5 Biodiversity and Non-target Impacts: a Case Study of Bt Maize in Kenya

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Non-target Environmental Risk Assessment Model

Biodiversity is recognized in the Convention on Biological Diversity (CBD, 1992) as having multiple values, including a critical role for meeting the food, health and other needs of the growing world population. The ecological value of biodiversity can be related to ecosystem functions that are vital for crop production in sustainable agricultural systems. For example, species assemblages in an agroecosystem fulfil a variety of ecosystem functions, and a change in these assemblages can possibly harm the agroecosystem, including the farmer. The environmental risk assessment model described here assesses the possible risks of transgenic crops on biodiversity by selecting species from these assemblages, identifying the potential for risk, and proposing research protocols to assess these risks.

Any given cropping system will typically contain about 1000 or a few thousand species. Although it is possible to assess impacts on this biodiversity in its entirety, the pre-release assessment of transgenic crops will be in closed, controlled environments, such as the laboratory, greenhouse or small-scale field, which require selection of a relatively small number of species or species groups. Therefore, an important component of a case-specific risk assessment is that the most relevant species are selected for pre-release testing in a scientifically defensible and transparent way.

A species-based approach can be accomplished for above-ground ecosystems, but below-ground ecosystems require a different approach. Soils are complex biological, chemical and physical environments, containing a large

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number of species. Soil microbial and macro-faunal communities are extremely complex and largely undescribed. Hence, any individual species may be non-representative or poorly connected to significant ecological functions, and individual species are unlikely to be predictive of ecological impact. Instead, the approach to below-ground biodiversity taken here is based on aggregate soil ecosystem functions.

The non-target environmental risk assessment model developed here involves five steps: Step 1: categorizing and listing potential non-target species and ecological functions, and identifying important interactions; Step 2: prioritizing species or functions for pre-release testing according to maximum potential exposure and potential adverse effect; Step 3: conducting exposure pathway analyses; Step 4: describing hazard scenarios and formulating testing hypotheses; and Step 5: developing ecologically meaningful testing methods and protocols. Each of these steps will be described in more detail and then applied to the Kenya case study.

Step 1: Functional groups and categorizing non-target organisms and functions

A combination of qualitative field expertise and data from biodiversity assessments is crucial for determining the list of possible non-target species, their trophic relationships and relevant functions. The list will be specific to the crop and its cropping context and agroecosystem. For above-ground ecosystems: (i) functional groups are established; and (ii) the identified species are classified into these functional groups. For below-ground ecosystems: (i) is carried out generally, and (i) and (ii) can be carried out for macroorganisms where there is enough species information.

Establishing functional groups

Using ecological function allows one to focus on ecological processes and limit the number of species and functions tested. Two types of functional criteria can be used (Table 5.1) – anthropocentric functions and ecological functions (Andow and Hilbeck, 2004). Anthropocentric functions are related directly to human goals, and include secondary pest species, natural enemies, rare or endangered species, species used to generate income, and species of social or cultural value. Natural enemies are the organisms in agroecosystems (predators, parasitoids and parasites) that help maintain pest species at stable and often low levels, a function known as biocontrol. Ecological functions relate to ecosystem processes independent of human goals, and include primary consumption, secondary consumption, pollination, decomposition, nutrient recycling and seed dispersal. These functional groups are not mutually exclusive. For example, many species are both secondary pests and non-target primary consumers. Others are both natural enemies and secondary consumers.

Table 5.1. Functional classification of terrestrial non-target organisms in or near agricultural systems for pre-release testing of transgenic plants (Andow and Hilbeck, 2004; see also Box 5.1).

Functional group	Definition	Examples
Anthropocentric functions	*Functions that are of value (negative or positive) to humans*	
Alternate or secondary pests	Species that cause relatively minor damage because their abundance is restrained by the primary pests or other external factors (e.g. pesticides), but that can cause significant damage if these factors change or the primary pests are reduced	Sporadic pests (e.g. locusts), minor pests (e.g. leafhoppers)
Natural enemies	Species that consume/kill or damage pests and competitors of the crop	Predators, parasitoids, parasites, pathogens, weed-eating herbivores
Species of conservation concern	Rare or endangered species (e.g. IUCN Red List) or species of pre-determined value for biodiversity conservation	
Species that generate income		Honeybees, silk moths, fungi
Species of social or cultural value		Monarch butterflies, *Morpho* butterflies or honeybees
Ecological functions	*Species that perform functions that benefit the functioning of the ecosystem*	
Competitors	Species that compete for environmental resources with the crop (light, water, nutrients)	Weeds
Non-target primary consumers	Plant-consuming species that are not the target of the transgene	
Secondary consumers	Species that eat primary consumers	Predators, parasitoids, parasites
Pollinators	Species that visit flowers and carry pollen between them	Social and solitary bees, flies, beetles, ants
Decomposers	Species that consume plant residues	Ants, collembolans, bacteria, fungi, nematodes, earthworms, mites
Nutrient cyclers		Fungi, bacteria
Seed dispersers		Birds, small mammals, ants
Species of unknown function		Nearly half the arthropod species in a habitat

Classification of non-target species

The non-target species relevant to the agroecosystem of the crop are classified into the functional groups using the information and expertise available. However, a vast number of species found in agricultural fields probably cannot be classified into any one of these functional groups. Hence, it is critical to consider a category of species with unknown function, so that these species are not inadvertently overlooked because of lack of knowledge.

Step 2: Prioritizing non-target species or functions

This part of the process involves: (i) ranking the non-target species or functions according to ecological principles; and (ii) prioritizing a number of these for possible assessment, with particular emphasis on those that might be adversely affected by the transgenic crop and are significant for ecological or anthropogenic reasons.

Prioritization using ecological principles

In order to provide a rational and transparent approach to support the choice of species or functions, we developed a series of selection matrices. Each species is ranked for its maximum potential exposure (occurrence, abundance, presence, linkage), and for potential adverse effect (significance, such as potential secondary pest, disease vector) (see Box 5.1 for details). This will often differ in each agroecological zone where the crop is grown. Species, groups or functions given the highest priority (rank 1) are therefore the ones that have high maximum potential exposure (they are very abundant, present in the agroecosystem every year throughout the growing season and closely linked to the crop as host plant) and the ones that have a vital role in ecosystem functioning. In the workshop, species were only assigned to three ranks to simplify the ranking procedure because only rank 1 (highest priority) species or functions are likely to be considered for assessment. This approach overcomes the simplistic assumption of species abundance as a direct measure of ecological significance, as the final rank results from the combination of factors. It is important not to exclude species on the basis of only one criterion. Although many species have an unknown or uncertain ecological function or significance, our collective lack of knowledge does not imply that they have an insignificant ecological role. For example, the ecological and functional significance of microbial symbionts is only beginning to be appreciated (e.g. *Wolbachia*; Werren, 1997). To ensure a precautionary approach, we suggest that species with a high standing biomass or that are found in frequent association with the transgenic crop habitat should be prioritized for testing even if their significance is unknown.

Selecting the high-priority categories to be tested

Species and functions that were assigned to the highest priority become candidates for testing. The final selection process is an expert-driven process, but is transparent. It is possible to advocate that several species from each functional

Box 5.1. Criteria for prioritizing non-target species in each functional group to facilitate selection of species to evaluate in stage one tests; these criteria are consistent with Annex III of the Cartagena Protocol (CBD, 2002).

1. *Maximum potential exposure* – This is based on geographic range, habitat specificity, local abundance (Rabinowitz, 1981), prevalence (proportion of suitable habitat that is occupied by the species) and temporal association with the crop. In the prioritization support matrices (Tables 5.4, 5.5 and 5.6), these are referred to as occurrence (occurs in crop agroecosystem, geographic range and prevalence), abundance (local abundance and prevalence), presence (temporal association with the crop) and linkage (habitat specificity, association with maize). These criteria can be evaluated independently of the specific transgenic crop. Species with a broad geographic range, specificity to the crop habitat, high local abundance, high prevalence and high temporal overlap with the crop are likely to have greater exposure to the transgenic crop.
2. *Potential adverse effect* – This is based on the potential value of an adverse effect on a non-target species, should one occur, based on ecological and anthropogenic significance (see Table 5.1). In the prioritization support matrices (Tables 5.4, 5.5 and 5.6), this is referred to as significance, and in the primary consumers matrix, particular significance as disease vector or damaging pest is separately listed. Ecologically significant species fulfil significant ecological functions, such as biological control, pollination or decomposition. Economically significant species are likely to have an economic impact if their abundance changes. Examples include disease vectors or damaging pests. Threatened species include those listed on Red and Blue lists or who are otherwise threatened or endangered. Species of cultural significance could be symbolic species that appear repeatedly in public in symbolic ways (flags, logos, advertisements, news, etc.), or species with unique attributes (e.g. social organization, mass migration, stunning and rare beauty, strength).
3. *Potential likely exposure* – Species likely to be exposed to the transgene product or metabolites in the crop ecosystem. This assessment must take into account the specific transgenic crop and expression levels of the transgene product in each tissue. Species that are not exposed directly or indirectly are less likely to be affected by the transgenic crop, and if they are affected it will probably be through another species that is directly exposed to the transgene product or metabolite. See text for additional discussion.

group should be tested. It is also possible to advocate that single species, or one or several functional groups from a subset of the functional groups should be tested. Clearly, the greater the level of precaution required in the assessment, the larger the choice of species or functions should be. However, the size of the species list is also likely to be influenced by other factors, including economic and political ones. If the decision making is transparent and based on the consensus of diverse experts, the decisions will be defendable and likely to be acceptable to many.

Step 3: Exposure pathway analyses

This step analyses possible causal pathways of exposure to the genetically modified (GM) plant and toxin, and potential impacts of the GM plant

(including direct and indirect, intended and unintended effects) for each species or function identified as highest priority in the previous step. The purpose of this evaluation is to differentiate candidate test species likely and unlikely to be exposed to the Bt toxin, and for the former, to guide the design of the exposure system in the test protocols.

Potential likely exposure can occur through many pathways. Transgenic plant material and transgene products and metabolites may affect non-target species and ecosystem functional dynamics directly via plant residues (above- or below-ground) (Zwahlen *et al*., 2003), senescent leaves and sloughed roots, root exudates (Saxena *et al*., 1999; Saxena and Stotzky, 2000), pollen (Losey *et al*., 1999), and other plant parts that express the transgene, such as seeds, floral and extra-floral nectaries, guttation fluids and phloem sap (Hilbeck, 2002). Any non-target organism feeding on the transgenic plant or parts of the plant may come in contact with the transgene and its product. In addition, the transgene product might interact with existing plant compounds to affect non-target organisms (Birch *et al*., 2002). Transgenic plant material and transgene products can affect non-target species indirectly through another organism, such as an herbivore (Birch *et al*., 1999; Hilbeck *et al*., 1999) or honeydew from Homopteran species such as aphids, scales or whiteflies (Raps *et al*., 2001; Bernal *et al*., 2002). Non-target species could therefore be affected by: (i) transgene products in the plant, plant secretions, herbivore, herbivore excretions or other species containing transgene products; (ii) metabolites of the transgene products; or (iii) interaction effects of the transgene products with other plant or herbivore compounds that alter plant or herbivore composition or physiology (e.g. Saxena and Stotzky, 2001a; Birch *et al*., 2002). The number of possible pathways is immense. It has been estimated that there are over 250 different exposure pathways by which a transgene product or its metabolites could affect a secondary consumer, of which only a few are direct effects of the transgene product (Andow and Hilbeck, 2004). These include unintended changes in ecologically important plant primary and secondary metabolites in the transgenic plant. This multitude of potential exposure pathways has important implications for test methodology (see below), and complicates analysis of potential exposure (Hilbeck, 2002).

Soil ecosystems are driven by the types and amounts of carbon-containing compounds entering soils, providing both energy and nutrients (Wheatley *et al*., 1990, 1991, 2001). These inputs are from plant residues and organic compounds released by the roots of growing plants. The constituents of these inputs vary according to plant physiology and structure, and the stage of plant growth. The forms and availability of these compounds can define both rate and choice of microbial function (Wheatley *et al*., 2001). Thus, soil ecosystem dynamics can be influenced by the type of plant driving it, and the impact of any plant cultivar must be evaluated on the basis of changes in these plant inputs. To guide the analysis of possible exposure or indirect effects we developed a series of questions that will be detailed in Step 3 of the Kenya case study non-target assessment further in this chapter.

Step 4: Hazard identification and hypothesis development

The information from the previous steps guides hazard identification and the development of testable assessment hypotheses. For example, will a particular non-target herbivore species be affected if it ingests a GM product via flower feeding (i.e. direct effect)? If it is not lethally affected, will it pass the GM product on to higher trophic levels, possibly affecting any of its natural enemies (i.e. tri- or multi-trophic effect)? Will a natural enemy species providing an important ecological service elsewhere decline in numbers because a main non-target herbivore prey source is adversely affected? For the soil ecosystem, hazard identification needs to address the possible effects of the transgene products and any other changes in plant inputs.

This step in the risk assessment is particularly important when exposure (direct or multi-trophic) is unlikely yet indirect effects are still possible. Such indirect effects can arise through changes in the population dynamics of a non-target organism and/or through a shift in the species composition of a particular community assemblage. This step requires careful examination of known impact pathways on species and functional interactions, and relies a great deal on the ecological field expertise available to the process. Functional dynamics in plant–soil ecosystems have been shown to be highly variable on both a temporal and spatial basis, and affected by many factors, such as temperature, rainfall and cultivation practices. Consequently, comprehensive baseline data will be required to determine natural variation within comparable soil ecosystems to be able to detect any effects of transgenic crops.

Step 5: Protocols and measurement endpoints (parameters to be measured)

The next step is to develop appropriate methodologies and protocols to assess risk. Such methods can be designed once it is evident which life stage(s) of a non-target organism is likely to be exposed and through what route it is likely to receive the GM product, directly and/or mediated indirectly, through other non-target prey. Similarly, when and where soil-ecosystem functions may be vulnerable can be used to design better assessment methods. Any ecologically meaningful experimental design will mimic identified exposure routes, test identified hazard scenarios and introduce ecological realism as far as possible.

Methodology/protocol

Two kinds of methodology are necessary. Firstly, conventional ecotoxicology methodologies can be modified to assess effects of exposure to the transgene products, although the majority of exposure pathways will be ignored, which creates ambiguity in the interpretation of the results (Hilbeck, 2002; Andow and Hilbeck, 2004). Secondly, a 'whole plant' methodology is required. In such a method, the effects of the whole transgenic plant are evaluated, not just the transgene product. Such methods require the use of appropriate experimental controls that mimic natural exposure, as it would occur in the field.

Appropriate genetic and ecological controls

An ideal genetic control is a plant verified as being fully isogenic to the transgenic plant. Isogenic means that the control is genetically identical to the transgenic plant except for the inserted transgene. In practice, isogenic controls cannot be used as such isogenic plants do not exist for commercial or commercializable varieties, or may not be truly isogenic. Near-isogenic controls are available for some commercial or commercializable crop varieties, but these may differ from the transgenic variety by as much as 4% of the genome. Moreover, all such transgenic varieties have had some selection for agronomic characteristics that their near-isogenic varieties have not experienced, which can result in genetic and phenotypic differences from their near-isogenic lines. Consequently, there can be systematic differences in agronomic characters between the transgenic plant and its near-isogenic control plants.

One way to address the lack of rigorous genetic controls is to run multiple comparisons between several pairs of transgenic varieties or events and their isogenic or agronomic controls. Agronomic controls would consist of locally grown varieties. If these pairs of varieties are sufficiently different from each other, then they will be less likely to share many genetic differences except for the difference in the transgene. Hence, running the same experiment on several transgenic control pairs (so long as multiple transgenic lines containing the same transgene exist), under similar environmental conditions, allows more definitive conclusions about the effects of a transgene.

An ideal ecological control is a plant variety that would be grown in a production system in the region of interest (Andow and Hilbeck, 2004). This does not have to be a commonly grown variety. However, a plant variety that has not been screened for performance in a local production system would not be a useful ecological control. In addition, it is crucial that the plant is presented to the test species in a way that mimics the way the species would experience the plant in the field. In many cases, these studies will be done in the laboratory, using greenhouse-grown plants, and laboratory-reared herbivores and natural enemies. If appropriate care is not taken, the plants could be etiolated (elongated) with low specific leaf weights, have atypical primary and secondary plant metabolism, and the test species could be inbred, physiologically stressed, physiologically variable or even diseased. Metabolism of excised plant tissues quickly changes radically, so laboratory bioassays using excised plant material should be short in duration (*c.* 24–48 h maximum) and do not mimic effects of the growing GM crop in the field.

Conducting lab tests and demonstrating no negative effects on natural enemies (particularly using artificial diets and purified toxins) should not be used as evidence that no further testing is needed and that the transgene product and GM crop is safe for natural enemies under field conditions. Other types of controls (for testing Bt maize) are strongly recommended. These include any available conventional pest-resistant varieties, a conventional maize variety with Bt sprays and a conventional variety protected with a commonly used pesticide regime for the region.

Measurement endpoint

An appropriate experimental endpoint (parameter to be measured) for initial testing is generational relative fitness or some component of relative fitness. Generational relative fitness is the relative lifetime survival and reproduction of the non-target species. Hence, survival experiments should last at least through all of the developmental stages of the non-target species, and adult life stage parameters should be measured, including age-specific mortality and female fecundity. In principle, the duration of the test should correspond to the time the non-target species would be exposed to the transgenic plant, plant parts and residues, and the temporal pattern of expression and persistence of the transgene product and its metabolites. Generational relative fitness is a particularly useful endpoint, because it relates directly to risk. If the transgenic plant were to adversely affect a non-target species in the environment, its effects would come through some component of relative fitness. Hence, the results from such initial testing would guide the design of further tests, by identifying the fitness components that would possibly be affected by the transgenic plant in the environment.

Although a general testing methodology cannot be specified in detail, an ecologically realistic experiment should meet several key criteria so that results are scientifically sound and ecologically interpretable (Andow and Hilbeck, 2004). Initial testing experiments minimally would include:

1. Food (e.g. ecologically relevant plant and or prey species) that is used by test species in their relevant habitat should be used in laboratory tests. If transgene product is used, it should be identical to what is produced in the transgenic plant.
2. Verification that the food offered to the species actually contained the administered material at the intended concentration or dose throughout the investigation.
3. Verification that all life stages of the species are exposed appropriately to the transgene product and actually contact the product in relevant ways.
4. Either use intact plants or plant parts in the experimental system with verification that the plant parts used contain the transgene product, or use the transgene product at concentrations or doses much higher than normally expressed in the plant as a worst-case scenario for short-term exposure.
5. Have a proper scientific control or controls.
6. Have sufficient replication and sufficient numbers of individuals screened, so that statistical power of the experimental design is not an issue for interpretation of results (Marvier, 2001). It is strongly recommended that professional statisticians be consulted *before conducting the experiments, as well as for analysis and interpretation*.

