Annex 1: Literature Report Environment

BEETLE

Biological and Ecological Evaluation
towards Long-Term Effects

*Long-term effects of genetically modified (GM) crops on health, biodiversity and the
environment: prioritisation of potential risks and delimitation of uncertainties*

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1.Introduction

The first step in information collection was a structured literature review of published peer-reviewed data. For practical reasons health and environmental aspects were separated within the BEETLE project into different reports. The literature report presented here addresses specifically environmental including biodiversity aspects.

The results of the literature review were integrated into the next assessment steps involving a broader scientific audience: the Peer Review Committee of BEETLE, an online survey and a CREA Space Workshop.

About 700 peer reviewed papers (see References and Annex L1), reports and internet resources were taken into consideration.

The report introduces briefly the used methodology used and presents an evaluation of the literature relevant to identifying processes which might influence the environment including biodiversity in the long term.

The BEETLE study assessed four crops from which GM varieties are on or close to the EU market for the purpose of cultivation and/or import and use: maize, oilseed rape, sugar beet, and potato. Additionally, four crops of which also GM varieties exist outside the EU were considered only in general, since they are currently of lesser relevance for future cultivation in the EU; they are: rice, wheat, soybean, and cotton. Crop/trait combinations with insect resistance (IR), herbicide tolerance (HT), and starch modification (SM) were assessed specifically as these are considered to be the most important genetic modifications within the EU.

The literature report is not a concluding analysis resulting in a prioritisation and identification of uncertainties.

2. Material and methods

2.1 Literature research sources

The literature research was based on various sources related to genetically modified organisms. Starting points were the existing project partners' expertise and internal BVL libraries. Further sources of information were the internet, various library catalogues (e.g. central catalogue of German libraries, International Centre for Genetic Engineering and Biotechnology (ICGEB with more than 5.600 publications), Washington Library of Congress) and online databases (e.g. ISI Web of Sciences, PubMed (National Library of Medicine and the National Institutes of Health)). A list of keywords was drawn up and perpetually expanded during the search process. The 'classical' literature research with the help of recently published review articles was also performed. Additional unpublished data (e.g. reports from pre-marketing experiments, personal communications from scientific and regulatory experts from science and regulation) were analyzed and indicated accordingly in the report. Information resources (AGBIOS[1], USDA[2], US-EPA[3], Canadian Plant Biosafety Office[4], AAFC[5], CSIRO Australia[6]) from countries with longer experience of cultivation of genetically modified organisms were explored, as far as they were accessible. The search results were categorized and managed with CITAVI© (Academic Software Zurich GmbH) reference management software.

2.2 Literature classification according to risk categories

Environmental risk assessment (ERA) is defined in Article 2 (8) of Directive 2001/18/EC as '*the evaluation of risks to human health and the environment, whether direct or indirect, immediate or delayed, which the deliberate release or the placing on the market of GMOs may pose*'. Potential adverse effects on human health and the environment, which may in particular occur directly or indirectly, are accurately assessed on a case-by-case basis taking into account the environmental impact according to the nature of the organism introduced and the receiving environment. ERA is carried out in accordance with Annex II of the Directive and with Commission Decision 2002/623/EC, establishing guidance notes supplementing Annex II of the Directive. Annex II describes in general terms the objective to be achieved, the elements to be considered and the general principles and methodology to be followed to perform the ERA, taking into account the impact on human health and the

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[1] http://www.agbios.com/main.php
[2] http://www.aphis.usda.gov/biotechnology/index.shtml
[3] http://www.epa.gov/agriculture/tbio.html
[4] http://www.inspection.gc.ca/english/plaveg/bio/pbobbve.shtml
[5] http://www.agr.gc.ca/index_e.php
[6] http://www.csiro.au/pubgenesite/research/environment/ecorisk.htm

environment according to the nature of the organism introduced and the receiving environment.

The ERA should be carried out in a scientifically sound and transparent manner based on available scientific and technical data.

The ERA has to consider uncertainty at various levels. Scientific uncertainty usually results from five characteristics of the scientific method:

- the variable chosen,

- the measurements made,

- the samples taken,

- the models used, and

- the causal relationships employed.

Scientific uncertainty may also arise from a controversy on existing data or lack of (some) relevant data. Uncertainty may relate to qualitative or quantitative elements of the analysis. The level of knowledge or data for a baseline is reflected by the level of uncertainty; the notifier of an application for GM plant authorisation needs to provide information that will allow assessment of uncertainty (resulting from, e.g. lack of data, knowledge gaps, standard deviation, complexity, rare events) in comparison with the scientific uncertainties in current practice.

The potential impacts of GM crops on the environment, and especially on biodiversity, have been extensively assessed worldwide over the past 10 years of commercial cultivation of GM crops. According to Sanvido *et al.* (2006) substantial scientific data on environmental effects of the currently commercialized GM crops are available today, and more will be obtained. Hopefully, the research programmes underway in a number of countries may result in a substantial increase in experience regarding the long-term effects of GM crop cultivation. Several controversial issues are related to the interpretation of scientific data (e.g. Greenpeace 2007 versus Schuler 2006). Sanvido *et al.* (2006) relate these issues to the inherent fact that scientific data are always characterized by uncertainties, and that predictions of potential long-term or cumulative effects are difficult. Uncertainties can either be related to the circumstance that there is not yet a sufficient data basis provided for an assessment of consequences (the "unknown", Sanvido *et al.* 2007), or to the possibility that specific negative effects may appear due to unpredictable random events. The latter cannot be excluded for biological systems existing in labile environments. In addition, the uncertainties due to future unpredictable random events are out of reach for scientific methods (the "unknowable", Sanvido *et al.* 2007).

The ERA is performed before marketing of GMOs and the results may not always give definitive answers to all the questions considered because of lack of data. In particular for potential long-term effects, the availability of data is limited or completely missing. However, the aim of the literature reviews within BEETLE is to summarize the latest available literature data on potential long-term effects of cultivation of all the GM crops and traits. For orientation, information on the 9 points as listed in Directive 2001/18/EC (section D2 of Annex II) will be taken into account:

1. Likelihood of the Genetically Modified Higher Plant (GMHP) becoming more persistent than the recipient or parental plants in agricultural habitats or more invasive in natural habitats.

2. Any selective advantage or disadvantage conferred to the GMHP.

3. Potential for gene transfer to the same or other sexually compatible plant species under conditions of planting the GMHP and any selective advantage or disadvantage conferred to those plant species.

4. Potential immediate and/or <u>delayed</u> environmental impact resulting from direct and indirect interactions between the GMHP and target organisms, such as predators, parasitoids, and pathogens (if applicable).

5. Possible immediate and/or <u>delayed</u> environmental impact resulting from direct and indirect interactions of the GMHP with non-target organisms, (also taking into account organisms which interact with target organisms), including impact on population levels of competitors, herbivores, symbionts (where applicable), parasites and pathogens.

6. Possible immediate and/or <u>delayed</u> effects on human health resulting from potential direct and indirect interactions of the GMHP and persons working with, coming into contact with or in the vicinity of the GMHP release(s).

7. Possible immediate and/or <u>delayed</u> effects on animal health and consequences for the feed/food chain resulting from consumption of the GMO and any products derived from it, if it is intended to be used as animal feed.

8. Possible immediate and/or <u>delayed</u> effects on biogeochemical processes resulting from potential direct and indirect interactions of the GMO and target and non-target organisms in the vicinity of the GMO release(s).

9. Possible immediate and/or <u>delayed</u>, direct and indirect environmental impacts of the specific cultivation, management and harvesting techniques used for the GMHP where these are different from those used for non-GMHPs.

Table 1: Environmental risk assessments points to be legally considered and the categories examined within the BEETLE literature report

ERA point within Directive 2001/18/EC, Annex II, section D2	BEETLE literature report category
1 - 3	A, B
4	C
5	D
6, 7	*points are addressed in Health Literature Report (Annex A4 of the BEETLE report)*
8	E
9	F
'Abiotics'	G
'Stacked events'	H

All of the nine fields cited need to be considered in ERA for potential long-term effects as well; however, as the ERA is usually based on short term data, the knowledge of potential long-term effects is inevitably limited. For example, effects related to persistence, selective advantage or disadvantage as well as gene transfer events (items 1 - 3) may become apparent only after longer periods of cultivation.

Based on the experience with GM crops gained during the last decade, potential long-term effects were identified according to the nine points in Section 2.2 above and grouped into the Sections listed in Table 1 accordingly. One aim of the BEETLE project was to summarize the latest available information on the potential long-term effects of cultivating GM crops.

Further, potential interactions with the abiotic environment as mentioned in Annex II (section D) of Directive 2001/18/EC are addressed in Literature Section 3.G. Stacked events are addressed in section H of this document.

Taking into account the aspects to be considered for ERA of GM plants according to Annex II of the Directive 2001/18/EC the literature to be evaluated was differentiated into seven higher categories. The categories selected were:

A) Persistence and Invasiveness

B) Altered Gene Transfer

C) Effects on Target Organisms

D) Effects on Non-Target Organisms

E) Effects on Ecosystem Functions

F) Cultivation and Management

G) Effects on the Abiotic Environment

Furthermore these categories were differentiated into 26 processes and 62 scenarios related to the specific categories. The processes level represents the level of literature evaluation.

Table 2: Environmental categories and processes with potentially adverse long-term effects identified in the BEETLE literature study

Categories		Process or effect	
A	Persistence and Invasiveness	**A.1**	Increased fitness of the GM cultivar
		A.2	Outbreeding depression after hybridization with wild relatives
		A.3	GM crop/feral/wild hybrid long-term persistence
B	Altered Gene Transfer	**B.1**	Potential reduction of pollination
		B.2	Altered flower phenology
		B.3	Altered compatibility reducing or favouring outcrossing
		B.4	Altered fecundity increasing seed (gene) flow
		B.5	Increased frequency of horizontal gene transfer
C	Effects on Target Organisms	**C.1**	Effects on target pathogens
		C.2	Effects on target pests
D	Effects on Non-Target Organisms	**D.1**	Direct toxic effects on plant-associated NTO
		D.2	Effects on NTO due to altered nutritional composition of the GM plant
		D.3	Tritrophic interactions on NTO
		D.4	Effects on NTO due to accumulation of toxic compounds
		D.5	Effects on rhizosphere microbiota
		D.6	Effects on symbiotic NTO
E	Effects on Ecosystem Functions	**E.1**	GM traits affecting changes on soil functions
		E.2	Effects on biological control
		E.3	Are GM traits causing changes in pollination?
F	Cultivation and Management	**F.1**	Altered use of agrochemicals
		F.2	Indirect changes in susceptibility of crops against plant pathogens
		F.3	Adverse effects on agro-biodiversity
		F.4	Potential changes in fertilizer use
		F.5	Potential changes in landscape structure
G	Effects on the Abiotic Environment	**G.1**	Increased production of green house gases
		G.2	Increased mineral nutrient erosion and fertilizer leaching
		G.3	Altered chemical attributes of soil fractions

3. Potential long-term effects

A. Effects on persistence and invasiveness

There is a potential for interspecific hybridization, ferality and gene flow as pathways for environmental spread of GM crops in the EU. Recombinant genes introduced into crops conferring resistance to crop stressors could potentially lead to increasing fitness of the GM crop itself as well as of potential GM hybrids or of potential feralized GM crop plants (Chapman & Burke 2006, see also the review of Gressel 2005). Feral plants (in contrast to crops) can reproduce and persist on their own, without being dependent on managed cultivation. According to Gressel (2005) feral plants are more likely to evolve from volunteer weeds (plants that germinate in seasons after a crop had been cultivated) than from crops producing seeds before or during harvest.

In view of persistence and invasiveness, the first step is to look at potential ways of environmental spread including ferality and gene flow. The second step is the assessment of the potential consequences of persistence and invasiveness in a given GM plant species (Saeglitz & Bartsch 2002).

Gene flow (including seed flow plus flowering and pollination) and the formation of hybrids is a prerequisite for any transfer of a trait being beneficial for fitness to non-target crops of the same species or to potential hybrids (Ellstrand 2001; Snow *et al.* 2001; Snow *et al.* 2005).

In general, spontaneous (interspecific) hybridization between vascular plants has been documented worldwide for decades. Gene flow seems to be rather the rule than the exception for specific crop species, and information is based on evidence for gene flow between the crop and its wild relatives (Raybould & Gray 1994, Darmency *et al.* 1998, Ellstrand *et al.* 1999, Ellstrand 2001, Hails & Morley 2005, Allainguillaume *et al.* 2006, Simard *et al.* 2006). The rate of hybridization between different populations is highly dependent on gene flow and pollination parameters, like amount of pollen produced by the potential hybridization partners, rate of self-fertilization, duration of pollen fertility, concordance of flowering, success of fertilization, degree of relationship of the partners, climatic parameters, and the distance between the potential partners. Under environmental conditions all these parameters may affect hybridization between different partners (Ellstrand 2003a, b).

Two crop species are of particular interest in the EU: Feral populations of oilseed rape are common weeds of disturbed habitats including fallow land (Crawley & Brown 1995, Cureton *et al.* 2006). The same applies to sugar beet e.g. in south-western France and in UK (van Dijk 2004, Cureton *et al.* 2006, Darmency *et al.* 2007). In addition, these two major target crops for genetic modification in the EU are known for their ability to transfer new traits by

outcrossing to wild relatives. For both sugar beet and oilseed rape Europe is part of their center of origin (Zohary & Hopf 2000, Sukopp *et al.* 2005, Hall *et al.*, 2005).

Differentiations are necessary concerning the potential for outcrossing and hybridization with wild relatives in view to the five crop species (oilseed rape, sugar beet, potato, maize, soybean) taken into consideration more specifically in this review.

Oilseed rape (*Brassica napus*)

A number of hybridization studies are available for oilseed rape and its wild relatives. *Brassica napus* is able to cross with its two genetic ancestors *Brassica rapa* and *Brassica oleracea*. The allotetraploid doubled oilseed rape genome (AACC; 2n = 38) consists of the AA genome (2n = 20) of *B. rapa* and the CC genome (2n = 18) derived from biennial *B. oleracea* (Warwick *et al.* 2003, Chèvre *et al.* 2004). Thus, these three species can be regarded as an important crop/weed/wild plant complex for the potential environmental spread of GM traits.

In most published hybridization experiments concerning GM oilseed rape outcrossing experiments were performed with *Brassica rapa* since populations of both species in Western Europe grow sympatrically at several locations in Western Europe. Additionally, populations of wild *B. rapa* (as weeds as well as wild populations) are more common than wild *Brassica oleracea* populations (Wilkinson *et al.* 2003). Hybrids from *Brassica napus* and *B. rapa* have been studied in more depth in Denmark and the UK (Jørgensen & Andersen 1994, Ammitzbøll *et al.* 2006). However, from the seeds produced on *B. rapa* in UK less than 2% were hybrids (Scott & Wilkinson 1998). Wilkinson *et al.* (2003) estimated the formation of a total of about 49.000 *B. napus* x *B. rapa* hybrids per year for the whole area of the UK. Allainguillaume *et al.* (2006) studied the fitness of spontaneous F1 hybrids between oilseed rape and *Brassica rapa* as well as of F2 hybrids between F1 offspring and *Brassica rapa* (first backcross). The authors estimated a number of about 7.000 second generation hybrids (i.e. ~ 20% of F1 hybrids) occurring every year in UK. These would be transgenic if the crop crossing partner would express a GM trait. F2 offspring usually is of higher fitness than F1 since proportion of wild type genes have increased. The frequently reported long distance transport of oilseed rape pollen by insects may partly be involved in hybridization success (Rieger *et al.* 2002).

Genetically modified *Brassica rapa* hybrids are likely to become invasive if they confer a fitness increasing trait. Although Allainguillaume *et al.* (2006) estimate that there is a natural biological containment as the fitness of hybrids between *B. napus* and *B. rapa* often is declined, there is scope for certain GM traits to increase their own frequency. It is common

sense in ERA that if gene flow is possible, the consequences of introgression and on invasiveness need to be addressed (e.G. EFSA 2004).

There is evidence that populations of oilseed rape as well as hybrids between oilseed rape and *Brassica rapa* may persist outside of fields independent from the nature of the genetic modification (Pivard *et al.* 2007, Knispel *et al.* 2008). However, any fitness advantage derived from new traits, which would enhance the chance of gene flow to wild relatives, could increase the chance for persistence in nature. This is again an argument for assessing not only gene flow as such but more importantly the consequences of such gene flow.

It is striking that European publications on *Brassica napus* and its hybrids are regionalized. Most studies were carried out in northwest, central and western Europe presumably indicating the regional importance of oilseed rape cultivation as well as of the hybridization aspects. However, studies on hybridization of *Brassica napus* x *Brassica rapa* are very rare (Darmency 2008, pers. comm.) when looking at France.

