the toxins because many of these are detritivores, feeding on decaying plant tissues. The taxonomic levels in figure 1 go down to family level, whereas in the text these are more explicitly described, most often down to the species level. To simplify the overview of species, subsections per crop are used, based on large ecological groups (mostly Orders), with their natural enemies.

3.1.1 Oilseed rape (*Brassica napus*) (Figure 1)

The flowers of oilseed rape, producing both nectar and pollen, attract large numbers of insects, especially Lepidoptera. The crop is home to, and visited by a relatively large variety of non-pest arthropods, but has relatively few pest species (the most important ones are beetles, especially weevils and pollen beetles).

<u>Oilseed rape: pest Coleoptera and their natural enemies</u>

Adults of *Ceutorhynchus assimilis* (cabbage seed weevil), migrate into oilseed rape crops at the start of flowering, and bore a hole in the pod wall to lay eggs. On hatching, the larvae feed on the seeds. Feeding in the pods can cause severe losses with up to 60% of pods affected (http://www.pestspotter.co.uk/pests/7.htm). Larvae are often found parasitised by the parasitoid Trichomalus perfectus (Pteromalidae) (Alford, 2000).

Ceutorhynchus napi (rape stem weevil), and *C. pallidactylus* (cabbage stem weevil) feed on the stems of the plants, sometimes causing substantial damage. The larvae of the latter two weevil species are heavily attacked by the parasitoid *Tersilochus tripartitus* (Alford, 2000). Pollen beetles (*Meligethes* spp.) feed on flower buds, and can cause severe damage (Hansen 2003). Up to nine species of parasitoids have been recorded having parasitised pollen beetles in Europe, with species of *Phradis* and *Tersilochus* (Ichneumonidae) being most common: parasitism in excess of 80% have been recorded (Alford, 2000). Larvae that happen to fall to the ground are readily preyed upon by spiders, and (carabid and staphilinid) beetles. The cabbage stem flea beetle (*Psylliodes chryocephala*) attacks the cotyledons of emerging winter rape crops. During winter time, larvae of these beetles may cause damage to the stems of winter rape, leading to stunted plants in the spring.

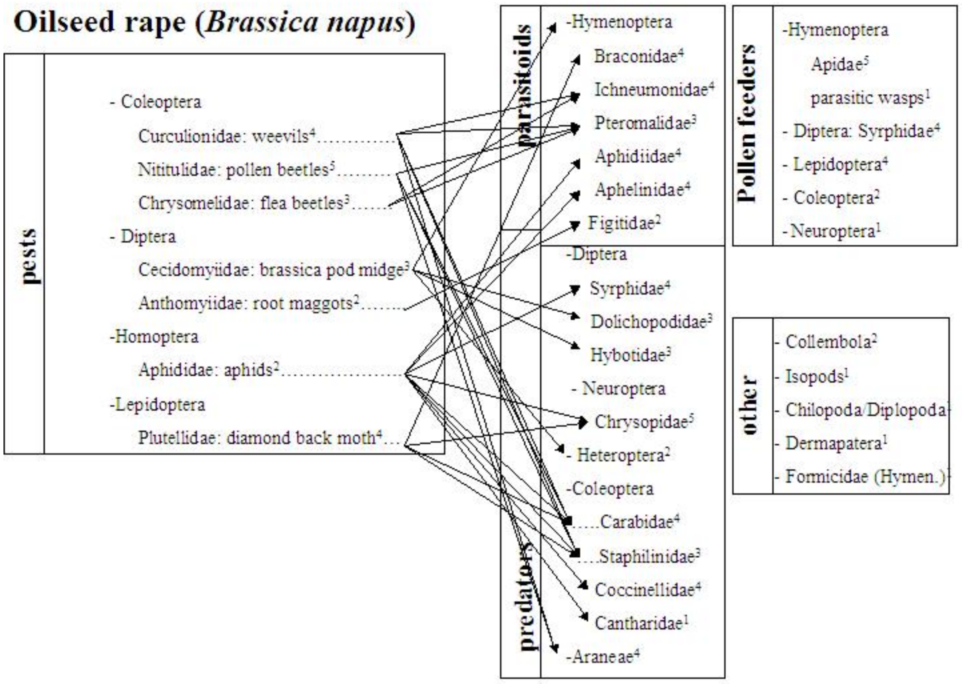

Figure 1. Simplified ecological foodweb of oilseed rape (*Brassica napus*). Each family has been assigned a number (1-5) which corresponds to its level of significance in terms of ecological, economical value, occurrence, and quantity of published data (1=low level of significance; 5= high level) (See section 4.1).

Like with the weevil larvae, also larvae of the cabbage stem flea beetle are parasitized by *Trichomalus perfectus* (Pteromalidae), and *Tersilochus tripartitus*, the latter parasitising flea beetles up to 61% in France, and 53% in Germany (Alford, 2000).

Oilseed rape: pest aphids and their natural enemies

Myzus persicae is a very important aphid pest that is able to feed on a wide range of crops. Affected cabbage or rape plants grow imperfectly and wither. Central flowers curl up and become discolored. Aphid feeding causes distorted flowers and may result in flower abortion. In addition to direct feeding damage the aphid is an important vector of plant viruses. This aphid can occur year round on a variety of herbaceous plants (including crops such as sugarbeet, other brassicas and potatoes). Also *Brevicoryne brassicae*, the mealy cabbage aphid is often found on oilseed rape plants. This latter aphid species is a specialist of Brassicaceous plants. Both species are often parasitized by aphid-specific parasitoids of the families Aphidiidae and Aphelinidae. Predators of aphids include the larvae of syrphid flies and of the green lacewing *Chrysoperla carnea* (Neuroptera: Chrysopidae).

Oilseed rape: pest Diptera and their natural enemies

The cabbage root fly, *Delia radicum* (Diptera: Anthomyiidae) is seen in nurseries and at all stages of plant development. It is an important pest in several *Brassica* crops. On the root vegetables, the larvae penetrate the fleshy parts and form galleries which render the plant unsuitable for consumption. On leaf-vegetables and rape, the root is more or less destroyed. Under good growing conditions, moderately attacked plants can send out new roots and produce a normal harvest (http://www.inra.fr/Internet/Produits/HYPPZ/RAVAGEUR/6delrad.htm). An important parasitoid of the cabbage root fly is *Tribliographa rapae* (Hymenoptera: Cynipidae) (Neveu *et al*., 2000). Brassica pod midges (*Dasineura brassicae*) lay their eggs within developing oilseed rape pods. On hatching, the small (1-2 mm) white or pale-yellow larvae feed on the inside of the pod wall, leading to distorted pods which eventually lead to pod-shatter and loss of seed. They are found especially at the edges of fields. Due to larval feeding, which causes swellings, valve deformation, precocious yellowing and bursting of pods, the seeds fall out. Damage, compared to other pest species, is considered moderate. Populations are heavily attacked by long-legged flies (Dolichopodidae) and dance flies (Hybotidae), Hemiptera (*Orius* spp.), carabid beetles, and over 20 known species of parasitoids have been recorded attacking the egg or larval stages (Prescher & Buechs, 1999; Alford, 2000).

Oilseed rape: pest Lepidoptera and their natural enemies

There are not many pest Lepidoptera on oilseed rape, if not for the diamondback moth (*Plutella xylostella*). This moth was long considered a relatively insignificant pest. Its increasing pest status has, for a large part, been due to its extensive insecticide resistance. Plant damage is caused by larval feeding. Although the larvae are very small, they can be quite numerous, resulting in complete removal of foliar tissue except for the leaf veins. This is particularly damaging to seedlings. The presence of larvae in florets can result in complete rejection of produce, even if the level of plant tissue removal is insignificant. Large larvae, prepupae, and pupae are often parasitized by the braconid parasitoid *Microplitis plutellae* (Capinera, 2000; Xu *et al*., 2001), the ichneumonid parasitoids *Diadegma spp.* (Wiech and Jankowska, 2000; Mustata, 2000), and the braconid parasitoid *Cotesia plutella* (Mustata, 2000).

Oilseed rape: flower visitors

Because the flowers produce both pollen and nectar (Pierre *et al*., 1999), oilseed rape flowers are visited by many different insect species. Honey bees (*Apis mellifera*) and bumblebees are among the most frequent visitors to collect and feed on the pollen of oilseed rape. The bumblebees that are encountered most frequently are *Bombus terrestis* and *B. lapidaries*, and, to a lesser degree, *B. pascuorum*, *B. pratorum*, and *B. hortorum* (Roy *et al*., 2003). Other pollen-and/or nectar feeders include cuckoo bees (*Psithyrus* spp.), solitary bees, adult (hover) flies, several adult beetle species, hymenopteran parasitoids, and butterflies. The latter group include the small white (*Pieris rapae*), the large white (*P. brassica*), the green-veined white (*P. napi*), the meadow brown (*Maniola jurtina*), the small tortoise shell (*Aglais urticae*), the peacock butterfly (*Inachis io*), gatekeeper (*Pyronia tithonus*), ringlet (*Aphantopus hyperantus*), and several others (Roy *et al*., 2003). Note that in oilseed rape the butterflies *Pieris rapae* and *P. brassica* are not, like their larval stages in several other crop plants, regarded as pest species.

Oilseed rape: soil-dwelling arthropods

Predatory carabid and staphilinid beetles and, to a lesser extent, wolfspiders, are continuously cruising the soil underneath the plants, in search for soil-dwelling insects (most often larvae), and insects that have fallen off the plant. This happens especially when there is considerable wind and during rain. This accounts not only for oilseed rape fields, but in agricultural fields in general, including potato and maize fields. Frequently encountered carabid species in cropping areas are *Poecilus cupreus*, *Bembidion quadrimaculatum*, *Metallina lampros*, *Pseudophonus rufipes*, *P. griseus*, *Orthomus expansus*, *Carabus granulatus interstitalis*, *Zaburs tenebrioides*, and *Anchomenus dorsalis* (Kalthof *et al*., 2001; Castenera and Orteg; Lozzia, 1999). Common staphilinid species include *Tachyporus obtusus* and *Oxytelus rugosus* (Kuhlmann and Van der Burgt, 1998; Kalthof *et al*., 2001). Other artrhopods that are present in cropping areas, some of which in large numbers, are springtails (Collembola), pillbugs (Crustacea: Isopoda), centipedes (Chilopoda) and millipedes (Diplopoda), earwigs (Dermaptera), and ants (Formicidae).