Scope of the Kenya Case Study Non-target Environmental Risk Assessment

This assessment focused on non-target and biodiversity effects associated with maize and nearby habitats. We did not attempt to extend the assessment to

cover potential effects associated with aquatic environments, or rare or fragile natural habitats in the landscape, or on intercropped plants mixed with maize (e.g. legumes; Muhammad and Underwood, Chapter 2, this volume). A further focus of these assessments was on initial risk assessment in closed, controlled environments prior to field releases of the transgenic crop. It is expected that our future activities will extend the analyses to cover experimental procedures for field assessments, guided by the data obtained in these initial tests. While the methodologies developed were focused on the Kenyan maize production context, they should extend beyond Kenya and be applicable in similar maize production systems of neighbouring countries (Andow and Hilbeck, Chapter 1, this volume). This will be addressed more formally in our future activities. At the workshop, we focused solely on evaluation of Bt maize, but the methods could be adapted to assess ecological impacts of other transgenic plants.

We assumed that taxonomic knowledge in Kenya is incomplete; therefore where individual species are not identified, species should be classified by ecological function, e.g. feeding guilds such as 'larval parasitoid' or 'egg parasitoid', rather than taxonomically.

Step 1: Functional groups and categorizing non-target species and functions in maize in Kenya

Based on the experts present and the available published arthropod surveys conducted in Kenya, Kenyan maize fields contain a diversity of arthropod species from at least 18 orders and 75 families probably comprised of hundreds of species (Table 5.2). This level of information is sufficient to categorize maize-associated species and functions. The following functional categories were used: non-target maize herbivores, natural enemies of maize herbivores, pollen feeders and pollinators, weeds, and soil functions. We did not have time to consider vertebrates (such as rats, squirrels and bush pigs; Muhammad and Underwood, Chapter 2, this volume), species of conservation concern (such as elephants or monkeys), species of cultural significance, soil macroorganisms (such as Melolonthinae larvae or cutworms; Muhammad and Underwood, Chapter 2, this volume), storage pests that are not found in the field (e.g. *Plodia interpunctella*, *Ephestia cautella* or *Ephestia kuehniella*; Fitt *et al*., Chapter 7, this volume) or plant pathogens (e.g. *Aspergillus flavus*, *Fusarium*; Munkvold *et al*., 1997; Muhammad and Underwood, Chapter 2, this volume).

Non-target maize herbivores

Non-target herbivores are potential secondary pests of the crop. While Bt maize may reduce target pest species to insignificant levels, other pests may increase and become damaging pests (secondary pests), decrease or be unchanged. Non-target herbivores may also function as vectors of diseases that may cause damage to the crop (e.g. leafhoppers/maize streak virus). In addition, they can serve as:

1. Food for biocontrol organisms (natural enemies) that regulate the target pest.
2. Carriers of plant compounds to higher trophic levels possibly inducing tritrophic effects.

Table 5.2. Number of individuals of listed arthropod orders and families recovered from pitfall, water and sticky traps, in farmers' maize fields in Kilifi, Kakamega and Masii during the short rains in 2000 (Josephine Songa, Nairobi, 2002, personal communication).

Arthropod order/family	Common name	Kilifi	Masii	Kakamega
Diptera				
Tachinidae	Tachinid flies	22	1	30
Sarcophagidae	Flesh flies	705	2,204	382
Syrphidae	Hover flies	9	11	26
Dolichopodidae	Long-legged flies	5	–	888
Stratiomyidae	Soldier flies	25	107	34
Sciaridae	Dark-winged fungus gnats	–	3	7
Calliphoridae	Blow flies	2	876	815
Muscidae	Muscid flies	260	1,194	5,518
Phoridae	Humpbacked flies	–	–	3
Diopsidae	Stalk-eyed flies	–	1	2
Drosophilidae	Vinegar flies	1	10	158
Otitidae	Picture winged flies	–	–	11
Tephritidae	Fruit flies	9	4	19
Asilidae	Robber flies	1	–	1
Rhagionidae	Snipe flies	–	–	11
Bombyliidae	Bee flies	1	–	–
Mycetophilidae	Fungus gnats	–	–	78
Lauxaniidae	Lauxaniid flies	–	–	2
Agromyzidae	Leaf miner flies	5	–	1
Anthomyzidae	Anthomyzid flies	23	22	70
Sepsidae	Black scavenger flies	–	–	2
Orthoptera				
Gryllidae	Crickets	12,187	522	10,838
Blattidae	Cockroaches	32	11	359
Acrididae	Short-horned grasshoppers	2,812	21	123
Tetrigidae	Pygmy grasshoppers	4	–	–
Tettigonidae	Long-horned grasshoppers	29	3	8
Gryllacrididae	Camel cricket	4	–	–
Mantidae	Mantids	164	1	1
Dermaptera				
Forficulidae	Common earwigs	7	5	173
Labiidae	Little earwigs	–	–	–
Hymenoptera				
Formicidae	Ants	6,365	361	7,996
Apidae	Honeybees	23	29	191
Ichneumonidae	Ichneumons	12	7	13
Vespidae	Vespid wasps	66	233	157
Pompilidae	Spider wasps	18	9	2
Sphecidae	Sphecid wasps	109	193	52
Cephidae	Stem sawflies	6	5	26
Eumenidae	Potter wasps	5	–	–
Braconidae	Braconid wasps	13	12	15
Chalcididae	Chalcidids	10	–	1
Megachilidae	Leaf-cutting bees	3	25	7
Tiphiidae	Tiphiid wasps	2	1	–

Continued

Table 5.2. *Continued.*

Arthropod order/family	Common name	Kilifi	Masii	Kakamega
Hymenoptera *(Continued)*				
Mutillidae	Velvet ants	1	–	–
Evaniidae	Ensign wasps	–	2	–
Ibaliidae	Ibaliids	–	8	–
Chrysididae	Cuckoo wasps	1	–	23
Halictidae	Halictid bees	14	–	9
Coleoptera				
Coccinellidae	Ladybird beetles	32	642	68
Carabidae	Ground beetles	1,465	64	90
Staphylinidae	Rove beetles	8	24	145
Tenebrionidae	Darkling beetles	2,646	5	35
Melyridae	Soft-winged flower beetles	725	24	779
Scarabaeidae	Scarab beetles	159	190	496
Mordellidae	Tumbling flower beetles	13	6	15
Chrysomelidae	Leaf beetles	55	181	134
Cerambycidae	Long-horned beetles	3	4	–
Curculionidae	Maize weevil	8	14	12
Elateridae	Click beetles	1	–	6
Lagriidae	Long-jointed bark beetles	4	–	2
Dasytidae	Soft-winged flower beetles	277	22	13
Bupestridae	Metallic wood boring beetles	9	2	1
Meloidae	Blister beetles	17	15	14
Hemiptera				
Miridae	Plant bugs	22	3	39
Cydnidae	Burrower bugs	10	5	3
Reduviidae	Assassin bugs	16	7	3
Berytidae	Stilt bugs	1	–	–
Pyrrhocoridae	Stainers	333	2	1
Pentatomidae	Stink bugs	3	6	16
Homoptera				
Cicadellidae	Leafhoppers	329	180	556
Cercopidae	Spittlebugs	2	–	–
Cicadidae	Cicadas	–	–	–
Membracidae	Treehoppers	16	14	31
Aphididae	Aphids	–	–	60
Isoptera				
Termitidae	Termites	1	84	25
Rhinotermitidae	Damp-wood termites	1	–	12
Thysanoptera	Thrips	–	2	–
Opiliones	Harvestmen	–	1	3
Lepidoptera	Moths and butterflies[a]	534	267	142
Araneae	Spiders	509	453	360
Diplopoda		3	3	–
Chilopoda		1	1	–
Isopopoda		217	4	–
Annelida		–	–	2
Acari		1	–	–
Coleoptera	Larvae	3	3	4

[a]Moths and butterflies could not be identified further as the scales had been removed in the water traps.

3. Biocontrol organisms of plants outside and/or inside the cropping system, e.g. when feeding on weeds, thereby helping to suppress their damaging effect.
4. Seed dispersers and seed predators.

Non-target herbivores and their natural enemies on beans and other legumes were not considered during the workshop, but were identified as an important intercrop of most maize cropping systems in Kenya (Muhammad and Underwood, Chapter 2, this volume), and should be considered subsequently. The workshop also did not look at potential impacts on species living on weeds or outside the maize field, e.g. on lepidopteran larvae on other plants associated with maize. The non-target herbivores evaluated are listed in Table 5.3.

Pollinating and pollen-feeding insects on maize

Maize is wind pollinated and produces copious quantities of pollen that is a significant food source for many insects that may be important pollinators of other crops, natural enemies, or otherwise significant. The working subgroup agreed that no systematic observations on flower-visiting species associated with maize have been conducted in Kenya. However, some pollen-feeders known to be present in significant numbers in maize in Kenya were included in Table 5.3.

Natural enemies of maize herbivores

Natural enemies are beneficial organisms that help reduce pest populations. Many synthetic pesticides have reduced the numbers of natural enemies in crop fields, and it has become apparent that sustainable agricultural production methods need to conserve natural enemies. Hence, new pest control technologies, such as Bt maize, need to be tested for their negative, neutral or positive effects on natural enemies, prior to large-scale use. Some of the parasitoid natural enemies attacking the four target stemborer pest species of Kenyan maize are fairly host specific, such as *Dentichasmias busseolae* (Heinrich), which attacks *Chilo partellus* (Swinhoe), but rarely attacks *Busseola fusca* (Fuller) (Zhou *et al*., 2003). Other parasitoid species are less host specific. Therefore, the reduction in stemborer prey on Bt maize is likely to lead rapidly to altered natural enemy diversity and abundance, and could affect their biocontrol function on other non-target herbivores. The deliberate introduction of several parasitoid species into Kenya is planned, and it is important to assess how Bt maize will affect them. Sufficient taxonomic and ecological information exists in Kenya to use qualitative ecological expertise to identify and prioritize parasitoids of maize herbivorous arthropods as potential species for non-target risk assessment. The main parasitoid species of stemborers are listed in Table 5.3, based on the expert knowledge available in the group and several recent publications (Bonhof *et al*., 1997; Songa *et al*., 2002; Zhou *et al*., 2003).

While the main generalist predators could not be listed, it is understood that they are also an important group that must be considered in a full risk assessment. Investigations by Songa in 2000 in coastal and western Kenya (IRMA, 2001) found potential predator groups to be ants, earwigs (Forficulidae),

Table 5.3. The categorized non-target organisms/functional groups included in the analyses of Bt maize in Kenya.

Non-target herbivores	Natural enemies	Weeds	Below-ground functions
Defoliators	Egg parasitoid	Grass and sedge weeds	Carbon decomposition
Grasshoppers/crickets	*Trichogramma* spp.	*Andropogon* spp.	Cellulose breakdown
Spider mites	*Trichogrammatoidea* spp.	*Brachiaria* spp.	Ammonification
Locusts	*Telenomus* spp.	*Cynodon* spp.	Nitrification
Spodoptera spp.	Larval parasitoids	*Cyperus* spp.	Denitrification
Sap feeders	*Cotesia flavipes* (Cameron) (introduced)	*Digitaria* spp.	Nitrogen fixation
Aphids	*Cotesia sesamiae* (Cameron)	*Eleusine* spp.	Phosphorus and micronutrient uptake
Leaf and planthoppers	*Goniozus indicus*	*Eragrostis* spp.	
Stinkbugs	Egg and larval parasitoid	*Hyparrhenia* spp.	
Grain feeders	*Chelonus curvimaculatus* (Cameron)	*Melinis* spp.	
Sitophilus zeamais (Motsch)	Pupal parasitoid	*Panicum* spp.	
Prostephanus truncatus (Horn)	*Pediobius furvus* (Gahan)	*Pennisetum* spp.	
Sitotroga cerealella (Oliver)	*Dentichasmias busseolae* (Heinrich)	*Rottboellia* spp.	
Silk and cob feeders		*Sorghum* spp.	
Cryptophlebia leucotreta (Meyrick)		Broad-leaf weeds	
Helicoverpa armigera (Hubner)		*Commelina benghalensis* (L.)	
Pollen feeders		*Bidens pilosa* (L.)	
Meloidae		*Tagetes minuta* (L.)	
Apis mellifera (L.)		*Senna obtusifolia* (L.) Irwin & Barneby	
Wild bee spp.		Parasitic weeds	
Ants		*Striga hermonthica* (Del.) Benth.	
Butterflies		*Striga asiatica*	
Root feeders		*Striga aspera*	
Gryllotalpidae		*Sorghum* spp.	
Cut worms			
Termites			
Melolonthinae			
Saprovores			
Mycetophagidae			
Carpophilus spp.			
Catatus spp.			
Tenebrionids			
Earwigs			
Total: 26	Total: 9	Total: 21	Total: 7

spiders, carabid beetles (Carabidae), ladybird beetles (Coccinellidae) and rove beetles (Staphylinidae). Many or most of these are also important predators of *Helicoverpa armigera* in maize and cotton, and of *Spodoptera* spp., since several of these predators are polyphagous (van den Berg and Cock, 1993, 1995; Watmough and Kfir, 1995). Other predator species identified to feed readily on *H. armigera* were various species in the families of Chrysopidae and Anthocoridae (van den Berg *et al*., 1993). Oloo (1989) found that predation may be a significant mortality factor for stemborer eggs and small larvae.

Maize-associated weeds

The weed community in Kenyan maize fields is very diverse. Grasses and sedges are important components of biodiversity – about 600 species of grasses alone have been reported in Kenya, suggesting that Kenya might be a centre of origin for many of the grasses found in East Africa (Ibrahim and Kabuye, 1987; Boonman, 1992). Weeds can contribute substantially to crop losses. In particular, the parasitic *Striga* weeds can be extremely damaging in parts of Kenya, and much scientific and financial effort has gone into seeking methods to control them in Africa (Muhammad and Underwood, Chapter 2, this volume). The weed species considered in the assessment are listed in Table 5.3.

Soil ecosystem functions

Species diversity in soils is as great as in any other ecosystem (Curtis *et al*., 2002), so that more than 90% of the biodiversity in agroecosystems is in the soil. A comprehensive assessment of the non-target impacts of GM crops on biodiversity must therefore include some assessment of impacts on soil biodiversity. Soil biodiversity is related to soil-ecosystem functions essential to plant production. Whether nutrients are made available for plant uptake, or lost to the environment, is entirely dependent on microbial functioning in the soil. Soil macroorganisms, such as insects and their larvae, nematodes and earthworms, also play a vital part in soil nutrient cycling by breaking down and redistributing organic material. Key microbial groups involved in nutrient cycling and soil fertility are good indicators of important ecosystem functions, such as nitrogen fixation, mineralization, nitrification or cellulose degradation. These can be rapidly assessed on large numbers of well-replicated soil samples, and are well-established parameters of soil health, particularly in low-input agroecosystems.

Seven microbial functions were selected as being particularly responsive to the amount and type of plant materials being introduced to the system (Table 5.3). These were: (i) carbon decomposition; (ii) cellulose breakdown; (iii) ammonification; (iv) nitrification; (v) denitrification; (vi) nitrogen fixation; and (vii) phosphorus uptake. Functions (i) to (vi) are within the carbon and nitrogen cycles, driven by plant residues, and are part of the nutrient cycling process vital to continued plant growth; (vi) and (vii) are of particular importance in maize cultivation because of the prominence of *Phaseolus* bean in the maize cropping system, and because of the intimate relationships of the mycorrhizal association between the plant roots and fungi. Indicator organisms were identified for each functional group.

Findings of Step 1

A total of 62 species, species groups and functions in five functional categories (non-target herbivores, pollen feeders and pollinators, natural enemies, weeds and soil functions) relevant to Kenyan maize production systems were compiled for further risk assessment. This list is not comprehensive and a number of categories, guilds and functions were not included, e.g. vertebrates, general predators, pathogens, soil macroorganisms, species of conservation concern and species of cultural or economic significance. It is recognized that these missing components are important, and a full risk assessment of Bt maize in Kenya would have to include these.

Step 2: Prioritizing non-target species or functions

To prioritize the listed species and functions, we used a selection procedure based on a prioritization and decision support matrix (selection matrix). This was done separately for non-target herbivores, natural enemies, weeds and soil functions. For soil functions, a modified decision support matrix was developed that was more suitable for the task. The species and functions prioritized as most important for testing were ranked 1. A full risk analysis of Bt maize in Kenya could also include species that received lower rankings.

We are not advocating that all the species/functions are tested; the aim of the workshop was to draw up options and identify knowledge gaps for future research effort, scaled and staged to available resources.

Non-target maize herbivores

The lepidopteran non-target herbivores identified as being most important in maize in Kenya are *H. armigera* and *Spodoptera* species (Table 5.4). *H. armigera* is a minor or sporadic pest of maize, but an important pest of several other crops. It is therefore considered an important potential secondary pest in Bt maize; particularly if the natural enemy population is altered to its benefit (see natural enemies below for a more detailed discussion). *Spodoptera* species were also ranked as very important because of their abundance in some Kenyan ecological zones and their high damage potential. These species were not discussed in detail; however, the group recommends that they be studied as a key non-target herbivore. Most of the protocols developed for testing of *H. armigera* can be adjusted readily for testing of *Spodoptera* spp.

Among the non-Lepidoptera, locusts, spider mites, leafhoppers, *Carpophilus* spp., and the grain feeders *Sitophilus zeamais* and *Prostephanus truncatus* were also given highest priority (Table 5.4). Locusts were considered sporadic pests, with outbreaks approximately every 10 years, but with significant damage caused during outbreaks. Spider mites were ranked highly as they may occur in high abundance at any time during the growth cycle of maize, and have a high damage potential. Leafhoppers (*Cicadulina* spp.) received the highest index of significance for their ability to transmit maize streak virus (MSV) disease to the maize plant, which causes significant crop

Table 5.4. Selection matrix for prioritizing non-target herbivore and saprovore arthropod species associated with maize in Kenya (species with blanks could not be covered during the workshop).