Sugar beet (*Beta vulgaris* ssp. *vulgaris*)

The ancestors of cultivated sugar beet (*B. vulgaris* ssp. *vulgaris*) are the sea beets (*B. vulgaris* ssp. *maritima*) naturally growing along drift lines of sea shores. Since wild sea beet and cultivated varieties have the same ancestor and sea beets are still used as plant genetic resources in breeding programmes, both forms are often difficult to distinguish (Ford-Lloyd & Williams 1975, Cureton *et al.* 2006). Cultivated *B. vulgaris* varieties, in contrast to wild varieties, are biennials, growing in the year of cultivation merely vegetatively as a result of breeding and selection. If the crop is not harvested in the first season, flowering usually occurs after the winter season. However, sugar beets are known as frost-sensitive species. Interestingly, the life span of wild sea beet exhibits very different periods. Wild sea beet may survive up to 11 years in Northern Brittany; whereas in Italy and south-western France the life time span ends usually after the second season (van Dijk *et al.* 1997).

Premature flowering of cultivated varieties may occur after sowing in spring due to low temperatures acting as late vernalization effect (Lavigne *et al.* 2002, Sukopp *et al.* 2005). In addition, sugar beets are capable of outcrossing to any other cultivated forms of *B. vulgaris* like red beet, leaf beet or table beet. Feralized annual sugar beets (weed beets) are known as volunteers growing in fields and as feral populations growing in disturbed habitats in areas with maritime climate, and gene flow from GM beet has experimentally been proven up to 300 m (Saeglitz *et al.* 2000, Darmency *et al.* 2007).

In summary, gene flow may occur (i) between flowering cultivated beets and wild beet in coastal beet breeding areas, (ii) after vernalization of living vegetative parts remaining on fields after harvest, (iii) by late vernalization of seedlings after sowing in spring, (iv) from

adventitious bolters occurring during cultivation (Bartsch *et al.*, 2003, Sukopp *et al.* 2005). GM traits could potentially be transferred from cultivation areas far away from natural sea beet habitats by weed beets acting as bridging plants (Fénart *et al.* 2007).

A close relationship to maritime climates in Europe is obvious for both oilseed rape and sugar beet crop/wild plant complexes.

Potato (*Solanum tuberosum* ssp. *tuberosum*)

Potatoes have been cultivated in Europe for about 300 years; and today they are a widespread crop used throughout Europe. The organs of interest are the tubers produced in the soil. Tubers are capable of vegetative reproduction (seed tubers) and sexual reproduction is possible via flowering. The known self-pollination rate is 80-100%. In addition, many cultivars show a reduced fertility (OECD 1997). Pollen mediated gene flow is only possible with other varieties of the cultivated potato species (*Solanum tuberosum*). Pollen transport is mostly limited to 3 m, and maximum distances are 10 m (McPartlan & Dale 1994). No gene flow has been found with the potato's wild relatives *Solanum nigrum* and *S. dulcamara* in the field (Eijlander & Stiekema 1994, McPartlan & Dale 1994, Conner 1997). However, very low frequency of hybridization is possible with *S. nigrum* under artificial conditions (OECD 1997). No data are available on potential transfer to other *Solanum* species, e.g. to *S. eleagnifolium*. In summary, the chances of successful hybridisation between cultivated *S. tuberosum* and other *Solanum* species are very unlikely based on empirical knowledge and literature reports.

Considering ferality, potato tubers and seeds are frost sensitive. They are destroyed by a frost period of 25 h at -2°C or by a frost period of 5 h at -10°C (OECD 1997). More recent papers are not available. In applications for deliberate releases of GM starch potatoes the applicants frequently report on potential altered winter survival of the new potato lines. There is no report on alterations in frost-hardiness or overwintering for amylose- or amylopectine enriched lines according to the results mentioned in the internal annual reports to BVL as national competent authority.

Maize (*Zea mays*)

Maize has been introduced to Europe - similar as potato - about 300 years ago. It is a highly domesticated annual plant and cannot survive temperatures below 0°C for more than 6 hours after having reached the 5-leaf stage; before reaching this stage the susceptible growing point is still below soil surface (OECD 2003). Survival of maize inside and outside cultivation in Europe is mainly limited by a combination of poor competitive ability, absence of a dormancy phase, susceptibility to diseases and to cold climate conditions. Maize is an open-pollinated cross-fertilizing species - the self-pollination rate is about 1-5% - and thus the

movement of genetically modified traits is not easily controlled. Maize pollen grains being about 90-100 µm in diameter are among of the heaviest and largest wind-dispersed pollen grains (Ma et al. 2004). Maize pollen has the potential for movement over great distances like any other biotic or abiotic particle of similar size and weight, mostly depending on wind conditions (Eastham & Sweet 2002, Henry *et al.* 2003, Devos *et al.* 2005). However, the longevity of maize pollen viability strongly differs according to air temperature and humidity. Under natural conditions the time span maize pollen keeps viable is ranging from 24 hours to several days. However, the pollen may be killed in hot dry weather after 1–2 hours: viability declines quickly with desiccation (Emberlin *et al.* 1999, Luna *et al.* 2001, Owen 2005).

According to Emberlin *et al.* (1999) percentages of airborne pollen concentrations downwind compared with concentrations at 1 m from the source are approximately 2 % at 60 m, 1.1 % at 200 m and between 0.75 and 0.5 % at 500 m. However, such numbers are just rough estimates since dispersal gradients would be altered by climatic conditions and local topography. After dehiscence pollen is maintaining viable for 1 - 2 hours depending on atmospheric humidity (Luna et al. (2001). In addition, gene flow may also occur via seeds (Abbott *et al.* 2003, Snow 2002a, b).

No wild relatives occur in Europe since maize is the only representative of the genus Zea. Vertical gene transfer is limited to other cultivated maize plants (OECD 2003).

Domesticated maize plants have lost their ability to survive in the wild and are not invasive. Due to seed collection in cobs maize needs human intervention to disseminate seeds (OECD 2003). However, as a result of the harvesting process individual kernels of maize are distributed in fields resulting in volunteers appearing in fields; sometimes due to transport losses volunteers also appear in roadsides. But maize never was able to establish outside of cultivation since under natural conditions seed dispersal from the cobs will not occur (Gould 1968). In Europe outcrossing to weedy wild relatives is impossible. Consequently additional introduction of wild traits being typical for weedy species will not occur.

However, the occurrence of maize volunteers in fields has been occasionally reported in Europe. Factors favouring maize volunteerism are harvesting techniques, infestations and tillage (Owen 2005). Volunteers of HT maize could lead to management problems if the GM maize is grown in rotation with other HT crops being tolerant against the same herbicide. This problem may increase if other HT crops also are cultivated in rows like HT soybean or HT sugar beet. Feral maize is unlikely to occur since the collection of seeds in cobs avoids seed spillage and is minimizing the ability to establish wild populations.

<u>Soybean (*Glycine max*)</u>

The cultivated soybean species *(Glycine max)* belongs to the subgenus *Soja* of the genus *Glycine*. The species originated from eastern Asia and is a highly domesticated crop (Lu 2005). In Europe, soybean is mainly cultivated in Italy, France and Romania and less in Hungary (FAOSTAT 2005). Weedy soybean has not been reported growing naturally outside its centre of origin. This holds true for areas such as the Americas and Europe, where only cultivated soybeans are found (Lu 2005).

Seed and pollen are potential sources of gene dispersal. However, soybean (*Glycine max*) is an annual almost completely self-pollinating crop in the field with a percentage of cross-pollination usually lower than 1% (Weber & Hanson 1961; Caviness 1966; Lu 2005).

<u>Evaluation criteria</u>

For the evaluation of the process-related results gained from the literature review these results were ranked applying criteria listed in Table 3. Based on the likelihood assessment, the final results were integrated in the next assessment steps involving a broader scientific audience: the Peer Review Committee of BEETLE, an online survey and a CREA Space Workshop.

Table 3: Likelihood options in the literature surveys and criteria applied by the BEETLE team for assessment

Likelihood of process	BEETLE team criteria
negligible	The likelihood of this process causing adverse long-term effects are negligible (can be excluded with a high confidence based on data available in the literature).
low	The likelihood of this process causing adverse long-term effects are possible, but to a low extent, and the process is only moderately important as a potential cause of environmental harm.
high	The likelihood of this process causing adverse long term effects is possible to a relatively high extent, and the possible effects on biological processes and / or ecosystem integrity could potentially be severe.

A.1   Increased fitness of the GM cultivar

Fitness is defined as the 'contribution made to a population of descendents by an individual <u>relative</u> to the contribution made by others in its present population'. It is the relative contribution that an individual makes to the gene pool of the next generation (Begon *et al.* 2005).

Fitness is thus a relative and not an absolute term. It depends specifically on the biotic and abiotic environment for a given plant species or plant population. Various numbers of stressors affect growth, yield, and reproduction of GM and non GM crops. Important environmental stressors are: weeds competing for space and nutrients on fields, pests feeding on crop plants like dwelling insects, infectious bacteria, fungi or viruses (Chapman & Burke 2006). Consequently, fitness improvement might be provided by any transgene that affords protection against the stressors mentioned. In addition, traits conferring advantages for crop cultivation under detrimental abiotic conditions would also have the potential for enhancing fitness (Hails & Morley 2005, Snow *et al.* 2005).

Where the genetically modified crop does not have any wild relatives in Europe, the assessment is simplified: only the fitness effect of the GM traits on the crop itself requires an assessment. Matters of environmental importance are e.g. occurrences of volunteer or feral populations.

Where the GM crop has - under natural conditions crossable - wild relatives in Europe, three types of populations need specific attention: (i) feral non-GM crop plants of the same species as the cultivated GM crop growing as volunteers in fields, (ii) wild relatives in (disturbed) rural areas close to cultivated fields, and (iii) wild relatives occurring in natural areas within reach of GM crop fields.

Abiotic stress tolerance and fitness

There are attempts to improve abiotic stress tolerance in GM crops. Literature reports are primarily available for the improvement of crop tolerance against salinity or drought, or tolerance against herbicidal agents.

Increased tolerance against salinity or drought

Drought and salinity are important environmental constraints to crop productivity in arid and semi-arid regions of the world. Currently, there are several attempts to improve stress tolerance in major crops like maize, potato, and soybean (Huang *et al.* 2000, Ahmad *et al.* 2007, Wu *et al.* 2008, Xue *et al.* 2007). The potential ecological impact of increased stress tolerance for sugar beet was discussed by Bartsch *et al.* (2003), but no experimental data on potential drought or salinity resistant GM sugar beet are yet available.

At the same time, basic research has been constantly carried out with model plants, e.g. GM plants exhibiting increased tolerance to salinity are developed for tomato (Foolad 2004) or perennial ryegrass (*Lolium perenne*; Wu *et al.*, 2005). In both cases the gene used is expressing a vacuolar anti-porter protein controlling Na^+/H^+ antiport derived from *Arabidopsis thaliana*. In potato drought resistance is achieved by introduction of the trehalose 6-phosphate synthase (TPS 1) gene from *Saccharomyces cerevisiae* (Yeo *et al.* 2000). By

introducing additional trehalose level controlling genes tobacco plants were genetically modified for purposes of achieving drought tolerance (Karim *et al.* 2007). Genetically modified plants overexpressing trehalose-6-phosphate synthase (TPS) for achieving drought tolerance often are not sufficiently protected against drought stress, which is related either due to short-term trehalose accumulation or due to pleiotropic growth aberrations. A better drought tolerance was achieved by introducing a double construct consisting of TPS and trehalose-6-phosphate phosphatase (TPP) from *Sacchoromyces cerevisiae* and combining it with specific regulatory elements from *Arabidopsis thaliana*. In this case plant growth was normal. Drought tolerance resulted from a still unknown water retention effect that was not found in the non-transformed varieties.

<u>Increased tolerance against herbicides</u>

Genes conferring tolerance against specific non-selective herbicides like glyphosate and glufosinate-ammonium are currently among the most common traits introduced into GM plants. When looking at herbicide tolerant maize, soybean, oilseed rape or sugar beet varieties, the tolerance trait will offer selective advantages if the complementary non-selective herbicide is applied due to minimizing competition from weeds. It is likely that volunteer HT plants will occur in successive years on the cultivation fields, and first long-term experience (>10 years) has been gained for HT oilseed rape in Canada (Warwick *et al.* 2007). However, the problem of herbicide-tolerant volunteers would be of lesser importance if GM crops were cultivated in rotation expressing different herbicide tolerance traits or if one specific HT crop were cultivated with non-herbicide tolerant crops in rotation.

The situation is more complex when looking at potential herbicide resistance development of weeds: Resistance development is not only dependent on frequency of the herbicide application in the rotation. In addition, aspects like applied tank mixture, dosage of active ingredients, type of cultivated species could also lead to resistance development.

Today, there are three different HT systems available on the market: Two with introduced GM tolerance (glyphosate and glufosinate-ammonium) and one with an herbicide tolerance system based on non-GM technologies (Imidazolinone – Clearfield technology).

In principle all HT varieties (including HT oilseed rape or HT sugar beet) are likely to have also growth advantages on disturbed ground outside the cropping field if the specific herbicide is applied there. According to the specific national herbicide regulations this may apply for field and road verges or for railway tracks.

A rotation using several GM HT crops puts an increased selection pressure on weed populations in fields, with an increased likelihood of evolving herbicide resistance (Chapman and Burke 2006). Currently, herbicide resistance to glyphosate has been observed for weeds

like *Lolium rigidum* by Powles *et al.* (1998) and for *Conyza canadensis* by Koger *et al.* (2004). Up to now, more than 9 non-GM wild plants (weeds) resistant to glyphosate have been confirmed on a worldwide scale (Heap 2008, Powles 2008).

The phenomenon of development of weed resistance against (conventional) herbicides is a well-known problem. For example, Nováková et al. (2006) and Soukup et al. (2006) reported the development of chlorsulfuron resistance in *Apera spica-venti* (silky bent grass) in agricultural areas of the Czech Republic. Silky bent grass is a typical monocot pest of winter cereal crop cultivation. The resistance factor is located at the herbicide target site (Acetolactate-Synthase - ALS) leading to a reduction in the herbicide's effectiveness to 30 %. However, regarding their general behaviour the authors found herbicide resistant and sensitive populations to be similar. Another example of chlorsulfuron resistance is reported from the USA. Wetzel et al. (1999) found resistance of the weed *Amaranthus palmeri* to acetolactate synthase (ALS) herbicides. Since ALS resistance is known for several *Amaranthus* species the authors found pollinated *Amaranthus rudis* with pollen of ALS-resistant *Amaranthus palmeri*. The authors were able to confirm transfer by interspecific hybridization to *Amaranthus rudis*, another species belonging to the same genus.

During the risk assessment of GM potatoes, it was discussed whether the expression of proteins for the formation of amylose- or amylopectine-enriched starch in tubers would alter frost tolerance (EFSA 2006); however, literature reports on fitness enhancing starch modifications were not found. Potato competes poorly outside the cultivated environment but tubers can survive mild winter temperatures in soil. The experimental data supplied within the application documents indicated that the starch modified potato EH92-527-1 did - despite differences in sucrose contents - not differ from its non-GM comparator with respect to frost tolerance and susceptibility to diseases and pests. These studies showed no evidence of enhanced competitiveness or over-wintering survival to indicate increased weediness or invasiveness of starch modified potato EH92-527-1 (EFSA 2006). However, synthesis or degradation of altered starch components could theoretically influence the concentration of soluble carbohydrates, like sucrose, glucose or fructose. As a consequence, the osmotic value could be affected resulting in decreased frost sensitivity. Lower frost sensitivity could result in enhanced survivability (Begon et al. 2005).

Biotic stress tolerance and fitness

The development of resistance against biotic stressors in GM plants is constantly improving. Studies on the consequences can be grouped into three areas:

<u>Increased pest tolerance leading to higher number of progeny</u>

Field experiments with Bt maize expressing resistance genes against infestations of European Corn Borer (ECB) in infestation areas usually revealed high protection of the crop (Koziel *et al* 1993). Beneficial effects in terms of yield are highly dependent on the local situation. In Europe, yield increases in infestation areas of up to 10% have been noticed. Therefore, the resistance against pest attacks of Bt protein expressing maize has increased under the pressure of the herbivore. This is also true for potential volunteers. If Bt protein expressing maize seeds would spread into non-agricultural habitats, they would also be protected against insect attacks. This situation is principally not different from the ecological situation as it was before the occurrence of the insect pests. Maize cultivation areas were largely free of any lepidopteran pest for quite some time and many areas are still not infested. Therefore, in comparison to non-infested areas this observation supports the view that there is no increase of fitness in Bt maize as consequence of insect resistance traits.