3.1.2 Potato (*Solanum tuberosum*) (Figure 2)

The three insect orders that contain species considered most harmful to potato are Coleoptera, Lepidoptera, and Hemiptera. Comparing the non-target taxa of potato with those of maize and oilseed rape, potato differs especially in its lack of flower visitors. This is due of poor nectar and pollen production of potato flowers.

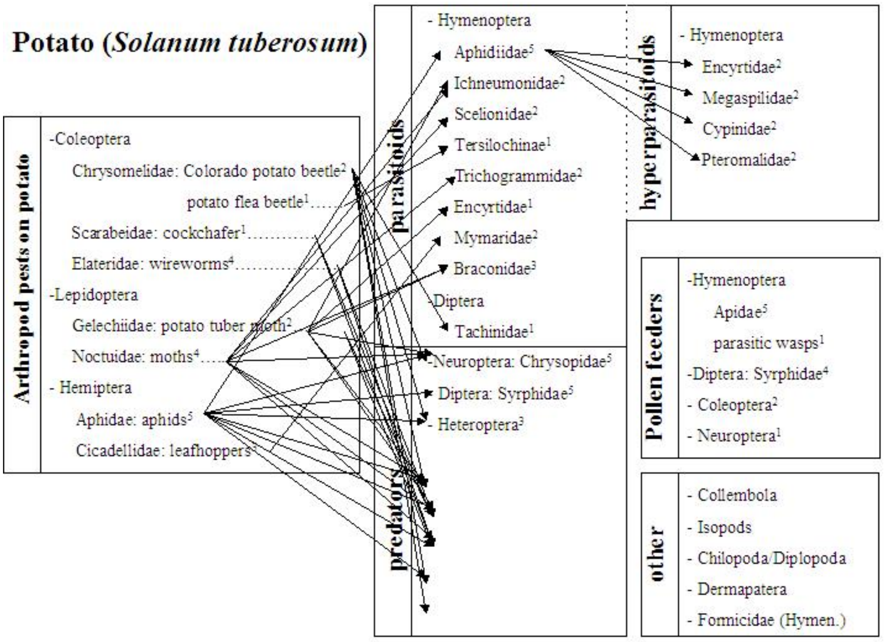

Figure 2. Simplified ecological foodweb of potato (*Solanum tuberosum*). Each family has been assigned a number (1-5) which corresponds to its level of significance in terms of ecological, economical value, occurrance, and quantity of published data (1=low level of significance; 5= high level) (See section 4.1).

Potato: pest Coleoptera and their natural enemies

Probably the most infamous insect in history regarding potatoes is the Colorado potato beetle (CPB, *Leptinotarsa decemlineata*, Coleoptera: Chrysomelidae). This species is able to defoliate whole potato plants, and has done so, to the horror of thousands of growers. Nowadays, in Western Europe, the CPB is well controlled and, although present, is not considered a pest. However, growers have a healthy ‘respect’ for this beetle, and are continuously on guard for an outbreak. In its native areas (Middle America), CPB’s eggs are parasitized by the parasitoid *Edovum puttleri* (Hymeoptera: Eulophidae) (Hu *et al*., 1999). In Europe, larvae are parasitized by several tachinid species (Diptera), and preyed upon by many different predatory arthropods: the green lacewing (Neuroptera), vespid wasps (Hymenoptera: Vespidae), several Heteropterans (stink bugs [Pentatomidae], damsel bugs [Nabidae], big-eyed bugs [Lygaeidae], and assassin bugs [Reduviidae]), ladybird beetles (Coleoptera: Coccinellidae), and several spiders (crab spiders, dwarf spiders, and an *Opiliones* sp.)(Groden *et al*., 1990 ; Drummond *et al*., 1991 ; Lacey *et al*., 2001 ; Karley *et al*., 2003 ; Chang and Snyder 2004). Occasionally, another chrysomelid beetle, the potato flea beetle *Psylliodes affinis* is encountered. This beetle eats from the leaves, leaving small holes. However, the damage is usually not large. The species is parasitized by *Tersilochus microgaster* (Hymenoptera: Tersilochinae). In the past, cockchafers (*Melolontha melolontha*) were considered important pest insects: the larvae of these beetles live belowground, feeding on the roots. Nowadays they have become rare and today cause almost no damage at all. This is most probably due to the widespread use of mechanical cultivation which kills the fragile larvae, as well as to the change in production systems. In contrast, the European wireworms *Agriotes lineatus* and *A. obscurus* (Coleoptera: Elatiridae) are increasing in importance (Parker and Howard, 2001). Wireworms are extremely polyphagous, and feed on roots of numerous plant species. Potato tubers are attacked at maturity, revealing narrow, shallow galleries which greatly reduce their economic value. Natural enemies of wireworms include soil-dwelling predators such as carabid and staphilinid beetles.

Potato: pest Lepidoptera and their natural enemies

The caterpillar of the cosmopolitan potato tuber moth (PTM), *Phthorimaea operculella,* (Lepidoptera: Gelechiidae) develops usually in potato tubers, but also occurs in the fruit, stem and even in the leaves of *Solanaceae*. In the event of a heavy infestation, 5 to 6 larvae may occur in one potato tuber; however, one caterpillar is enough to spoil and destroy a tuber. Also fungi and mites develop inside the galleries of the tuber moth, causing the decomposition of the tuber and the release of an unpleasant smell. Natural enemies of the PTM include ladybird beetles (Coleoptera: Coccinellidae), the green lacewing *Chrysoperla carnea*, predatory bugs of the genus *Orius* (Heteroptera: Anthocoridae), ants (Hymenoptera: Formicidae), and several ichneumonid and braconid parasitoids (Col *et al*., 2000; Cabellero *et al*., 1991). The caterpillars of the cutworms (Lepidoptera: Noctuidae) *Agrotis ypsilon* (darksword-grass moth) and *A. segetum* (turnip moth), *Autographa gamma* (silver-Y-moth), and *Euxoa nigricans* (garden dart moth) feed on above- as well as belowground plant parts, and can be particularly destructive to early season plantings. Cutworms are notorious for their epizootics that are difficult to predict. Natural enemies of cutworms consist of the predators mentioned above for the PTM. Important parasitoids include the ichneumonids *Meloboris collector* and *Campoletis annulata*, and the braconids *Apanteles telengui*, *A. ruficrus*, *Aleiodes gasteratus* and the trichogrammid *Meteorus rubens* for *Agrotis* spp., and *Apanteles kazak* (Braconidae), *Telenomus* sp. (Scelionidae), and *Trichogramma cordubensis* (Trichogrammatidae) for *Autographa gamma* (Cabellero *et al*., 1991,1992; Garcia *et al*. 1998).

Potato: pest Homoptera and their natural enemies

Aphids are regarded important pest insects on potato, not so much because of their direct feeding damage, but especially because of their indirect damage, by transmitting plant pathogenic viruses. In northern Europe, the most important potato viruses are Potato leafroll virus (PLRV) and strains of Potato virus Y (PVYO, PVYN) (Robert *et al*., 2000). Important aphid species include *Myzus persicae* (green peach aphid), *Aulacortum solan* (glasshouse & potato aphid), *Macrosiphum euphorbiae* (potato aphid), *Aphis gossypii*, and (occasionally) *Aphis fabae* (black bean aphid). Natural enemies of aphid in potato are many. Among the predators are *Chrysoperla carnea* (Neuroptera), hoverflies (Diptera: Syrphidae), the heteropteran damsel bugs (Nabidae), big-eyed bugs (Lygaeidae), *Orius* spp. (Anthocoridae), and assassin bugs (Reduviidae), and the coleopteran ladybird (Coccinellidae) and predatory ground beetles (Carabidae and Staphilinidae) (Coll *et al.*, 2000; Lacey *et al.*, 2001; Karley *et al.*, 2003; Meyling *et al.*, 2004). Important parasitoids belong to the family of Aphidiidae, e.g. *Aphidius matricariae* , *A. colemani*, *Diaeretiella rapae*, and *Praon gallicum* (Grasswitz and Resse, 1998; Lacey *et al.*, 2001; Freuler *et al.*, 2001). There are several hyperparasitoids of aphid parasitoids, e.g. *Syrphophagus aphidivorus* (Encyrtidae), *Dendrocerus carpenteri* (Megaspilidae), *Alloxysta victrix* (Cypinidae), *Asphahes vulgaris*, *A. suspensus*, and *A. lucens* (Pteromalidae) (Freuler *et al.*, 2001; Buitenhuis *et al.*, 2004). Regarding the selection of non-target arthropods for pre-market risk-assessment laboratory tests of GM crops, it is proposed that hyperparasitoids are only tested when it has been shown that their hosts (the parasitoid of a pest species) are affected negatively by the GM crop other than by a mere absence of hosts. Such effects on hyperparasitoids have been reported for non-GM plants (Harvey *et al.*, 2003).

The leafhopper *Empoasca solani* (Homoptera: Cicadellidae) can cause considerable damage. Eggs are parasitized by hymenopteran parasitoids of the family Mymaridae (Anonymous, 2001; Kuroli *et al.*, 2003).

Potato: flower visitors

In the context of pollen feeders, the degree to which flowering of potato occurs, the duration of flowering and the response of flowering behaviour to environmental conditions is greatly influenced by cultivar. Flower development does not ensure fruit set. Pollen sterility is frequently encountered, in which case pollinating insects may not be attracted due to poor nectar production. This includes Hymenoptera (Sanford & Hanneman, 1981), and Lepidoptera (O'Brien *et al.*, 2003).

Potato: soil dwelling arthropods

As for the predatory ground beetles (Carabidae and Staphilinidae), spiders, and other soil-dwelling arthropod communities present in cropping areas, see description above under oilseed rape.