Feeding guild	Assemblage	Maximum potential exposure				Possible adverse effect			Rank
		Occurrence	Abundance	Presence	Linkage	Significance	Disease vector	Damage	
Defoliators	Grasshoppers/crickets	Likely	Low	Vegetative	Sometimes			Low	3
	Locusts	Occasional	Abundant	Anytime	Sometimes			Heavy	1
	Spider mites	Occasional	Abundant	Anytime	Sometimes			Heavy	1
	Spodoptera spp.	Occasional	Abundant	Anytime	Sometimes			Heavy	1
Sap feeders	Aphids	Certain	Medium	Anytime	Sometimes	Potential pest	Maize dwarf mosaic virus	Sometimes	2
	Leaf and planthoppers	Likely	Medium			Potential pest	Maize streak virus (MSV)		1
	Stinkbugs	Certain if near cotton	Low				MSV		3
Grain feeders	*Sitophilus zeamais*	Certain	Abundant	Reproductive	Always	Significant potential pest, also postharvest			1
	Prostephanus truncatus	Certain	Abundant	Reproductive	Always	Significant potential pest, also postharvest			1
	Sitotroga cerealella	Likely	Abundant	Reproductive	Always	Potential pest, also postharvest			2
Silk and cobs	*Cryptophlebia leucotreta*	Certain	Medium	Reproductive	Always	Significant potential pest			2
	Helicoverpa armigera	Certain	Medium	Reproductive	Always	Significant pot pest			1
Root feeders	Gryllotalpidae (mole crickets)	Occasional	Medium	Vegetative	Sometimes				3
	Cut worms	Occasional	Medium	Vegetative	Always				2
	Termites	Occasional	Abundant, site specific	Anytime but more important in maturity	Sometimes	Potential pest			2
	Melolonthinae (e.g. *Schizonycha, Phyllophaga*)								

Continued

Table 5.4. *Continued.*

Feeding guild	Assemblage	Maximum potential exposure				Possible adverse effect			Rank
		Occurrence	Abundance	Presence	Linkage	Significance	Disease vector	Damage	
Saprovores	Mycetophagidae (dusty brown beetles)	Likely	Low	Anytime	Sometimes	Potential pest		Low	3
	Carpophilus spp.	Certain	Abundant	Reproductive	Sometimes	Storage contaminant	*Aspergillus flavus*		1
	Catatus spp.	Likely	Medium	Reproductive	Sometimes				3
	Tenebrionids (darkling beetles)	Likely	low						3
	Earwigs	Certain	Medium	Reproductive	Always	May be mainly predator			2

losses in Kenya (Muhammad and Underwood, Chapter 2, this volume). *Carpophilus* spp. were also given the highest priority because of their role in spreading the fungus *A. flavus*, which causes high aflatoxin levels in maize grains (Sétamou *et al*., 1998). *S. zeamais* and *P. truncatus* were prioritized because of their ubiquity and because of their abundance and the importance of postharvest losses for Kenyan farmers (Muhammad and Underwood, Chapter 2, this volume).

In summary, of the total of 26 herbivore species and groups identified as important in Kenyan maize production systems in Step 1, eight species received the highest ranking (i.e. '1'). Some of these will be used in subsequent analyses, to illustrate procedures that should be applied to the other highly ranked species.

Pollinating and pollen-feeding insects on maize

Given the lack of information presently available (Step 1), the selection matrix could not be used to prioritize and select these species but was used to identify knowledge gaps (Table 5.5). In the face of this uncertainty, general ecological information about species associated with maize pollen in other parts of the world was used to suggest at least four species be included in subsequent analysis, including at least one species of wild, native bee, honeybee, and two species of the more important pollen-feeding predator species, e.g. from the orders Forficulidae (earwigs) and Coccinellidae (ladybird beetles). We focused on the honeybee (*Apis mellifera* L.) because honeybee biology is well known, specific expertise is available at the local level, they are important pollinators, they visit maize as a preferred source of pollen in other countries (Nowakowski and Morse, 1982; Vaissiere and Vinson, 1994), and previous evaluations of Bt maize have focused on the European subspecies, while in East Africa the African subspecies dominates.

Natural enemies of maize herbivores

The main parasitoid species of maize stemborers in Kenya are ranked for the highlands where *B. fusca* dominates and the lowlands where *C. partellus* dominates (Table 5.6). *Trichogramma* spp. were identified as an important egg-parasitoid species also attacking *H. armigera*, one of the most important identified non-target herbivores in maize in Kenya. The most common identified Kenyan species are *Trichogramma* sp. nr. *mwanzai* (Guang and Oloo, 1990), *Trichogramma* sp. nr. *exiguum* (Ochiel, 1989) and *Trichogramma buornieri* (Abera *et al*., 2000). The final choice of which species to test should be based on a more detailed analysis of their relative importance and abundance in the agroecological zone of interest, which could not be completed during the workshop. The selected species is therefore referred to as *Trichogramma* spp. in the further assessment.

The two *Cotesia* spp. (larval parasitoids) also received the highest ranking and will be used in subsequent analyses. Larval and pupal parasitoids of stemborers are important in reducing the carryover population in crop residues

Table 5.5. Selection matrix for pollinator and pollen-feeding species associated with maize in Kenya.

		Maximum potential exposure				
Feeding guild	Assemblage	Occurrence	Abundance	Presence	Linkage	Possible adverse effect
Pollen feeders:	Meloidae (pollen beetles)	Likely		Reproductive		Natural enemies: parasitic on grasshopper eggs
Flower visiting	*Apis mellifera*					Pollinators of other crops
	Wild bee spp.					Pollinators of other crops
Other than flower visiting	Ants	Likely	Abundant	Anytime	Sometimes	Natural enemy
	Earwigs	Certain	Medium	Reproductive	Always	May be mainly predator
	Coccinellids	Certain	Medium	Reproductive	Always	Predaceous
Not on maize	Butterflies	Certain	Medium	Reproductive	Through dispersed pollen	Not feeding on crop

Table 5.6. Selection matrix for prioritizing parasitoid natural enemy species associated with maize in Kenya (differentiated for lowland and highland maize ecosystems).

Guild	Species	Maximum potential exposure				Rank
		Occurrence	Abundance	Presence	Linkage	
Lowland Kenya ecosystem (*Chilo partellus* dominates)						
Egg parasitoid	*Trichogramma* spp.	Certain	Medium	All season	Strong	1
	Trichogrammatoidea spp. (uncompleted)					
Larval parasitoid	*Cotesia flavipes*	Certain	Medium	All season	Strong	1
	Cotesia sesamiae	Certain	Low–medium	All season	Strong	2
	Goniozus indicus (uncompleted)					
Egg and larval parasitoid	*Chelonus curvimaculatus* (uncompleted)					
Pupal parasitoid	*Pediobus furvus*	Certain	Low	All season	Strong	2
	Dentichasmias busseolae, native	Occasional	Low	All season	Strong	3
Predators	(Uncompleted)					
Highland Kenya ecosystem (*Busseola fusca* dominates)						
Egg parasitoid	*Trichogramma* spp.	Likely	Medium	All season	Strong	2
	Trichogrammatoidea spp. (uncompleted)					
	Telenomus spp. (uncompleted)					
Larval parasitoid	*Cotesia sesamiae*	Certain	Medium	All season	Strong	1
	Cotesia flavipes	Occasional	Low	All season	Strong	3
Pupal parasitoid	*Dentichasmias busseolae*	Occasional	Low	All season	Strong	3
	Pediobus furvus	Certain	Low	All season	Strong	2
Predators	(Uncompleted)					

that may give rise to the initial infestation of the next growing season. In addition, new parasitoid species that have recently been released (*Xanthopimpla stemmator* Thunberg) or are being evaluated for release in Kenya (*Telenomus isis* Polaszek) as biocontrol agents of stemborer species will also need to be considered in a programme of impact testing.

Maize-associated weeds

FREE-LIVING GRASS AND SEDGE WEEDS. Species ranks were based primarily on occurrence, abundance and significance (Table 5.7). 'Presence' and 'Linkage' were always high and general, because weeds are a pre-selected subset of plants that are present throughout most of the cropping season and linked generally to the crop plant. Most species with high occurrence were abundant in both lowland and highland areas, but for others, abundance seemed to differ regionally. For example, while *Bracharia* spp. and *Pennisetum* spp. are at low densities in the lowlands, they are at high densities in the highlands. Several species are significant as potential alternative host plant species for maize stemborers (Fitt *et al*., Chapter 7, this volume), and may function as sources or sinks of pests and natural enemies. The attractiveness of some grasses to stemborers is used in the 'push–pull' strategy to suppress stemborer populations in maize (Khan *et al*., 1997). However, recent evidence suggests that *B. fusca* is hardly present in wild hosts, and that *C. partellus* is specific to some wild plant species, while others are very poor hosts for maize stemborers (B. Le Ru, Nairobi, 2004, personal communication). The exact mechanism of host switching and host suitability of wild plants for stemborers is identified as an important information gap (consequences for resistance management/refugia selection; Fitt *et al*., Chapter 7, this volume). Some species are used as animal fodder.

Based on these considerations, four grass and sedge species groups – *Sorghum* spp., *Pennisetum* spp., *Panicum* spp. and *Rottboellia* spp. – were ranked 1. Because of their additional importance within the push–pull strategy and as possible refuge species for Bt maize, *Sorghum* spp. and *Pennisetum* spp. were analysed further.

BROAD-LEAF WEEDS. Common broad-leaf weeds include *Commelina benghalensis*, *Bidens pilosa*, *Tagetes minuta* and *Senna obtusifolia.* A selection matrix was not constructed because the expertise was not present at the workshop. The species should be included in a full risk assessment of Bt maize in Kenya.

PARASITIC WEEDS. *Striga* spp. were immediately identified as the single most important weed species in those areas where it occurs in Kenya, and as a high priority for testing the non-target effects of Bt maize (Table 5.7). *Striga* spp. occur in the moist mid-altitude zone in western Kenya (Muhammad and Underwood, Chapter 2, this volume). In infested areas, they are highly abundant, widely present and strongly linked to the maize crop (obligatory parasite), and can cause significant reductions in yields.

Table 5.7. Selection matrix for prioritizing weeds associated with maize in Kenya (differentiated for low- and highland maize production systems).

	Maximum potential exposure				Potential adverse effect	
Weed species	Occurrence	Abundance	Presence	Linkage	Significance	Rank
Grass and sedge weeds						
Andropogon spp.	H3/L2	H3/L1	H/L1	General	AH/NE-Reserv.	2
Brachiaria spp.	H2/L3	H1/L3	H/L1	General		3
Cynodon spp.	H1/L2	H2/L3	H/L1	General		3
Cyperus spp.	H/L2	H/L2	H/L1	General		2
Digitaria spp.	H/L2	H1/L2	H/L1	General	Important weed	2
Eleusine spp.	H/L1	H1/L2	H/L1	General		2
Eragrostis spp.	H/L1	H/L1	H/L1	General	Important weed	2
Hyparrhenia spp.	H1/L3	H1/L2	H/L1	General	AH/NE-Reserv.	2
Melinis spp.	H2/L3	H2/L3	H/L1	General	Insect repellent used in PP-strategy	3
Panicum spp.	H/L1	H/L2	H/L1	General	AH/NE-Reserv.	1
Pennisetum spp.	H/L1	H1/L3	H/L1	General	AH/NE-Reserv. used in PP-strategy	1a
Rottboellia spp.	H/L1	H/L2	H/L1	General	AH/NE-Reserv.	1
Sorghum spp.	H/L1	H/L2	H/L1	General	AH/NE-Reserv. used in PP-strategy	1a
Broad-leaf weeds: not completed						
Parasitic weeds						
Striga spp.	L2	L2	L1	High	Damaging, difficult to eradicate	1

H, highlands; L, tropical lowlands; 1, high–likely; 2, intermediate; 3, low–unlikely; AH, alternative host plant to stemborers; NE-Reserv., can serve as a reservoir for natural enemies of stemborers. Some species of these genera are alternative host plants for all of the four most important stemborer species (Khan *et al.*, 1997).

Soil ecosystem functions

Because of the high species and process diversity in plant–soil systems, the selection procedure was pursued simultaneously with a function-based approach for microorganisms and a species-based approach for macroorganisms. Horizontal gene transfer in the soil was not considered in this workshop, but will be in future. A function selection matrix was developed and applied to prioritize soil ecosystem functions (Table 5.8). Exposure to Bt maize material and linkage to plant roots were criteria used to define 'maximum potential exposure'. Criteria related to 'potential adverse effect' were the importance in nutrient cycling for maize production, and as indicator values for soil health.

Higher priority was given to functions involved in the degradation of maize residues (carbon compound and cellulose breakdown) with the coincident release of plant nutrients. The organisms involved in these functions (bacteria, fungi and soil micro- and macroorganisms) may be continually exposed to the Bt toxins. Soil-ecosystem dynamics are limited by energy availability, and functions are responsive to plant inputs. Bt maize may have more lignin in stems than the non-Bt counterparts (Saxena and Stotzky, 2001a), which may lead to slower degradation. This in turn may affect other soil micro- and macroorganisms, e.g. an increase in populations of grey maize leaf spot (*Cercospora zea-maydis*), which survives between cropping seasons on plant residues.

As Kenyan maize fields receive little fertilizer, higher priority was also given to functions essential for nitrogen and phosphate supply. *Phaseolus* bean, with nitrogen-fixing bacteria in its roots, is a primary inter- and rotation crop with maize in Kenya. The quantity and the composition of root exudates probably play a role in the colonization of roots by specific *Rhizobia* spp. and other bacteria (Parker *et al*., 1977). Although maize is not the host plant, changes in its root exudates could affect neighbouring *Rhizobia* spp., and other free-living nitrogen fixers. Mycorrhizas are associated with the provision of phosphate to plants, and are often found in plants grown in low-phosphate or nutrient-poor soils, under a range of tillage systems (McGonigle *et al*., 1999). Maize roots have been frequently shown to be colonized by arbuscular mycorrhizas (AM) (e.g. McGonigle *et al*., 1999; Fries *et al*., 2000), over a large range of climates and soils. AM associations have also been reported to enhance crop yield on the acid sandy soils of West Africa (Bagayoko *et al*., 2000), and in maize cultivation in Kenya (Beatrice Anyango, Nairobi, 2002, personal communication). So it was considered probable that maize–AM associations are important factors relating to crop yield in Kenya. As they have a particularly intimate intracellular association with maize roots, on which they are dependent for carbon, they require evaluation.

There was insufficient expertise at the workshop to complete a selection matrix for soil macroorganisms, but some options are offered. Cry toxins persist associated with plant residues, and so effects could continue after the modified crop has been grown and harvested. Interactions can be complex. It is recommended that soil macroorganisms be prioritized by:

Table 5.8. Selection matrix for prioritizing soil functions for Bt maize in Kenya.

		Potential exposure		Potential adverse effect				
				Importance in nutrient cycling for plant production		Soil health indicator	Impact of adverse effect on maize production	
Function	Indicator organisms	Source of Bt toxin and metabolites	Link to plant roots	Cycle	Importance			Rank
Carbon decomposition	Macrofauna, fungi, bacteria	Residues (leaf, stalk and roots) and exudates	Low	C	Essential	High	High	1
Cellulose breakdown	Fungi, bacteria	Residues and exudates	Low	C	Essential	High	High	1
Ammonification	Bacteria	Residues and exudates	Medium	N	Important	High	Medium	2
Nitrification	Bacteria	Residues and exudates	Medium	N	Important	High	Medium	2
Denitrification	Bacteria	Residues and exudates	Medium	N	Important	Low	Medium	2
Nitrogen fixation	*Rhizobia* spp.	Residues, exudates and root associations	High	N	Essential	High	High	1
Phosphorus and micronutrient uptake	Mycorrhizas	Residues, exudates and root associations	High	P	Essential	High	High	1

1. Abundance – common species may be more likely to play significant ecological roles.
2. Functional significance – e.g. direct consumers of plant residue (e.g. earthworms, beetles, termites, mites, Melolonthinae larvae, e.g. *Schizonycha*, *Phyllophaga*) that degrade large pieces of residue into smaller pieces thereby facilitating/enhancing microbial degradation; organisms that are important for soil physicochemical structure, such as soil macro- and micropores, soil crumble structure (e.g. earthworms, termites).
3. Trophic relationships – predatory or saprophagous soil organisms important for regulation of soil pest species (e.g. predatory mites, nematodes, collembolans).

Findings of Step 2

From a total of 62 species, species groups and functions, 24 were assigned highest priority and analysed further: eight non-target maize herbivore species, five natural enemies of maize herbivores, four pollen feeders, two weed species and four soil functions. The selection matrices proved to be a valuable tool that allowed us to make efficient, transparent, science-based decisions on which species and function to proceed with in the further assessment. The information required to complete a selection matrix depends on the cropping system and local environment, but does not rely on any information associated with the transgenic crop. For soil functions and weeds, the selection matrix was modified to accommodate the particular system characteristics.

Step 3: Exposure pathway analysis

The purpose of this evaluation is to differentiate candidate test species/functions likely and unlikely to be exposed to the Bt toxin, and for the former, to guide the design of the exposure system that should be used in the test protocols (Step 5). Information on Bt toxin expression in maize tissues is not fully available (Andow *et al*., Chapter 4, this volume). Most of the promoters are constitutive, and it is expected that Bt toxins are expressed in all growing maize tissues, including pollen, cobs, silk, roots and root exudates.

Non-target maize herbivores

Because expression information is not available, all herbivores feeding on any Bt maize tissue should be expected to ingest Bt toxin. One exception may be exclusively phloem-feeding insects, such as aphids, because no Bt proteins have been detected either in the insects or in their excreted honeydew when feeding on Bt maize (Head *et al*., 2001; Raps *et al*., 2001; Dutton *et al*., 2002). However, in rice, Bernal *et al.* (2002) found some evidence for presence of Bt toxin in the honeydew of the brown leafhopper. Thus, although expression in the maize plant phloem is probably unlikely, it should be verified in each transgenic Bt crop event in case new events express toxin in this tissue, intentionally or unintentionally. Leafhoppers feed on phloem but also on mesophyll cells that will contain the Bt

toxin in many maize events, and are therefore likely to be exposed (Bosque-Pérez, 2000). They are important transmitters of virus diseases.