There was a discussion in the reviewed literature for highly domesticated crops whether the overall fitness characteristics, like low competitive ability, missing seed dormancy and frost sensitivity would be significantly altered. This included a discussion of altered dissemination or multiplication characteristics.

Stewart *et al.* (1997) found higher numbers of seeds produced by insect protected Bt oilseed rape than by non-GM oilseed rape under the pressure of herbivores. Fitness of GM hybrids was also enhanced by Bt traits. At a Nebraska and a Colorado site Snow *et al.* (2003) crossed Cry1Ac expressing cultivated *Bt* sunflower (*Helianthus annuus*) with wild sunflower. This model is realistic as wild sunflower often grows in vicinity to cultivated sunflower and gene flow is possible in the USA. After crossing and backcrossing, the (GM) hybrid sunflower was more resistant to attacks of the lepidopteran species *Suleima helianthana* (*Tortricidae*, sunflower bud moth), *Cochylis* spp. (*Tortricidae*, banded sunflower moth), *Isophrictis similiella* (*Gelechiidae*) and *Plagiomimicus spumosum* (*Stiriinae*, frothy moth). The GM hybrid plants, produced an average of 55% more seed per plant in comparison to non-GM controls at the Nebraska site but only 14 % more seeds per plant in Colorado. Since wild sunflowers even suffered from herbivory by lepidopterans, the transgene transferred to the hybrids had a significant resistance effect in the infested region. In Nebraska stem damage by first-generation *Suleima helianthana* larvae was five-times more frequent than in non-transgenic controls compared to the transgenic plants. In Colorado this effect was not examined. Damage by *Cochlys* spp. was significantly lower in transgenic hybrids in Nebraska as well as in Colorado. Flower head damage by *Plagiomimicus spumosum* was more common in non-transgenic plants at both sites.

Similar results were obtained by Vacher *et al.* (2004) when testing the fitness of F1 hybrids between *Bt* protein expressing oilseed rape and *B. rapa*. Interestingly, the *Bt*-hybrids produced 6.2-fold fewer seeds than non trangenic plants in the absence of herbivores resulting in a remarkable decline of *Bt*-hybrids in the following generation. If herbivores were present the GM-hybrids produced 1.4 times more seeds than non-*Bt* hybrids. The authors conclude that *Bt* expressing hybrids will primarily benefit from the recombinant gene in terms of invading into wild floras when herbivore pressure is of high importance.

<u>Decreased pathogen susceptibility with consequences on offspring</u>

Burke & Rieseberg (2003) examined GM sunflower expressing the oxalate oxidase (OxOx) gene protecting the recombinant plants against the fungal pathogen *Sclerotinia sclerotiorum*. After backcrossing the gene into wild sunflowers over three generations no fitness effects in the recombinant hybrids could be detected, despite severe pathogen pressure. Seed production was not altered. Even non transformed and infested control plants were able to produce seeds at the same level as the recombinant plants. However, the effects were site dependent, giving rise to the assumption that environmental parameters have influenced the results. No altered survivability (including the offspring) have been reported from studies with GM potatoes with increased pathogen tolerance (Rasche *et al.* 2006a, b and references therein).

Experiments with genetically modified and near isogenic virus (*Rhizomania*) resistant hybrids of sugar beet x wild sea beet demonstrated that biomass production depended on the intensity of virus infections and on the intensity of competition by other weeds (Bartsch *et al.* 1996). However, the virus resistance offers a selective advantage only under specific conditions i.e. a high degree of virus infection. Wild forms of cultivated beets and wild sea beets seem to be naturally resistant. Advantages of the GM trait are not likely in the area of natural occurrence of sea beets since the virus seems not to exist in the semi-saline habitats of natural sea beets (see review by Bartsch *et al.* 2003).

<u>Conclusions regarding increased fitness of the GM cultivar</u>

Literature data are currently scarce with respect to long-term effects of GM crops or GM hybrids expressing salinity or drought resistance genes, in particular in view of potentially enhanced fitness. Currently, information for the ERA needs to be derived from analogous data on the behaviour of conventional crop varieties selected for salinity or drought resistance. Biotic stress tolerance was studied more intensively in the past. According to the often cited case of fitness enhancement of GM sunflower hybrids, the preliminary assumption can be drawn that the importance of fitness enhancement by a specific GM trait would be case-specific for environments with selective pressure in favour of the GM trait. Specific

differentiation is needed for hybrids between domesticated and wild populations (Arnold & Hodges 1995, Chapman & Burke 2006).

In some areas, oilseed rape (France, UK, and Germany) and sugar beet (South-western France) can naturally establish feral populations outside of cropping fields. According to literature this tendency seem to be - for oilseed rape - more striking in northern, western, and western Mediterranean areas. Herbicide tolerance genes will only enhance fitness in cropping systems where the complementary herbicide is applied. Insect resistance in maize will not enhance fitness as domestication and missing adaptation to EU climate conditions are counteracting any invasive behaviour.

A.1 Overall assessment:

The following process was identified from the literature screening in principle:

Recombinant new fitness enhancing traits may lead to increased fitness of the GM cultivar. Consequently, the GM cultivar may persist inside and outside fields, become invasive as a question of time and/or changing environmental conditions, and finally affect other plant species. The process is favoured by (i) increased stress tolerance (e.g. towards temperature, water, salinity), (ii) increased number of progeny, (iii) decreased pathogen susceptibility, (iv) increased pest tolerance/resistance, and/or (v) increased tolerance against herbicides.

According to the published information, the likelihood of this process for the currently used GM crops in the EU is:

- **High** for HT oilseed rape or HT sugar beet in complementary herbicide crop rotation and in non-agricultural habitats if the herbicide is applied, and
- **Negligible** for HT maize, HT Soybean, SM potato, or Bt maize.

A.2 Outbreeding depression after hybridization with wild relatives

From the population biology point of view, the term "outbreeding depression" describes hybrids being offspring from crosses between individuals of a source and a recipient population that have lower fitness than progeny from crosses between individuals from the same population. The drop in fitness may be caused by insufficient homology of the genomes resulting in detrimental effects on the functioning of metabolic pathways or other unfavourable parental gene combinations and undermining the hybrid's vigour (Hails & Morley 2005). As final consequence these effects may lead to extinction of small wild species populations (Ellstrand *et al.* 1999). Outbreeding depression is of increased interest particularly within conservation genetics of rare and endangered wild plant populations. In general, knowledge of the consequences of inbreeding and outbreeding depression in wild

populations as well as the capacity of small populations to adapt to local environmental conditions is urgently needed (Pertoldi *et al.* 2007).

Since crop-wild hybridization ability is the prerequisite condition, these effects are specifically reviewed for the two crops with potential for hybridization with wild relatives in the EU: oilseed rape and sugar beet.

Potential outbreeding is most important for repeated gene flow into small populations of wild plants, specifically from large scale cultivation of GM crops (Rhymer & Simberloff 1996, Ellstrand *et al.* 1999). Small wild populations could be affected significantly due to gene swamping. An adverse effect is more likely to occur if indigenous populations cannot avoid repeated pollinations by high amounts of "detrimental" pollen. However, the risk of extinction of small crop-related populations will be higher if the specific crop is introduced into new areas since the wild populations still exhibit a higher degree of genetic 'integrity' than wild populations growing in production zones (Ellstrand 2001). The receiving environment is of specific interest for the evolutionary consequences of outbreeding depression (Campbell *et al.* 2006).

Altered number of progeny / altered fecundity

Oilseed rape and relatives:

Under permanent herbivore pressure Vacher *et al.* (2004) reported a significantly higher number of offspring from F1 hybrids between Cry protein expressing oilseed rape and *B. rapa* than from conventional *B. rapa* populations. The results of a hybridization experiment carried out by Hauser *et al.* (1998a; b) with non-GM hybrids of *B. napus* x *B. rapa* demonstrate, that the fitness of F1 plants was intermediate in comparison to their parents and declined further in F2 and backcross hybrids.

Mikkelsen *et al.* (1996) detected high similarity of GM *B. napus* x *B. rapa* hybrids to wild *B. rapa* plants with respect to morphology and chromosome number. At least F1 hybrids of *B. napus* x *B. rapa* had a relatively high fertility in comparison to the wild ancestor. However, the male fitness of the F1 hybrids was low in another hybridization experiment with glufosinate-tolerant oilseed rape and *B. rapa* (Pertl *et al.* 2002). Similar results were published by Halfhill *et al.* (2005) when crossing Bt protein expressing *B. napus* x *B. rapa*. The authors found the F1 hybrids and the backcrosses to be of similar nitrogen efficiency as oilseed rape. The hybrids were weakened in terms of competitive ability in comparison to wild *B. rapa*. Hence the assumption can be drawn that a diminished competitive ability would decrease their chances to spread and thus to persist in nature.

In addition, hybridization between oilseed rape and wild radish (*Raphanus raphanistrum*) or cabbage (*Brassica oleracea*) may lead to viable offspring, even though to a relatively low

extent (Darmency *et al.* 1998, Al Mouemar & Darmency 2004, Chèvre *et al.* 2004, Halfhill *et al.* 2004, Ford *et al.* 2006). F1 hybrids derived from *B. napus* x *R. raphanistrum* exhibit low fertility (Chèvre *et al.* 1997). Guéritaine *et al.* (2002) backcrossed GM oilseed rape to *R. raphanistrum* six times. Depending on the direction of the backcrosses, the fitness of lines differed. Hybrids derived from backcrossing with the wild relative kept wild characteristics to higher extents. However, in the presence of the transgene, fecundity of the hybrids was generally reduced by ~ 50%.

Ammitzbøll & Jørgensen (2006) tested the gene flow between *R. raphanistrum* or *Raphanus sativus* and male sterile *B. napus*. The wild relatives originated from France, Switzerland and Denmark. The pods of the hybrids mostly just contained less than 1 seed per pod and 0.02 to 0.6 seeds were confirmed as hybrids. Hybridization success was highly dependent on the regional origin of the wild populations. In general, offspring of all combinations had very low pollen fertility (0-15%). Lee & Snow (1998) revealed the importance of pollinator preferences for the fate of *Raphanus* hybrids since the hybrids often show white coloured flowers, which are less attractive to pollinators.

Cultivated sugar beet and wild forms

Outbreeding depression was indirectly studied in experiments with GM (*BNYVV*[7]resistant hybrids of sugar beet x wild sea beet and near isogenic control hybrids (Bartsch *et al.* 1996). Decreased biomass production and competitiveness were observed for one of the two GM events studied in the absence of a selective advantage (see review by Bartsch *et al.* 2003). The most likely explanation is an unintended effect due to the genetic transformation as such. The observed effect was likely to be independent of the specific trait transferred.

These results underline the importance of selection pressure for any fitness effect of GM traits for oilseed rape and sugar beet. Without selection pressure, hybrids of interspecific crosses often exhibit reduced fitness (Arnold & Hodges 1995, Allainguillaume *et al.* 2006). Outbreeding depression is thus a more general concern for crop-wild populations, for GM crop ERA the characteristics conferred by the new traits could be of importance.

Other potential effects resulting from outbreeding depression

Theoretically, other consequences of outbreeding might be related to the transferred traits as mentioned in other chapters of this review: decreased stress tolerance, pest tolerance/resistance and increased pathogen susceptibility.

Most of the introduced cases above gave evidence that outbreeding depression may be caused by disturbances in physiological and genetic interactions in the recipient hybrids

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[7] BNYVV = Beet Necrotic Yellow Vein Virus; the virus causes a disease called Rhizomania in sugar beet

related to the 'conventional' genetic background of the parental plants. In case of hybrids containing a GM trait conferring tolerance against a specific stressor, the load for expressing the extra protein might be neutralized by the selective advantage due to the GM. However, any fitness advantage is likely to disappear if the pest or abiotic stressor is absent (Chapman & Burke 2006, Snow *et al.* 1999). The results of several studies on that issue gave rise to classifying the GM plant expression of non-essential proteins as "fitness costs" or "cost of resistance" (Coley *et al.* 1985). Bergelson (1994) found reduced fecundity of transformed lines in comparison to untransformed plants in cases where the stressor was absent. However, the resistance costs vary substantially. Strauss *et al.* (2002) found costs ranging from 6-45%. Additionally, it was found in the above mentioned investigations by Vacher *et al.* (2004) on hybrid fitness of *Bt* protein expressing *B. napus* x *B. rapa* hybrids, that vitality of the *Bt* protein containing hybrids was reduced in the absence of the herbivores. In general, available literature on GM effects on outcrossing is scarce, specifically as it comes to mechanisms related to specific GM traits.

In addition, outcrossing rates between oilseed rape and its wild relatives seem to differ regionally. Ammitzbøll & Jørgensen (2006) tested spontaneous hybridization rates between male-sterile GM *Brassica napus* spp. *oleifera* with *Raphanus raphanistrum* as wild relative species. The populations of wild radish came from France, Switzerland and Denmark. The offspring of the crosses with the French wild radish population was 100 % confirmed as being hybrids. From the cross with the Swiss population only 53 % were hybrids and only 2 % of the offspring crossed with the Danish population were confirmed as hybrids. Thus, the outcrossing barrier between different but related species seems to vary regionally. However, such differentiation in the ability of crop-wild plant hybridization is not observed in the European *Beta vulgaris* complex. Sugar beet breeders use wild populations of *Beta vulgaris* quite commonly in their breeding programs (Bartsch et al. 2003).

Conclusions regarding outbreeding depression

The few data available on effects resulting from outbreeding depression make a general assessment on potential long-term effects difficult. The "costs of resistance" are case-specific and data on the mechanisms of "outbreeding depression" are rarely found in the GM crop literature. This phenomenon might be related to common practise where any genotype that exhibits an outbreeding depression in crossings with other crop lines will be eliminated from breeding programs.

Based on general knowledge about outbreeding depression, a loss of vitality and competitive ability is theoretically possible and may lead to decreased stress tolerance, decreased pest resistance or increased pathogen susceptibility in wild plant populations.

Modern crop varieties have, in general, characteristics that are likely to reduce fitness in wild cross-compatible populations. It can be assumed that several wild populations have already suffered from outbreeding depression with their related crops for a long time, and may already in extreme cases have become extinct. On the other hand, coastal wild beet populations in the Italian sugar beet seed production areas seem to be more genetically diverse due to crop gene introgression. This phenomenon has yet not led to any population decline. An acceleration of outbreeding depression resulting from GM varieties of commonly used crops may occur if the new trait does not offer any advantage to the GM hybrid and its expression would lead to additional physiological load.

A.2   Overall assessment:

Based on the literature reviewed, the following process was identified as a potential long-term effect in principle:

Introgression of potentially fitness decreasing traits into wild relatives e.g. of oilseed rape or sugar beet may cause reduced GM hybrid fitness by outbreeding depression. With continuous gene swamping into the recipient wild population, the genetic barrier may further decline and if more GM hybrids were released, the wild species could become less fit in natural or semi-natural habitats so that the size of populations could decrease significantly. The process will be favoured if the GM trait leads to (i) decreased stress tolerance (e.g. towards temperature, water, salinity), (ii) decreased number of progeny, (iii) increased pathogen susceptibility, and/or (iv) decreased pest tolerance/resistance.

According to the published information, the likelihood of this process for the currently used GM crops in the EU is:

- **Low** for HT oilseed rape or HT sugar beet, and
- **Negligible** for HT maize, HT soybean, SM potato, or Bt maize.

A.3   GM crop/feral/wild hybrid long-term persistence

The introduction of fitness enhancing recombinant genes into crop species might influence crop persistence and also potentially lead to gene flow and introgression in crop/wild plant complexes. Thus the consequence of GM crop/trait combinations for long-term persistence needs particular attention. Under European conditions ferality and long-term persistence scenarios are restricted to genetically modified oilseed rape and sugar beet. The same is true for ferality and long-term persistence scenarios regarding hybridization with wild relatives. Any persistence of GM hybrids will be restricted to the same two species as these two are the only EU species with cross-compatible wild relatives in the European flora.

Persistence of oilseed rape and sugar beet

Oilseed rape is generally a common weed in agriculture, but seems to be a poor competitor in undisturbed habitats (Stewart *et al.* 1997). Crawley *et al.* (1993) carried out a crop study with HT oilseed rape that was sown in wild habitats to study the potential persistence and invasiveness of GM oilseed rape. The authors found significantly lower seedling establishment for GM oilseed rape than for the conventional lines. Generally, the HT oilseed rape lines varied in fitness across sites and years. In a 10-year study, subsequent survival of HT oilseed rape at 3 out of 12 experimental sites was lower, at 2 sites higher compared to control. No difference was observed at the other sites. Seed survival did not translate into higher persistence and invasiveness of GM oilseed rape. The HT trait did not offer selective advantage outside cultivation (Crawley *et al.* 2001). Within the same experiments, no difference was found for seedling survival of HT maize in comparison to conventional maize. The studies performed with HT sugar beet showed a dependency of survival on the genetic background of the seeds. In all cases non-GM sea beets were more competitive. The authors conclude that only in those cases where an ecological impact might become apparent, performance under field conditions could enhance. Traits potentially offering advantages to crops running wild are traits directed against biological or abiotic stressors, like pests or drought (Crawley *et al.* 2001).