3.1.3 Maize (*Zea mays*) (Figure 3)

Maize suffers from a larger variety of pest arthropods than potato and oilseed rape. Most damage is caused by cornborers, aphids, corn root worms and wireworms. Maize plants produce enormous amounts of pollen, resulting in many pollen-feeding insects that visit the maize fields.

Maize: pest Lepidoptera and their natural enemies

The pest in maize in north-western Europe that is considered most harmful in maize is probably the European corn borer (ECB) (*Ostrinia nubilalis*), although this species does not occur in the Netherlands. Natural enemies include *Orius* sp. (Hemiptera: Anthocoridae), green lacewings, *Chrysoperla* spp. (Neuroptera: Chrysopidae), and several ladybird beetles (Coleoptera: Coccinellidae). Insect predators often eliminate 10 to 20% of corn borer eggs (Capinera, 2000). Important hymenopteran parasitoids belong to the Trichogrammidae: *Trichogramma ostriniae*, *T. nubilale*, *T. vanescens* and *T. maidis*/*T. brassicae* (Wang *et al.*, 1999), the ichneumonids *Sinophorus* *turionus* and *Eriborus terebrans* (Cagáň and Bokor, 1998, 1999a) and the braconid *Microgaster tibialis* (Bokor and Cagáň, 1999b).

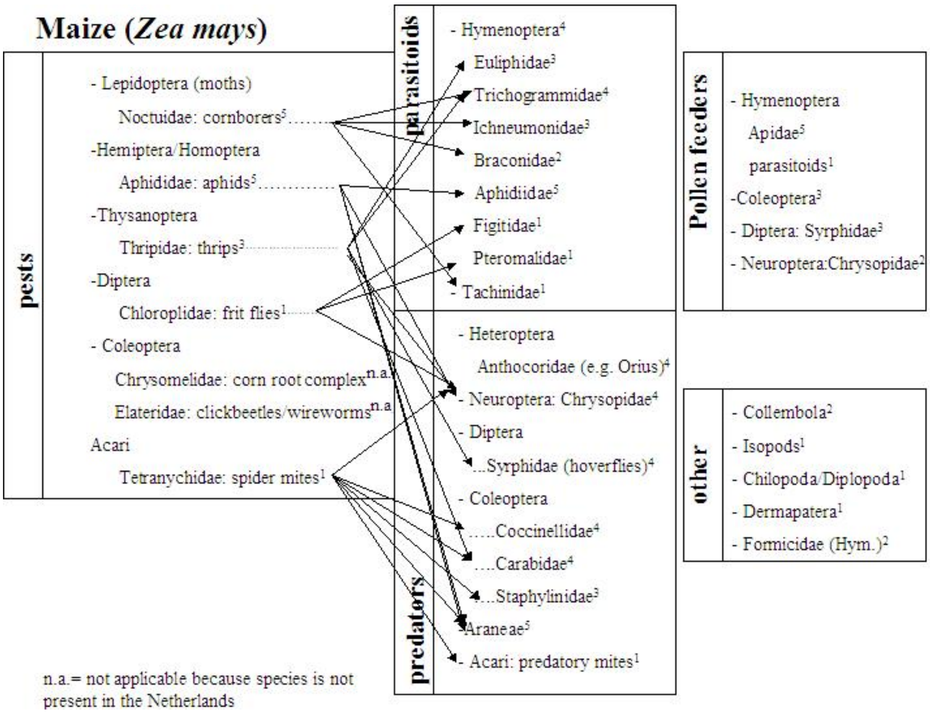

Figure 3. Simplified ecological foodweb of maize (*Zea mays*). Each family has been assigned a number (1-5) which corresponds to its level of significance in terms of ecological, economical value, occurrance, and quantity of published data (1=low level of significance; 5= high level) (See section 4.1).

Occasionally, larvae are parasatized by the Dipteran Tachinids *Lydella thompsoni*, *Pseudodoperichaeta nigrolineata*, and *Drino inconspicua* (Bourget et al., 2002; Dippel and Hilker, 1998; Manachini, 2003). Other Lepidopteran pest insects of maize that cause heavy damage in other parts of Europe are the noctuids *Heliothis armigera* and *Agrotis ipsilon*. Although these species have been observed in large, increasing numbers in the Netherlands (De Vos, 2003), no damage has (yet?) been reported.

Maize: aphid pests and their natural enemies

Just as on potato and oilseed rape, aphids are known to cause damage on maize. Occasionally, damage can be extensive, such as in 1997 in Belgium, caused for a large part by the English grain aphid *Sitobion avenae* and the rose-grain aphid *Metopolophium dirhodum* (Depoorter, 2005). Another species that is considered important is the bird cherry-oat aphid (*Rhopalosiphum padi*) (Cabanettes, 1985; Schmitz and Bartsch, 2001). Predators and parasitoids of aphids are discussed above for oilseed rape and potato. Other common Homoptera, which, despite their feeding on maize plants are generally considered non-target organisms rather than pest species, are leafhoppers (Cicadellidae). Common species are *Zyginidiae scutellaris* and *Macrosteles* sp., and, to a lesser degree, *Psammotettix alienus*, *Empoasca pteridis*, and *Laodelphas striatellus* (Waloff, 1994; Rauschen *et al*., 2004).

Maize: thrips pests and their natural enemies

Thrips (*Frankliniella occidentalis* and *Haplothrips aculeatus*) suck the contents from plant cells by rasping, scraping and puncturing the plant surface. Thrips are vectors of various plant viruses, and can cause considerable damage to maize. Thrips are preyed upon by the green lacewing *Chrysoperla carnea*, hoverflies (Diptera: Syrphidae), predatory mites (e.g. *Amblyseius* spp.), and predatory bugs *Orius* spp. (Heteroptera: Anthocoridae) (Schmitz and Bartsch, 2001; Dutton *et al*., 2003; Tomassini, 2003). Both eggs and larvae are regularly parasitized, respectively by Trichogrammidae and Euliphidae. The latter family contains a few *Ceranisus* species that are known to parasitize *F. occidentalis* (Loomans, 2003).

Maize: pest Diptera and their natural enemies

Frit flies (Diptera: Chloropidae), the most common species being *Oscinella frit* and *Geomyza tripunctata* occasionally heavily infest maize crops. Infection percentages of up to 40% of the plants have been recorded from the UK (Heard *et al*., 1981), although normally infection rates are much lower (Anonymous, 1999). Although *O. frit* is found in Germany (Schmitz and Bartsch, 2001), there are no recent damage reports from the Netherlands. Frit flies are predated by the green lacewing.

Maize: pest Coleoptera and their natural enemies

The western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae), is the most destructive pest of maize in North America, and began to successfully invade Central Europe in the early 1990's. It is the question ‘when’ rather than‘whether’ this species will invade the Netherlands. The larvae of this beetle feed on the roots of maize plants. Under heavy rootworm pressure, root systems can be completely destroyed, but damage usually is less than this. Yield losses due to rootworm feeding are difficult to determine, as many corn varieties can compensate for some root damage. In general, economic losses occur after one or more primary roots are chewed to within 2.5 cm of the plant. Grain yield losses have averaged about 10 percent over the last decade (Peairs and Pilcher, 2004). A survey for natural enemies in Europe (Toepfer and Kuhlmann, 2004) revealed that no effective indigenous natural enemies have been found on *D. v. virgifera* in Europe (Toepfer and Kuhlmann, 2004). A recent study by Rasmann *et al*. (2005), however, showed that an indigenous entomopathogenic nematode (*Heterorhabditis megidis*) of Europe attacks *D.v. virgifera*. Also the larval stages of another beetle: wireworms (*Agriotes* spp.) (Coleoptera: Elateridae) are known to feed on the roots of maize plants, causing damage in central and southern parts of Europe (Gomboc, 2003; Chaton *et al*., 2003), but not in the Netherlands. There is almost no information regarding natural enemies of wireworms other than the ‘regular’ predatory beetles present in most agricultural cropping areas.

Maize: spider mite pests and their natural enemies

The two-spotted spider mite (*Tetranychus urticae*), is a very polyphagous herbivore, attacking more than 200 different plant species, including maize. To feed, the mite attacks the leaves and imbibes cellular sap. Direct damage is due to feeding punctures: the leaves become spotty, and then dry out. If attacks are heavy, the plant may die. Spider mites have been studied with respect to *Bt* transgenic plants in order to assess the intake of *Bt* proteins by *Chrysoperla carnea* when feeding on phytophagous arthropods such as spider mites (Dutton *et al*., 2001). The results showed that, compared with the other prey herbivores *Rhopalosiphum padi* and *Spodoptera* *littoralis*, *T. urticae* contained the highest amounts of *Bt* proteins. Although relatively commonly occurring (Carbonelle *et al*., 2000), spider mites are seldom considered primary pest species in maize in north-western Europe. Predators of spider mites include predatory mites, the green lacewing *Chrysoperla carnea*, small ladybird beetles (Cocinellidae), minute pirate bugs (*Orius* spp.), big-eyed bugs (Lyaidae, *Geocoris* spp.), and predatory thrips (Thysanoptera: Aeolothripidae) (Cranshaw and Sclar, 2004). These predators are exposed to *Bt* toxins when feeding on spider mites that fed on *Bt*-maize.

Maize: flower visitors and their natural enemies

Maize pollen is produced in enormous quantities. Estimates for the numbers of pollen grains produced by an average-sized plant range from 14 to about 50 million (Emberlin *et al*., 2001). Pollen is spread from plant to plant through physical contact between neighboring plants and by wind. Insects do not play a considerable role in pollination because female flowers are little incentive for pollinating insects (Messéan, 2003). Nevertheless, the large quantities of pollen attract numerous insects, especially honeybees, bumblebees, and solitary bees (Nowarowski and Morse, 1982). Moreover, large amounts of wind-transported pollen are deposited near the field margins on other plants, where they are consumed by insects (Lang *et al*., 2004). Pollen feeders other than bees on maize plants (and surrounding field margins) include the adult stages of a large range of insects, among which flies (e.g. hover flies), beetles, and parasitoids.