LEPIDOPTERAN HERBIVORES. Both *H. armigera* and several *Spodoptera* spp. feed on maize leaves or other plant tissues that will contain Bt toxin. Both species are susceptible to Cry1Ab and Cry1Ac proteins (Hilbeck *et al*., 1998; Dutton *et al*., 2002), although considerably less so than the target pest species. Sublethal or possibly even lethal effects can be expected and should be quantified. Both species spend their entire life cycle on maize, *Spodoptera* feeding primarily on leaves and green tissues, whereas *H. armigera* feeds preferably on maize ears, which may contain lower Bt toxin concentrations.

NON-LEPIDOPTERAN HERBIVORES. Bt proteins are very complex, highly bioactive toxins, and it cannot be excluded that they exert subtle, sublethal effects on non-lepidopteran herbivores (non-target). Hence, direct toxicity from direct exposure to the Bt protein should not be the only criterion for making the final selection of non-target herbivore species to be tested, and it is important to also consider non-lepidopteran herbivores that are important in maize in Kenya for risk assessment. Locusts are defoliators, and spider mites ingest the cell contents of leaves and other tissues where the Bt toxin is expressed; therefore both groups will be directly exposed to Bt toxin (Dutton *et al*., 2002), possibly affecting life cycle and behaviour and therefore population dynamics, and indirect effects are also possible. These species were not evaluated further in the workshop, but should be considered in the complete assessment.

GRAIN FEEDERS. *S. zeamais* and *P. truncatus* feed on the maize cob and kernels and will therefore probably be directly exposed to tissues expressing Bt toxin, both before and after harvest. The amount of Bt toxin in the cob and kernel of each event needs to be determined to check if exposure is likely. Exposure is likely to be long term in storage, even if the dose of Bt toxin is low.

SAPROVORES. *Carpophilus* spp. beetles feed primarily on the frass of lepidopteran herbivores such as *H. armigera*. Raps *et al.* (2001) reported that the frass of a non-target lepidopteran pest, *Spodoptera littoralis* (Boisd.), contained fairly high concentrations of Bt toxins when fed on Bt maize. It is therefore assumed that *Carpophilus* spp. may be exposed to the Bt toxin via the frass of exposed lepidopteran herbivores, such as *H. armigera* and *Spodoptera* spp.

Pollinating and pollen-feeding insects on maize

Domesticated honeybees and wild bees visit maize flowers to collect pollen. In diverse systems, bees can collect from many different plant species. Honeybee foragers and other pollen feeders (e.g. parasitoid wasps such as *Trichogramma* spp., see below; Long *et al*., 1998) regularly visit maize. It is not known but likely that the Bt protein will be expressed in maize pollen with the currently used promoters (Andow *et al*., Chapter 4, this volume). Foraging bees carry the pollen to the bee colony, potentially exposing both larvae and adults to the Bt toxin. Maize plants do not have true nectaries, but they secrete guttation

fluids from vascular tissue sources, which are used by many insects (D.A. Andow, Nairobi, 2002, personal communication), although it is unlikely that these guttation fluids contain substantial Bt toxins. A number of natural enemies, including species of the Coccinellidae and Forficulidae, are also known to feed on pollen as adults and sometimes as larvae. Pollen accumulates on maize leaves at the collar and, if it gets moist, will ferment, with yeasts being a dominant component of the fermentation process. Alternatively, if there is not much moisture, pollen will tend to be scattered on the upper leaf blade and persist there, often colonized by fungi, including some facultative maize pathogens, providing a rich food source (D.A. Andow, Nairobi, 2002, personal communication). Pollen can also be consumed by herbivore species (e.g. butterfly larvae) on other plants around the maize field receiving drifting pollen.

Natural enemies of maize herbivores

Natural enemies can be exposed to the effects of Bt toxin via multiple pathways. It is therefore important to consider not only the primary tritrophic exposure route through the prey or host, but also bitrophic exposure through direct feeding on plant tissues, or feeding on herbivore excretions such as honeydew or frass. A series of questions were developed to facilitate exposure analysis for the *Trichogramma* and *Cotesia* species groups (Box 5.1).

EGG PARASITOIDS OF NON-TARGET LEPIDOPTERA. *Trichogramma* spp. parasitize eggs of lepidopteran species on maize, including *H. armigera*, and are known for their importance as biocontrol agents (van den Berg and Cock, 1993; Sithanantham *et al.*, 2001). The hatched parasitoid larvae feed on the egg contents, pupate inside the egg and leave the egg after adults emerge. *Trichogramma* spp. spatio-temporal occurrence overlaps well with that of their host species. Further data are required to check if lepidopteran spp. feeding on maize also feed and survive on neighbouring uncultivated plants. Recent evidence suggests that *B. fusca* is rarely found on wild plants, and that other maize stemborers are present only in certain weeds, which could prevent parasitoids from finding alternate hosts if Bt maize eliminates stemborers on maize (B. Le Ru, Nairobi, 2004, personal communication). Many unidentified noctuid species exist on wild grasses and sedges (B. Le Ru, Nairobi, 2004, personal communication), and are likely to be alternate hosts to some maize stemborers in some regions, but more information is needed (knowledge gap). In addition, we need to know what happens to parasitoids when maize stemborers are sublethally affected by Bt toxin (e.g. low dose exposure or low susceptibility to the toxin). In general, little information exists on the importance of egg parasitoids in East and southern Africa (identified information gap).

Bitrophic exposure for *Trichogramma* spp. (Box 5.2): Adult *Trichogramma* spp. are usually highly mobile and seek to feed preferably on plant nectar, but also on maize pollen and guttation fluids (Long *et al.*, 1998). Other direct plant tissue feeding has not been reported for these species. If Bt toxin is expressed in the pollen or guttation fluids of Bt maize in Kenya, then adults will be exposed. This potential exposure pathway can be readily evaluated.

Box 5.2. Likely potential exposure analysis for egg parasitoids *Trichogramma* spp. and larval parasitoids *Cotesia* spp., based on questions (Q) and responses given to the best of knowledge of the experts present and the information available.

Exposure analysis for egg parasitoid *Trichogramma* spp. and larval parasitoid *Cotesia* spp.

Bitrophic exposure

Q1: What is the spatio-temporal overlap of *Trichogramma* or *Cotesia* spp. feeding period and the Bt maize growth cycle?

⇒ *Trichogramma* spp.: In most parts of Kenya, maize growth stages overlap largely with *Trichogramma* spp. occurrence.

⇒ *Cotesia* spp.: Whole season, i.e. overlap is complete.

Q2: Does *Trichogramma* or *Cotesia* spp. feed on the parts of the maize plant containing the Bt toxin?

⇒ *Trichogramma* spp.: Adult *Trichogramma* spp. feed on pollen, which could contain Bt toxin, depending on the promoter used. The nectar-like guttation fluid may be fed upon but is unlikely to contain Bt toxins.

⇒ *Cotesia* spp.: Possibly, on pollen (depending on promoter used and on field confirmation that pollen is a food source for *Cotesia flavipes*) and on nectar-like guttation fluid (which is less likely to contain Bt toxin than pollen).

Q3: Is the Bt toxin and/or metabolites detectable in *Trichogramma* or *Cotesia* spp. or its excretions after feeding on the plant?

⇒ *Trichogramma* spp.: Possible, but no data available. We assume exposure through this route negligible or unlikely (see above).

⇒ *Cotesia* spp.: No data available but considered possible if Bt-containing pollen is consumed.

Q4: Does *Trichogramma* or *Cotesia* spp. feed on host products/excretions, e.g. honeydew, faeces?

⇒ *Trichogramma* spp.: Possible, but little data – *Trichogramma* spp. feed on aphid/planthopper honeydew but to date Bt toxins have not been detected in phloem or xylem tissues of maize. *Trichogramma* adults are not known to feed on Lepidopteran faeces.

⇒ *Cotesia* spp.: Possibly feeds on frass of hosts and honeydew from aphids or leaf and planthoppers.

Q5: Is the Bt toxin and/or metabolites detectable in the host products/excretions e.g. honeydew, faeces?

⇒ *Trichogramma* spp.: Unclear whether maize guttation fluids or honeydew contains Bt toxin.

⇒ *Cotesia* spp.: Possibly feeds on frass of hosts and honeydew from aphids or leaf and planthoppers.

Q6: Is the Bt toxin and/or metabolites detectable in *Trichogramma* or *Cotesia* spp. or its excretions after feeding on the host products?

⇒ *Trichogramma* spp.: Unclear. See previous response under Q5.

⇒ *Cotesia* spp.: No data available (but see responses above).

Tritrophic exposure

Q7: What is the spatio-temporal overlap of *Trichogramma* or *Cotesia* spp. parasitism on the host Lepidoptera eggs on Bt maize?

⇒ *Trichogramma* spp.: In most parts of Kenya, oviposition by the host Lepidoptera overlaps through most of the crop growth stages with *Trichogramma* spp. oviposition.

Continued

Box 5.2. *Continued.*

⇒ *Cotesia* spp.: Only certain life cycle stages of the host (third to fifth instar) are suitable for attack, usually during medium crop growth stage. *But* if the crop is at different growth stages in adjacent fields then third to fifth instars could be available all season.

Q8: Is the Lepidoptera host feeding on the plant tissues containing the Bt toxin and/or metabolites?

⇒ *Trichogramma* spp: Yes, *H. armigera* feed on leaves and ears that contain the Bt toxin.

⇒ *Cotesia* spp.: Yes.

Q9: Is the Bt toxin and/or metabolites detectable in the host?

⇒ *Trichogramma* spp.: Possible only if Bt toxin is transferred from caterpillar to adult and from adult to egg (food for *Trichogramma* spp. larva).

⇒ *Cotesia* spp.: Yes, most likely, although no data exist to date specific to the Kenyan case. However, published data exist where Bt toxin concentrations was measured in *Spodoptera* spp. feeding on Bt maize (Raps *et al*., 2001; Dutton *et al*., 2002).

Q10: Is the *Trichogramma* or *Cotesia* spp. feeding on hosts that contain the Bt toxin and/or metabolites?

⇒ *Trichogramma* spp.: Unclear. No data on Bt toxin content of host eggs.

⇒ *Cotesia* spp.: Yes, most likely, although no data exist to date specific to the Kenyan case (see response to Q9 above).

Q11: Is the Bt toxin detectable in the *Trichogramma* or *Cotesia* spp. after feeding on the host?

⇒ *Trichogramma* spp.: Unclear. No data.

⇒ *Cotesia* spp.: No data yet (but see responses above).

Q12: If the Bt toxin is not detectable in the *Trichogramma* or *Cotesia* spp., can it be indirectly affected by Bt maize?

⇒ *Trichogramma* spp: Not certain. It is possible that the plant composition or constituents, which are not of toxicological concern, but of insect nutritional value for the parasitoid, could be affected through altered host-egg suitability for development.

⇒ *Cotesia* spp.: Not certain. It is possible that the plant composition or constituents that are not of toxicological concern, but of nutritional value for the parasitoid, could affect suitability for development through altered host larvae. Additionally, if host larva is lethally affected by the Bt toxin, *C. flavipes* larva will die with the host, increasing *C. flavipes* population mortality rates.

Higher level exposure

Q13: Do *Trichogramma* or *Cotesia* spp. cannibalize their own species and therefore might be exposed to the Bt toxin, its toxins or its effects via its own spp. hosts?

⇒ *Trichogramma* spp.: Not known so far whether *Trichogramma* spp. parasitize their own eggs in a host (gap in knowledge).

⇒ *Cotesia* spp.: *C. flavipes* develops at the expense of *C. sesamiae* (Ngi-Song *et al*., 2001).

Q14: What feeding preference or other behaviour could increase exposure of *Trichogramma* or *Cotesia* spp.?

⇒ *Trichogramma* spp.: changes in oviposition behaviour and oviposition rates to favour Lepidopteran eggs on Bt maize, reactions to changes in host population density.

⇒ *Cotesia* spp.: The hyperparasite *Aphanogmus fijiensis* was identified as a next higher trophic level organism possible being exposed to the Bt toxin via its *C. flavipes* host. Further analysis is necessary to assess what the role of this natural enemy is and whether there is reason for concern if Bt-containing hosts would adversely affect this species.

Tritrophic exposure for *Trichogramma* spp. (Box 5.2): The lepidopteran host larvae are known to feed on Bt maize and ingest the Bt toxin. However, it has not yet been determined whether the ingested Bt toxin is passed on to the lepidopteran adult stage and subsequently to the eggs that the lepidopteran adults produce. This would constitute the most likely tritrophic route through which *Trichogramma* spp. larvae could come into contact with Bt toxins or their metabolites. It is also possible that adult *Trichogramma* spp. could be exposed if the honeydew produced by aphids and leafhoppers, a food source for *Trichogramma* spp. (McDougall and Mills, 1997) is found to contain Bt toxin.

LARVAL PARASITOIDS OF NON-TARGET LEPIDOPTERA. The specialized *Cotesia flavipes* (Cameron) and *Cotesia sesamiae* (Cameron) together account for 83% of the parasitized borers in southern coastal Kenya (Zhou *et al*., 2003). *C. flavipes* is an exotic parasitoid introduced to control *C. partellus*, and will accept equally all four main Kenyan stemborer species; however, it fails to develop in *B. fusca* due to encapsulation of the eggs (Ngi-Song *et al*., 1995), unless the *B. fusca* larva is parasitized by both *Cotesia* species at the same time, in which case *C. flavipes* can develop at the expense of *C. sesamiae* (Ngi-Song *et al*., 2001). Both *Chilo* stemborer species and *Sesamia calamistis* (Hampson) are suitable hosts for *C. flavipes*, though mortality is higher in *S. calamistis*. When third, fourth, fifth and sixth instars of *C. partellus* were exposed to *C. flavipes* females, parasitoid development was least successful in third-instar hosts, and most successful in fifth-instar hosts, and developmental time was longer (on average 24 days in third instar, 16.2 days in sixth instar). In contrast, *C. partellus* is most likely to die when parasitized as third instar and least likely as sixth instar.

Bitrophic exposure for *C. flavipes* (Box 5.2): Where *C. flavipes* occurs, it completely overlaps with the maize growth period. Adult *C. flavipes* are highly mobile, with a lifespan of a few days. They possibly feed on maize guttation fluids or pollen, or plant sap from wounds, as they are known to have a longer lifespan in the laboratory when fed honey (Potting *et al*., 1997). They might also feed on the fresh frass of the stemborer host larvae; however, this must be confirmed by field studies on the feeding behaviour of adult *C. flavipes* in Kenyan agroecosystems. If the Bt toxin is expressed in the pollen, phloem or guttation fluids, adults can be exposed. Stemborer frass has been reported to contain Bt toxins in fairly high concentrations, so it is possible that *C. flavipes* would be exposed via this route (Raps *et al*., 2001).

Tritrophic exposure for *C. flavipes* (Box 5.2): Stemborer host larvae are known to feed on Bt maize and ingest Bt toxin, therefore *Cotesia* spp. larvae are very likely to be tritrophically exposed to Bt toxin and/or metabolites as long as the stemborers can survive on Bt maize.

Maize-associated weeds

An exposure analysis was not considered useful for weeds. The group decided to incorporate exposure analysis into the hazard identification section (Step 4 below).

Soil ecosystem functions

Exposure analysis of soil ecosystems is complex as the organisms are exposed to a combination of degrading and living transgenic plant material and root exudates. Furthermore, the novel transgenic products/proteins will interact physically and chemically with soil constituents such as humic acids, clay minerals and or colloids. Far less is known about the fate of complex organic molecules like proteins in soils than of smaller organic or inorganic chemicals such as pesticides or industrial pollutants. Therefore, the following parameters need to be determined for an exposure analysis.

Firstly, routes of movement and transport of transgenic plant material and products need to be identified (e.g. root exudates, movement of plant residues, including roots, release of proteins from the plant residues, etc.). This requires a good working knowledge of protein expression, the soil–plant interface, and residue movement and management. Bt toxins enter the soil in several ways: (i) via root exudates; (ii) release during senescence; and (iii) via leachates from degrading plant material and injuries.

Secondly, the fate of these plant materials and associated Bt proteins needs to be understood and quantified (e.g. adsorption to clay minerals (Tapp *et al*., 1994), or humic acids, and potential accumulation of the protein (Tapp and Stotzky, 1995b), immobilization or leaching of transgenic proteins). It is essential to know how long the toxin persists in plant residues, and whether it remains active. Saxena and Stotzky (2001a,b), and Saxena *et al*. (2002) reported that active Bt toxin from transgenic maize root exudates and degrading Bt maize biomass persisted in soil for up to 350 days, the longest time studied. In studies that have examined the persistence of Bt toxin from transgenic cotton, the toxin was still detectable when the experiments were terminated after 28–140 days (Palm *et al*., 1994, 1996; Sims and Ream, 1997). Other studies have also reported the persistence of purified Bt toxins in soil for up to 234 days, when the trials were terminated (Tapp and Stotzky, 1995a; Palm *et al*., 1996; Tapp and Stotzky, 1998). Soil micro- and macroorganisms may therefore be exposed to Bt toxin over long periods.

Findings of Step 3

Table 5.9 lists all 16 maize-associated arthropods prioritized for pre-release non-target impact testing in Kenya, with a summary of significance and exposure. For six species or species groups, testing protocols were developed in Step 5. For seven of the arthropod species or groups that were analysed, direct bitrophic exposure to Bt toxin must be expected. For four species analysed there did not exist sufficient information on transgene expression in pollen, nectar or phloem to confirm or refute bitrophic exposure. This re-affirmed the importance of a thorough characterization of the Bt maize for risk assessment (Andow *et al*., Chapter 4, this volume). For *Cotesia* spp., a knowledge gap was identified on adult feeding behaviour. *Carpophilus* spp. and *Cotesia* spp. may be exposed via lepidopteran frass. Tritrophic exposure is unlikely to occur for *Trichogramma* spp., unless lepidopteran eggs or aphid/planthopper honeydew contain Bt toxin or metabolites and are consumed. However, other important

Table 5.9. Summary of non-target above-ground maize-associated arthropods prioritized for testing for non-target impacts of Bt maize in Kenya.