Knispel *et al.* (2007) found evidence for long-term survival of HT *Brassica napus* populations growing outside of fields. The results differed on trait depending fitness increase of hybrids between *Brassica napus* and *Brassica rapa*. Although the HT trait usually does not offer fitness advantages outside of fields high percentages of HT *Brassica napus* hybrids were found expressing two or three herbicide tolerance conferring proteins. It remains unclear whether the persistence of these multi-herbicide-tolerant hybrids depends on intensive cultivation and accordingly to a continuous HT gene input from fields cultivated with HT tolerant oilseed rape. However, gene flow and introgression obviously leads to natural recombination of GM HT genes.

Establishment of conventional feral oilseed rape populations on road verges are reported by Pivard *et al.* (2007). The authors found persistence of conventional feral oilseed rape seeds in soil seed banks on road verges located in central France in a 4-year study. About 35-40% of the roadside *B. napus* populations found were derived from seed bank seeds. Local recruitment was responsible for just 10% of the populations. Crawley & Brown (2004) examined the population dynamics of feral oilseed rape populations outside of fields. They found that persistence is mainly associated with two factors: soil disturbance and continuous seed input. These factors are locally dependent, whereas climatic conditions play only a role on large scales.

The most important prerequisite for persistence of recombinant genes in hybrid populations is successful introgression through backcrossing over several generations leading to stable incorporation of a new gene (Hails & Morley 2005, FitzJohn *et al.* 2007). However, the dissemination and invasion of hybrids expressing new traits usually occurs after a longer lag-phase that depends on the specific species and the environment (Ellstrand & Schierenbeck 2000). Principally, hybridization and subsequent introgression followed by stable expression of new traits could be the stimulus for speciation (Ellstrand 2003a).

By means of a specific genetic marker, Hansen *et al.* (2003) revealed extensive introgression of crop genes after hybridization between *B. napus* x *B. rapa*. The wild oilseed rape relative *B. rapa* acted mainly as maternal partner. In the offspring of the hybrids the amount of oilseed rape DNA diminished from year to year. Since oilseed rape as pollen donor was absent in later years the genetic background of the wild form became more and more important in the hybrids. In spite of missing oilseed rape as pollen donor, the majority of the plants at the investigation site still exhibited hybrid character.

Johannesen *et al.* (2006a, b) carried out a backcrossing experiment between F1 hybrids from transplastomic oilseed rape x *B. rapa* (with oilseed rape as maternal partner) and *B. rapa* (transplastomic F1 x *B. rapa*). The authors found that the BC1 offspring was negatively affected in terms of biomass production and of seed weight due to the density of cultivated *B. napus*. High densities as well as low densities of one partner enhanced the opportunity for the formation of BC1 offspring. The authors conclude that success of introgression of genes from one to the other hybridization partner depends on the density of populations.

Warwick *et al.* (2007) examined persistence of genetically modified hybrids between glyphosate tolerant (HT) *B. napus* x *B. rapa* (wild form) from 2002 to 2005. The authors found a strong decline of hybrids from 200 plants at the beginning of the study to ~85 in 2002 and to 5 in 2005. Both F1 and backcross hybrid generations were detected. However, despite the reduced pollen viability the hybrids produced significant amounts of seeds. Of the next generation nearly 50% still carried the transgene. Thus, a 'ecologically neutral' trait like HT in non-herbicide spray areas may still persist over time in spite of missing selective pressure and potential fitness costs.

The sympatric growth of wild beets in coastal sugar beet cultivation zones will play an important role for potential spread of transgenes (Darmency *et al.* 2007, Darmency & Richard-Molard 2008). Weedy forms of *Beta vulgaris* inhabit disturbed places, either agricultural land, field verges, ruderal sites or coastal drift lines. These weed beets can invade fields with dicotyledonous crops, in particular with beet cultivars where they cannot be controlled by selective herbicides, except when GM HT sugar beet is used. There is still

much discussion about the genetic mechanisms that change sugar beet cultivars into feral weed beets (Sukopp *et al.* 2005).

The persistence of GM traits from sugar beet is thus strongly dependent on (natural) recombination with feral or wild genetic background. GM weed beet growing in virus infested areas will potentially benefit from virus resistance genes, whether they are of genetically modified or of classical breeding origin. This will also apply to herbicide resistant weed beets in crop rotations using the complementary herbicide (Bartsch *et al.* 2003).

<u>Persistence in other crop complexes</u>

If genetically modified crops are released in regions where wild relatives grow, spontaneous hybridizations are very likely to occur. In case GM traits are advantageous they will generally persist and introgress into the natural populations (Ellstrand 2003b). Enhanced weediness of a hybrid will depend on the amount of fitness increase and current environmental conditions (Ellstrand *et al.* 1999). Potential fitness advantage remains the best predictor for gene spread in the wild, overall gene flow alone is of lesser importance (Hails & Morley 2005, Chapman & Burke 2006).

<u>Conclusions regarding GM crop/feral/wild hybrid persistence</u>

So far no clear rules can be derived concerning outcrossing between related species and the fate of a transferred GM trait . Obviously, introgression and persistence of transferred genes in a recipient population are dependent on a number of random factors. The dissemination of new traits in wild populations seems to become more likely if the hybrids still exhibit sufficient weedy characteristics. After a gene has introgressed into a hybrid population the fate of the new trait in the environment might develop increasingly into a problem of intraspecific gene flow (Chapman & Burke 2006). However, potential intraspecific gene flow between the hybrid population and wild populations could be disturbed if the crop as main pollen source and pollinator is still present.

<u>A.3   Overall assessment:</u>

Based on the available information, the following process was identified for potential long-term effects:

GM hybrids may persist in and outside fields if they exhibit weedy characteristics. Where the new GM trait causes increased fitness after gene flow and introgression into wild relatives (hybrids), GM hybrids might become invasive over time or in response to changing environmental conditions, and finally affect other plant species. The process is favoured by a GM trait that leads to (i) increased stress tolerance (e.g. towards temperature, water,

salinity), (ii) increased number of progeny, (iii) decreased pathogen susceptibility, and/or (iv) increased pest tolerance/resistance.

Based on the available literature, the likelihood of this process for the currently used GM crops in the EU is:

- **High** for HT oilseed rape and HT sugar beet in crop rotation or in disturbed areas outside cultivation where the complementary herbicides are applied,
- **Low** for HT oilseed rape and HT sugar beet in crop rotation or in disturbed areas outside cultivation where the complementary herbicides are not applied, and
- **Negligible** for HT maize, HT soybean, SM potato, and Bt maize.

B. *Altered gene transfer*

B.1 Potential reduction of pollination

Attractiveness to pollinators is an important prerequisite for reproduction of strongly insect-pollinated plants. The main factor for attracting pollinators is the colour of petals. Stanton *et al.* (1986) studied the insect-attractiveness of white or yellow coloured flowers of the self-incompatible species *Raphanus raphanistrum*. According to their results, white coloured petals attracted fewer insects than yellow coloured. However, the maternal function in terms of fruit and seed production was not affected when comparing the seed set of white or yellow coloured flowers. Thus, the attractiveness of flowers and the frequency of insect visits do not seem to be sufficient explanation for female reproductive success when the male function of a flower is retained.

Raphanus sativus (cultivated radish) and *R. raphanistrum* (wild radish) are frequently used as a model system for studying the potential fate of GM traits in hybrids (Klinger & Ellstrand 1994). Lee & Snow (1998) carried out a study on hybridization between cultivated radish (as potential target for genetic modification) and wild radish in view of potential fitness effects on hybrids. Cultivated radishes were white-coloured; the wild partner *R. raphanistrum* had yellow flowers but is known as polymorphic for flower colour (white or yellow). The hybrids invariably had white or pink coloured flowers like the crop partner, thus visualizing the transfer of crop genes. From pollinator studies, the authors found that the white coloured hybrids were significantly less frequently visited by pollinators than the wild yellow coloured parental plants. Consequently, a lower seed production was observed in the hybrids. The discrimination tendencies for the white-coloured hybrids were of substantial importance when hybrids were of great numbers. The results give evidence that pollinator preferences may potentially affect the likelihood for reproduction and survival of GM hybrids. However, as found by Stanton *et al.* (1986), the frequency of pollinator visits alone is not a sufficient

estimate for reproductive performance. This example derived from the non-GM crop-wild *Raphanus* complex can be regarded as an analogous model for GM crops with (intentionally) altered flower colour.

Beside petal colours as factor for attracting pollinators, also volatiles emitted from the flowers to ambient air play an important role for orientation of insects and plant pollination. Plants synthesize and emit a large variety of volatile organic compounds; some are probably common to almost all plants whereas others are specific to only one or a few related taxa (Pichersky & Gershenzon 2002). Plant flavours are not only produced to attract pollinating insects; several plant species emit volatile substances acting as repellents to deter pests or herbivores from attacking the specific plants (e.g. scents of distress = "Herbivore-induced Plant Volatiles" – HIPV). Additionally, HIPV are attracting the herbivores enemies (predators) in terms of providing biological defence (Turlings & Ton 2006).

Pierre & Pham-Delègue (2000) demonstrated in a study on honey bee that the attractiveness of GM oilseed rape, constitutively expressing a protease inhibitor or HT (glufosinate), will not alter the attractiveness of such plants compared to non-GM plants. The analyses of nectar and flower volatile compounds from protease inhibitor plants discovered differences in the chemical composition between GM and non-GM oilseed rape. However, this did not change the attractiveness of the flowers for bees.

Internal reports from field trials with GM sugar beet, oilseed rape, and maize carried out in Germany do not reveal any qualitative effect on pollination during the last 15 years.

Conclusions

Theoretically, some specific genetic modification might alter the chemical composition of flower scents of oilseed rape. That could lead to a potential reduction of pollination due to decreased flower attractiveness for pollinators (altered colour, altered scent), but the given *Bt*, HT and SM traits are not likely to produce such effects in the major crops studied in this review.

B.1   Overall assessment:

Based on the literature and field trial database review none of the important crop/trait combinations currently used in the EU is likely to reduce pollination. However, due to the overall importance, the following process was identified for expert consultation on potential long-term effects due to potential reduction of pollination:

A GM trait may reduce pollination, e.g. due to a decreased attractiveness for pollinators (altered colour, altered scent).

According to the published information, the likelihood of this process for the currently used GM crops in the EU is:

- **Negligible** for all HT, Bt and SM crops.

B.2   Altered flower phenology

Phenology of plant flowering depends to a great extent on day length (Mestel 2000). For many annual plant species, additional factors like vernalization of seeds (e.g. sugar beet) and hormone levels are necessary to trigger germination and later flowering. Regular flowering is an important factor in their short life cycle (phenology) since flowering and seed setting enforce survival. Synchronous flowering time is decisive for all individuals of a population in case of self-incompatibility (Begon *et al.* 2005). However, different flower phenologies may also be important if a small population of individuals exhibit poor competitiveness. In this case, the likelihood of extinction would be further enlarged if pollen from a cross-compatible widespread species were the only main pollen source (Marques *et al.* 2007). Today, genetic engineering has been used for basic research on principles and regulation of flowering genes mostly in model plants ("flowering on command", Mestel 2000).

There are some scarce internal study reports to competent authorities indicating potential unintended alterations in the flower phenology of some GM plants. In one published study, a delayed flowering of *Bt* crops was observed (Hoheisel & Fleischer 2007). The authors studied the effects of concurrent introduction of 3 vegetable *Bt* cultivars (sweet corn, potato, winter squash) with respect to coccinellid, aphid and pollen diversity. For all of the three GM varieties, the authors found a delay in pollen production in comparison to the isogenic non-genetically modified controls. Small differences were detected for sweet corn; larger differences were revealed for potato and squash. The production of substantial pollen amounts was one to three weeks delayed in the genetically modified lines. For the *Bt* potato event the peak of pollen production was one week delayed. The number of flowers was reduced in the *Bt* varieties but higher amounts of pollen were produced; the reason for this unintended effect is not known, and somaclonal variation might be one possible explanation.

In the vast majority of publications and internal reports for GM plant notifications for the placing on the market, such effects cannot be found. Altered flower phenology could potentially lead to genetic isolation due to delayed pollination or missing pollinators, a phenomenon known from ecological textbooks (Begon *et al.* 2005). On the other hand natural variations in flowering time are well known also for non-GM crops due to different planting dates, etc. This is also true for the mainly vegetatively reproducing potato (non-GM or GM varieties).

<u>Conclusions</u>

GM traits may theoretically cause altered flower phenology, which could, after gene introgression, lead to genetic isolation of wild relatives. However, studies demonstrating an introgression of GM traits from oilseed rape or sugar beet into compatible wild relatives did not measure, report, or assess possible changes in pollination success up to now.

<u>B.2　Overall assessment:</u>

Based on the literature review, the following process was identified for expert consultation on potential long-term effects due to altered flower phenology:

Expression of GM trait may cause altered flower phenology, after gene introgression leading potentially to genetic isolation of wild relatives.

According to the published information, which is not very comprehensive, the likelihood of this process for the currently used GM crops in the EU is

- **Low** for Bt maize, and
- **Negligible** for all HT and SM crops.

B.3　Altered compatibility reducing or favouring outcrossing

Male sterility is a tool in plant breeding to direct a one-way gene flow and potentially avoid outcrossing. For decades plant breeders have used this system to produce hybrid lines by means of conventionally bred no-pollen plants. The advantage of this system is that it enables controlled pollination which is necessary to produce well defined hybrids.

Additionally, this system serves as a strategy for preventing pollen escape in genetically modified crops by e.g. incorporation of the "barnase" system (the gene is derived from *Bacillus amyloliquefaciens*) with a tapetum-specific promotor leading to cell death and thus bringing about the avoidance of pollen production. By means of hybridization with a second GM line expressing the "barstar" gene (restorer gene) the sterility system may be switched off. In oilseed rape, an advantage of this system is that due to the inability for pollen production the seed amounts obtained from barnase expressing lines after pollination are significantly higher than from self-pollinating oilseed rape lines (Bisht *et al.* 2007). This approach or similar systems, as for example the cytoplasmic sterility system (CSM), have also been used for preventing pollen flow from genetically modified plants (Feil *et al.* 2003, Sandhu *et al.* 2007).

Cultivated GM plants expressing male sterility systems could theoretically pose a risk to cross-compatible wild populations: plants emerging from spilled seeds could be male fertile (up to 50% progeny) with the chance to transfer the heterozygous male sterility gene to other

populations, in particular via seed gene flow (Sleper & Poehlman 2006). In the following generations, the development of male sterile (wild) offspring could potentially occur and thus could alter the intra-specific population gene flow of wild plants. In contrast, Ribartis et al (2007) used a dominantly inherited pollen sterility system, which is based on the combination of a tapetum-specific promoter with metabolic starvation: homozygous plants are completely male sterile, heterozygous hybrids (upon fertilization with non-GM pollen) and will produce 50% fertile WT-Pollen whereas the 50% GM pollen will die due to metabolic starvation in early steps of development. However, CSM has been used in conventional plant breeding of oilseed rape and sugar beet for decades without any report of adverse effects on related wild plant populations (Biancardi *et al.* 2005, Poppy & Wilkinson 2005). However, the situation may change if these traits are combined with additional recombinant genes conferring fitness enhancing characteristics.

Conclusions

Male sterility may theoretically lead to an altered compatibility between GM crops and conventional varieties or between GM crops and their wild relatives, e.g. reducing or favouring outcrossing. However, the given *Bt*, HT and SM traits of non male-sterile lines are not likely to produce such effects in the major crops studied in this review.

B.3   Overall assessment:

Based on the literature review, the following process was identified for expert consultation on potential long-term effects due to altered compatibility reducing or favouring outcrossing:

The GM trait may cause an altered compatibility between GM crops and conventional varieties or between GM crops and their wild relatives, e.g. reducing or favoring outcrossing.

According to the published information, which is not very comprehensive, the likelihood of this process for the currently used GM crops in the EU is

- **Negligible** for all HT, Bt and SM crops.