Maize: soil dwelling arthropods

On the ground, predatory carabid and staphilinid beetles and, to a lesser extent, wolf- and jumping spiders, are continuously cruising in search of soil-dwelling insects (most often larvae), and larvae that have fallen off the plant. This happens especially when there is considerable wind and during rain. Ants are generalist predators that occur both on the soil and on plants. Other soil-dwelling arthropods that are present in cropping areas, often in large numbers, are springtails (Collembola), pillbugs (Crustacea: Isopoda), centipedes (Chilopoda), millipedes (Diplopoda), and earwigs (Dermaptera). With the exception of centipedes, these arthropods feed on decaying plant material, and as such may be exposed to *Bt* proteins, especially when the roots of GM plants such as maize are left in the soil after harvest.

4 SELECTING NON TARGET ORGANISMS

4.1 What criteria to use in the selection process?

According to the National Library of Medicine (NLM), a non-target organism is defined as 'an organism which may be affected by an interaction for which it was not the intended recipient'. There are some complications implying this definition. For example, with regard to agricultural pests versus non-target organisms: it does not specify a limited area, i.e. species A may be considered a non-target organism in crop B, but this same species A may be a pest species in a neighboring crop C (e.g. larval *Pieris rapae* is considered a pest species in cabbage, but not so in oilseed rape). Also, the definition does not take into account that different developmental stages may fill different ecological niches: in many cases where insects are considered pests in their larval stages, these become non-target insects in their adult stages, most often as pollinators, although it should be noted that these same adult stages are also responsible for producing (damaging) offspring.

Unlike many broad spectrum chemical insecticides, that are not specific towards certain arthropods, GM crops containing *Bt*-genes are, to a large extent, specific to insects at the Order level. Moreover, while chemical insecticides are sprayed on plants and cuticle-insecticide contact alone may be enough to kill insects, *Bt* genes in a GM plant are expressed within plants, so that only those insects that ingest the toxins and their natural enemies may be affected. In contrast to the USA, where GM-*Bt*-plants are considered as yet another insecticide with according risk-assessment studies (EPA protocols), in Europe, the above differences between transgenic plants and conventional pesticides are considered an important reason to view GM plants as new entities and the need for appropriate risk-assessment is acknowledged. This acknowledgement includes the necessity for appropriate non-target organisms in risk-assessment studies. In fact, one of the obstacles encountered by the COGEM when assessing applications for field trials of GM crops were inappropriate/irrelevant non-target species and tests being used in laboratory risk-assessments. In several cases, standard non-target species were used, while these do not occur in the particular ecosystem of that crop-plant, or the tested species are unlikely to get exposed to the *Bt*-proteins. In this context, Dutton *et al.* (2003) proposed to select non-target organisms for pre-market risk-assessment of GM crops based on the following criteria: 1) the ecological and economical significance of the non-target organism for that particular crop, and 2) the probability of exposure to the insecticidal trait. For example, it seems to make little sense to use ladybird beetles (Coleoptera) as a non-target organism for a GM-crop with an inserted *Bt* gene coding for Cry1 proteins (specific against Lepidoptera), because they are not likely to be susceptible to the toxin. Instead, it makes more sense to test species that 1) are most susceptible: in this case a non-pest Lepidopteran (e.g. pollinator), and 2) are likely to be exposed to high levels of the toxin, such as natural enemies of arthropods likely to have fed on that particular *Bt*-crop plant. In general, the species that should be selected in the first place are those that are susceptible and which may be exposed to the toxin (heavy weight of criterion 1). However, upon the situation where a susceptible species is not likely to get exposed to the toxin, it should not be selected. On the other hand, it may be desirable to select an ecologically and economically important species that is probably less susceptible, but is expected to be at high exposure 'risk'. In this case, criterion 2 prevails over 1.

When selecting non-target species for laboratory tests, different species are to be compared to select the species most relevant to the particular GM crop ecosystem.

This applies especially to the above-mentioned criterion of ‘significant’ ecological/economic role. In order to be able to compare, it is desirable that this criterion is somehow quantified. We have attempted to do so by assigning each taxon in the ecological food-web (on family level) a number between 1 (not important) and 5 (very important) (Figure 1). This number is based on a combination of factors, the most important ones being economic and ecological value, and occurrence. For example, honey bees in general are considered economically important because of their large quantities, their pollinating properties and honey production. A high ecological value is assigned when it is likely that without that particular taxon an important ecological regulator is absent, resulting in a high probability of a pest epizootic. Examples are natural enemies that keep population levels of potential pest species under the economic threshold level. We have tried to assign levels of ‘significance’ based on published data on damage levels (regarding pest species), recorded data on predation/parasitization levels (regarding natural enemies), occurrence, or pollen and/or nectar feeding properties of the taxon. These cover both economic and ecological values. Another criterion, used also by Dutton *et al*. (2003) in compiling the table on phytophagous arthropods in maize, was the quantity of data available, both published in scientific journals, as from other sources, including the internet. The rationale for the latter criterion was that when there is little written about a certain species, it is probably not as important as a species for which a lot of published data exist. These significance levels, based on a combination of several criteria, not all of which are easily quantifiable, remain subject to interpretation and, by definition, cannot be completely objective. They should therefore not be taken strictly but more as an indication. Additionally, there are some complicating factors: First, there is always temporal as well as spatial variation in the level of occurrence or activity of natural enemies and pest species (Rott and Godfray, 2000). Second, a species may be economically very important, but ecologically much less so, which makes ‘level-assignment’ difficult. Third, a generalist predator may feed heavily (and thus be ‘very important’) on pest species A, but may feed less so on species B. Regarding species B it should be assigned a low number, but a high number regarding species A. In such cases, the assigned number was weighted to the importance level given to the pest species, i.e. if pest species A was more important, the level of importance of the predator was given a high number as well. A fourth consideration relates to the level of importance of a natural enemy that is very host/prey-specific, e.g. a parasitoid. It may be that this parasitoid is important in maintaining the population level of its host under control, and may thus be considered important. If, however, its host is not considered an important pest species in that particular crop system, the parasitoid, seen in the framework of the cropping system, has a low importance, and was therefore given a low value. Therefore, it should be stressed that the assigned levels of significance are only an indication, to be used in the selection procedure of non-target organisms in laboratory experiments, for comparison between several different non-target organisms. They are by no means strict. They serve not as a novel way to quantify importance of pest species and natural enemies (and should not be viewed like that), but they are merely an aid in the selection procedure of which non-targets organisms to choose for laboratory studies.

4.2 A key, to assist in selecting non-target organisms

Here we present a tool to select non-target arthropods to be tested (Annex I). It is based on the rationale described above, and has the form of a ‘key’; It is a series of questions, the answers to which direct the applicant to a set of 4-6 non-target organisms that should be tested in laboratory tests. In the current study the key is limited to GM plants (potato, maize, and oilseed rape) with *Bt* traits in north-western Europe, but the same rationale can be applied to select non-target organisms of other plants, geographical areas, and GM traits as well.

In the case of *Bt* crops, there are many species that are considered to be non-target species, including non-pest herbivores (e.g. those herbivores that are present and feed on the crop plant, but, due to their limited presence or damage, are not considered pests). In fact, since only a small minority of the arthropods present is defined/considered as pests, the majority of organisms that is present should be regarded as non-target organisms. The bulk of the damage inflicted on the crops comes from a relatively small number of species that, however, can sometimes be present in large numbers. The activities of these pests attract natural enemies. Yet, the fact that other herbivores do not have a pest status may be caused by regulation by their natural enemies. Elimination of the latter natural enemies may result in the non-pest reaching pest status as a result of the elimination of the regulating factor.

While setting up the key, it was recognized that the inserted GM trait will have consequences for the naturally occurring enemy fauna of that crop in that specific area. For example, when growing an area with *Bt* maize plants containing genes coding for Cry1 toxins (toxic only against Lepidoptera), there will be low numbers of Lepidoptera in the crop. It is expected that few or none of the specialist parasitoids of maize-related Lepidoptera will enter the area. This lack of parasitoids in the crop is therefore not directly due to the toxic effects of the plants, but to the lack of hosts. When setting up the key, these expected effects were taken into consideration. Although recognizing the fact that most of the entomofauna (including herbivores) present in the cropping areas are non-target species, the selection of non-target organisms here, concerns species that are either key-species in controlling the pest (natural enemies), pollinators, or pollen-feeders. The latter group was incorporated since many insects come into contact with pollen by feeding on it (beetles, hoverflies, bees, etc). Cropping areas in the concerned countries in the EU are scattered, and of relatively small sizes compared to those in e.g. northern America, resulting in relatively large off-crop areas. Although we acknowledge that the probability is not high, the possibility of rare and/or endangered Lepidoptera to encounter these cropping areas is present, and, in our view, cannot be neglected. For this reason we included one protected butterfly species in the list.

4.3 Selecting non-target test species of crops with inserted *Bt* genes

The outcome of the selection process described per crop below in the sections 4.3.1-4.3.3 has been summarized in Table 1.

Table 1. Lists of sets of 4-6 non-target arthropods to be tested in first tiered laboratory tests of maize, potato and oilseed rape cultivars containing *Bacillus thuringiensis* (*Bt*) genes coding for Insecticidal Cry (1-5) endotoxins.