Selected species (above-ground arthropods)	Feeding guild	Rationale	Exposure	Protocol developed?
Helicoverpa armigera	Silk and cob feeder	Significant potential pest	Very likely to be exposed by feeding behaviour, susceptible to toxin	Yes
Spodoptera spp.	Defoliator	Abundant, cause heavy damage	Very likely to be exposed by feeding behaviour, susceptible to toxin	Yes
Spider mites	Defoliator	Abundant, cause heavy damage	Very likely to be exposed by feeding behaviour	No
Locusts	Defoliator	Cause heavy damage when present	Very likely to be exposed by feeding behaviour	No
Sitophilus zeamais	Grain feeder	Significant potential pest, always present	Very likely to be exposed by feeding behaviour	No
Prostephanus truncatus	Grain feeder	Significant potential pest, always present	Very likely to be exposed by feeding behaviour	No
Plant and leafhoppers	Sucker	*Cicadulina* spp. vector of maize streak virus	Likely to be exposed by feeding on mesophyll cells	No
Carpophilus spp.	Saprovore	Vector of *Aspergillus flavus*	May be exposed by feeding on frass of *Helicoverpa armigera*	Yes
Honeybee (*Apis mellifera*)	Pollen feeder	Important pollinator of other crops	Will be exposed if Bt toxin is present in pollen or guttation fluids	Yes
Wild bee spp.	Pollen feeder	Pollination function	Will be exposed if Bt toxin is present in pollen or guttation fluids	No
Coccinellidae spp.	Pollen feeder, larval predator	Important natural enemy	Will be exposed if Bt toxin is present in pollen or guttation fluids	No
Forficulidae spp.	Pollen feeder, egg predator	Important natural enemy	Will be exposed if Bt toxin is present in pollen or guttation fluids	No
Trichogramma spp.	Egg parasitoid	Important natural enemy of lepidopteran species	Will be exposed if Bt toxin is present in pollen, phloem or honeydew, guttation fluids or lepidopteran eggs	Yes
Cotesia flavipes	Larval parasitoid	Important natural enemy of *Chilo partellus*	Will be exposed to Bt toxin in host larvae; may be exposed if Bt toxin is present in pollen or lepidopteran frass	Yes

Continued

Table 5.9. *Continued.*

Selected species (above-ground arthropods)	Feeding guild	Rationale	Exposure	Protocol developed?
Cotesia sesamiae	Larval parasitoid	Important natural enemy of stemborer species	Will be exposed to Bt toxin in host larvae; may be exposed if Bt toxin is present in pollen or lepidopteran frass	No
Predators, e.g. ants, anthocorids, chrysopids	Egg or larval predator	Important natural enemies of lepidopteran eggs and larvae	Will be exposed to Bt toxin in lepidopteran larvae, will be exposed if Bt toxin is present in lepidopteran eggs	No

indirect impact routes or effects on important disease vectors, natural enemies or weeds make it necessary to include species that are unlikely to be directly exposed to the Bt toxin. The identified soil functions will all be exposed to Bt toxin in plant residues and root exudates.

Step 4: Hazard identification and hypothesis development

Hazards relate to the effects of the Bt toxin, its metabolites or any combination effects with plant secondary metabolites on the life history and fitness parameters of the non-target organism, such as development time, survival and reproduction. Behavioural parameters such as preferential host, or host plant and oviposition choices, are also of great ecological importance. From the exposure analyses, hazard scenarios can be identified and used to frame hypotheses relevant for risk assessment. In the following sections, hazards will be identified first, and subsequently the research hypotheses that address the identified hazards will be listed.

Non-target maize herbivores

Eight non-target maize herbivore species are recommended for inclusion in a pre-release non-target effects testing programme on Bt maize in Kenya. All are likely to be directly exposed to the plant-expressed Bt toxins. Identification of hazards and research hypotheses was carried out for the three species below, and was not completed for *S. zeamais*, *P. truncatus*, leafhoppers, locusts or spider mites.

LEPIDOPTERAN HERBIVORES. The hazard associated with these species is that they might become significant secondary pests on Bt maize. Resistance risks associated with them are discussed by Fitt *et al*. (Chapter 7, this volume). *H. armigera* is closely associated with maize when cotton is not readily available. *Spodoptera* spp. are more polyphagous and can survive on a broader range of host plants. Therefore, understanding the possible changes in the fitness of these herbivores with the introduction of a new GM crop will determine their pest status. The following research hypotheses were developed:

1. Non-target lepidopteran herbivores (*H. armigera* and *Spodoptera* spp.) will have higher reproductive rates and/or immature survival on Bt maize.
2. These herbivores will prefer to oviposit on Bt maize.

SAPROVORE AND DISEASE VECTOR *CARPOPHILUS* SPECIES. Aflatoxin infections of maize are a serious health threat for subsistence farmers in Kenya. *Carpophilus* spp. may alter transmission and prevalence of *A. flavus* fungi on Bt maize. Consequently, it will be important to quantify whether the spread of *A. flavus* is increased, decreased or unchanged in Bt maize. The following research hypothesis was developed:

3. *Carpophilus* spp. transmits *A. flavus* from maize ear to maize ear when it consumes the frass of lepidopteran target and non-target species feeding on Bt maize.

Pollinating and pollen-feeding insects on maize

In this section, the focus was on the honeybee, *A. mellifera*, which is an important economic species and essential for pollination in many crops such as fruit trees. Hazards for honeybees can arise at different organizational levels, affecting individual fitness parameters but also the colony as a whole. Feeding on Bt maize pollen expressing the Bt toxin may have subtle, long-term impacts on any of the relevant parameters. Similar hazard scenarios – except on the colony level – are also possible for other pollen-feeding organisms. The following research hypotheses were developed:

4. Individual fitness parameters of pollen-feeding/pollinating species will be reduced by Bt maize compared with the non-transgenic varieties.
5. Bt maize is more attractive to honeybees than non-transgenic maize varieties.
6. Pollinator effectiveness will differ on Bt maize from that on non-transgenic varieties.
7. Colony development will be adversely affected by Bt maize compared to the non-transgenic varieties.

Natural enemies of maize non-target herbivores

PARASITOIDS. The hazard associated with the selected parasitoid species is that Bt maize causes a decrease in their reproductive fitness on both target and non-target pests, thus preventing them playing a role in resistance management (Fitt *et al*., Chapter 7, this volume) and reducing their biocontrol capacity on non-target pests and on neighbouring non-Bt maize. For both parasitoid species groups, bitrophic exposure is considered likely if the Bt toxin is present in pollen, phloem and/or guttation fluids. For the larval parasitoid *Cotesia* spp., tritrophic exposure is also possible. Tritrophic exposure is unlikely to occur for *Trichogramma* spp. but is possible if they consume lepidopteran host eggs or aphid/planthopper honeydew that contain Bt toxin or metabolites.

EGG-PARASITOID *TRICHOGRAMMA* SPECIES. *Trichogramma* spp. parasitize lepidopteran eggs, including *H. armigera* and *Spodoptera* spp. eggs. Plant secondary metabolites (volatile compounds) influence the host search and oviposition behaviour of gravid female parasitoids (Bouwmeester *et al.*, 2003). Such behavioural changes can have a profound impact on their biocontrol capacity (Turlings *et al.*, 1995; Hoballah and Turlings, 2001; Hoballah *et al.*, 2002). Little is known to date about possible changes in the volatile patterns produced by transgenic Bt maize and how this could affect both herbivore and parasitoid behaviour. Further, certain parasitoid species are known to track the population dynamics of their host species in a density-dependent fashion. It is conceivable

that reductions in target and non-target lepidopteran species in Bt maize will lead to a significant decline in egg parasitoids and therefore reduced biological control of other pests. The following research hypotheses were developed:

8. Suitability of host eggs for *Trichogramma* spp. parasitoids is reduced on Bt maize.
9. Oviposition preferences of *Trichogramma* spp. parasitoids are altered on Bt maize.
10. Bt maize is less attractive than non-transgenic maize for *Trichogramma* spp. parasitoids.

LARVAL PARASITOID *COTESIA* SPECIES. Negative effects on *Cotesia* spp. could arise either from direct effects of the Bt toxin in host larvae, or indirectly, through altered nutritional composition of host larvae for *Cotesia* spp. larvae. Overall, parasitoid population density could be affected by the premature death of *Cotesia* spp. larvae if the host larvae die. Sublethal effects on the host larvae could signify reduced value of the host for survival, development and reproduction of the parasitoid. Lepidopteran adult oviposition behaviour is affected by cues from their host plants as *Trichogramma* are (Pivnick *et al.*, 1994). Bt maize could therefore affect parasitoid host-finding ability, thus affecting its biocontrol capacity. In laboratory experiments, female *C. flavipes* are attracted to odours from infested and uninfested maize, sorghum and Napier grass, but the parasitoid is significantly more attracted to maize infested with stemborers than to artificially damaged maize, larvae alone, host frass or uninfested maize, indicating that these parasitoids use both plant volatiles released from damaged maize plants and volatiles from *C. partellus* frass as key host-finding signals (Potting *et al.*, 1993, 1995; Ngi-Song *et al.*, 1996). The production of volatiles attractive to the parasitoids is not restricted to the infested stem-part but occurs systemically throughout the plant (Potting, 1997). Learning does not seem to play a role in host microhabitat location for *C. flavipes*, and no intraspecific variation in host selection behaviour has been found (Potting, 1997). However, different strains did show variation in reproductive success and *C. sesamiae* are unable to locate aestivating hosts (Mbapila and Overholt, 1997). The relative proportions of stemborers parasitized on maize and on wild grasses or other crops could also change (e.g. if maize stemborers are eliminated on Bt maize and cannot survive on nearby wild plants of low host quality), which could have implications for resistance management strategies (Fitt *et al.*, Chapter 7, this volume). In dual choice tests, *C. flavipes* and *C. sesamiae* cannot, however, discriminate between maize infested with *C. partellus* and maize infested with *B. fusca* (Potting *et al.*, 1995). Unidirectional incompatibility, possibly caused by *Wolbachia*, was found between coastal and inland populations of *C. sesamiae* (Ngi-Song *et al.*, 1998), and a *C. sesamiae* population from the coast was found to be infected with *Wolbachia* (Mochiah *et al.*, 2002). *Wolbachia* are known to affect the phenotype of the carrier through several mechanisms, including male killing, cytoplasmic incompatibility, induction of parthenogenesis, feminization and altered fertility. The effect of Bt maize on these complex interactions are

unknown but should be investigated. *Wolbachia* has never been found in *C. flavipes* (A. Ngi-Song, Nairobi, 2002, personal communication), therefore *C. sesamiae* is proposed as the test parasitoid. The following research hypotheses were developed:

11. Bt maize reduces the host-finding ability of *C. flavipes.*
12. Host suitability for *C. partellus* for *C. flavipes* is reduced when hosts feed on Bt maize.
13. *Wolbachia* has different effects on *C. sesamiae* when hosts have fed on Bt maize compared to non-transgenic control varieties.
14. Adult *C. flavipes* feed on maize pollen and guttation fluids under field conditions.

Maize-associated flora

FREE-LIVING WEEDS. While weeds live in association with maize plants, they do not live on or from the maize plants. Therefore, an exposure analysis as for the arthropod food web section was not useful for weeds. Indirect competitive effects are the most likely routes of hazard. With widespread production of Bt maize, the density of some stemborer species may decrease releasing weeds from stemborer damage. Based on the criteria used in the prioritization and decision support matrix (Table 5.7), two weed species, *Sorghum* spp. and *Pennisetum* spp., were selected for pre-release risk assessment, based on the hypothesis that stemborers have a significant impact in their fitness. For example, *C. partellus* has been found to utilize and be widespread on wild *Sorghum* spp. as alternative host plant (B. Le Ru, Nairobi, 2004, personal communication). *Sorghum halepense* (Johnson grass), also native to Africa, is a minor weed in maize in Kenya, but in other parts of the world it is among the most noxious weeds. Since *Sorghum* spp. are an important alternative host for stemborers, it is important to check if a possible decline in the abundance and densities of the stemborer complex in Bt maize and surrounding areas would result in an increased fitness of *Sorghum* weeds and, consequently, in a worsening of their weed status. The following research hypothesis was formulated:

15. Stemborer feeding reduces the fitness of *Sorghum* weeds.

PARASITIC WEEDS. In the previous analysis, *Striga* spp. were identified as a priority test species for risk assessment (Muhammad and Underwood, Chapter 2, this volume). *Striga* spreads only through its seeds, which are moved primarily by humans, particularly in infested soil, contaminated crop fodder and crop seeds. The seeds are tiny and produced in large numbers. They will only germinate under favourable environmental conditions (e.g. correct moisture, temperature) and in the presence of a germination stimulus, usually a root exudate from the crop plant. This stimulant ensures that *Striga*, which stores minimal food reserves in the seed, does not germinate until it has received a signal that a host root is nearby. Various compounds have been identified as stimulants. Strigol is a major *Striga* seed germination stimulant in

maize and a minor one in *Sorghum* root exudates (Siame *et al*., 1993). However, there is no definite proof that one single signal compound or class of compounds induces germination of parasitic weeds in the field. In fact, strigol was shown to act as a germination stimulant in maize but was also found in plant root exudates of medicinal plants where no parasitism by *Striga* was reported (Yasuda *et al*., 2003).

Once stimulated, the *Striga hermonthica* germination strategy is based on the regulation of ethylene biosynthesis (Sugimoto *et al*., 2003). The *Striga* radicle grows directly towards the source of stimulant (Chang *et al*., 1986). The radicle must penetrate the maize seedling within 3–5 days or the *Striga* seedling will perish. Once penetration has occurred, an internal feeding structure (haustorium) is formed, and the parasite establishes host xylem connections. The host photosynthate is then diverted to the developing parasite, which also utilizes the host root system for water and mineral uptake. The relative success of each stage of the life cycle governs the volume of seed production. Any of the steps in this process could be altered by the expression of the Bt toxin or other unintended modifications, e.g. secondary metabolites. There is substantial variation among and between sorghum and maize cultivars with regard to tolerance to *Striga* (Showemimo, 2002); therefore, it will also be important to evaluate cultivar effects in Bt maize. From this, the following research questions regarding possible *Striga* and Bt maize interactions were identified that should be addressed in pre-release risk assessment:

16. Production of biologically active germination stimulant of *Striga* is higher in Bt maize than in non-transgenic cultivars.
17. Bt maize improves the fitness of the *Striga* species compared to the non-transgenic varieties.
18. Bt maize reduces the effectiveness of the *Striga*-control component of the push–pull strategy.

In Table 5.10, the above-ground maize-associated flora prioritized for non-target testing are summarized.

Soil ecosystem functions

A healthy sustainable soil ecosystem requires the maintenance and stability of biodiversity. One potential hazard of Bt maize may be a shift towards particular microbial groups, such as ligninase organisms. Changes in the dynamics of specific functional groups or marker species may relate to changes in functional microbial diversity**.** Organic matter is decomposed by microbial exo-enzymes and Bt toxins may affect the functioning of such enzymes.

Nitrogen-fixing bacteria are not directly associated with maize plants but maize roots are in close association with both legume roots and free-living nitrogen fixers in the soil. So any Bt toxin released from transgenic plants may adversely affect the functioning of nitrogen-fixing bacteria. Similarly, as the nitrogen-fixing bacteria rely on carbon flow from the plants, their functioning rate may be changed when the transgenic plants provide an altered carbon input (pleiotropic effect).

Table 5.10. Summary of above-ground maize-associated flora prioritized for testing for non-target impacts of Bt maize in Kenya.

Selected species	Guild	Rationale	Hazard identification	Protocol developed?
Sorghum spp.	Competitive weed	Significant weed competitor in maize	Alternative host for stemborer – implications for resistance management; currently under biocontrol through stemborer?	Yes
Striga spp.	Parasitic weed	Parasite causing significant crop losses	Possible exacerbation of negative impact on maize yield through effects of Bt maize	Yes

Mycorrhizal associations involve an intimate intracellular physical association between the fungus and plant cells, so intracellular fungal structural development may be affected by the Bt toxins. In mycorrhizal associations, the fungus obtains carbon, for energy, from the plant. Since the plant's physiology has been changed to produce the Bt toxin, the pattern of carbon flow through the root may also be changed. This could have consequences for the growth and function of the fungus, and subsequently on its ability to provide nutrients to the plant.

Functional dynamics in plant–soil ecosystems are highly variable, temporally and spatially, and affected by many factors, such as temperature, rainfall and cultivation. Consequently, in order to be able to detect any effects of transgenic crops, comprehensive baseline data will be required to determine natural variation within comparable soil ecosystems.

So the following hazard scenarios and hypotheses for soil ecosystem functions were identified:

19. Inputs from Bt maize will alter microbial genetic diversity in soils, compared to non-transgenic maize.
20. Organic matter decomposition rates will be slower in soils from Bt maize than in soils from non-transgenic maize.
21. How long does the soil-incorporated Bt toxin from roots and degrading plant materials persist in soils? How much of it persists? How does this vary between different Kenyan soil types?
22. Nitrogen fixation rates, both of nodulated intercropped plants and free-living bacteria in the soil, will be reduced in the presence of the Bt toxins.
23. Is the rate of conversion of plant residues to inorganic nitrogen for plant uptake affected by Bt maize residues in soils compared to non-Bt maize residues?
24. Mycorrhizal fungal development, colonization and subsequent function will be reduced in Bt maize plants compared to non-transgenic maize plants.

Table 5.11 lists the soil ecosystem functions prioritized for the impact assessment of Bt maize in Kenya.

Table 5.11. Summary of the soil ecosystem functions prioritized for testing non-target impacts of Bt maize in Kenya.

Selected function	Rationale	Hazard identification	Protocol developed?
Carbon decomposition	Essential for supplying energy and nitrogen for microbial function; plant organic input dependent	Changed root exudate/residue composition and production in Bt maize may cause changes in carbon decomposition, affecting all other microbial functions	Yes
Cellulose breakdown	Essential for supplying energy and nitrogen for microbial function; plant organic input dependent	Changed root exudate/residue composition and production in Bt maize may cause changes in cellulose decomposition, affecting all other microbial functions	Yes
Nitrogen fixation	Essential for supplying nitrogen for plant production in low-input maize; plant organic input dependent	Changed root exudate/residue composition and production in Bt maize may cause changes in rhizobia stimulation, affecting nitrogen supply for maize	Yes
Phosphorus and micronutrient uptake	Essential for supplying nutrients for plant production in low-input maize on poor soils; plant organic input dependent	Changes in Bt maize may cause changes in mycorrhizal associations and activity, affecting nutrient supply for maize, particularly phosphate	Yes

Findings of Step 4

The exposure analysis from Step 3 was used to develop ecological hazard scenarios and relevant research hypotheses. Ecological hazard scenarios were identified for most of the highest priority species and functions. Some could not be completed in the workshop (e.g. spider mites, locusts) but should be included in a full risk assessment for Bt maize in Kenya. Some relevant risk-related hypotheses for risk assessment of Bt maize in Kenya were formulated.