B.4   Altered fecundity increasing seed (gene) flow

An example for increased fecundity of a GM hybrid is described by Snow *et al.* (2003). The authors crossed Cry1Ac expressing *Bt* sunflower crop (*Helianthus annuus*) with wild sunflower growing in vicinity to cultivated sunflower. After crossing and backcrossing, the hybrid sunflower harbouring the *Bt* gene exhibited resistance to lepidopteran insects similar to the GM crop plants. These hybrids produced an average 55% more seed per plant in comparison to non-GM control sunflowers. The authors ascribe these results to the missing

infestation by herbivores. In contrast to plants harbouring the *Bt* gene, wild sunflowers (control) suffered from herbivory by lepidopterans.

Campbell & Snow (2007) studied the performance of the F3 generation of non-GM *Raphanus raphanistrum x Raphanus sativus* hybrids in semi-natural agricultural environments. They found decreasing seed numbers per fruit and fewer fruits per plant for the hybrid. However, concomitantly with increasing competition in the habitats, the relative number of fruits and seeds of the wild ancestors decreased; this was not found for the hybrids. Thus, the establishment and spread of hybrids as well as of wild ancestors in semi-natural habitats is highly dependent on level of competition in specific plant communities.

The study of Snow *et al.* (2003) is one of the rare available examples of fitness and fecundity advantages of a *Bt* expressing (hybrid) variety under ongoing herbivore stress, indicating environmental advantages for herbivore resistant plants. No published reports are available for *Bt* maize, HT oilseed rape; HT sugar beet, HT soybean and SM potatoes.

Conclusions

New traits conferring stress tolerance are potentially able to alter fecundity by altering the number of seeds produced, which may cause increased seed (gene) flow from GM crops to wild plant populations. However, the given *Bt*, HT and SM traits are not likely to produce such effects in the major crops studied in this review.

B.4   Overall assessment:

Based on the literature review, the following process was identified for expert consultation on potential long-term effects due to altered fecundity increasing seed (gene) flow:

A GM trait may alter fecundity resulting from altered number of seeds produced, which may cause increased seed (gene) flow from GM crops to wild plant populations.

According to the published information, which is not very comprehensive, the likelihood of this process for the currently used GM crops in the EU is

  - **Negligible** for all HT, Bt and SM crops.

B.5   Increased frequency of horizontal gene transfer

A non-sexual exchange of genetic material between organisms belonging to the same or different species is referred to as horizontal gene transfer (HGT). HGT is a naturally occurring process that was first demonstrated to occur between bacteria (Wellington & van Elsas 1992; Nielsen *et al.* 1998). The impact of the process depends on the likelihood of its occurrence and the magnitude of associated adverse outcomes (Nielsen *et al.* 1998; Droge *et al.* 1998).

In soil, large DNA strands may be stabilized through the adsorption to soil constituents like clay, minerals or humic substances. Adsorbed DNA may persist for weeks or months (Gebhard & Smalla 1999, Nielsen *et al.* 1997). Additionally, DNA may be preserved in dead plant tissue and kept biologically available to soil bacteria for hours, days or longer time spans.

The most likely scenario for the transfer of DNA from (GM) plants to bacteria takes into account the transformation of naturally competent bacteria with free plant DNA released into the soil or into the digestive tract of humans or animals (Nielsen *et al.* 1998). In this process, several events must occur sequentially, the likelihood of which depends on the availability of intact homologous DNA, the ability of bacteria to undergo transformation with the specific DNA, and the competitiveness of the transformed bacteria. Accordingly, the likelihood of horizontal gene transfer and incorporation of eukaryotic DNA by prokaryotes is extremely low due to genetic incompatibilities and to barriers which prokaryotes evolved to suppress this kind of gene transfer (de Vries & Wackernagel 2005).

Evidence for horizontal gene transfer regarding recombinant plant DNA transferred to bacteria has not been obtained up to now under natural conditions. Gebhard & Smalla (1998) observed uptake and integration of transgenic plant DNA and of plasmid DNA into competent *Acinetobacter* sp. strain BD413. The authors studied the ability of Acinetobacter sp. strain BD413(pFG4nptII) to take up and integrate transgenic plant DNA based on homologous recombination under optimized laboratory conditions. A recombinant nptII-gene (neomycine-phosphotransferase-gene) conferring kanamycin resistance being integrated into the genome of GM sugar beet leaves was selected as marker for potential HGT. The recipient *Acinetobacter* strain was kanamycin sensitive before the transfer process. Bacteria being resistant to kanamycin when cultivated together with a homogenate of GM sugar beet leaves were taken as proof for HGT. The frequency of horizontal transfer of sugar beet DNA to *Acinetobacter* sp. strain BD413 was calculated as 1.5×10^{-10}.

Another approach for testing HGT under optimized conditions was carried out by de Vries *et al.* (2004) using a specific illegitimate recombination system. Competent cells of *Acinetobacter* were exposed to plant DNA from leaf and root tissue of GM tobacco plants. The authors used the spectinomycin/streptinomycin-resistance gene as "anchor" for the transfer of segments of the tobacco plastid DNA. Horizontal transformations of genes connected to the anchor gene were found at a frequency of 1.2×10^{-7} per cell. The relatively high frequency was due to specific GC-rich microhomological sites in the *Acinetobacter* genome. These GC-rich sites may act as hot-spots for stimulating illegitimate recombinations. The specific (artificial) conditions selected in the experiments (anchor-

sequences plus existence of microhomological site in rector bacteria) were the prerequisites for achieving relatively high frequencies of HGT.

In considering the probability of functional gene transfer from plants into bacteria in the environment or human/animal gut, several aspects need to be taken into account (with npt II as example, see EFSA 2007):

1. DNA is released from plant material by normal digestion processes that take place in the gastrointestinal tract, or by activities of nucleases present in various organisms in the environment.

2. The probability that bacteria will be exposed to DNA stretches long enough to contain the intact nptII. gene is very low because of the above mentioned digestion and degradation processes (Lorenz and Wackernagel, 1994).

3. The nptII gene from plant material can only be taken up by competent bacteria via natural transformation, a process that occurs infrequently in many bacteria and in most environmental conditions (Davison, 1999).

4. If the intact nptII gene enters the bacteria, it will be rapidly degraded by restriction endonucleases in many bacterial cells which possess DNA restriction systems in order to destroy foreign DNA (Davison, 1999).

5. If the intact nptII gene does indeed survive, the probability of its incorporation into the bacterial genome is very low unless there are homologous regions already present in the bacterial genome. Gene transfer from plants to bacteria has only been demonstrated under laboratory conditions when regions of homology were already present in the recipient bacterium (Bennett et al., 2004, de Vries et al., 2001, de Vries and Wackernagel, 2002, Kay et al., 2002, Tepfer et al., 2003).

6. Expression of the incorporated nptII gene is unlikely considering that in GM plant material the nptII gene is under the control of a promoter with preferential expression in plants, which does not support its efficient expression in bacteria.

7. Stable integration and inheritance of the nptII gene in the host bacterium is not likely in the absence of selective pressure from a relevant antibiotic.

When all of the above mentioned aspects are taken into account, the probability of functional gene transfer from plants into microorganisms is extremely low. According to EFSA (2007) it is not surprising that transfer of an antibiotic resistance marker from GM plants to bacteria has still not been observed under natural conditions (Gay and Gillespie, 2005).

Principally, the same constraints as for HGT from GM plant material to competent bacteria are effectual for incorporation of transgenes from the pollen of GM crops into the genomes of

bacteria living in the gut of pollen feeding insects like bees. Mohr & Tebbe (2007) chose the following approach to detect HGT in gut bacteria: Gut bacteria were collected from the gut of bee larvae fed with pollen collected from a field cultivated with glufosinate tolerant oilseed rape. Subsequently, the gut bacteria were tested for glufosinate tolerance. 60,4% of the 96 strains tested were sensitive to glufosinate applications. The others were tolerant to differing concentrations of the herbicide. However, this surprisingly high proportion of resistant gut bacteria is not *per se* an indication for horizontal gene transfer. More likely, the bacterial populations already exhibited a natural resistance; therefore this approach does not seem to be appropriate for demonstrating HGT.

Several authors share the view that studies on HGT should consider specific conditions potentially enhancing HGT despite the very low likelihood of its occurrence. In addition, the appropriateness of detection methods needs attention. Nielsen *et al.* (1998) recommended enhancing the understanding of selection processes in the environment (e.g. in soil). Any prediction of possible consequences of the introduction of novel traits into the environment would be impossible without a thorough understanding of selection events. If specific transgenes are derived from e.g. prokaryotes or plastoms, the likelihood of HGT after degradation in soil could be higher (Monier *et al.* 2007). According to Nielsen & Townsend (2004), the sampling methodology and sample sizes need substantial improvement in order to ensure the collection of representative samples, reflecting e.g. the biological conditions at the sampling sites and the natural variability of microbial communities.

The structure of DNA originating from GM plants is usually not fit to allow for expression in prokaryotes (e.g. due to different promoter elements). De Vries & Wackernagel (2005) emphasize that the probability of effects of HGT to occur would increase if the potentially transferred foreign DNA conferred selective advantage to the recipient. Nielsen *et al.* (2007) emphasize the importance of the acquisition of extracellular foreign DNA for the evolution of bacteria. In contrast to long strands of DNA, DNA of limited size seems to physically persist in natural media over time. The question is still not answered whether this kind of DNA is more accessible to competent bacteria than long strands of intact DNA. Heuer & Smalla (2007) address the necessity of checking "mobile genetic elements" (MGE), typical elements of bacteria including GM bacteria, for their potential to enhance HGT between bacterial species.

In summary, the probability for HGT from GM plants to bacteria could potentially increase if the specific transgene (i) conferred a specific selective advantage, (ii) its structure was prokaryote-related and (iii) its strand length was not too long. Under these circumstances, the probability for the spread of a transgene in prokaryotes would be enhanced.

<u>Conclusions</u>

A GM trait may theoretically increase the frequency of horizontal gene transfer from plants to populations of microorganisms, thereby introducing new traits into microbial communities. However, a high degree of homology between plant DNA and bacterial genes is the prerequisite for increasing frequency of horizontal gene transfer. When all available information (including the origin of promoters) is taken into account the probability of functional gene transfer from plants into microorganisms is extremely low and of negligible relevance for long-term effects. The given *Bt*, HT and starch modification traits are not likely to increase the likelihood of HGT for the major crops studied in this review.

<u>B.5   Overall assessment:</u>

Based on the literature review, the following process was identified for expert consultation on potential long-term effects due to increased frequency of horizontal gene transfer:

The GM trait may be the prerequisite for increased frequency of horizontal gene transfer from plant to microorganism populations introducing new traits into microbial communities.

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Negligible** for all HT, Bt and SM crops.

C.   Effects on Target Organisms

Target organisms are defined as pests (mostly insects) or pathogens (fungi, bacteria, viruses, protozoa, nematodes) which are targets of plant protection measures. For example genetically modified *Bt*-plants expressing Cry1Ab protein (the measure) killing corn borer larvae (the target organism). The situation of weeds growing in fields cultivated with HT crops is different to the effects of Bt crops on target insects. Weeds are affected by complementary herbicides applied in HT cropping systems; not from the HT GM crops as such. Potential effects of the use of non-selective herbicides within HT GM crops are addressed in Section 3.F (indirect effects of cultivation and management).

Resistance development of pest or pathogens against plant protection measures is well documented. For example, about 500 arthropod species and 100 pathogens developed resistance against pesticides in the last decades (Eckert 1988, Whalon *et al.* 2008). It is not liekely that sooner or later pests or pathogens will also develop resistance against GM-crops (Andow & Zwahlen 2006).

C.1 Effects on target pathogens

According to Tepfer (2002) and AGBIOS (2008), only a few crops with pathogen virus resistance are on the market, mainly in the USA and Canada. These GM crops are papaya with resistance against ringspot virus (PRSV), squash with resistance against cucumber mosaic virus (CMV), zucchini yellow mosaic (ZYMV) and watermelon mosaic virus (WMV); potatoes with resistance against potato virus Y (PVY) and potato leafroll virus (PLRV) and plum with resistance against plum pox virus (PPV). None of these crops are on the EU market. Lheureux *et al.* (2003) and Fernadez-Cornejo & Caswell (2006) report a number of crops with resistance genes against fungal or bacterial diseases to be under development.

Conclusion

After screening the available literature, no report was found demonstrating development of pathogen resistance against GM crops. Whether viral pathogens are able to develop resistant against GM-crops is subject to controversial discussion (Tepfer 2002). However from the experience with other plant protection measures long-term resistance development cannot be precluded.

C.1 Overall assessment:

Based on the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Negligible** as no GM crops - targeting pathogens - are currently on the market.

C.2 Effects on target pests

The vast majority of insect resistant (IR) GM plants (maize, cotton, potato, tomato) placed on the market express Cry-Proteins against lepidopteran pests (Cry1Ab, Cry1Ac, Cry1F, Cry2Ab, Cry9C1) or coleopteran pests (Cry 3A, Cry 3Bb1, Cry34Ab1, Cry35Ab1) (AGBIOS 2007). At the end of 2007, only MON810 maize expressing Cry1Ab was available on the market for cultivation purposes in Europe. However, Ranjekar *et al.* (2003) reported that other IR-crops have been developed expressing proteins against target pests like proteinase inhibitors, amylase inhibitors, chitin degrading enzymes or plant lectins. In addition synthetic Cry proteins in various crop plants were reported to be under investigation Ferré & van Rie (2002). In general, sufficient data are currently not available for non-*Bt* approaches in IR GM plants in view of their potential for resistance development of target organisms. For the EU, the literature review is currently of relevance only for GM crops expressing *Bt*-protein.

The toxic pathway involves (i) ingestion of Cry protein by susceptible insects, (ii) solubilization of crystals, (iii) release of protoxins, (iv) processing of protoxins by midgut

proteases into a protease-resistant core fragment (the toxin), (v) passing of the toxin through the peritrophic membrane, (vi) binding to a specific receptor located on the brush border of the midgut cells, (vii) partial insertion of the toxin into the membrane, (viii) pore formation, (ix) cell lysis followed eventually by cell death (Schnepf *et al.* 1998).

McGaughey (1985) published the first report on *Bt*-resistance development in 1985. The Indian meal moth from grain bins developed a 100-fold level of resistance against conventional *Bt*-insecticides after laboratory selection. Ferré & van Rie (2002) gave a broad overview of further *Bt* resistant pest strains evolving during the following decades, specifically for several lepidopteran pests. Here, the first case of field resistance was observed in Hawaii. Populations of the diamondback moth *Pluttela xylostella* showed a reduced susceptibility to *Bt*-sprays (Tabashnik et al 1990). A decreasing susceptibility and increasing resistance or increasing frequency of resistance genes against Bt-protein expressed in GM cotton was observed for lepidopteran pests in the field several years after cultivation in Australia and China (Zhao *et al.* 1996, 2000, Shen *et al.* 1998, Gunning *et al.* 2005, Downes *et al.* 2007), whereas no resistance development in cotton was reported from India and the USA (Fakrundin *et al.* 2003). A new resistance development in *Bt* maize for an African Lepidopteran species (*Busseola fusca* - African Stem Borer) was reported for South Africa by Van Rensburg (2007). The author argues that the speed of resistance development was favoured by the practise of irrigating the cultivated maize fields. Since the African Stem Borer prefers humid atmosphere the target moth lays their eggs at negligible frequency on the purely rain-fed maize fields serving as non-GM refugia grown in the vicinity of the irrigated maize fields. The conclusion can be drawn that partial irrigation of maize fields could have favoured resistance development of target organisms despite using the recommended refugia strategy.

Potentially, this aspect is of importance for resistance management especially in the Mediterranean region since maize cultivation usually is connected with artificial irrigation. If farmers there were also to stop irrigating refugia stands cultivated with non-*Bt*-maize, the development of resistance in target lepidoptera could increase.

Other GM *Bt*-plants did not show resistance development of pests in the field so far (Fox 2003). In particular, for different *Bt*-maize cultivar resistant to lepidopteran or coleopteran pests no resistance development in the field was observed either in the USA or in Europe (Farinós *et al.* 2004, Eizaguirre *et al.* 2006, Siegfried *et al.* 2005, Stodola *et al.* 2006, Huang *et al.* 2007). However European Corn Borer populations with a decreased susceptibility to *Bt*-proteins were selected in the laboratory by Huang *et al.* (1997), Bolin *et al.* (1999), Chaufaux *et al.* (2001) and Alves *et al.* (2006).

From the breeder's point of view the potential development of resistance of target organisms is a serious problem since their products would be useless in specific regions. To delay evolution of pest resistance breeders introduced multiple (stacked) Cry toxins into maize lines. The strategy relies on the assumption that simultaneous evolution of resistance to slightly differently acting cry-proteins would be highly unlikely. An example is the transformation of specific *Bt*-cotton lines with Cry1Ac and Cry2Aa genes for improving long-term effects on *Heliothis virescens* (Tobacco budworm; Jurat-Fuentes *et al.* 2003). Another strategy for avoiding evolution of resistance in pest insects is the construction of synthetic Cry-genes whose proteins are equipped with multiple binding domains. An example is the synthetic Cry1.105. It consists at least of binding domains of Cry1Ab, Cry1Ac and Cry1F. All of them are directed against lepidopteran larvae (MON 89034 http://www.efsa.europa.eu/EFSA/DocumentSet/gmo_02_partii_summary,0.pdf).