Cry gene	Specific against	oilseed rape (*Brassica napus*)	potato (*Solanum tuberosum*)	maize (*Zea mays*)
Cry 1	Lepidoptera	- lepidopteran pollinator - endangered Lepidoptera* - parasitoid of non-pest Lepidoptera - hymenopteran pollinator - predatory ground beetle	- endangered Lepidoptera - parasitoid of non-pest Lepidoptera - wolf spider - predatory ground beetle	- pollinating Lepidoptera - endangered Lepidoptera - parasitoid of non-pest Lepidoptera - predatory ground beetle - hymenopteran pollen feeder
Cry 2	Lepidoptera, Diptera	- lepidopteran pollinator - endangered Lepidoptera - parasitoid of non-pest Lepidoptera - predatory Diptera (larval hoverfly) - pollen feeding Diptera (adult hoverfly) - predatory ground beetle	- endangered Lepidoptera - parasitoid of endangered Lepidoptera - pollen-feeding Diptera (adult hoverfly) - predatory Diptera (larval hoverfly) - predatory ground beetle	- endangered Lepidoptera - parasitoid of non-pest Lepidoptera - predatory Diptera (larval hoverfly) - pollen feeding Diptera - predatory Neuroptera (lacewing)
Cry 3	Coleoptera	- predatory ladybird beetle - predatory ground beetle - wolf spider - hymenopteran pollen feeder	- predatory ground beetle - predatory ladybird beetle - wolf spider - hymenopteran pollen feeder	- predatory ground beetle - predatory ladybird beetle - predatory Neuroptera (lacewing) - wolf spider - hymenopteran pollen feeder
Cry 4	Diptera	- predatory Diptera (larval hoverfly) - predatory ground beetle - wolf spider - hymenopteran pollen feeder	- not applicable	- not applicable
Cry 5	Lepidoptera, Coleoptera	- lepidopteran pollinator - predatory ladybird beetle - predatory ground beetle - wolf spider - parasitoid of non-pest Lepidoptera	- endangered Lepidoptera - parasitoid of non-pest Lepidoptera - predatory ladybird beetle - predatory ground beetle - wolf spider - hymenopteran pollen feeder	- endangered Lepidoptera - parasitoid of non-pest Lepidoptera - predatory ground beetle - predatory ladybird beetle - predatory Neuroptera (lacewing) - hymenopteran pollen feeder

* Endangered Lepidoptera are non-pest Lepidoptera that are either protected and/or endangered.

4.3.1 Oilseed rape (*Brassica napus*)

The arthropod pests on oilseed rape in countries of north-western Europe (Benelux and UK) that are considered most important belong to the Coleoptera (pollen beetles, stemflea beetle, seed- and stem weevils), Homoptera (aphids), and Diptera (brassica pod midge). The selection of non-target arthropod species for laboratory tests should be based on the type of genetic modification, the extent to which the non-target organism is expected to get in contact with the trait (its ecological function), and its economic and ecological importance in the crop system.

Let us first consider the case of oilseed rape plants with a Cry 1 gene (specific against Lepidoptera). The only Lepidoptera species of this crop that is considered to be a pest is the diamondback moth. Since the expected population density of this target lepidopteran will be very low as a result of the Cry 1 gene, there will also be very few individuals of its most important specialist natural enemies, the Hymenopteran parasitoids *Cotesia plutella*, and *Diadegma spp.*. For this reason, *Cotesia plutella* and *Diadegma spp.* are not expected to be highly exposed to the Cry1 toxin and are therefore not incorporated in the list of species to be tested in laboratory studies. Since nectar does not contain *Bt* toxin (Raps *et al.*, 2001), the many adult lepidopterans encountered on this crop are not directly at risk. However, a Lepidopteran is selected because pollen, which does contain Cry toxin, is likely to be deposited close to the field margins on neighboring plants, where they may be ingested by caterpillars (including non-target Lepidoptera) while feeding (pollen may be transported either by wind, or insects that had previously visited the GM plants, most likely (bumble) bees and butterflies). These ingested toxins may also affect parasitoids developing in these non-target Lepidoptera, which is why these two groups should be tested.

The phenomenon of negative effects of *Bt* toxins on a natural enemy, as a result of indirect exposure to these toxins has been reported previously (Hilbeck *et al.*, 1998a,b, but see also Romeis *et al.*, 2004). It was shown that a Cry1A/b toxin, supposedly specific to Lepidoptera only, negatively affected a natural enemy (*Chrysoperla carnea*: order Neuroptera) that preyed extensively on a Lepidopteran pest. In order to assess whether this type of toxic effects in natural enemies, indirectly acquired from extensive contact with the host species may have negative effects, the rationale of such ecological events is used in selecting non-target species for laboratory studies. For example, the choice to include a predatory beetle for laboratory tests is that these beetles, like spiders, are generalist predators. They are present in the undergrowth or on the soil, searching for soil-dwelling insects and insects that have fallen off the plant, including e.g. aphids, caterpillars and beetle larvae. They are thus likely to feed on herbivorous insects that have ingested the Cry toxins which did, or did not, affect the herbivore itself, but, indirectly, may affect the predatory beetle (criterion 2, described under §4.1). In the current case of a Cry1 oilseed rape crop, predatory beetles are selected because in the field they may feed on toxin-affected caterpillars that fell on the ground. A hymenopteran pollinator was included because of the sheer numbers of bees and bumblebees that visit flowering oilseed rape plants, the large amounts of pollen they acquire and may distribute to other plants, and their ecological and economical importance.

In the case of an oilseed rape plant with an inserted Cry2 gene, a gene producing specific toxins that are toxic to Lepidopterans and Dipteran, a different set of species is requested for laboratory tests. Since Lepidoptera and Diptera are vulnerable to these toxins, the selection of non-target species should be based on species that belong to these taxa, and/or are highly exposed to the toxins, and are considered to be of economic and/or ecological importance in this crop. The choice to select a Lepidopteran pollinator, a protected Lepidopteran species, a predator of a non-target herbivore, and a Hymenopteran parasitoid of a non-pest Lepidopteran species is based on the rationale described above regarding Cry1. To meet the requirements to include a non-target species that either is, or has a close ecological connection with Diptera, a selective predatory syrphid fly is selected, because larvae of this species prey intensively on aphids. Aphids are not specifically considered a major pest on oilseed rape, and due to their feeding strategy (sucking phloem, which contains only traces of the *Bt* delta-endotoxins) they pertain low exposure risk to the insecticidal trait. However, since recent research showed that endotoxins, although at low concentrations, were found in aphids (Dutton *et al.*, 2004; Burgio *et al.*, *in prep*), and aphids and syrphid flies are common on oilseed rape, it is important to include the predatory larval stage of a hoverfly to the list of non-targets to be tested in laboratory studies. The reason for selecting an adult syrphid fly is because, like bees, they feed on pollen, and are thus exposed to Cry2 endotoxins.

In oilseed rape in north-western Europe, the most destructive pest species are Coleoptera species, especially cabbage seed weevils, rape stem weevils, cabbage stem weevils, pollen beetles, and cabbage stem flea beetles. It is, therefore, not unlikely that GM oilseed rape with Cry3 genes will be developed, which will produce toxins specifically directed against Coleoptera. The species to be selected for laboratory tests should therefore either be, or have close ecological links to Coleoptera. Ladybird beetles are included in the list for the following reason. As described above, aphids, although not primary pests of canola, do occur on this plant. Since aphids may ingest Cry3 endotoxins while feeding, it is possible that non-target coccinellid beetles (both larvae and adults feeding extensively on aphids) are affected. Ground beetles are included in the list because they prey on a large range of phytophagous insects that are found on the soil, including those that have fallen off from crop plants such as e.g. aphids and diamondback moth larvae, that are likely to contain Cry endotoxins. Although there are no data to support the suspicion that Aranea are directly affected by Cry3 endotoxins, they are very common in oilseed rape crops, feeding extensively on both pest and non-pest insects. We included a wolf spider in the list based on the rationale that they may feed on (possibly moribund) beetle larvae, and as such may ingest substantial quantities of the toxins. The choice to select one Hymenopteran pollinator is described above under ‘Cry 1’.

In the case of Cry4 genes, endotoxins are produced that are specifically toxic to Diptera. The non-target species most at risk either are, or have close ecological links to Dipterans. As a first test species, hover flies (Syrphidae) should be tested in laboratory studies, because the larval stage of this Dipteran species preys extensively on aphids. The choice to include both a ground beetle and a wolf spider, are based on the same reasoning as explained above for Cry3. Although spiders and beetles are not expected to be susceptible to Cry4 toxins, they are ecologically linked to susceptible species, and are thus likely to get indirectly exposed to, and possibly affected by the toxins. The choice to select one Hymenopteran pollinator is described above under ‘Cry1’.

As for the examples described above, the selection of the 5 non-target species in case of oilseed rape containing genes coding for Cry5 proteins (specific against Lepidoptera and Coleoptera) are based on the rationale that the non-target species should either be, or have close ecological relations to Lepidoptera and/or Coleoptera. These include a Lepidopteran pollinator, and two entomophagous Coleoptera (ground beetle and a coccinellid beetle), all three of which are common in an oilseed rape cropping system. Because it is expected that parasitoids of pest Lepidoptera and Coleoptera will not be present in the cropping area of Cry5 oilseed rape due to a lack of herbivores, these parasitoids are not included in the list. However, Cry5 proteins in the crop could affect the third trophic level through other, non-target, herbivores, such as non-target Lepidopterans that ingest toxin-containing pollen, deposited on off-crop plants due to wind/insect transportation such as described above under Cry1. To assess this risk, a parasitoid of a non-pest Lepidoptera (pollinator) is included in the list.

4.3.2 Potato (*Solanum tuberosum*)

The pest species most encountered in the literature and which has been extremely damaging on potato plants in north-western Europe in the past is the Colorado potato beetle (CPB). Nowadays the CPB is not anymore considered the most damaging pest insect for this crop in north-western Europe, although its potential danger is widely acknowledged and outbreaks remain possible. The taxa considered most damaging in potato include aphids, flea beetles, and the larval stages of several moth species. As described above for oilseed rape, the selection of non-target arthropod species for laboratory tests should be based on the type of genetic modification, the extent to which the non-target organism is expected to get in contact with the trait (its ecological function), and its economic and ecological importance in the crop system.

Let us consider the selection procedure applied for Cry1 genes in potato plants. Although potato may be reproduced by seed, the commercial crop is propagated from tuber cuttings (Rowe, 1993). Flower development does not ensure fruit set. In general, pollen sterility and poor nectar production is frequently encountered, in which case nectar-feeding, pollinating insects (e.g. Lepidoptera) may not be attracted. Recognizing the low probability pollinating Lepidoptera will visit the crop area (O'Brien *et al.*, 2003), this group is not selected from the non-target list for laboratory studies. However, like it was described for Cry1 oilseed rape above, caterpillars of protected/ecologically vulnerable Lepidopterans may feed on pollen deposited on off-crop plants, which is the reason that this group is represented in the selected list, as well as its parasitoid. A carabid (predatory) beetle and a wolf spider are selected because they are likely to feed on moribund or dead lepidopteran larvae and non-target herbivores that have fallen to the ground after having fed from the plant and thus may ingest large quantities of the toxins.