Step 5: Methods and protocols

Research methods and experiments were developed for most of the potential hazards identified in step 4. This section:

1. Poses the research hypothesis.
2. Provides a brief rationale on why this hypothesis is important for risk assessment.
3. Describes the proposed protocols.
4. Defines the measured endpoints (parameters to be measured).
5. Describes the appropriate statistical analysis.

Non-target maize herbivores

NON-TARGET LEPIDOPTERAN HERBIVORES (E.G. *H. ARMIGERA*, *SPODOPTERA* SPP.). For sublethally affected lepidopteran herbivore species, it is important to find out whether or not their pest status will change on Bt maize compared to the non-transgenic maize varieties. It is also important to understand their role and capacity to induce food-chain effects at higher trophic levels.

Research question 1: What is the level of susceptibility of non-target lepidopteran herbivores to the expressed Bt toxin in transgenic maize? What biological parameters are affected?

Rationale: Information on this question will help to predict whether either one of the two species will become a more damaging or less damaging pest on Bt maize.

Proposed protocols: (i) Laboratory feeding trials using artificial diet containing different concentrations of microbially produced, purified Bt toxin of the same type(s) that is (are) expressed in the transgenic Bt maize, compared to artificial diet without toxin. Five replications per trial using at least 30 first-instar larvae should be carried out. Larvae are allowed to develop through adult eclosion, and at least two consecutive generations are to be tested. (ii) Laboratory feeding trials using greenhouse or field-grown Bt maize and non-transgenic control plants. Ideally, the 30 first-instar larvae are allowed to feed on the intact, growing plants by caging them on leaves or cobs. If no living plants can be used, cut leaf or cob material must be replaced every day, or every other day, to simulate the continuous exposure that the larvae would experience on the plant itself. For all tests with *Spodoptera* spp., use maize plants when they still have 'soft' leaf material, as this is the preferred tissue of this species.

Measured endpoints: Mortality rate, growth rate, stage-specific developmental time, fecundity (indirect measures using pupal weight is acceptable), adult emergence, sex ratio. The first three parameters should be recorded for each larval stage and for the entire immature life stage (hatch of larvae until eclosion of adults).

Statistical analysis: Life history parameters are derived for each treatment, as well as determining LD_{50}. Biological parameters are subjected to analysis of variance (ANOVA) to test for differences between Bt and non-Bt treatments. Insect pest survival/mortality curves can be compared between treatments using the lifetest procedure.

Research question 2: Does oviposition preference of *H. armigera* and *Spodoptera* spp. differ between Bt maize and non-transgenic maize?

Rationale: H. armigera is more closely associated with maize as a host plant, at least when cotton is not readily available, while *Spodoptera* spp. are more polyphagous and can survive on a broader range of host plants. Differential oviposition behaviour will significantly influence the pest status of each species.

Proposed protocols: Plants should be grown in 2–4-l pots, with one seed/plant per pot. At least 75 pots per maize line, i.e. Bt and non-transgenic maize cultivars, should be included. Both choice and no-choice experiments are recommended. For all tests with *H. armigera*, start the experiments when

the plants are at the soft dough stage, which is when *H. armigera* is commonly found on maize plants (see Bernal and Sétamou, 2003, for details).

Choice experiment: At least eight plants per replication and cage are arranged in a circle of 1.5 m in diameter, with transgenic and non-transgenic plants alternating. Gravid *H. armigera* or *Spodoptera* spp. females are released into the cage at the rate of one female per plant, i.e. eight females for eight plants. Plants must be dissected to record the number of eggs per plant within 48 h after release of the females.

No-choice experiment: The same experimental set-up and procedure as above should be used, except only one maize-line will be provided at a time, i.e. either only Bt maize or only non-transgenic maize.

Measured endpoints: Number of transgenic and non-transgenic plants on which eggs were deposited, number of eggs per plant and/or cob.

Statistical analysis: Non-parametric test to discriminate between oviposition of non-target lepidopteran species on Bt maize vs. non-transgenic maize.

Research question 3: How are *Carpophilus* spp. affected by consumption of the frass of *H. armigera* feeding on Bt maize and how does this influence the distribution of *A. flavus* and other fungi and their associated aflatoxin levels?

Rationale: Carpophilus spp. beetles feed primarily on the frass of lepidopteran herbivores such as *H. armigera* and are therefore likely to be exposed to Bt. Aflatoxin infections of maize are a serious health threat for subsistence farmers in Kenya. Thus, it is crucial to understand whether the ability of *Carpophilus* spp. to transmit and distribute *A. flavus* fungi will be altered on Bt maize.

Proposed protocols: H. armigera should be reared on both Bt maize and the control. Both maize lines are to be kept in separate cages. Cobs are to be collected and disinfected using a 0.5% hypochlorite solution for 5 min and thoroughly rinsed afterwards. Infest the cobs with second-instar *H. armigera* and wait until the larvae reach the fourth instar (purple frass produced). Fresh frass from the colonies should be collected every day and 5 mg of frass weighed into a clean capsule. Five *Carpophilus* beetles should be released into each of the experimental containers. *Carpophilus* have to be collected from the field, as it is difficult to raise them in the laboratory. Mortality should be recorded daily. At least 500 individuals should be tested per treatment. One week after infestation, a subsample of 20 individuals per treatment is randomly selected and tested for mobility. Each individual is placed in a large Petri dish and its behaviour observed for 5 min. The time spent in movement and direction of movement is recorded (e.g. video tracking).

Measured endpoints: Mortality rate, growth rate, stage-specific developmental time, fecundity (indirect measures using pupal weight is acceptable), adult emergence, sex ratio. Mortality rate, growth rate and stage-specific developmental time should be recorded for each larval stage and for the entire immature life stage (hatch of larvae until eclosion of adults).

Statistical analysis: Life-test analysis will be used to compare the survival curve of individuals fed Bt vs. non-Bt maize. A *t*-test will be used to compare the time in movement, and the time to 50% or 95% mortality in each treatment.

For studies on *A. flavus* infection and aflatoxin levels, the cobs must be first infested with *H. armigera* eggs at the milk stage (at least two per cob). When the grain reaches the soft dough stage, each line of maize (Bt or non-Bt) will be subjected to four different treatments. The four treatments comprise a combination of: (i) artificial infestation with five *Carpophilus* sp. adults per cob or no infestation; and (ii) inoculation with *A. flavus* spores or no inoculation, in a factorial experimental design. At maturity of maize, five cobs per treatment have to be collected and, from each cob, ten randomly selected kernels taken. The kernels are shelled, mixed and ground for fungal and aflatoxin analysis. At least five replications of 100 kernels are plated per treatment in each maize line and three subsamples analysed for aflatoxin content.

Measured endpoints: The percentage of kernels infected with *A. flavus* and other fungi, as well as the amount of aflatoxin B levels in samples, recorded per treatment and maize type.

Statistical analysis: The percentage of kernels infected with *A. flavus* is arcsine-transformed and aflatoxin levels are log-transformed before analysis. A factorial ANOVA is used to evaluate the effects of both Bt maize and presence of *Carpophilus* sp. on the increase of *A. flavus* infection and aflatoxin levels. The increase in aflatoxin levels due to the presence of *Carpophilus* sp. is calculated via linear contrasts, and a *t*-test used to compare this increase in Bt vs. non-Bt maize.

Pollinating and pollen-feeding insects on maize

Research question 4: Will individual fitness parameters of pollen-feeding/pollinating species be affected by Bt maize compared to the non-transgenic varieties? If yes, how?

Rationale: Possible long-term exposure of pollen feeders/pollinator species has been identified. Protocols are proposed at the individual level for honeybees and for other pollen-feeding species (Forficulidae and Coccinellidae).

(i) Proposed protocols at organismal level: Assessment of anti-metabolic effects of the toxin on immature honeybees can be conducted in laboratory experiments that allow complete control of food source and quality, toxin concentration and distribution, and an optimal environmental situation. Bioassays can be conducted on artificial diet that allows honeybee larvae to develop until maturity (Brødsgaard *et al*., 1998). This provides a reliable method for testing the effects of Bt toxins at the concentrations that one can expect larvae to be exposed to in the field, after pollen incorporation into diet in hives. Larvae are reared in sterile tissue culture multi-wells and grafted daily to new wells with food; handling is reduced to one time per day. Toxins can be mixed into the standard food at the appropriate concentrations. Bee larvae and pupae are monitored once daily until adult emergence.

Measured endpoints: Larval development time is calculated at the 'spinning' stage of the fifth larval instar (when larvae stop feeding and defecate to begin pupation) and at adult emergence. The newly emerged adults are also weighed to investigate possible differences in body mass. It is advisable to use

first instars to start the experiment, as young individuals are likely to be the most sensitive ones to a large array of proteins.

(ii) Proposed protocols for other pollen feeding species (Forficulidae and Coccinellidae): Laboratory feeding bioassays using pollen from transgenic Bt maize and the appropriate control are recommended. Life history parameters should be measured for the whole lifespan, including immature and adult life stages, for two consecutive generations.

Measured endpoints: Mortality rate, growth rate, stage-specific developmental time, fecundity (indirect measures using pupal weight is acceptable), adult emergence, sex ratio. The first three parameters should be recorded for each larval stage and for the entire immature life stage (hatch of larvae until eclosion of adults).

Statistical analysis: Life history parameters are derived for each treatment. Biological parameters are subjected to ANOVA to test for differences between Bt and non-Bt treatments. Insect pest survival/mortality curves can be compared between treatments using the lifetest procedure, as well as determining LD_{50}.

Research question 5: Will attractiveness of Bt maize for honeybees differ from that of non-transgenic maize varieties?

Rationale: Unintended effects of genetic modification might affect the volatile production or flower appearance in plants. The attractiveness of transgenic plants compared to their controls can be measured by observing the pre-foraging behaviour of honeybees.

Proposed protocols: These choice experiments can be done in semi-field conditions (shaded house). In this case, foragers are given the choice between Bt and non-Bt plants. The experiments can also be conducted either at the individual or colony level. For the first case, individual foragers are released in the arena. For the second case, free-living colonies in shaded houses are given the choice of transgenic and control flowering maize plants. Colonies should be checked for infections (e.g. *Nosema*) before starting experiments.

Measured endpoints: For the individual-level experiments, the individual forager bee is observed and her choices tracked and recorded. For the colony-level experiments, visual observations of flower visits have to be made and the number of foragers arriving in a given time span recorded. In both cases, a baseline record of the actual number of flowering plants per unit area is needed.

Statistical analyses: To analyse behavioural data, non-parametric statistical tests should be considered, as these data are generally not normally distributed. A minimum of 100 individuals should be observed. More-detailed behavioural observations might also be conducted, in order to clarify differences during foraging activity, depending on the outcome of the above-proposed experiments.

Research question 6: Will pollinator effectiveness differ on Bt maize from that on non-transgenic varieties?

While insect pollination is not very relevant for maize, it is crucial for the reproductive success and yield of other crops. It is possible to monitor pollination effectiveness both directly (via visual observations) or indirectly (like fruit set). We therefore recommend these activities to be part of the impact assessment for other transgenic crop plants.

Research question 7: Will colony development be affected by Bt maize compared to the non-transgenic varieties?

Rationale: Colony development can be studied. Several indicators of colony response to environmental stressors such as Bt maize can be measured by hive examination (as opposed to adult lifespan).

Proposed protocol at colony level: Smaller size colonies can be prepared to reduce the work needed for experimental observations, allowing more replications to increase the reliability of the results. Colony development can be monitored in closed systems or under semi-field conditions, using a shaded house with a field crop of appropriate size and covered with meshing nets. Each treatment should be in a different unit so that the bees are not to be given the choice between Bt maize and isogenic food. In this way, colonies are only exposed to the appropriate treatment. Maize plants should be the main food source within the shaded houses. We assume the colonies have the same origin and basically the same age and composition.

Measured endpoints: Number of combs occupied by bees and the degree (%) of brooding and occupation of each single comb must be recorded. Larval mortality through regular examination of young larvae from all combs using a dissecting microscope (at least twice per week) should be carried out. Queen fertility and fecundity should also be monitored to assess potential sublethal effects. Pupal weight should be recorded at least on a weekly base. Colony activity should also be monitored by recording (automatically or even manually) the number of flights in and out of the hive in a time span.

Statistical analyses: ANOVA will furnish good estimates of variation sources, provided adequate numbers of observations are made.

Natural enemies of maize herbivores

Whilst we acknowledge that the workshop assessment was not comprehensive across all natural enemies, the focus for the following research protocols is on the highest priority parasitoids in maize in Kenya, *Trichogramma* spp., *C. flavipes* and *C. sesamiae*. However, if modified to accommodate differences in biology, these methods can serve as examples for other non-target natural enemies.

Protocols to test the selected egg-parasitoid Trichogramma *species*

Research question 8: Are there differences in suitability of host eggs from non-target lepidoptera (*H. armigera* and *Spodoptera* spp.) compared with host eggs from target Lepidoptera (*C. partellus* and *B. fusca*) to *Trichogramma* spp. parasitoids on Bt maize or non-transgenic maize?

Rationale: Whilst eggs are not directly exposed, adults that produce them have developed from larvae that consumed the host plant tissues (leaves,

stems) potentially throughout their entire larval stage. Although it is not known whether Bt toxin is transferred to the egg, other unintended physiological changes in the transgenic plants might affect host egg suitability for the egg-parasitoid *Trichogramma* spp. that merits this question. *Trichogramma* spp. are very important natural enemies and any change in their biocontrol capacity would have significant implications for pest control, in particular in subsistence maize production, where farmers have few or no other options. Research should investigate if the eggs resulting from a generation of non-target pest larvae that was highly exposed to the host plant differ in their suitability for parasitism (oviposition and initial development) and progeny emergence (complete development to adults).

PROPOSED PROTOCOLS FOR NO-CHOICE EXPERIMENTS. In laboratory no-choice experiments, parasitization success of the candidate trichogrammatid species can be measured by presenting them with eggs or egg masses from the different pest lepidopteran hosts either fed as larvae on a diet including Bt or on a Bt-free diet. The larvae can be fed on either artificial diet plus Bt maize vs. artificial diet, or on conventional maize plus Bt maize vs. conventional maize. Cut batches of about 40 eggs from the *C. partellus* eggs (laid on pieces of card), *H. armigera* eggs (laid on pieces of cloth), and *B. fusca* and *Spodoptera* spp. eggs (normally laid on strips of translucent paper). The eggs are viable for parasitism for up to 72 h, so must be used for the experiment within 24 h after oviposition. Present the egg batches of each lepidopteran species separately to 1-day-old mated, individual females of each of the chosen trichogrammatid species in 70×20 mm glass vials. Plug the vials immediately with cotton wool, and provide minute streaks of honey (diluted by adding distilled water and gelatine in the ratio of 66:33:1 respectively) on photocopier paper as diet. After 24 h, transfer the eggs to fresh vials and discard the females. The experiment should be repeated with 20 different female individuals (as replicates) for each trichogrammatid species and for each pest Lepidoptera being tested. Keep the vials until the exposed eggs produce either larvae or parasitoids. Remove hatched larvae daily to prevent them from feeding on the parasitized eggs. Keep emerged parasitoids for a maximum of 2 weeks before data is taken.

Measured endpoints: (i) Percentage of eggs parasitized (from a sample of *c.* 20 egg masses per plot received after 3 days of exposure); (ii) number of progeny produced (number of female and male adults in the progeny produced; to be estimated from a sub-sample of 50/100 adults, if there are more progeny).

Statistical analysis: Data are subjected to ANOVA (Proc GLM, SAS Institute, 1988) followed by the Student Newman–Keul mean separation test when the ANOVA was significant ($P<0.05$). The proportion of blackened (i.e. parasitized) eggs is arcsine transformed before being subjected to the ANOVA (Sokal and Rohlf, 1995).

Research question 9: Are there differences in oviposition preferences of *Trichogramma* spp. parasitoids when given the choice between non-target vs. target host eggs on Bt or non-transgenic maize?

PROPOSED PROTOCOLS FOR DUAL CHOICE EXPERIMENTS. This experiment tests host preference of the different *Trichogramma* spp. for non-target lepidopteran species (*H. armigera*, *Spodoptera* spp.) fed on Bt as larvae or not fed on Bt, compared to *C. partellus*/*B. fusca* as the target pest hosts partly fed on Bt as larvae (i.e. sublethally affected) or not fed on Bt. Use only egg masses from first-generation adults whose larvae were raised on Bt or non-transgenic maize plants. This research question could be explored either in a small plot cage exposure experiment (2×2-m fine mesh cages), or, alternatively, without field release permission, it can be carried out in closed, controlled environment systems (greenhouse or climate chamber). Dual choice experiments in the laboratory can be conducted by presenting pairs of egg batches to single mated female *Trichogramma* individuals in vials, as in the no-choice experiment above. The rearing host should be a storage lepidopteran (e.g. *Ephestia* sp.) to avoid any influence of rearing host on subsequent parasitoid behaviour.

Measured endpoints: (i) Percentage of eggs parasitized (from a sample of *c.* 20 egg masses per plot recovered after 3 days of exposure); (ii) number of progeny produced (number of female and male adults in the progeny produced; to be estimated from a sub-sample of 50/100 adults, if there are more progeny); (iii) sex ratio of progeny from each female adult.

Statistical analysis: Data are subjected to ANOVA (Proc GLM, SAS Institute, 1988) followed by the Student Newman–Keul mean separation test when the ANOVA was significant ($P<0.05$). The proportion of blackened (i.e. parasitized) eggs is arcsine transformed before being subjected to the ANOVA (Sokal and Rohlf, 1995).

Research question 10: Will effects of host plant constituents/volatiles on behaviour and performance of adult *Trichogramma* spp. parasitoids differ between Bt maize and non-transgenic cultivars under semi-field conditions?

Rationale: Trichogramma spp. egg parasitoids are known to be more sensitive/responsive to the attributes of host plant than to that of the host insect (pest) (Smith, 1996). It is useful to check if any subtle changes in the physical and/or chemical attributes of the foliage of the transgenic host plant could potentially affect the behaviour and field performance of the adult parasitoids, mainly in terms of parasitism of host eggs.