Conclusion

Summarizing the available literature, resistance development of lepidopteran species against Bt-protein has not been observed in Europe (as at end 2007); this also holds true for Bt-maize cultivation worldwide with the exception of South Africa. So far, the applied insect resistance management seems to be a successful measure for delaying or preventing resistance development during seven years of large scale cultivation (Tabashnik *et al.* 2003, Bates *et al.* 2005). Additionally knowledge about the potential for resistance development has been gained due to extended studies about basal susceptibility and the frequency of resistance alleles in Europe (e.g. Chaufaux *et al.* 2001, Ferré & van Rie 2002, Bourguet 2004, Saeglitz *et al.* 2006, Schuphan 2006). However, several of these authors regard resistance development also for *Bt* crops to be a question of time.

C.2   Overall assessment:

Based on the literature review, the following process was identified for expert consultation:
Bt proteins can cause resistance development in target pests, which results in a loss of environmentally desirable plant protection tools. Due to the unequal distribution of many insect pests in Europe resistance development in target insects may occur only in specific regions.

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Low - High** for Bt maize, and
- **Negligible** for all HT and SM crops.

D.   Effects on Non-Target Organisms

The potential impact on non-target organisms is a substantial part of any environmental risk assessment of GMOs, and this issue was discussed in the literature long before the first commercial use. According to one of the first reviews (Ehler 1990), the environmental impact is defined as any measurable effect of an introduced species on a non-target species. With respect to unintended effects on non-target species a number of review papers particularly emphasize the importance of GM plants that produce insecticidal proteins (Hails & Raymond 2004, Dolezel *et al.* 2005, Lövei & Arpaia 2005, O'Callaghan *et al.* 2005, Andow & Zwahlen 2006, Hilbeck & Schmidt 2006, Schuler 2006, Sanvido *et al.* 2007, Greenpeace 2007, Marvier *et al.* 2007, Widmer 2007, Woiwod & Schuler 2007).

An assessment is required of the possible immediate and/or delayed environmental impact resulting from direct and indirect interactions of the GM plant with non-target organisms, including the impact on population levels of competitors, herbivores, symbionts (where applicable), predators, parasites and pathogens (EFSA 2004). Tests on non-target organisms covering bi- and tri-trophic interactions including direct and indirect effects, are widely accepted in risk assessment and results are widely published in the literature.

An extensive body of research data has been assembled on non-target impacts of the Cry1Ab expressing maize events MON810, 176 and Bt11. One important lesson according to Schuler (2006) is that negative effects observed in the laboratory do not necessarily translate into impacts in the field where many other factors affect the impact on non-target species (including climate, food availability and predation). The majority of studies reviewed by Schuler (2006) do not show any unexpected negative effects on non-target insects.

A tiered process of toxicity testing is generally used to assess the non-target effects posed by traditional insecticides because it is suitable for assisting the decision-making process in an effective and rigorous way (Romeis *et al.* 2008). The application of tiered approaches is widely accepted (Rose 2007, EFSA 2004), but differences appear e.g. how to use the results for decision making. There is considerable disagreement about the most appropriate framework for using ecological approaches (Andow *et al.* 2006, Andow & Zwahlen 2006; Romeis 2006, Romeis *et al.* 2006a), and a major difference between the approaches is related to confidence and certainty in decision making within the tiered framework.

Birch *et al.* (2007) provided a detailed discussion of the role of laboratory, greenhouse and field scale experiments in understanding the interactions between GM plants and soil ecosystems. They concluded that results were not predictive between the three experimental scales, but have value when used with feedback loops between the scales, which can be used to address questions raised by results from any level of experimentation and also for

putting GM crop risks into context with current agricultural practices in regionally differing agro-ecosystems.

The review of Andow & Zwahlen (2006) provides an overview on ERA development since the end of the 1980s. The authors synthesize previous models for tiering risk assessment and propose a general model for tiering. Future genetically modified crops are likely to pose greater challenges for risk assessment, and meeting these challenges will be crucial in developing a scientifically coherent risk assessment framework. Scientific understanding of the factors affecting environmental risk is still nascent, and environmental scientists need to help improve environmental risk assessment.

In conclusion, 'tiering' provides a very useful concept to group the published literature into the following five categories (*see Annex L2 directly attached to the end of this literature review*).

Tier 0	Literature reviews or modelling approaches
Tier 1	Laboratory studies with purified insecticidal protein
Tier 2	Laboratory or glasshouse studies with GM insecticidal plants (or parts of plants)
Tier 3	Semi-field studies (contained environment) with GM insecticidal plants
Tier 4	Field studies with GM insecticidal plants (open environment)

D.1 Direct toxic effects on plant-associated NTO

Genetically modified crops that express insecticidal Cry proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) have been grown on a steadily increasing area worldwide since their first introduction in 1996. However, no studies greater than three to four years are published so far concerning effects of GM crops on NTO.

Bt proteins are known to be highly specific to the target species groups (O'Callaghan *et al.* 2005). GM Bt plants usually express lepidopteran and/or coleopteran specific proteins in all plant organs (including pollen and roots) during the whole season. Therefore, larvae of target species (e.g. European Corn Borer (ECB) or Western Corn Rootworm (WCR)) as well as larvae of non-target species (e.g. butterflies or beetles) are exposed. In the EU, lepidopteran or coleopteran specific Bt proteins have only been used in maize until now (mainly for imported products), and only a Cry1Ab expressing maize (MON810) is cultivated.

It is well documented that a range of lepidopteran species larvae may be affected by Bt proteins (Losey *et al.*1999, Felke & Langenbruch 2002, Lang & Vojtech 2006, Prasifka *et al.* 2007). Results on detrimental effects are mainly derived from laboratory tests; some

appeared in maize fields (Hellmich *et al.* 2001, Schmitz *et al.*, 2003; for a review see Evans 2002). However, exposure of any populations of lepidopteran larvae to the toxin is restricted to those consuming the Bt plant or its products. In the vicinity of the Bt maize field additional larvae may be exposed to the Bt protein when Bt maize pollen is deposited on plants on which they are feeding. This was proven by a huge number of semi-field and field experiments conducted in USA and Europe (Oberhauser *et al.* 2001, Pleasants *et al.* 2001, Sears *et al.* 2001a, b, Stanley-Horn *et al.* 2001, Wraight *et al.* 2000, Dively *et al.* 2004, Lang 2004, Gathmann *et al.* 2006a). In all studies no or minor effects for lepidopteran non target species were reported according to the authors.

Similar to the lepidopteran example potential detrimental effects for NTO coleopteran species could be expected from crops expressing coleopteran Bt-protein e.g. Cry3Bb1. Beetle species feeding on plant parts as well as carnivorous species such as rove beetles or carabid beetles are exposed to the Bt protein (Harwood *et al.* 2005, Zwahlen & Andow 2005, Obrist *et al.* 2006a, b). In lab tests, three studies showed increased mortality or sublethal effects e.g. on longevity of ladybird or carabid beetles (Bai *et al.* 2005, Meissle *et al.* 2005, Schmidt *et al.* 2004). It has to be considered that these studies were conducted in the laboratory under worst-case scenarios. Under field conditions Wold *et al.* (2001) found for the ladybird beetle *Coleomegilla maculata* in only one year fewer individuals compared to conventional maize fields. In the following year this result was not reproducible. The authors concluded: "In summary, few statistical differences and inconsistent numerical trends might suggest that Bt has no adverse effects on beneficial insects in the field". Other studies regarding coleopteran species detected no effects on coleopteran species either for coleopteran (e.g. Lundgren & Wiedemann 2002, Bhatti *et al.* 2005, Mullin *et al.* 2005, Lundgren *et al.* 2005, Ahmad *et al.* 2006a, Duan et al 2006, Ferry *et al.* 2007, Raybould *et al.* 2007) or for lepidopteran specific Cry proteins (e.g. Lozzia 1999, Bourguet *et al.* 2002, Candolfi et al 2004, Toschki *et al.* 2007).

Effects on NTOs not related to the Lepidoptera or *Coleoptera* and associated to Bt-plants are only reported from tier 1 or tier 2 studies for aphids (Deml et al 1999, Ashouri *et al.* 2001, Liu *et al.* 2005), acari (Obrist *et al.* 2006a), Heteroptera (Ponsard *et al.* 2002) and bees (Ramirez-Romero et al 2005) (see also review of Hilbeck & Schmidt 2006). However, none of these studies could clearly identify the Bt protein as stressor responsible for the effect. The methodology and experimental design of some of the studies has also been subject to controversial discussion. Further studies report on adverse effects with regard to predators and parasitoids fed with prey or hosts reared on Bt protein containing diet or plants (e.g. Hilbeck *et al.* 1998, Lövei & Arpaia 2005, Hilbeck & Schmidt 2006). These studies are discussed under Section D.3 (tri-trophic interactions).

A number of laboratory semi-field and field studies on non-target effects on bees reported no effects of Bt protein (Malone *et al.* 2001, Hanely 2003, Malone 2004, Bailey *et al.* 2005, Babendreier *et al.* 2005, Rose *et al.* 2007, Marvier *et al.* 2007), or for different groups of phytophagous and predatory arthropods (e.g. Riddick *et al.* 2000, Bourguet *et al.* 2002, Jasinski *et al.* 2003, Candolfi *et al.* 2004, Carter *et al.* 2004, Rauschen *et al.* 2004, Sisterson *et al.* 2004, Alvarez *et al.* 2005, Dively 2005, Eckert *et al.* 2006, Meissle & Lang 2005, Naranjo *et al.* 2005a, Obrist *et al.* 2005, O'Callaghan *et al.* 2005, Pons *et al.* 2005, Poza *et al.* 2005, Eizaguirre et al 2006, Habustova *et al.* 2006, Ludy & Lang 2006a, Habustova *et al.* 2007, Rose & Dively 2007, Toschki *et al.* 2007).

Additionally, potential toxic effects of Bt-proteins were also assessed on NT soil organisms. Results of studies on the effects of Bt proteins on nematodes are controversial. Direct toxic effects on nematodes were observed by Marroquin *et al.* (2000) and Wei *et al.* (2003) using Cry5B, Cry 6, Cry 12, Cry 14 and Cry 21 proteins which are different from lepidopteran or coleopteran specific proteins currently used in GM plants. No significant differences in the abundance of nematodes in the rhizosphere of Bt maize and non-Bt maize were reported by Saxena & Stotzky (2001a). In contrast, Manachini *et al.* (2004) observed a shift in nematode community structure in soil cultivated with Bt oilseed rape compared to the non-Bt oilseed rape control. An overall comparison of Bt versus non-Bt maize across three different field sites in different European regions revealed significantly reduced numbers of nematodes under Bt maize compared to non-Bt maize (Griffiths *et al.* 2005). The authors judged this effect as small and within the normal variation range expected in the considered agricultural systems. In a greenhouse study no negative effects on nematode communities were observed, the nematode population sizes under Bt maize were even higher than in soils with non-Bt maize (Griffiths *et al.* 2006). The reasons for these contradictory results remain unclear. Lang *et al.* (2006) studied the effects of Bt maize on two nematode species in field trials: No adverse Bt-effects were observed with respect to population parameters of *Pratylenchus spp*, whereas growth and reproductive fitness of *Caenorhabditis elegans* was negatively affected.

In other studies only sublethal effects of Bt protein such as reduced weight, food selection or behaviour were observed. Conclusions on toxic effects from these studies are difficult to draw due to potential side effects e.g. caused by use of different experimental designs: According to Bakonyi *et al.* (2006) Bt-maize (producing Cry1Ab protein) was a less preferred food source for *Folsomia candida* than the isogenic one. No similar phenomenon was found in the case of two other species (*Heteromurus nitidus* and *Sinella coeca*). In a further study Heckmann *et al.* (2006) found no adverse effect on the collembolan *Protoaphorura armata*. Zwahlen *et al.* (2003b) reported a 200-day study investigating the impact of genetically

modified Bt maize event Bt11 (expressing Cry1Ab) on immature and adult *Lumbricus terrestris* in a single worst-case laboratory study and in a single small scale field test. At the end of the laboratory test the earthworms showed a significant weight loss of 18% (compared with their initial weight) when fed with Bt maize litter whereas a weight gain of 4% occurred with non-GM control maize. No difference was found in the higher tier small scale field test. Further two studies reported that Cry1Ab had no apparent effect on earthworms or nematodes in a 45-day combined laboratory and field study (Saxena & Stotzky 2001a, Ahmad *et al.* 2006). Laboratory studies to investigate effects of genetically modified Cry1Ab (MON810) maize leaf material on the terrestrial isopods report a difference in isopod growth (Escher *et al.* 2000, Wandeler *et al.* 2002, Clark *et al.* 2006). A further study reports on sublethal effects of Bt-proteins on saprophytic diptera larvae (Büchs *et al.* 2005)

In contrast, no effects found in the ECOGEN project could be attributed to the Bt maize on snails, microarthropods or mycorrhizal fungi in mesocosm and field experiments (Cortet *et al.* 2007, de Vaufleury *et al.* 2007, Griffith *et al.* 2007a, b, Krogh *et al.* 2007). However, the detection of Bt protein in snails and their faeces was identified as a novel route into the soil food web (Harwood *et al.* 2005, Harwood & Obrycki 2006, de Vaufleury *et al.* 2007).

Harwood & Obrycki (2006) studied the impact of Bt maize on molluscs. Although the slug, *Deroceras reticulatum* is a major agricultural pest throughout the world, Bt crops were not intended to target these species. Molluscs are readily consumed by many generalist predators; if these Cry1Ab proteins are taken up by slugs during feeding on genetically modified plants, predators would therefore be exposed to elevated Bt protein concentrations. Using a biochemical assay, the authors tested the hypothesis that slugs fed genetically modified maize would accumulate detectable quantities of Cry1Ab proteins for prolonged periods of time. Characterization indicated that at low dilution rates, Cry1Ab proteins were detectable in slugs fed Bt-maize but no reactivity was elicited by specimens fed non-GM food. It was possible to detect Cry1Ab proteins in slugs for 95.9 h after consumption of Bt-maize. Although quantities were small, these long detention periods indicated potential exposure of generalist predators to low concentrations of genetically modified insecticidal proteins in the field. In addition it was observed in a further study that *Bt* had an adverse effect on snails parasitized by nematodes whereas snails showed no reaction on Bt when healthy (Kramarz *et al.* 2007a, b).

At the beginning of the 1990s, the potential harm *Bt* insecticide posed to aquatic ecosystems was investigated (Kreutzweiser *et al.* 1992, Richardsen & Perrin 1994 Kreutzweiser *et al.* 1994, Kreutzweiser & Capell 1996). The results from these studies indicated that significant adverse effects of *Bt* protein on aquatic macroinvertebrates were not likely. Additionally, the study of Douville *et al.* (2007) showed that aquatic organisms could be exposed to *Bt* protein

also from genetically modified plants due to residues in the sediments. The laboratory study of Rosi-Marshall *et al.* (2007) showed significant adverse effects on caddiesflies at high pollen exposure level, but the relationship to *Bt* maize events or dose-response level of Cry1Ab is unclear from the data presented in the publication.

Additionally, studies with GM crops expressing other proteins such as protease inhibitors, chitinase or lectins report adverse effects on bees, predators and parasitoids (e.g. Malone *et al.* 1999, Burgess *et al.* 2002, Down *et al.* 2003, Otsu *et al.* 2003, Dechaume-Moncharmont *et al.* 2005, Lövei & Arpaia 2005, Romeis *et al.* 2006b). However, none of these GM crops has yet been authorised.

Reports looking at toxic effects caused by herbicide tolerant GM crops to NTO are rare. The low number can be expected due to missing theoretical indications for toxicity of the specific transformation event. Finally, none of the available studies report adverse effects on NTOs (e.g. Volkmar *et al.* 2003, Huang *et al.* 2004)

One additional aspect regarding potential target organism effects from introduction of *Bt* genes into maize has come up in the last years. In several applications for marketing of *Bt* maize lines multiple Cry-proteins are expressed. This is realized for example. in *Bt* cotton expressing Cry1Ac and Cry2Aa proteins both acting against lepidopteran pests (Jurat-Fuentes *et al.* 2003) or in MON89034 expressing the Cry1.105 toxin.