Potato plants with Cry2 genes (specific against Lepidoptera and Diptera) inserted: A protected Lepidopteran herbivore and its Hymenopteran parasitoid are selected for the same reasons as described above under 'Cry1 for potato'. Regardless of whether the plant produces sterile pollen or not, they dó produce small quantities of pollen, which may affect pollen-feeding insects, including syrphid flies, and Coleoptera. For this reason, adult syrphid flies should be tested in laboratory studies. From the same species, the larval stage should be tested for the reason described above under 'Cry2 for oilseed rape'. A carabid (predatory) beetle is selected for the reason described above under 'Cry1 for potato'.

Cry3 genes (specific against Coleoptera) inserted: Aphids are considered important pest species on potato. Like mentioned before, aphids feed on phloem and are therefore not expected to ingest the endotoxins. However, the fact that small quantities of *Bt*-endotoxins have been isolated from aphids that had fed on Cry1 oilseed rape (Burgio *et al.*, *in prep.*, but see also Raps *et al.*, 2001) contradicts this assumption, and keeps open the possibility that ladybird beetles, that feed exclusively on aphids, are affected. A wolf spider, being a non-coleopteran, is not expected to be negatively affected by ingested toxins. It is, however, selected from the list of non-target organisms because it preys heavily on a large range of herbivorous insect species, especially 'soft-cuticled' insects, such as beetle larvae. It is therefore not unlikely for them to ingest considerable amounts of endotoxins, possibly affecting this ecologically important arthropod. A Hymenopteran pollen feeder is included for the reasons described above under 'Cry 1 for Lepidoptera'.

Cry 4 genes (specific against Diptera) inserted: there are no herbivorous Dipteran species on potato in north-western Europe that are considered pests, so it is unlikely that a company will develop a potato plant variety with an inserted Cry 4 gene.

Cry5 genes (specific against Coleoptera and Lepidoptera): the rationale for selecting one protected Lepidoptera and its Hymenopteran parasitoid is the same as described under Cry1 for potato. The reasons for selecting a ground beetle, a ladybird beetle, and a wolf spider are the same as those explained under Cry3 for potato. A Hymenopteran pollen feeder is included for the reasons described above under Cry 1 for Lepidoptera.

4.3.3 Maize (*Zea mays*)

The pest arthropods in maize in north-western Europe considered most harmful are the European corn borer (*Ostrinia nubilalis*), thrips (*Frankinella* spp), leafhoppers, aphids, the two-spotted spider mite (*Tetranychus urticae*), and the western corn root worm (*Diabrotica virgifera*).

Cry 1 genes (specific for Lepidoptera). Maize produces large amounts of pollen, but no nectar and is therefore visited more by pollen-feeders such as beetles and adult syrphid flies rather than butterflies. However, a study by Schmitz *et al.* (2003) in western Germany, showed that about 7% of German Macrolepidoptera species occur in farmland areas and were selected as being potentially affected by *Bt* maize pollen exposure. They list over 90 species that were found in or near maize fields, including species such as the green-veined white (*Pieris napi*), small toroiseshell ('kleine vos') (*Aglais urticae*), red admiral ('atalanta') (*Vanessa atalanta*), Osiris blue (*Cupido osiris*), and the protected Jersey Tiger (*Euplagia quadripunctaria*). The green-veined white (*Pieris napi*), a protected Lepidopteran species (*Euplagia quadripunctaria*) and their Hymenopteran parasitoids are therefore selected as non-target species to be tested. A Hymenopteran pollen feeder (bee or bumblebee) is selected because the large amounts of pollen produced by maize attracts many bees and bumblebees. Although Cry1 genes are said to be specific for Lepidoptera, a carabid beetle is selected because this is likely to feed on toxin-containing phytophagous insect larvae that fell to the ground, which, similar to the effects reported for (Neuropteran) lacewings (Hilbeck *et al.*, 1998a,b, 1999), may negatively affect ground beetles. A honey bee is selected because they are present in large numbers in maize fields, feed on pollen, and have a high economic value.

Cry2 genes (specific for Lepidoptera and Diptera). The reason why a Lepidoptera and its parasitoid are selected is described above under Cry1 for maize. Hoverflies are selected because in the adult stage they feed on maize pollen, and during the larval stage it is a non-target insect because it feeds on softbodied insects, e.g. the maize pest insects aphids and leafhoppers. A lacewing (*Chrysoperla carnea*) is selected as a non-target species because it is not unlikely that by eliminating the primary pest (European corn borer), secondary pests may become more important, such as e.g. aphids or spider mites, both of which are preyed upon by this lacewing.

Cry3 genes (specific for Coleoptera). A wolf spider and a predatory ground beetle are selected because these are likely to be exposed to the toxin by feeding on phytophagous insects, including e.g. larvae of the western corn root worm *Diabrotica virgifera* (Kuhlmann & Van der Burgt, 1998) and other maize-feeding beetles, caterpillars, and leafhoppers. Since a Cry3 gene is involved, it is more likely that the predatory ground beetle rather than the spider will be affected. A predatory ladybird beetle is selected because this non-target Coleoptera feeds exclusively on aphids, and may be negatively affected by traces of the toxin(s) present in aphids. A lacewing is selected because, among others, it feeds on spider mites, that are frequently found on maize and contain relatively large concentrations of *Bt* toxins when living on *Bt*-transgenic maize plants (Dutton *et al*., 2004). A Hymenopteran pollen feeder (bee or bumblebee) is selected because the large amounts of pollen produced by maize (Emberlin *et al*., 2001) attracts many bees and bumblebees (Nowarowski and Morse, 1982).

Cry4 genes (specific for Diptera). Apart from the frit fly, which is far less important as a pest species than e.g. the European corn borer, thrips or aphids, there are no significant Dipteran pest species on maize in north-western Europe. It is therefore unlikely that a maize cultivar will be genetically modified by inserting Cry4 genes.

Cry5 genes (specific for Lepidoptera and Coleoptera). The rationale for selecting a Lepidopteran that is found in/near maize fields (*Pieris napi*), a parasitoid of *P.napi* has been described above under ‘Cry1 for maize’. The rationale for selecting a predatory ground beetle, a lady beetle, and a lacewing are described above under ‘Cry3 for maize’. The rationale for selecting a Hymenopteran pollen feeder is described above under ‘Cry1 for maize’.

5 NON TARGET SPECIES LIST

Applying this reasoning behind the selection procedure of non-target organisms could result in an academic, rather unpractical situation for applicants with an unlimited array of non-target species to test, some of which previously unknown to laboratory tests, and possibly difficult to keep or rear in an insectary. To avoid this, and instead in order to try to benefit from the decades of experience in pesticide risk-assessment studies, it was proposed by Joerg Romeis (personal communication) to set up a list of a limited number of non-target organisms from which 4-6 species should be selected (using the selection criteria described above) per GM plant (table 2). Preferably, these should be organisms that have been used in risk-assessment studies before, or at least species that are commercially available or easy to keep and rear. In the current study this idea was further developed for the three crop species. Criteria that were used to select the species for this list were their abundance and their significant ecological and/or economic role in at least one of the three crops in north-western Europe, as described in section 4 (Selecting non-target species). To keep the number of species in this list acceptably low for pragmatic reasons, we have preferentially selected generalist predators and parasitoids rather than selective natural enemies: preferably species that occur in all three ecological food webs described above. Here, a short explanation is given for each non-target arthropod that has been included in the list (table 1). Information on species refers to the geographical area of north-western Europe.

Table 2. List of arthropod species. Applicants should select 4-6 different species from this list for laboratory tests. A key system may be used to facilitate this selection.

ecological group	Taxon	family	species	common name
pollen/nectar feeders				
	Lepidoptera	Pieridae	*Pieris napi*	green-veined white
	Lepidoptera	Satyridae	*Maniola jurtina*	meadow brown
	Hymenoptera	Apidae	*Apis mellifera*	honey bee
	Hymenoptera	Apidae	*Bombus terrestris*	bumble bee
predators				
	Coleoptera	Coccinellidae	*Adalia bipunctata*	two-spotted ladybird beetle
	Coleoptera	Carabidae	*Pseudophonus rufipes*	ground beetle
	Diptera	Syrphidae	*Syrphus corollae*	hover fly
	Neuroptera	Chrysopidae	*Chrysoperla carnea*	green lacewing
	Aranea	Lycosidae	*Pardosa agrestis*	wolf spider
	Aranea	Lyniphiidae	*Oedothorax apicatus*	‘hangmat spin’
	Acari	Phytoseiidae	*Phytoseiulus persimilis*	predatory mite
parasitoids				
	Hymenoptera	*Braconidae*	*Cotesia glomerata*	larval parasitoid of non-target Lepidoptera
	Hymenoptera	Trichogrammidae	*Trichogramma brassicae*	egg-parasitoid of non-target Lepidoptera
	Hymenoptera	Braconidae	*Aphidius ervi*	parasitoid of aphids
	Hymenoptera	Ichneumonidae	*Tersilochus tripartitus*	parasitoid of Coleoptera
soil organisms				
	Collembola	Isotomidae	*Folsomia candida*	springtail
	Isopoda	Oniscidae	*Pocellio scaber*	‘pill bug’ woodlouse
	Millipede	Diplopoda	*Julus scandinavius*	millipede
Protected species				
	Lepidoptera	Arctiidae	*Euplagia quadripunctaria*	Jersey tiger

5.1 Pollinators

* *Pieris napi* (green-veined white). *P. napi* is one of the most abundant species, also in/close to extensive agricultural areas (Saarinen, 2002). The species is easy to rear, and is not a pest species. Larvae are present from May until September (Groenendijk *et al*, 2002).