Proposed protocols: This research question could be explored either in small plot cage exposure experiment, or, alternatively, without field release permission, it can be carried out in closed, controlled environment systems (greenhouse or climate chamber). Test plots of Bt maize and non-Bt maize are planted in close vicinity and should be caged using preferably a fine mesh size and cage size of 2×2 m. The *Trichogramma* spp. egg parasitoid should be a representative species/strain of *Trichogramma* relevant to the ecology of the target stemborer species. One-day-old mated females should be used. To provide for host eggs, adult moths of the species to be compared are released into the caged plots for 24 h to oviposit on the maize plants. They must be removed after that. There should be at least 100 freshly laid eggs in each cage. Subsequently, the freshly laid eggs will be exposed as hosts for 24 h to *Trichogramma* spp. females released into the cages for recording the field

performance. The release ratio is one female *Trichogramma* to 100 freshly laid eggs. They are released for 24 h and then removed. After 6 days, the parasitized eggs become black (nigrescence), and the ratio of parasitized to unparasitized eggs gives the parasitism rate. *Note*: some stemborer eggs can host two *Trichogramma* individuals. Counting the eclosion (exit) holes on the surface of the parasitized eggs after 12 days will give adult number.

Measured endpoints: Percentage parasitized eggs (mean for each exposure) and progeny produced (mean per adult parasitoid released) (see above).

Protocols to test the selected larval parasitoid species C. flavipes *and* C. sesamiae

Research question 11: Does Bt maize alter host-finding behaviour of *C. flavipes*?

Rationale: Host-finding behaviour of *C. flavipes* is complex and influenced by many factors. One important factor is volatiles emitted by the host plant; here this is maize. Both the quantity and the composition of the emitted volatiles influence *Cotesia* spp. host finding (Steinberg *et al*., 1993; Hoballah *et al*., 2002). These are emitted from infested or uninfested plants, respectively. In Kenya, Nwanze and Nwilene (1999) found plant volatile-mediated differential reaction of parasitoid activity to various sorghum genotypes.

Proposed protocols: A colony of *C. flavipes* collected from *C. partellus* in the coastal zone of Kenya should be used. After parasitization, maintain stemborers on artificial diet (Ochieng *et al*., 1985; Onyango and Ochieng-Odero, 1994) at 25°C, 65–70% relative humidity (RH) and 12:12 (L:D) photoperiod. Collect parasitoid cocoons in glass vials and keep in a clean Perspex cage until emergence of adults. Provide adult parasitoids with a 20% honey/water solution as diet. Use 1-day-old, mated, naive female parasitoids in all experiments. Similarly, *C. partellus* collected from maize fields near the Kenyan coast should be used for the experiments. Rear larvae of *C. partellus* on an artificial diet, as described by Ochieng *et al*. (1985). Prior to any experiment, remove stemborer larvae from the artificial diet and feed them on fresh maize stems for 48 h. Grow maize Bt-expressing lines and control lines in 20-l plastic buckets in a nursery. Potted plants can be kept under large field cages (2×2×2 m) covered with fine mesh (400 μm) netting to protect them from insect attack. Additional maize plants are grown in the field.

Behaviour assays can be conducted using a Y-tube olfactometer (described by Steinberg *et al*., 1992). The odour sources are placed in two Perspex chambers (30×30×120 cm) sufficiently large to accommodate whole plants (2–3 months old). One of the square ends of the chamber is left open. For the system to be airtight, the open end of the each box is placed over the test material, which stands in water held in a plastic basin. The open end is submerged 15 cm below the meniscus. The two chambers are connected to the arms of the Y-tube with Tygon tubing from the top of the chambers. An inlet, through which clean air enters the chamber, is drilled 30 cm from the bottom of the chamber on one side. A vacuum pump (Cole–Parmer Air-Cadet) draws and pushes air through the closed system.

Air is pushed through activated charcoal filter into the two chambers and drawn into the Y-shaped glass tubing of the olfactometer. The airflow is set at 2.5 l/min for each arm. Parasitoids are released individually in the stem of the Y-tube and allowed 5 min to choose one of the arms. When the parasitoid remains more than 15 s beyond the finishing line (4 cm past the intersection), it is recorded as a choice. The number of non-responding females is also recorded. The connections of the odour source chambers to the arms of the olfactometer has to be reversed after testing five insects, to rule out the effect of asymmetrical bias in the olfactometer or its surroundings. A cream-white curtain can be used to separate the experimental area from the surroundings. Tests are typically conducted at 23–26°C, 65–75% RH and light intensity of 350–450 lux. Time of day for tests should also be standardized. All tests should be replicated at least three times with 20 parasitoids per replicate.

Different series of experiments can be conducted with this set-up using uninfested or stemborer-infested maize plants.

1. Response to volatiles from uninfested maize plants: Uninfested 8–10-week-old Bt maize and control maize plants should be used in the experiments. All plant genotypes should be tested in single and dual choice experiments, in the Y-tube olfactometer. One series of experiments can be conducted to determine the attractiveness of odours from uninfested plants. Bt maize and control maize are tested in single-choice experiments. Individual parasitoids are given a choice between odour from potted Bt or control maize and air drawn over a pot with soil only. Another series of tests can be conducted to determine the parasitoid's preference for odours from different uninfested maize genotypes (Bt maize vs. control maize). Plants are placed in chambers connected to both arms of the olfactometer. An approximately similar biomass of plants must be used for each arm. Attractiveness of volatiles from potted Bt maize is compared with attractiveness of volatiles from potted control maize. Field materials must be checked for damage before each test and dissected after a test to confirm that it was uninfested.

2. Response to volatiles from plants infested by one stemborer species, *C. partellus*: In the Y-tube olfactometer, parasitoids are given a choice between odours from Bt maize or control maize plants infested with *C. partellus.* Each one is tested against the control (uninfested plants of the same type). Infest potted maize plants with two fourth instars by boring two holes in the maize stem (1 cm deep) with a 4-mm cork borer and placing one larva in each hole. Larvae are allowed to feed overnight and tests are conducted 18–20 h after infestation. Make holes in control plants, without introducing any larvae. Transfer the plants into the chambers connected to the Y-tube in the pot in which they were grown for 30 min before observations are made (to allow time for volatiles to be released in the chamber). Pot-grown plants are used in this test.

A second series of tests can be conducted to determine the parasitoids preference between Bt or control maize plants infested by different stemborer species. The following treatments can be tested:

1. Bt maize infested with *C. partellus* larvae vs. Bt maize infested with *Chilo orichalcociliellus* larvae (repeat for control maize).
2. Bt maize infested with *C. partellus* larvae vs. Bt maize infested with *S. calamistis* larvae (repeat for control maize).
3. Bt maize infested with *C. partellus* larvae vs. Bt maize infested with *B. fusca* larvae (repeat for control maize).

Measured endpoints: The number of parasitoids selecting each odour zone is recorded.

Statistical analysis: Data is analysed via Friedman's one-way ANOVA by ranks, to determine orientation and choice preference of parasitoids.

Research question 12: Is the host suitability of *C. partellus* for *C. flavipes* altered if it feeds on Bt maize?

Rationale: Intended and unintended physiological changes in the transgenic plants might affect host egg suitability for the larval parasitoid *C. flavipes* that merits exploring this question. *C. flavipes* is a significant natural enemy of *C. partellus* in the Kenyan coast and Dry Mid-altitude zones, and any change in its biocontrol capacity would have significant implications for pest control in subsistence maize production where options are limited. Research should investigate whether or not non-target lepidopteran larvae fed with Bt maize differ in their suitability for parasitism (oviposition and initial development) and progeny emergence (complete development to adults) from non-transgenic control maize.

Proposed protocol: Rearing procedures for *C. flavipes* are described by Ngi-Song *et al*. (1996). Insects are kept at 25°C, 65–70% RH and at a photoperiod of 12:12 h (L:D). Adult parasitoids are provided a 20% honey/water solution as diet. One-day-old naive female parasitoids should be used in the experiments. *C. partellus* were reared on a medium developed by Ochieng *et al*. (1985). Prior to experiments, stemborer larvae are fed fresh maize stems for 48 h.

(i) Host suitability and parasitoid efficacy trials in the laboratory: The suitability of *C. partellus* previously fed on Bt maize or control maize for the development of *C. flavipes* is assessed by exposing single fourth-instar stemborer larvae to individual adult parasitoids as described by Ngi-Song *et al*. (1995). Parasitized larvae are then reared on artificial diet in an incubator at 28±1°C, 30–55% RH and a 12:12 (L:D) photoperiod, and inspected daily for mortality or parasitoid emergence (Onyango and Ochieng-Odero, 1994).

Measured endpoints: Brood size, sex ratio, developmental time of progeny (egg to adult), mortality inside the host, female weight and the proportion of hosts from which parasitoids emerged and produced cocoons are recorded. This is compared to the life history factors of parasitoids from the controls as host plants for the herbivorous host of the parasitoid.

Statistical analyses: Data are subjected to ANOVA (Proc GLM, SAS Institute, 1988) followed by the Student Newman–Keul mean separation test when the ANOVA was significant ($P<0.05$). Insect counts are square root transformed before being subjected to analysis (Sokal and Rohlf, 1995). The

data on sex ratio, mortality and proportion of hosts from which parasitoids emerged and produced cocoons are arcsine transformed before being subjected to the ANOVA (Sokal and Rohlf, 1995).

(ii) Host suitability and parasitoid efficacy trials in the field: Experimental plots are planted in a randomized block design with four blocks of two plots each. Each plot consists of 15 rows; rows are 5 m long separated by 0.3-m alleys. The between-plot distance is 5 m within each block and blocks are located 5 m apart from each other. Walk-in cages (2.5×2.5×2.5 m) are randomly placed in each plot at the reproductive stage of maize. The field cages are constructed of Nitex nylon mesh (362 µm mesh size) with a metal frame and 1-m long zippered opening. Each cage contains 24 maize plants (either Bt maize or control maize). Ten plants are randomly selected in each cage and artificially infested with two fourth-instar *C. partellus* larvae, previously reared on artificial diet (with or without Bt toxin at a defined concentration). Two holes are drilled in each stalk, one at 0.5 m above the ground and the other at 1.5 m, and one larva placed in each hole. Holes are plugged with glass vials to prevent larvae escaping. Two days after infestation, the glass vials are removed and ten pairs of 1-day-old *C. flavipes* are released into each cage. Infested plants are cut 1 week after parasitoid release and dissected to recover stemborer larvae. These are then individually reared on artificial diet (no Bt toxin) until formation of parasitoid cocoons or moth pupae (Sétamou *et al*., 2002).

Measured endpoints: The proportions of recovered and parasitized larvae, numbers of parasitoid cocoons, and numbers and sex ratio of parasitoid adults per host will be recorded.

Statistical analysis: Log-likelihood ratio tests are used to compare the percentages of larvae recovered and parasitized, and parasitoid sex ratios between Bt and non-Bt maize. Mean brood sizes per larva recorded in both maize lines will be compared via *t*-tests.

Research question 13: Does *C. sesamiae* have a different interaction with *Wolbachia* when parasitizing herbivores fed on Bt maize or on its non-transgenic control varieties?

Rationale: Wolbachia are known to affect the phenotype of the carrier through several mechanisms, including male killing, cytoplasmic incompatibility, induction of parthenogenesis, feminization and altered fertility. The *C. sesamiae* populations exposed to Bt or control maize via a bitrophic or tritrophic route (see above) are tested for differences in the presence of *Wolbachia* infections.

Proposed protocol: Extract DNA from five individuals of each population that have been stored in 99% ethanol since emergence. Rehydrate individuals by shaking in 100 µl of TE buffer for 1 h. Remove the TE buffer and homogenize the parasitoids using a pestle in an Eppendorf tube (500 µl). Next, add 200 µl of Tris buffer (10 mM Tris, 2.5 mM $MgCl_2$ and 50 mM KCl, pH=7.6), 10 µl of 20% sodium dodecyl sulphate and 200 µl of phenol and zirconium beads of 0.1 and 0.5 mm diameter. Shake the Eppendorf tube in a Bead Beater homogenizer for 3 min and then centrifuge for 10 min. Transfer

the supernatant into a 1.5-ml Eppendorf tube and add 200 µl of chloroform/isoamyl alcohol (24:1). Shake vigorously for 30 s and then centrifuge the vial again for 5 min (14,000 rpm). Transfer the supernatant to a new 1.5-ml Eppendorf tube. Precipitate the DNA with 400 µl of absolute ethanol and 20 µl of 3 M NaAc (pH=4.8) and keep it overnight in a −20°C freezer. Next, centrifuge the Eppendorf tubes for 20 min (14,000 rpm) at 8°C, and wash the precipitate using ice-cold 70% ethanol. Vacuum dry, then resuspend the precipitate in 50 µl of water. Use this solution as the template in the PCR. Concentration and quality of the DNA templates for PCR are checked using the conserved D-extension of the 28S rDNA (Campbell *et al*., 1994). In subsequent tests for the presence of *Wolbachia* in the wasp DNA, approximately equal amounts of template DNA are used. Several primer combinations are used, including the Holden FTSZ primers and the A and B primer combinations of Werren *et al*. (1995). PCR programmes as described by Werren *et al*. (1995) are used. After PCR, the samples are run out on 1% agarose gels to determine if a product of the appropriate size had been obtained.

The above plant-based protocol can also be adapted to test the host-mediated effects of purified Bt toxin, incorporated into the diet of *C. partellus* at a range of sublethal concentrations.

Measured endpoints: Development time, longevity, egg load, fecundity and larval survival of *C. sesamiae* are recorded to discover any change in the influence of *Wolbachia* on the life table statistics of *C. sesamiae*, mediated via the herbivorous host feeding on maize.

Statistical analysis: Data on development time, longevity and fecundity are subjected to ANOVA (Proc GLM, SAS Institute, 1988), followed by the Student Newman–Keul mean separation test if the ANOVA was significant ($P<0.05$). Insect counts are square-root transformed before being subjected to analysis (Sokal and Rohlf, 1995). The proportion of hosts from which parasitoids emerged and produced cocoons are arcsine transformed before being subjected to ANOVA (Sokal and Rohlf, 1995).

Research question 14: Do adult *C. flavipes* feed on maize pollen and guttation fluids under field conditions?

Rationale: This has been identified as a gap of knowledge in the exposure analysis. If *C. flavipes* adults feed on pollen and guttation fluids of Bt maize they will be bitrophically exposed, as well as being tritrophically exposed.

Research protocol: Field observations on the feeding behaviour of adult *C. flavipes* should be made in representative climatic zones (e.g. coastal, highlands) and maize agroecosystems (e.g. small-scale, intercropped, push–pull, intensive large-scale).

Maize-associated flora

The analysis of potential hazards suggests that the effects of Bt maize on germination, competitive ability, weed fitness and compatibility with the push–pull system should be assessed.

Research question 15: What is the fitness impact of stemborer feeding on *Sorghum* weeds?

Rationale: Stemborers also feed on *Sorghum* weeds where they act as biocontrol agents. If overall density levels of stemborers are reduced because of large-scale production of Bt maize this could lead to a release of *Sorghum* weeds from a main natural enemy.

Proposed protocol: Fitness of selected *Sorghum* weeds with and without various levels of stemborer feeding pressure should be tested under field conditions. *Sorghum* weed plants will be cultivated in field plots containing at least 100 plants in a non-competitive field arrangement (depending on soil fertility). The plots should be arranged in a completely randomized block design with a minimum of four replications. This experiment should be repeated at different locations in the target region for Bt maize release(s) where *Sorghum* weeds occur. Varying levels of feeding pressure are achieved by releasing different numbers of stemborer larvae per plant – varying from 0 to 20 larvae per plant. Wild hosts of stemborers do not support high populations compared with maize, so the numbers released have been increased to counter natural mortality on suboptimal host plant species (Ofomata *et al*., 2000). The trials should be conducted with the locally most abundant, economically more important stemborer species known to feed on *Sorghum* weeds. The control treatment will be *Sorghum* weed plots that are protected from stemborer feeding by spraying repeatedly the plants with Bt insecticides. Systemic insecticides could be an alternative, but they will also kill other herbivore species that are not affected by the Bt toxin.

Measured endpoints: It is suggested that three main plant features are analysed: (i) plant growth; (ii) seed and rhizome production; and (iii) propagule viability. Fifteen plants should be collected every 21 days and analysed for: plant height, shoot number, dry matter weight of leaves, stems, rhizomes, and roots, leaf area, rhizome length and bud number, seed number and seed viability.

Statistical analyses: Seed viability should be analysed every 6 months for 2 years after each experiment. Plant growth characteristics should be analysed by using selected growth parameters; e.g. leaf area index (LAI), absolute growth rate (AGR), relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR), specific leaf area (SLA). Seed number, rhizome length and bud number should be analysed using ANOVA. Seed viability should be fitted (time vs. germination index) and the parallelism in the curves be tested by a suitable statistical model.

Research question 16: Does *Striga* germination stimulant production and its biological activity differ in Bt cultivars compared to the isolines and the most commonly used cultivars?

Rationale: Striga seeds will not germinate in the absence of a chemical stimulant. These stimulants can be classified as host root exudate, non-host root exudate, natural leachates/compounds and synthetic germination stimulants.

Proposed protocols: Protocols were taken from the IITA Manual (1997), developed by *Striga* scientists.

Collection of germination stimulants: To prepare stimulants of maize root exudates, the simplest way is to grow plants in a 'double pot' system. This requires two tapered pots of the same size. Perforate the bottom of the one pot and fit it into a second unperforated pot. Plant Bt and non-Bt varieties in sand in the upper pot. Water will percolate through the sand and the holes in the upper pot to collect in the bottom pot. After growing the seedlings for 7–14 days, discard the water in the bottom pot, refill with 25 ml of water and collect the subsequent leachate. This root exudate should be refrigerated and can be used to stimulate *Striga* seed germination. In addition to this root exudate, synthetic germination stimulants (any of the strigol analogs) can be used as checks, since properly conditioned *Striga* seed (see below) should be stimulated to germinate with these compounds. A 10 mg/l solution in water should be used but these compounds first have to be dissolved in a small volume of acetone since they are water insoluble. After dissolving them in acetone, the compounds can be mixed with the final desired volume of water.