Conclusions

An extensive body of research data has been assembled e.g. on non-target organism impacts of IR maize. Where the available literature suggests toxic effects of crops expressing Bt protein within short term Tier 1 and Tier 2 studies, these effects are rarely – if at all – also found in Tier 3 and Tier 4 studies, i.e. negative effects observed in the laboratory do not necessarily translate to field conditions. However, conclusions from short-term ecotoxicological experiments cannot entirely eliminate uncertainty regarding long-term environmental effects. In particular, the observed sublethal effects could have the intrinsic potential to affect NTOs in the long run. The majority of laboratory studies and all the field studies reviewed have not revealed any unexpected adverse or long-lasting effect on NTOs. There is also no indication for direct adverse effects on NTOs caused by HT crops.

D.1 Overall assessment:

Based on the literature review, the following process was identified for expert consultation on potential long-term effects for Bt maize:

GM traits may have adverse effects on plant-associated non-target organisms (e.g. on herbivores, pollinators, soil organisms, predators, parasitoids) due to sublethal toxicity (chronic exposure) by consumption of pollen and plant tissue.

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Low** for Bt maize lines expressing **high levels of Cry1Ab protein in pollen**, and
- **Negligible** for all HT and SM crops.

D.2 Effects on NTO due to altered nutritional composition of the GM plant

Saxena and Stotzky (2001b) found higher lignin contents in three maize events (maize Bt11, Bt176, and MON810) genetically modified to express the *Bacillus thuringiensis* Cry1Ab protein potentially derived from a pleiotropic effect. Poerschmann *et al.* (2005) confirmed the occurrence of pleiotropic effects with regard to lignin biosynthesis in stems of Bt maize as described by Saxena and Stotzky (2001b), although to a lesser extent. Another study suggests that the extent of lignification of Bt maize (several lines derived from MON810 and Bt11) does not differ from the non-GM controls (Jung and Sheaffer 2004). However, lignin is well-known for its capability to influence palatability and digestibility of plant material to herbivores and decomposers (Zwahlen *et al.* 2003a).

Some studies reported that herbivores might prefer genetically modified plants as food more than their conventional counterparts or developed faster on GM crops than on non-GM crops. This was observed in lab studies for acari and isopods (Escher *et al.* 2000, Wandeler *et al.* 2002, Zemková-Rovenská *et al.* 2005) and in field studies for aphids, thrips and saprophytic beetles (Bourguet *et al.* 2002, Lumbierres *et al.* 2004, Eckert *et al.* 2006). However, it is not clear whether these effects are caused by the genetic modification, by potential epigenetic effects or by different genetic backgrounds of the GM crop and its near isogenic counterpart.

During the ERA of starch modified potato, concern was raised that plant-associated organisms (e.g. invertebrates) might be affected by altered nutritional composition of the hosting crop (EFSA 2006). From field studies carried out in Sweden, Germany and The Netherlands, data were available on the impact of the modified crops on plant-associated organisms. According to EFSA (2006) the results of field studies suggest neither greater susceptibility nor greater resistance to pests (e.g. aphids, leafhoppers, potato cyst nematodes (sp *Globodera)*) and diseases (e.g. late blight (*Phytophthora infestans*), potato early blight (*Alternaria solani*), *Erwinia* rots) than non-GM potato lines. There was no evidence of changes in sensitivity to the plant-associated viruses PVY, PLRV, PMTV, and

TRV. In view of this and the equivalent composition of the GM potato plant, EFSA (2006) considered that no adverse effects on plant-associated organisms would be expected from cultivation of the potato EH92-527-1. However, altered starch composition caused by genetic modifications (e.g. amylose or amylopectine content) of potato tubers was considered to be addressed within the ERA.

Potential adverse effects of GM trees with various higher or lower lignin contents were studied by Halpin *et al.* (2007). The lignin modifications had no unexpected biological or ecological impacts. Interactions with leaf-feeding insects, microbial pathogens and soil organisms were unaltered although the short-term decomposition of genetically modified roots was slightly enhanced (Halpin *et al.* 2007).

Conclusions

The nutritional value of GM crops may potentially be altered with respect to their quality for herbivores by the newly expressed proteins (e.g. by Bt). There is no indication that altered starch composition may potentially alter fitness of plant associated herbivores or decomposers e.g. due to the genetic modification (amylose or amylopectin content) of potato tubers. In general, very few studies support any assumption that herbivorous insects favour GM in contrast to non-GM plants. Consequently, data regarding altered herbivore attractiveness of GM crops with changed nutritional composition are scarce so far. However, the available studies report no adverse effects or negligible effects being within the normal variation (see also Section 3.D.1).

In conclusion, there is a lack of experience so that the knowledge of potential long-term effects remains poor. Chronic and sublethal effects are not well investigated so far (Clark *et al.* 2005).

D.2   Overall assessment:

Based on the literature review, the following process was identified for expert consultation:

GM traits may alter nutritional composition of plants, leading to reduced fitness in plant-associated (non-pest) herbivores or decomposers

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Low** for Bt maize and SM potato (with some **uncertainty**), and
- **Negligible** for all HT crops.

D.3 Tritrophic interactions on NTO

Tri-trophic interactions are intensively discussed in ERA of GMP since first evidence was found for Bt maize and the green lacewing *Chrysoperla carnea*. Recently, Hilbeck & Schmidt (2006) reviewed the published laboratory feeding studies on effects of Bt proteins and Bt plants on *Chrysoperla carnea*.

From their results obtained in direct and prey-mediated Bt feeding trials, Romeis *et al.* (2004), Dutton *et al.* (2003) and Rodrigo-Simon *et al.* (2006) concluded that the observed mortality in Bt-fed lacewing larvae is solely due to lower nutritional quality of the sublethally affected prey without the Bt protein having a role in it. Based on a literature review, Romeis *et al.* (2006) emphasize the laboratory and glasshouse studies have revealed effects on natural enemies only when Bt-susceptible, sublethally harmed herbivores of bad nutritional quality were used as prey or host, with no indication of direct toxic effects. Conversely, Hilbeck and Schmidt (2006) claim this unlikely and a too limited interpretation. For them, the direct effects of the Bt protein feeding studies clearly document the sensitivity of *C. carnea* larvae at higher concentrations, which cannot be explained by reduced prey quality as Bt protein was fed directly to the predator using a specific lacewing diet.

In general, tri-trophic interaction could be affected by changes in prey availability, altered food quality and toxicity of the prey or host to the predator or parasitoids. The use of an adequate methodology is highly important for generating reliable results from tri-trophic experimental studies.

The potential spread of Bt proteins in the food chain was demonstrated by several studies. The exposition of different predator species to Bt protein was demonstrated for carabid beetles, Heteroptera and Chrysopidae (Harwood *et al.* 2005, Obrist *et al.* 2006a, Zwahlen & Andow 2005) with surprisingly high concentrations in some higher trophic level organisms while not in others. Of all herbivores tested, only spider mites (*Tetranychus urticae*) revealed a high concentration of Bt, which was three times higher than in Bt176 maize leaves the mites had fed upon (Obrist *et al.* 2006b).

Increasing prey or host abundance was found in some studies (see Section 3.D.2), and thus an increase of prey/host dependent predators or parasitoids was not observed. Vice versa herbivores may change their host plant preferences, which lead to a decreased prey or host availability (e.g. Zemková Rovenská *et al.* 2005). Goal of GM IR plants is to reduce effectively the abundance of the target organisms. Consequently, effects on the abundance of monophagous or oligophagous predators or parasitoids could be expected which could lead to a decreasing abundance of these species in particular on the long term (e.g. Riddick *et al.* 1998, Bourguet *et al.* 2002, Pilcher *et al.* 2005). A changed preference of prey was

described by Zemková-Rovenská *et al.* (2005) showing that predatory mites preferred feeding on mites not reared on genetically modified Bt egg plants.

As mentioned above host quality may affect tritophic interactions. In particular the parasitoids or hyperparasitoids developed in Bt fed host were significantly smaller, have longer development time or less fecundity or are less successful in parasitizing (Romeis et al 2003, Prütz *et al.* 2004, Lövei & Arpaia 2005, Prütz & Dettner 2004, Romeis *et al.* 2006b, Sanders *et al.* 2007, Ramirez-Romero *et al.* 2007). Birch *et al.* (1999) showed for predatory 2-spotted ladybirds feeding on aphids reared in potatoes expressing snowdrop lectin that reduced longevity and fecundity of the beetles.

In contrast, Down *et al.* (2003) found for the same prey and host species no effects on longevity and a trend for improved fecundity up to 70%. Furthermore Schuler *et al.* (2003) showed that number of emerging parasitoids was higher on Bt-plants compared to wildtype plants. Faria *et al.* (2007) reported in laboratory studies an effect of Bt maize on the performance of the maize leaf aphid *Rhopalosiphum maidis*, which in turn enhanced the performance of parasitic wasps that feed on aphid honeydew from GM plants.

A tri-trophic study was performed with GM oilseed rape expressing a protease inhibitor (oryzacystatin – OC-1), the grey field pest mollusc *Deroceras reticulatum* as herbivore and the beneficial predator *Pterostichus melanarius* as predator (Harwood & Obrycki 2006). It was demonstrated that the protease inhibitor had no acute detrimental effect on the beneficial beetle consuming the pest mollusc, which was exposed to OC-1.

Trophic interactions between GMP and birds were studied by Gibbons *et al.* (2006) and by Chamberlain *et al.* (2007) based on the farm scale evaluation (FSE) in the UK (see Firbank *et al.* 2003a). The study aimed to compare bird abundance between GMHT and conventional crop treatments. Depending on assessed species, GM crop and crop management for a decrease or increase of bird abundance were recorded. The observed differences were in accord with likely differences in food availability ascertained from previous research carried out under the FSE (see also Section 3.F).

<u>Conclusions</u>

GM protein susceptible herbivores (2nd trophic level organisms) feeding on host plants expressing GM protein (1st trophic level organism) have the potential to show a reduced nutritional value leading to a reduced fitness of predators or parasitoids. Long-term adverse effects might be expected on populations or diversity of predators or parasitoids feeding on larvae with altered nutritional value. On the other hand, the vast majority of available studies report no adverse effects or negligible effects of predators or parasitoids being within the normal variation (see also Section 3.D.1).

<u>D.3   Overall assessment:</u>

Based on the literature review, the following process was identified for expert consultation:

GM traits may cause altered nutritional value of host or prey organisms in trophic interactions, which affects trophic interactions in higher trophic levels. Adverse effects become apparent in non-target-organism communities (e.g. predators, parasitoids)

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Low** for Bt maize, and
- **Negligible** for all HT and SM crops.

D.4   Effects on non-target organisms due to accumulation of toxic compounds

Some scientific publications indicate that the Bt protein may persist in soil during cultivation of Bt maize and may accumulate in sequential crops. This might affect soil organisms. Therefore, both direct and indirect impacts of the protein or the Bt maize (e.g. potential increase of lignin content in combination with a possible delay in decomposition) on non-target organisms and soil function should be considered in risk assessment (Saxena *et al.* 2002a, Zwahlen *et al.* 2003a)

Nguyen & Jehle (2007) monitored the tissue-specific expression and seasonal abundance of Cry1Ab protein in Mon810 maize plants in Germany. The results showed that the Cry1Ab contents varied strongly between different plant individuals. The observed variation exceeds variation levels reported previously and may be due to the large number of analysed samples and different growing years. They suggest a certain plant to plant variation in Cry1Ab expression. The authors conclude that the variations observed are within a biologically explainable range and could explain partly the variation of Bt protein between years in soil.

Concentrations of Cry1Ab in the Bt-varieties were sufficient to effectively control corn-borer larvae within the ECOGEN project conducted over three years in field plots in Denmark and France (Andersen *et al.* 2007). Cry1Ab protein concentrations found in the soil were increased in the plots with Bt-varieties but they did not seem to increase from year to year. The quantification of Bt-protein in soil was confounded by the low concentrations in soil and interference from soil factors, as suggested by the seasonal variation in the amounts of Bt-protein apparently detected even under non-Bt maize.

Douville *et al.* (2007) examined the occurrence and persistence of the Cry1Ab gene from conventional Bt spray (Btk) and genetically modified Bt maize in aquatic environments near fields where Bt maize was cultivated in Canada. For sediments, the Cry1Ab gene from Bt

maize was still detected after 40 days in clay and sand-rich sediments. Field surveys revealed that the Cry1Ab gene from genetically modified maize and from naturally occurring Bt was more abundant in the sediment than in the surface water. The Cry1Ab DNA sequence was detected as far away as 82 km downstream from a maize cultivation plot, suggesting that there were multiple sources of this gene and/or that it undergoes transport by the water column. Sediment-associated Cry1Ab gene from Bt maize tended to decrease with distance from the Bt maizefield. The data indicate that DNA from Bt maize and conventional Btk were persistent in aquatic environments and were detected in rivers draining farming areas. However, the levels of Cry1Ab protein in samples were below the detection limit most of the time.

According to Rauschen & Schuphan (2006) it is likely that genetically modified Bt-maize will be fed into agricultural biogas facilities. The fate of the entomotoxic protein Cry1Ab from MON810 maize was therefore investigated in silage and biogas production-related materials in the utilization chains of two farm-scale biogas facilities. The Cry1Ab content in silage exhibited no clear-cut pattern of decrease over the experimental time of 4 months. After fermentation in the biogas plants, the Cry1Ab content declined to trace amounts of around 3.5 ng g^{-1} in the effluents. The limit of detection of the employed ELISA test corresponded to 0.75 ng Cry1Ab g^{-1} sample material. Assays with larvae of *O. nubilalis* showed no bioactivity of the reactor effluents. The authors conclude that the utilization of this residual material as fertilizer in agriculture is therefore deemed to be ecotoxicologically harmless.

Field studies were done in Germany to assess how much of the genetically modified, insecticidal protein Cry1Ab, was released from Bt-maize (MON 810) into soil. Additionally, potential differences were analyzed for bacterial communities inhabiting the rhizosphere of MON 810 maize in comparison to those of the rhizosphere of non-GM maize cultivars (Baumgarte & Tebbe 2005). The concentrations of Cry1Ab protein in the rhizosphere did not accumulate during the growing season, despite the affinity of the Cry1Ab protein for soil particles. The concentrations of the Cry1Ab protein in soil from Bt-maize fields are in the range between 0.1 and 10 ng g^{-1} in bulk soils and rhizospheres. Baumgarte & Tebbe (2005) were not aware of any non-target or target organism that would directly respond to such low concentrations as a bioindicator. In addition, the bacterial community structure was less affected by the Cry1Ab protein than by other environmental factors, i.e. the age of the plants or field heterogeneities.

A four year study on the decay of genetically modified maize Bt protein was published by Hopkins and Gregorich (2003). The Bt protein decay followed the rate at which the Bt-maize leaves decomposed in soil from a field in which Bt-maize had been cultivated for four years. In addition, Hopkins and Gregorich (2005) determined the concentrations of the Cry1Ab

protein in organic residues from MON 810 maize plants at increasing stages of ageing and decay, and the subsequent decomposition in soil of these residues and the Cry1Ab protein in them. The Cry1Ab protein decomposes faster than the bulk organic carbon in residues and it is likely to fall below the detection limit by ELISA within months of entering the soil. The results suggested that much of the Cry1Ab protein in crop residues is highly labile and quickly decomposes in soil, but that a small fraction may be protected from decay in relatively recalcitrant residues.

Icoz & Stotzky (2007) studied the potential accumulation of the Cry3Bb1 protein which is insecticidal to the corn rootworm complex (*Diabrotica* spp.). *Bacillus thuringiensis* (Bt) subsp. *kumatomoensis* was released in root exudates of genetically modified Bt maize (event MON863) in sterile hydroponic culture and in nonsterile soil throughout growth of the plants. Soils were analyzed for the presence of the protein every 7 to 10 days with a western blot assay (ImmunoStrip) and verified by ELISA. The protein was detected for only a maximum of 21 days. For the authors, these results indicate that the Cry3Bb1 protein does not persist or accumulate in soil and seems to be degraded rapidly.

In another study published in 2008 the authors are reporting very different results regarding the persistence of Cry-proteins in soils (Saxena & Stotzky 2008). Half-lives of cry1Ab protein range from 1,6 days (Sims & Holden 1996) up to 34 days (Wang *et al.* 2006b). Also long-term persistence of cry1 proteins in soils is variable. Cry1Ab proteins in low concentrations were detected up to 56 days (Donegan *et al.* 1995) or up to 234 days (Tapp & Stotzky 1998) or up to 180 to 350 days in residues of Bt maize (Saxena & Stotzky 2002b).

As mentioned in Section D.3 Bt protein was accumulated in prey organisms and may lead to increased exposure to predators or parasitoids (Obrist *et al.* 2006a, b). Studies with other GM proteins than Bt concerning potential accumulation in soil or water are not available yet.