* *Maniola jurtina* (meadow brown/bruin zandoogje). This butterfly is one of the most abundant non-pest macro-lepidopteran species in north-western Europe and is found in all kinds of ecological settings. The species is easy to rear (http://www.helsinki.fi/science/fragland/tmr_idx.html). Larvae are present from August until May (Groenendijk *et al*., 2002).

* *Apis mellifera* (honeybee). Generally considered one of the most important pollinators, with high ecological and economical value (as a pollinator and as producer of honey). Standard species used as non-target insect for pesticide-tests, and are also already being used for transgenic non-target tests (Pierre and Pham-Delegue (2000).

* *Bombus terrestis* (bumblebee, aardhommel). Like the honeybee, this species is generally considered as one the most important pollinators and as such has a high ecological and economical value. Like honeybees, bumblebees are standard non-target species for pesticide tests, and are already being used for transgenic non-target tests as well (Arruego *et al*., 2000). They are commercially available in the Netherlands from e.g. Koppert, and PPO (WUR), and in Belgium from Biobest.

5.2 Predators

* *Adalia bipunctata* (two-spotted ladybird beetle). Feeding exclusively and extensively on aphids, both larval and adult stages of this beetle are generally seen as important regulators of aphid populations. They are abundant in natural ecosystems, are relatively easy to rear and commercially available in the Netherlands from Entocare and Koppert, and in Belgium from Biobest.

* *Pseudophonus rufipes* (ground beetle). Unlike ladybird beetles, that forage on plants, ground beetles roam the ground under plants, searching for and feeding on insects that either have fallen off from the plants (e.g. during rain), or are soil-dwelling insects themselves. Among other species, *P. rufipes* is reported as one the most abundant ground beetles in agricultural areas (Langmaack *et al*., 2001). Although commercially not available, they can be reared (Goulet, 1975).

* *Syrphus corollae* [*Eupeodes corollae*] (terrasjes komma zweefvlieg). This hoverfly species is very common in a variety of ecological areas, from April until November. While larvae feed extensively on aphids, the adults feed on pollen. The species is easy to rear (Scott and Barlow, 1984). The species has been proposed previously to be used as a beneficial insect for non-target pesticide tests. (*Episyrphus balteatus*, another syrphid species, is easy to rear (Hart and Bale, 1997), and is commercially available in the Netherlands from Koppert).

* *Chrysoperla carnea* (lacewing). The larvae of this common neuropteran insect prefer to prey on aphids, but will also feed on leaf-hoppers, mites, and other soft-

cuticled insects on plants. The adult stage feeds on pollen, nectar, and honeydew. They are considered to have a high ecological value, and are standard insects tested in non-target toxicology tests for pesticides. They are easy to rear, and are commercially available by several companies (e.g. Koppert, Entocare, and Biobest).

* *Pardosa agrestis* (wolf spider). Wolf spiders prey actively on a large range of insects, both on plants, and on the ground. The large numbers of *P. agrestis* in agricultural areas is an indication that this species may have a high ecological value. Although cannibalism occurs regularly, mass rearing is possible (Dinter, 2004; Tóth & Koshbayar, 2000).

* *Oedothorax apicatus* (hangmat spin). With over 60 species, Lyniphiidae (hangmat spinnen) constitute the most abundant spider family in north-western Europe. They do not actively hunt like wolf spiders do, but depend on their web for obtaining prey. For mass rearing, see Dinter (2004).

* *Phytoseiulus persimilis* (predatory mite). This common acarid feeds extensively on spider mites, and is easy to rear (also commercially available in the Netherlands from Entocare and Koppert.

5.3 Parasitoids

* *Cotesia glomerata* (parasitic wasp: Braconidae). This species is a common caterpillar parasitoid of Lepidopterans, including non-target Lepidopterans such as e.g. *Pieris napi*. The species can be reared (Steinberg *et al*., 1992).

* *Trichogramma brassicae* (parasitic wasp: Trichogrammidae). This egg-parasitoid lays its eggs in the eggs of a large range of Lepidoptera, including many (non-target) pollinators and endangered/protected butterfly species (Babendreier *et al*. (2003). The species is easy to rear on European corn borer eggs and commercially available from Koppert (NL), Laboratory of Entomology (Wageningen University, NL) and Biocare (D). Also Entocare (NL) sells *Trichogramma* spp., although it is not specified which species.

* *Aphidius ervi* (parasitic wasp: Braconidae). This species is an important regulator of aphid populations. It is easy to rear and commercially available (from e.g. Koppert, Biobest, Entocare, and Syngenta) for biological control of aphid pests.

* *Tersilochus tripartitus* (parasitic wasp: Ichneumonidae). The wasps of this genus are important parasitoids of Coleoptera, including stembeetles, weevils (Curculionidae), pollen beetles (Nitulidae), and flea beetles (Chrysomelidae), arriving to natural parasitization levels of up to 95% (Alford, 2000). Rearing is possible (Klingenberg and Ulber, 1994), but they are not commercially available.

5.4 Soil organisms

* *Folsomia candida* (common springtail). This springtail is regarded as the 'standard test springtail', i.e. it is the species that is often used as non-target insect for toxicology tests of pesticides and metals, and to assess soil quality (e.g Fountain and Hopkin, 2004). The species is easy to rear (ISO, 1999).

* *Pocellio scaber* (pill bug). This very common (Berg and Hemerik, 2004) soil dwelling crustacean is well studied in several different research laboratories, is easy to rear (Abzhanov and Kaufman, 1999), and is often being used in pesticide non-target toxicity tests.

* *Julus scandinavius* (millipede: Diplopoda). This species is among the most abundant millipedes in the Netherlands, and is one of the standard non-target species in heavy metal toxicity tests (e.g. Koehler and Triebskorn, 1998).

5.5 Protected/endangered butterfly

* *Euplagia quadripunctaria* (Jersey Tiger). This Arctiid butterfly occurs in central Europe, with northern borders in the southern parts of the Netherlands, and has the status ‘endangered’ in the EU (Vlinderstichting, 2005). The larvae feed on a variety of common plants, and even on shrubs, while the adults visit flower-rich areas, including field margins of agricultural fields, in search of nectar. Although there are no official reports on rearing of this species, it is feasible, according to specialists (Dr. Groenendijk, Dutch Butterfly Association, *personal communication*).

6 WHAT'S NEXT?

The current study presents an ecologically based selection procedure of non-target organisms for first tier laboratory tests, the first step in pre-market risk-assessment. The next step would be to define precisely whích laboratory tests should be carried out, how these should be carried out, and by whom.

It is recommended to produce a clear and transparant format, to benefit both the applicants and the legislators. Laboratory tests of *Bt*-crops should include 1) toxicity/sensitivity tests, 2) behavioural studies and 3) physiological studies. The results of these tests should be interpreted on a case-by-case scenario and based on their relevance in the entire cropping (eco) system. Toxicity and sensitivity tests should determine to what extent the non-target species are affected by the toxins. Unlike with pesticides, many of which are insecticidal upon contact with the cuticle without the need of ingestion, *Bt*-toxins are effective only upon ingestion of the non-target arthropod, either by feeding on plant parts (regarding herbivores and detritivores) or by feeding on herbivores that had fed on *Bt*-plant tissues (predators and parasitoids). Therefore, standard pesticide tests cannot be applied for risk-assessment of GM plants. Instead, appropriate plant tissues (leaves for leafeating herbivores; [decaying] root and leaf material for detritivores, pollen for pollenfeeders and caterpillars [*Bt* pollen deposited on off-crop plants]) should be used. Toxicity/sensitivity tests should comprise a dose-response test and exposure to the relevant *Bt*-tissue (either plant material or *Bt*-toxin containing herbivores). E.g. a ladybird beetle should feed on aphids that themselves had fed on the *Bt*-plant in question. Important parameters of the non-target organisms that should be tested in the toxicity/sensitivity tests should be a) developmental time, b) adult longevity, c) body weight and/or d) fecundity. What the most relevant parameters are is dependent on the species. Selection of the most relevant parameters can be done based on the literature on the biology of the selected or closely related species. Often, these parameters do not cover all characteristics that may affect the non-target arthropods' ecological effectiveness, and therefore behavioral studies and/or physiological studies should give additional information. For example, parasitoids may be affected in their host-seeking behaviour, the very characteristic that is often important in regulation of herbivore populations.

To keep the array of potential laboratory tests practicably acceptable, these should be standardized per non-target species on a case by case basis. As such, the list of non-target organisms, presented in this study, should be accompanied with a set of protocols per species of laboratory experiments that are based on ecologically sound reasoning. These standardized tests should be carried out by one or a limited number of renown laboratories/institutes that have extensive experience in experimental studies on insect-plant interactions.

7 CONCLUSION

Unlike the testing of a pre-fixed set of non-target arthropods as in pesticide risk-assessment, the proposed process of selecting non-target organisms for *Bt* crops is based on ecological reasoning. While the choice of this principle could lead to the selection of non-target organisms from a theoretically unlimited number of species, we have selected a restricted set of species for pragmatic reasons. Our aim was to select a set of species that represents different insect orders and ecological groups and yet is amenable to laboratory tests. This resulted in a list of non-target organisms that occur in north-western Europe, that are common, have considerable ecological and economic value, and are easy to rear (or commercially available) (See section 5). The current study was a first step, and was limited to *Bt*-transgenic potato, maize and oilseed rape, but can relatively easily be expanded to other crops. The application of the method developed here can also be extended to transgenic traits other than *Bt* toxin production, i.e. based on ecological reasoning concerning the transgenic trait and the most important groups of organisms that may be affected by the transgenic trait. For each transgenic trait the first consideration should be whether the trait is expected to have effects on non-target organisms, similar to the procedure we have developed to select the non-target organisms to be included in a risk assessment evaluation. We limited this study to north-western Europe, based on the ecological food webs of that region. However, the next step would be to apply the same approach for other regions of the EU. Lists of non-target organisms relevant for other regions can be compiled and for each crop an EU-wide set of 4-6 species can be selected, based on the same principles and criteria used in the current study for the Benelux and neighboring countries. If no negative effects on this set of species are found, second or third level tiered tests can be carried out, according to the methodology proposed by Dutton *et al*. (2003).