(i) Determination of Striga seed germination rate using collected germination stimulants: Striga seeds will not germinate in the absence of a chemical stimulant or unless they have been conditioned (see IITA Manual, 1997, for detailed protocols). After harvest of *Striga* seeds, there is a period of 4–6 months when the seed are truly dormant and generally cannot be conditioned to germinate. After this time period, it takes 7–21 days of exposure to moisture to precondition the seeds so that they will respond to germination stimulant. *Striga* seed surface must be disinfected to eliminate microbial contamination by washing the seeds in a 1% sodium hypochlorite solution. Place the surface-disinfected *Striga* seeds into 30 ml of sterile water in a sterile Petri dish. Stir the seeds to force them to sink. Put the Petri dish in a dark place for 14 days. During this period, the water must be exchanged every 2 days. After this period, spread the seeds on moist filter paper in another Petri dish. A small paintbrush works well to spread the seeds evenly over the surface of the filter paper. After spreading the seeds, add enough stimulant to barely cover all of the seed. After 48 h, screen the Petri dish for germinated *Striga* seeds.

Measured endpoints: Number of germinated seeds is counted. From this, the germination percentage is determined and analysed after arcsine transformation.

(ii) Testing the biological activity of the collected stimulants with maize plants: Use 2-l pots that are perforated in the bottom and cover the bottom hole with filter paper to avoid losses of *Striga* seeds. Fill with clean topsoil to a level of 8 cm below the desired soil surface. Sprinkle the *Striga* seeds on to the soil. Use between 1500–2000 seeds per g of soil (the seed germination must be checked before this trial, protocol see above). Then add the remaining 8 cm of soil. Irrigate the pots carefully after sowing, in order to avoid the *Striga* seeds moving down the soil profile. The next irrigation must be carried out after 4 days. Leave 3 more days without sowing maize. Use between 30 and 50 maize plants of each tested cultivar (at minimum Bt maize and the isogenic control). Sow two to three maize seeds per pot, and 7–10 days after planting thin to leave only one healthy maize plant per pot. Add 20 ml of NPK

(4–14–8) fertilizer diluted in water per pot on to the soil surface at a rate of 60–90 kg/ha. Allow the crop to grow for 5–6 weeks with minimal watering every 2 days. When the first *Striga* emerges, carefully remove the maize plant from the pot and wash the soil off the roots. The roots must not be squeezed because the *Striga* seedling can easily become dislodged from the maize roots. To account for potentially dislodged *Striga* seedlings, the remaining soil in the pot and the soil washed off should be sieved and *Striga* seedlings counted.

This experiment can also be conducted as a small field trial if a permit for Bt maize field release is granted.

Measured endpoints: Numbers of *Striga* seedlings per pot and maize plant.

An alternative testing method for *Striga* is described in Khan *et al*. (2002).

Research question 17: Could Bt maize improve or decrease the fitness of *Striga* spp. compared to the non-transgenic varieties?

Rationale: Above-ground *Striga* mortality and seed production capacity is influenced by *Striga* plant vigour. Therefore, it is useful to measure how vigorous the plant is at various stages and how this is affected by any difference between Bt maize and the control maize. For example, *Sorghum* produces larger *Striga* plants with more flowers and seed capsules than maize. Seed production is a direct result of development of the seed-bearing stalks and organs, which are influenced by a range of environmental conditions as well as by biological factors, such as host plant resistance.

Proposed protocols: The pot experiment or small field trial described in detail above will be repeated, except maize plants will not be removed for *Striga* seedling counting at first emergence of *Striga*. Instead, maize and *Striga* will be allowed to continue their growth until *Striga* flowers and sets seeds. *Striga* plants do not all mature at the same time. They should be collected just as they mature which requires frequent, if possible daily, checking. The most common vigour and fitness measurements include biomass of *Striga*, and height. *Striga* can be harvested, dried and weighed at any stage of interest. Typically, this is done 10 weeks after maize planting. *Striga* height is highly correlated with biomass and capsule number per plant. Harvested capsules should be left drying for 10–14 days. After that, the capsules should be gently threshed by tapping the floral heads on plastic sheeting to force seed shed. After threshing, the material should be screened by passing it through a 150–250-μm sieve. Sieving helps remove most of the plant trash in the seed lot.

Measured endpoints: Numbers of flowers and produced capsules should be recorded for every *Striga* stalk. Total biomass can be weighed fresh and after drying for seed threshing. Collected seeds should be weighed to determine total seed production per *Striga* plant and per maize plant and pot.

Research question 18: Will Bt maize interfere with the *Striga*-control component of the push–pull strategy?

Rationale: One component of the push–pull strategy is to suppress *Striga* infestation. This is largely dependent on the combination of host and non-host plants used. In order to optimize the push–pull strategy and minimize the risk of adverse interference, Bt maize should be tested in the field for its suitability and

susceptibility to *Striga* infestation within the context of the other plants employed in the push–pull strategy.

Proposed protocols: Regular field trials for optimal use of various plants within the push–pull strategy should include one treatment using Bt maize instead of the conventional maize used. Everything else should be conducted according to regular protocols for testing and optimizing that strategy (Khan *et al*., 2002).

Measured endpoints and statistical analyses: According to standard protocols for testing the push–pull strategy (Khan *et al*., 2002).

Soil ecosystem functions

Approaches to soil ecosystem research have tended to look at measures of soil activity such as respiration, nitrification and ammonification, and then to extrapolate these data to known microbial groups associated with that function. More recently, DNA-based molecular tools have opened up new opportunities to look at the dynamics of microbial species. The study of the complexity of soil macroorganisms and their trophic interactions also presents difficulties, but several techniques such as assemblage studies enable adequate surveys.

Changes in microbial community structure caused by transgenic plant inputs are best defined by using molecular techniques such as PCR-denaturing or temperature gradient gel electrophoresis (DGGE/TGGE), terminal restriction fragment length polymorphism (T-RFLP) and mRNA/cDNA studies. These studies are complex and resource consuming, and should be highly targeted. The methods are rapidly improving and will provide powerful tools to precisely assess changes in species composition, both type and numbers, and in functional activity. However, many of these methods are still not routine and require considerable experience to run properly: no one molecular test will give a definitive answer. We therefore consider the best approach is to combine a specific test, either function or species-specific, with a more general microbial activity assessment such as basal or SIR respiration. In all cases, protocol design requires a detailed appraisal of all elements in the system, and constant revision to incorporate novel techniques.

Soil microbial communities

Research question 19: Inputs from Bt maize will alter the genetic microbial diversity in soils compared to non-transgenic maize.

Rationale: Plant inputs drive the soil microbial community. As different plants differ in their system inputs the resultant microbial communities may differ. Bt proteins could either have a toxic effect or be a novel food source. Changes in soil microbial diversity may adversely affect functional dynamics in the plant–soil system.

Proposed protocols: Molecular techniques can describe the microbial population as a whole, constituent parts (e.g. fungi) or functional groups (e.g. nitrifiers). Procedures can be modified to provide more options by using specific primers. These methods overcome all the previous problems inherent in using culture techniques for identification.

PCR-based community-profiling techniques produce information on the structure of the whole community or specifically targeted portions. The primer set used determines specificity (Pennanen *et al*., 2004). Microbial diversity is defined by PCR amplification of whole soil DNA then separation of the DNA bands down a gel by applying techniques such as DGGE/TGGE and/or T-RFLP. With DGGE/TGGE, separation of the 16S fragments is achieved on GC content; whereas with T-RFLP, after endonuclease restriction of the fragment, the labelled terminal fragment is observed in a way analogous to amplified fragment length polymorphisms (AFLPs). These banding patterns can then be compared between and across samples to provide a description of the soil microbial community of each soil (Pennanen *et al*., 2004).

Measured endpoint: The degree of difference is compared to the null hypothesis: there is no difference in biodiversity in soil in which Bt maize has been grown compared with soil in which conventional maize has been grown.

Research question 20: Organic matter decomposition rates will be slower in soils from Bt maize than in soils from non-transgenic maize?

Rationale: The primary step in the functioning of soil ecosystems involves the breakdown of plant residues to provide energy. Microbial functional dynamics are dependent on the quality of this input. As the physiology of the GM plant will be different to that of the non-transformed plant, these inputs will change and so it is necessary to compare function rates between soils receiving GM and non-GM plant inputs. At the same time, nitrogen-containing compounds required for many other microbial functions are released. Approaches to this can be divided into:

1. Assessments of the effects of freshly incorporated, and therefore relatively intact, plant material, stems, leaves and roots.
2. Studying the effects of somewhat degraded material that has been incorporated into the soil-organic matter component of the soil.
3. Effects of the Bt toxins, which are proteins, both as a potential substrate and as a possible toxin.

Proposed protocols:

1. Decomposition rates can be estimated:
   (i) Via loss of organic content from leaf litter confined in nylon bags, with a 1-mm mesh. Bury 10 g of dried leaves from both transgenic and non-transgenic Bt maize in the soil. Collect samples every 20 days, dry at 105°C and ash at 600°C for 4 h. Calculate the loss of organic matter using the equation described by Santos and Whitford (1981).
   (ii) By hydrolysis of cellulose and its derivatives. Cellulolytic enzymes hydrolyse β-D (1–4) glucoside bonds. Estimate cellulase activity by using carboxymethyl-cellulose as a substrate for soils from GM and non-GM plants with five replicates, following the protocol of Gilligan and Reese (1954), and by determining rates of reducing sugar formation using the procedures described in Miller (1959).

2. Microbial biomass (microbial population size) is responsive to inputs, particularly plant residues, exudates, etc.:

(i) Biomass determinations, to show any effects of the Bt crop on the 'standing crop' of microbes, by fumigation extraction (FE) method (Ritz *et al*., 1992); soil samples, 10 g fresh weight, fumigated with ethanol-free chloroform for 18 h at 25°C, control samples stored at 5°C. After removal of the chloroform, soils are extracted with 40 ml 1 M KCl (soil/solution 1:4) on a roller bed (Wheatley *et al*., 1989) followed by centrifugation (2500 *g* for 10 min) and filtration through a Whatman GF/F filter (Ritz *et al*., 1997). Analyse filtrate for dissolved organic carbon in a persulphate reagent using a segmented flow auto-analyser.

Measured endpoint: Biomass carbon is calculated as the concentration of carbon in the fumigated samples minus that in the appropriate control soil. This gives an indication of the microbial biomass, and so whether the various inputs to the soil from Bt maize affect microbial population development differently from the isoline.

3. Microbial activity in soils is limited by availability of energy, and influenced by other inputs such as proteins (Wheatley *et al*., 2001):

(i) Substrate-induced respiration (SIR) indicates the effects of inputs on microbial activity. Use five replicates of each soil from comparable plants, repeated at least five times across the field. Add glucose to 10 g fresh weight of soil to a final glucose concentration of 2000 μg C/g. Incubate in sealed containers for 4 h at 20°C (Anderson and Domsch, 1978). Measure concentrations of CO_2 in the headspace of the incubation bottles using a gas chromatograph fitted with a thermal conductivity detector. Calculate rates of C release per g of soil and compare.

Measured endpoint: Microbial activities in soil are driven by the fixed carbon inputs from plants. These measurements will demonstrate whether inputs from the Bt maize affect microbial activity levels in a different way to the isoline.

Research question 21: How long does the soil-incorporated Bt toxin persist in soils? How much persists, and does this vary between different Kenyan soil types?

Rationale: Exposure to the Bt toxins, exuded from roots and released during the decay of Bt maize plant material may adversely affect soil micro- and macroorganism dynamics. Bt toxins persist in soils, so continual effects must be determined.

Proposed protocols: Soil samples should be collected from each soil type in each agroecological zone of interest, and brought to the research facility for experimentation.

(i) Bioassays with applied Bt toxins: Spray a range of different concentrations of the microbially produced, activated Bt toxin on to the soils. Five replicates should be used, and untreated soil must be included as a control. Take soil samples at 0, 2, 4, 8, 16, 32 and 64 weeks after adding the Bt toxin. Extract and analyse soil samples by ELISA, as described in Zwahlen *et al*. (2003).

Measured endpoints: Decline in Bt toxin concentrations over the 64 weeks, therefore the persistence of the toxin, which has implications in cropping management systems.

(ii) Bioassays on soil in which plants have grown: Grow transgenic and non-transformed maize plants to maturity in greenhouse and field plots, of the different soil types. Any possible effects of the production of the inactive Bt toxin by indigenous *Bacillus thuringiensis* in the soils will be revealed in the control soils. Mix different quantities of soil into a standard artificial, i.e. material from a non-transformed plant, diet for lepidopteran larvae. Allow the larvae of *C. partellus*, the target pest of Bt maize in Kenya, to feed for 4–5 days.

Measured endpoints: Determine the LD_{50} (Saxena and Stotzky, 2001b). This can then be assessed against the long-term implications of cropping Bt-transformed plants on the macrofauna.

If the laboratory or greenhouse trials indicate that the plant material and Bt toxins persist in Kenyan soils, extended, field-realistic testing periods should be considered. If field release trials can be conducted, soil samples should be monitored for at least 3 years of continuous cultivation of Bt maize.

Research question 22: Will nitrogen fixation rates, both of nodulated intercropping plants and free-living bacteria in the soil, be reduced by the presence of the Bt toxins?

Rationale: Nitrogen fixation, the conversion of atmospheric nitrogen to ammonium, by a variety of organisms, is a major function of the nitrogen cycle and a major provider of nitrogen to plants in most agricultural systems. Nitrogen provision from nitrogen fixation is of particular importance in Kenyan intercropping maize systems. Both the nitrogen-fixing bacteria in root nodules and other free-living microorganisms in the soil are driven by energy supplies from the plant, and influenced by signal compounds produced by the roots. So it is important that the effects of GM plants on N-fixation dynamics are compared to non-transformed plants, to assess any negative impact.

Protocol: Remove nodulated roots and attached soil of legumes grown in association with transgenic and non-transformed plants. Assess nitrogen fixation rates of nodules and field soil with the acetylene-reduction method; incubate replicated nodules and soil samples in an atmosphere amended with acetylene, then analyse headspace samples on a gas-chromatograph fitted with a flame-ionization detector.

Measurement endpoint: Relative rates of nitrogen fixation can be deduced from the rates of ethylene formation, to indicate whether the amounts of nitrogen made available to the maize in intercropping systems is affected in any way by the Bt transgene.

Research question 23: Is the rate of conversion of plant residues to inorganic nitrogen for plant uptake adversely affected by Bt maize residues in soils compared to non-Bt maize residues?

Rationale: The recycling of inorganic N from plant residues for further crop production is a very important function of the soil ecosystem. This process

involves the interaction of a vast array of microflora and higher trophic groups of organisms, such as the micro- and mesofauna, in the soil food web. These interactions over a range of trophic levels are particularly relevant in the first step in the nitrogen cycle, ammonification, but because of the complexity of this function, and time and expertise limitations, it was decided to progress this at future workshops and to include a comprehensive review and suggested protocols in the final workshop report. Nitrification is the next step in the N cycle, during which the immobile ammonium form is converted to the mobile nitrate form, with consequent implications for environmental management. As previous work (Wheatley *et al*., 2001) had shown nitrification rates to be particularly susceptible to changes in carbon inputs, particularly proteins, this function was chosen for study.

Nitrification assays: Potential nitrification rates can be estimated by the method of Belser and Mays (1980). Amend 25 g of each soil sample with $(NH_4)_2SO_4$ and $NaClO_3$ solutions to give a final concentration of 4 and 15 mM respectively. Incubate at 20°C for 48 h.

Measurement endpoint: Nitrification rates are calculated from the rate of accumulation of NO_2^-N over time. This will give a comparison of the relative impacts of Bt maize on the system, in particular the rate at which nitrogen becomes available to the plant.

Research question 24: Will mycorrhizal fungal development, colonization and subsequent function be reduced in Bt maize plants compared to non-transgenic maize plants?

Rationale: The fungal structures of mycorrhizas function within the root cells of the Bt maize plant, and so will be continually exposed to the Bt toxins. Therefore, it is conceivable that the Bt toxin or any other physico-chemical alteration in the transgenic plant may adversely affect the efficacy of the mycorrhizal association.

Proposed protocols: Remove roots and associated soil from transgenic and non-transformed maize plants grown in the field, greenhouse and nursery. Carefully separate intact root systems and attached hyphae from the soil by immersing in a tub of water and gently agitating. After washing, keep the root samples moist in plastic bags. If necessary, refrigerate (approximately 5°C) for several days. Process and preserve roots in 50% ethanol in tightly sealed plastic vials for transport and storage (Brundrett *et al*., 1996). The washed roots are cut into small sections, mixed and subsamples removed, and weighed. Make the roots translucent by autoclaving for 15–20 min at 121°C in 10% KOH (w/v), then stained with Chlorazol Black E (CBE) in a lacto-glycerol solution (Brundrett *et al*., 1984) in an autoclave for 15 min at 121°C, or by standing in the solution for several days. Roots can also be stained with trypan blue (Bevege, 1969; Philips and Hayman, 1970). Roots can be destained again by immersing them in 50% glycerol for several days prior to observation, to removes excess stain. Root colonization and root length are measured simultaneously with mycorrhizal colonization by a gridline intersection procedure (Giovanetti and Mosse, 1980) in which roots are randomly dispersed in a 9-cm diameter Petri plate with grid lines. Intersections between gridlines

and roots designated as either mycorrhizal or non-mycorrhizal are quantified with a dissecting microscope. Roots can also be mounted on slides and viewed with a compound microscope (McGonicle *et al*., 1990).

Measured endpoint: Examination of this intimate association between plant and fungus will show if fungal development has been affected by continuous exposure to relatively high levels of the Bt toxins, or to changes in nutrient supply from the roots. Any changes in mycorrhizal function have implications for plant uptake of phosphorus and micronutrients, particularly in low-input systems.

Findings of Step 5

Scientifically rigorous laboratory-based testing methodologies and protocols were designed that addressed most of the hazards and research questions identified for the selected species and functions in the previous step 4.

There are several specific functions involved in the release and transformation of nitrogen for plant growth in soil. All of these are responsive to the gross inputs of plant material, as a source of C and N. The choice of which function occurs is also influenced by substrate availability and type. The rates of these functions can also be affected by relatively small changes in the inputs of specific compounds (Wheatley *et al*., 2001).

It is highly recommended that the maize plant residues be allowed to decompose for a long time in all of the incubation experiments – essentially until they are completely decomposed. Zwahlen *et al*. (2003) reported that Bt toxin was detectable in plant material as long as it was present, in any state of decomposition. Hence, all soil organisms degrading such material will be continuously exposed to the Bt toxin.

A full risk assessment programme for Bt maize in Kenya should include the development of protocols and methodologies for the remaining species that could not be addressed at this workshop.

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