8 RECOMMENDATIONS

Ongoing and intensifying international developments in genetic modification of crops and the use thereof will confront the EU with increasing pressure for evaluating applications for deliberate releases of GM crops. It has been agreed in the EU that its policy regarding GM crops should be based on scientific data and reasoning (EC 2001, 2004, 2005). This requires appropriate risk-assessment studies. The current study, by proposing an ecologically based procedure to select non-target organisms to be evaluated in laboratory tests, provides a first step in this direction. A logical next step is to address the following objectives:
1) whát laboratory pre-market risk-assessment studies should be carried out,
2) hów should these should be carried out, and
3) by whom should the studies be carried out.
Like the selection procedure of the current study, it is recommended that these questions are approached from an ecological viewpoint, and that the tests are ecologically relevant. Although transgenic techniques are relatively new, risk-assessment data being reviewed and assessed by committees such as the COGEM, should be viewed in the framework of long-term ecological effects.

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ANNEX I

Key to select non-target arthropods on Bt-transgenic oilseed rape, potato and maize in Benelux countries.

	Go to
1. What is the GM crop?	
a) *Brassica napus* (oilseed rape)……	2
b) *Solanum tuberosum* (potato)……..	3
c) *Zea mays* (maize)…….	4
d) Other…….	5
2. Which trait has been inserted in the crop plant?	
a) a trait to reduce insect damage (e.g. insecticidal or anti-digestive)	6
b) herbicide tolerance	
c) disease resistance	
d) male sterility	
e) altered oil characteristics	
f) nitrogen fixation	
g) other	
3.Which trait has been inserted in the crop plant?	
a) a trait to reduce insect damage (e.g. insecticidal or anti-digestive)	13
b) herbicide tolerance	
c) disease resistance	
d) male sterility	
e) prolonging storage life	
f) increased starch production	
g) nitrogen fixation	
h) other	5
4. Which trait has been inserted in the crop plant?	
a) a trait to reduce insect damage (e.g. insecticidal or anti-digestive)	19
b) herbicide tolerance	
c) disease resistance	
d) male sterility	
e) prolonging storage life	
f) increased corn production	
g) reduced pollen production	
h) nitrogen fixation	
i) other	5
5. This key is not suitable to determine the test species. Please communicate with the relevant legislation authority of the country of interest for advice.	
6. What kind of gene has been inserted to reduce insect damage?	
a) *Bacillus thuringiensis* gene(s)	7
b) plant genes, coding for anti-digestive proteins that are part of plant natural defense systems (including digestive enzyme inhibitors, enzymes, and lectins)	

c) gene coding for the production of cholesterol-oxidase
d) other 5

7. The inserted *B.t.* gene codes for

a)	Cry 1 proteins (specific against Lepidoptera)	8
b)	Cry 2 proteins (specific against Lepidoptera and Diptera)	9
c)	Cry 3 proteins (specific against Coleoptera)	10
d)	Cry 4 proteins (specific against Diptera)	11
e)	Cry 5 proteins (specific against Lepidoptera and Coleoptera)	12

8. (Cry 1) The non-target organisms that need to be tested in the laboratory should include

- 1 lepidopteran pollinator
- 1 protected/endangered Lepidoptera
- 1 hymenopteran parasitoid of a non-pest Lepidoptera (pollinator)
- 1 hymenopteran pollinator
- 1 predator of a non-target herbivore (carabid beetle)

9. (Cry 2) The non-target organisms that need to be tested in the laboratory should include

- 1 lepidopteran pollinator
- 1 protected/endangered Lepidoptera
- 1 hymenopteran parasitoid of a non-pest Lepidoptera
- 1 entomophagous Diptera (larval hover fly)
- 1 dipteran pollinator (adult hover fly)
- 1 predator of a non-target herbivore (carabid beetle)

10. (Cry 3) The non-target organisms that need to be tested in the laboratory should include

- 2 entomophagous Coleoptera (a ground beetle, lady beetle)
- 1 Aranea (wolf spider)
- 1 hymenopteran pollinator

11. (Cry 4) The non-target organisms that need to be tested in the laboratory should include

- 1 entomophagous Diptera (hover fly)
- 1 entomophagous Coleoptera (ground beetle)
- 1 Aranea (wolf spider)
- 1 hymenopteran pollinator

12. (Cry 5) The non-target functional groups that need to be tested in the laboratory should include

- 1 Lepidopteran pollinator
- 2 entomophagous Coleoptera (a ground beetle and a coccinellid beetle)
- 1 Aranea
- 1 Hymenopteran parasitoid of lepidopteran pollinator

13. What kind of gene has been inserted to reduce insect damage?

a) *Bacillus thuringiensis* gene(s) 14
b) plant genes, coding for anti-digestive proteins that are part of plant natural defense systems (including digestive enzyme inhibitors, enzymes, and lectins)
c) gene coding for the production of cholesterol-oxidase
d) other 5

14. The inserted *B.t.* gene codes for
a) Cry 1 proteins (specific against Lepidoptera) 15
b) Cry 2 proteins (specific against Lepidoptera and Diptera) 16
c) Cry 3 proteins (specific against Coleoptera) 17
d) Cry 5 proteins (specific against Lepidoptera and Coleoptera) 18

15. (Cry 1) The non-target functional groups that need to be tested in the laboratory should include

- 1 protected/endangered Lepidoptera
- 1 Hymenopteran parasitoid of a non-pest (protected) Lepidoptera
- 1 predator of a non-target herbivore (aranea)
- 1 predator of a non-target herbivore (carabid beetle)

16. (Cry 2)The non-target functional groups that need to be tested in the laboratory should include

- 1 protected/endangered Lepidoptera
- 1 parasitoid of a protected/endangered/vulnerable Lepidoptera
- 1 pollen feeding Diptera (adult hover fly)
- 1 entomophagous Diptera (larval hover fly)
- 1 predator of a non-target herbivore (carabid beetle)

17. (Cry 3) The non-target functional groups that need to be tested in the laboratory should include

- 2 entomophagous Coleoptera (a ground beetle and a lady beetle)
- 1 Aranea (wolf spider)
- 1 hymenopteran pollen feeder

18. (Cry 5) The non-target functional groups that need to be tested in the laboratory should include

- 1 protected/endangered Lepidoptera
- 1 hymenopteran parasitoid of non-target (protected) Lepidoptera
- 2 entomophagous Coleoptera (a ground beetle and a lady beetle)
- 1 Aranea (wolf spider)
- 1 hymenopteran pollen feeder

19. What kind of gene has been inserted to reduce insect damage?
a) *Bacillus thuringiensis* gene(s) 14
b) plant genes, coding for anti-digestive proteins that are part of plant natural defense systems (including digestive enzyme inhibitors, enzymes, and lectins)
c) gene coding for the production of cholesterol-oxidase
d) other 5

20. The inserted *B.t.* gene codes for
a) Cry 1 proteins (specific against Lepidoptera) 21
b) Cry 2 proteins (specific against Lepidoptera and Diptera) 22
c) Cry 3 proteins (specific against Coleoptera) 23
d) Cry 5 proteins (specific against Lepidoptera and Coleoptera) 24

21. (Cry 1) The non-target functional groups that need to be tested in the laboratory should include

- 1 pollinating Lepidoptera
- 1 protected/endangered Lepidoptera
- 1 hymenopteran parasitoid of non-pest Lepidoptera
- 1 predatory ground beetle
- 1 hymenopteran pollen feeder

22. (Cry 2) The non-target functional groups that need to be tested in the laboratory should include

- 1 non-target, protected/endangered Lepidoptera
- 1 hymenopteran parasitoid of non-target Lepidoptera
- 1 predatory Diptera (larval stage hoverfly)
- 1 pollinating Diptera (adult stage hoverfly)
- 1 predatory Neuroptera

23. (Cry 3) The non-target functional groups that need to be tested in the laboratory should include

- 1 predatory ground beetle
- 1 predatory ladybird beetle
- 1 predatory Neuroptera (green lacewing)
- 1 wolf spider (Aranea)
- 1 hymenopteran pollen feeder (honey bee)

24. (Cry 5) The non-target functional groups that need to be tested in the laboratory should include

- 1 protected/endangered Lepidoptera
- 1 hymenopteran parasitoid of non-target Lepidoptera
- 1 predatory ground beetle
- 1 predatory ladybird beetle
- 1 Neuropteran predator
- 1 Hymenopteran pollen feeder

ANNEX II

Onderzoeksopdracht COGEM

Introductie in het milieu: Landbouw

1. Titel: Standaardisering criteria ‘effecten op niet-doelorganismen’
Onderwerp: Bij aanvragen voor teelt van genetisch gemodificeerde gewassen in het kader van de centrale Europese procedure moet door de aanvrager gegevens verstrekt worden over mogelijke effecten op niet-doelorganismen. De COGEM heeft geconstateerd dat er geen standaardcriteria of richtlijnen zijn waaraan deze studies moeten voldoen. Dit heeft ertoe geleidt dat er ernstige vraagtekens zijn geplaatst bij niet alleen de kwaliteit maar ook de relevantie van de door aanvragers verstrekte gegevens.
Doel: Vaststelling welke criteria gehanteerd moeten worden bij studies ter bepaling van effecten van gg-gewassen op niet-doelorganismen ter verbetering van de methodiek. Overeenstemming over de criteria zal bij de aanvrager leiden tot een beter inzicht in welke gegevens noodzakelijk zijn voor de risicoanalyse en tot een transparanter vergunningverleningproces leiden.
Resultaat: Rapport waarin een overzicht wordt gegeven van de criteria waaraan dergelijke studies moeten voldoen, inclusief een overzicht welke niet-doelorganismen van relevantie zijn. Dit rapport moet uitmonden in een advies aan het Ministerie van VROM.
Onderzoek: literatuuronderzoek, interviews met experts en organisatie van een workshop.
Looptijd: 6 maanden
Additionele opmerkingen: Dit project sluit nauw aan bij een adviesvraag van VROM (CGM/031014-01) betreffende deze materie.