Conclusions

Bt proteins may theoretically accumulate in some host species (see Section D.3). Short term studies showed so far that the fate of Bt proteins in the soil is not fully understood in the low concentration range. The time-spans over which residues of Bt proteins have been detected vary enormously. Bt-protein concentrations measured using standard ELISA-tests in soil or water are very low indicating that direct toxic effects to soil or water organisms are presumably of low likelihood. However, it is still unclear whether soil persistence processes could be of higher importance and could lead to long-term effects on soil organisms and soil ecological function.

D.4   Overall assessment:

Based on the literature review, the following process was identified for expert consultation:

Accumulation of toxic compounds e.g. from Bt proteins in various environmental compartments (e.g. accumulation in soil) could result in decrease of abundance of (e.g. beneficial) NTO.

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Low** for Bt maize, but **uncertainties** remain concerning long-term effects on soil NTOs and soil ecological functions and concerning effects of specific Bt proteins if Bt maize is cultivated continuously in the same fields,
- **Negligible** for all HT and SM crops.

D.5   Effects on rhizosphere microbiota

According to Schuler (2006), scientists have expressed concern that understanding of the impacts of Bt protein on soil functions is still limited. Genetically modified products were introduced in soils through two pathways (i) decaying plant material (Baumgarte & Tebbe 2005) and (ii) release of Cry1Ab proteins by roots via root exudates (Saxena *et al.* 2002b).

A total of 60 studies were reviewed for the effects of seven different types of genetically engineered traits, i.e. herbicide tolerance, insect resistance, virus resistance, proteinase inhibitors, antimicrobial activity, environmental application, and biomolecule production by Widmer (2007). For Bt maize, 12 studies were analysed. The result of the overall 60 studies presented by the author indicates that the tools for sensitive detection of changes in soil microbiological characteristics are available; however, they also reveal that at present it is very difficult or impossible to define which alterations in these characteristics may represent unacceptable damage to a soil system. This limitation becomes evident from the scientific literature presented here, as no study reported damage of a soil system, but rather potentially adverse effects.

An overview of the ECOGEN results which were not considered by the review of Widmer (2007) was published by Krogh & Griffiths (2007). Maize expressing an insecticidal protein from *Bacillus thuringiensis* (Bt-maize) was chosen as the model GM crop due to its availability, while studies using GM herbicide tolerant (HT) maize were initiated in the latter stages of the project. Detailed measurements in mesocosms revealed that the slight effects of Bt maize or a conventional insecticide on nematodes, protozoa and microorganisms were less pronounced than effects due to soil and plant growth stage (Griffiths *et al.* 2006), and less than the variation seen between eight maize cultivars (Griffiths *et al.* 2007b). No effects could be attributed to the Bt maize on snails, microarthropods or mycorrhizal fungi in a separate mesocosm experiment, but the detection of Bt protein in snail faeces was identified

as a novel route into the soil food web (de Vaufleury *et al.* 2007). Field experiments were performed at four sites across three European climatic zones and showed the effectiveness of Bt maize against the European Corn Borer (Andersen *et al.* 2007). These field experiments point to the conclusion that Bt-maize (Mon 810 event) could have a significant, but small and transient, negative effect on soil protozoa, nematodes and microorganisms (Griffiths *et al.* 2005; 2007a) but no effects on organic matter (wheat straw) decomposition (Cortet *et al.* 2006). The fact that ECOGEN conducted experiments using the same organisms and soils across a range of scales (i.e. laboratory, glasshouse and field) allowed for a comparison of results from these scales and an assessment of their utility. While it was not possible to predict the outcome between scales, there was useful information and insights from each of the experimental approaches (Birch *et al.* 2007). The complexity of soil organisms and their functioning was collectively summarised in soil quality attributes and a multi-attribute model, and used in assessment of new agricultural technologies including GM crops. ECOGEN developed a quantitative, multi-attribute model to summarise the effects of the different cropping systems on soil quality (Bohanec *et al.* 2007), which has considerable potential for application for other aspects of soil management. The authors conclude that Bt-maize did not have deleterious effects on the soil biota. When effects were observed these were likely to be caused by differences between the maize varieties. Bt-maize studied in the agricultural systems did not decrease soil quality due to the GM crop itself, but changes in the agricultural techniques used along with the GM crop could improve (reduced tillage) or reduce (increased use of pesticides) the soil quality (see category F).

A further review by Lilley *et al.* (2006) focussed on studies of the effects of GM plants on soil systems. 25 peer reviewed studies were evaluated involving nine plant species (alfalfa, bird's-foot trefoil, black nightshade, potato, rice, maize, cotton, tobacco and oilseed rape) genetically modified in ten distinct ways for the expression of: α-amylase; lignin peroxidase; an organic acid (malate dehydrogenase); T4-lysozyme (anti-pathogenic); cecropin b (an antibacterial lytic peptide); Bt protein; insect resistance (proteinase inhibitor I); herbicide tolerance; opines; and lignin production. Effects on the soil community or soil system were noted in 16 of the studies. Although these effects were generally transient, they included effects on bacterial diversity, number and activity; fungal counts; effects on numbers of protozoa, nematodes and collembola; diversity of nematodes; and woodlice mortality. Most of these studies were limited to a single growth-season; although some post-experiment monitoring was reported and two- and three-year studies were reported in genetically modified herbicide tolerant canola and T4-lysozyme expressing potato. The conclusions of this review are as follows: (1) Considerations of the transgene product, its activity, site of expression and persistence are important guides to developing, on a case-by-case basis,

lines of investigation into probable and/or important potential effects. (2) Most genetically modified plants have detectable effects on the soil system, which are relatively minor compared with differences between cultivars or those associated with weather and season. Assays of the natural variation in the system provide valuable baseline references. (3) The response of soil systems when genetically modified plants are removed provides an important measure of impact. Studies generally find a quick return of many soil parameters to match those of the control soils. (4) Many apparent losses of taxa observed in field monitoring are probably changes in the relative numbers of different groups in the community. (5) Regular sampling is important because changes in community structures, through the season and plant development, confer an added level of complexity to comparing GM *vs*. non-GM effects. Many of these effects are context-dependent and not systematic in character through the season. (6) Arising from the case-by-case approach, specific targets for monitoring are selected, which have intrinsic and clear definitions of damage; however, there is a lack of monitoring activity, which is linked to a concept of damage to the system. Lilley *et al.* (2006) conclude that slowly accumulating effects, effects that become apparent when land-use conditions change, and effects detected only at commercial scales make it important that long-term monitoring should be installed for data collection, both of which can improve the science of risk assessment (see also Section 3.F).

Brusetti *et al.* (2004) investigated functions and communities of rhizosphere bacteria living in the rhizosphere of Cry1ab expressing Bt 176 maize in comparison to a non-Bt maize control. With respect to bacterial counts and catabolic profiling no significant differences were revealed. However, regarding rhizosphere and bulk soil bacterial communities differences were detected depending on the age of the two plant communities. Accordingly, the authors state that root exudates are able to determine the selection of specific bacterial communities.

Devare *et al.* (2007) studied the impact of Cry3Bb1 expressing MON863 on soil microbial activity and biomass in a 3-year field trial in the USA. Microbial biomass, nitrogen (N) mineralization potential, short-term nitrification rate, and respiration rate were measured in rhizosphere and bulk soil samples collected from three replicate field plots just before planting, at anthesis, and at harvest in each year. There were clear seasonal effects on microbial biomass and activity in the field soils as represented by the consistent changes in all measured variables across years and sampling times. Differences in the measured variables were also sometimes observed between bulk and rhizosphere soil. According to the authors there were no adverse effects of either the Bt or non-Bt maize with insecticide applied compared to the non-Bt controls. On the contrary, microbial biomass and soil respiration data suggested a stimulatory effect of the Bt genotype, particularly in comparison to the non-Bt isoline. The data suggest that cropping MON863 Bt maize is unlikely to

adversely affect soil ecology in the short term. Longer-term monitoring of cropping systems using GMP should assure that the biotic functioning of the soil is maintained as a part of studies on overall ecosystem integrity.

Direct adverse effects of HT plants to soil microorganisms are not reported so far. Effects on nitrogen fixing bacteria and pathogen fungi due to herbicide use and root exudates containing herbicide residues were found by Kremer *et al.* (2005) and Njiti *et al.* (2003). These aspects were taken into consideration in Section 3.F.

Conclusions

Decaying plant material or root exudates containing genetically modified products may affect population size and activity of rhizosphere organisms. Field experiments led to the conclusion that genetically modified products, in particular Bt proteins, could have a significant but transient negative effect on soil protozoa and microorganisms (e.g. O'Callaghan *et al.* 2005, Krogh & Griffiths 2007 (ECOGEN)). However, data are only available from short term experiments and predictions of potential long term effect are difficult to deduce.

D.5   Overall assessment:

Based on the literature review, the following process was identified for expert consultation:

GM traits are able to cause adverse effects on rhizosphere (plant-associated) bacteria and mycorrhizal fungi, e.g. due to altered root exudation impacting soil communities (population size and community structure)

According to the published information the likelihood of this process for the currently used GM crops in the EU is

- **Low** for Bt maize but **uncertainties** remain regarding mykorrhizal fungi, and
- **Negligible** for all HT and SM crops.

D.6   Effects on symbiotic NTO

Mycorrhizal symbiosis of crop plants provides a source of nutrients and constitutes an important functional component of the soil plant system (Leyval *et al.* 2002). Effects on Bt maize on mycorrhizal fungi have been reported by Castaldini *et al.* (2005) and Turrini *et al.* (2004) in microcosm and greenhouse experiments. Significantly lower level of mycorrhizal colonization in Bt maize roots was observed compared to non-Bt maize. On the contrary, de Vaufleury *et al.* (2007, ECOGEN) did not find any differences in mycorrhizal colonization or infectivity between Bt and non-Bt maize in microcosms.

Direct influence of HT crops on symbiotic organisms is not reported so far. Only indirect effects of herbicides on nitrogen fixing bacteria were reported which are addressed in Section 3.F.

Conclusions

Adverse long-term effects are likely on symbiotic activity of mycorrhizal fungi in Bt-maize plants if these varieties are continuously cultivated on one field over several years expressing lepidopteran-specific or coleopteran-specific proteins

D.6   Overall assessment:

Based on the literature review, the following process was identified for expert consultation:

Bt traits may cause specific root exudations which could lead to fitness changes in NTO-involved symbiotic organisms e.g. involved in nitrogen-fixing activities, mycorrhizal fungi.

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Low** for Bt maize with **uncertainties** due to effects on mykorrhizal fungi and to effects observed in Bt176 maize, and
- **Negligible** for all HT and SM crops.

E.   Effects on ecological functions

Some species or groups of species being potentially affected by GM plants participate in ecological processes that may be key species for fundamental ecological functions like soil fertility maintenance, biological control of pests, and pollination (e.g. Lövei 2001). In addition to their broad ecological relevance, these functions are useful or necessary for agricultural production. However studies relating to potential effects on single species or species groups to ecosystem functions are rare.

E.1   GM traits affecting changes on soil functions

The maintenance of soil fertility is a major ecological function. Besides physical factors like climate, soil type, weathering and water supply it is influenced strongly by the soil biota mediated processes of nutrient cycling, decomposition of organic matter, and biological nitrogen-fixation (e.g. Begon *et al.* 2005). There is concern that impact of GM plants on non-target organisms including microorganisms (see Section 3.D) which are involved in these processes, will cause negative alterations of soil functions (e.g. Wolfenbarger & Phifer 2000).

GM plants release exudates into the rhizosphere. Additionally, GM crop residues decompose in soil. Through both processes genetically modified products will enter the soil environment.

For the Bt protein of GM insecticidal resistant crops this has been demonstrated in several studies (Saxena & Stotzky 2001a, Saxena *et al.* 2002b, Zwahlen *et al.* 2003a, Saxena *et al.* 2004, Stotzky 2004, Baumgarte & Tebbe 2005, Icoz & Stotzky 2007). Beside root exudates and plant residues pollen is another source of Bt proteins entering soils (e.g. Losey et al 1999, Obrycki *et al.* 2001). Additionally, Bt proteins were found in the faeces of wildlife, livestock, and biogas facilities after feeding or use of Bt maize, thus entering the soil environment (Einspanier *et al.* 2004, Pont & Nentwig 2005, Harwood & Obrycki 2006, Rauschen & Schuphan 2006, de Vaufleury 2007).

GM herbicide tolerant crops are of minor interest in this case, because herbicide tolerant crops are considered not to have direct toxic effects on organisms as the enzymes conferring herbicide tolerance are normally present in plants or microorganisms and are not known to be toxic (e.g. Carpenter 2001).

The stability and persistence of the Bt proteins in soil is a key factor for determining exposure and potential effect on soil biota related to soil function. Studies have shown that Bt proteins can bind to soil particles (e.g. Pagel-Wieder *et al.* 2007, Stotzky 2004) and persist for at least half a year (Stotzky 2004, Zwahlen *et al.* 2003a), thereby retaining its insecticidal activity (Saxena *et al.* 2002b), even though the Bt concentrations were rather low after a few months compared to the initial amounts (Baumgarte & Tebbe 2005, Zwahlen et. al. 2003a). In another field trial, the Cry1Ab protein was detected in soils also during four consecutive years of Bt maize cultivation in very low levels (Icoz *et al.* 2008).

Several studies examined the persistence of Bt proteins in soils. Most studies in soil microcosms have suggested that Bt proteins do not persist and degrade within days or few weeks in soil (Donegan *et al.* 1995, Sims & Holden 1996, Head *et al.* 2002, Ahmad *et al.*2005, Dubelman *et al.* 2005, Icoz & Stotzky 2007). These variable degradation times for Bt proteins in soil might be the result of the different crops and Cry proteins observed and of different factors such as nutrient concentrations, temperature, pH-value, type and amount of clay minerals, organic matter concentration (for a broad discussion see Icoz & Stotzky 2008). Additionally different methodological approaches may partly account for conflicting results (Bruinsma *et al.* 2003, Dolezel *et al.* 2005). The variations in the reported Bt protein contents could also be explained by the binding of the Bt proteins on surface-active particles (Tapp & Stotzky 1998, Saxena & Stotzky 2001b, Blackwood & Buyer 2004, Pagel-Wieder *et al.* 2007). The amount of surface-active particles, mainly clays and humic acids, seems to play a major role in the ability of the Bt proteins to persist in soils. Once bound to these particles, their availability to microbial degradation is reduced, but insecticidal activity is retained (Crecchio & Stotzky 2001).

The persistence of Bt proteins in soil at low levels may potentially lead to a prolonged exposure of the microbial and invertebrate communities in soils. The soil biota including microorganisms mediates crucial ecological processes being part of nutrient cycling, e.g. decomposition and N-fixation. Impacts of GM plants on these processes may be due to direct toxic effects on organisms involved or due to differences in the amount and composition of root exudates and plant residues (Motavalli *et al.* 2004) (see also Section 3.D.)

From studies dealing with potential impacts of Bt maize on soil processes and communities, some reveal a lowered decomposition rate of residues of Bt crops compared to non-Bt crops (Flores *et al.* 2005), while others do not (Hopkins & Gregorich 2005, Zwahlen *et al.* 2007). Devare *et al.* (2004, 2007) reported no differences in N-mineralizing potential, nitrification rates and soil respiration between fields planted with either Bt or non-Bt maize. From field-incubation trials Cortet *et al.* (2006) reported, that mineralization and decomposition were mainly driven by climatic parameters with no adverse effect of Bt proteins on these processes. In a further study Mulder *et al.* (2006) reported a short-term increase in microbial respiration activity in mesocosms with Bt maize straw. The author stated that these rates of CO_2 production suggest different mineralization patterns of Bt maize straw in comparison with conventional maize straw. However, questions remain about the result of Mulder et al. (2006) as the observed differences in microbial respiration might have been caused by differences in soluble sugar content between the used GM and non-GM maize material.

Unexpected changes in plant compounds could affect residue composition (Raubuch *et al.* 2007) and hence the rate of decomposition that is often inversely related to the C:N ratio (Begon *et al.* 2005, Taylor *et al.* 1989). There have been inconsistent reports of lignin contents of Bt-crops (mainly maize) compared to the correspondent non-Bt hybrids (Flores *et al.* 2005, Jung & Sheaffer 2004, Mungai *et al.* 2005, Poerschman *et al.* 2005, Saxena & Stotzky 2001c). Griffiths *et al.* (2007b) investigated in a glasshouse experiment plant growth parameters including C:N ratios of eight varieties of maize each with its corresponding near-isogenic variety. The only plant parameter which showed a difference between Bt varieties and near-isogenic counterparts was the shoot C:N ratio; this was observed for only two of the eight varieties, and was assumed not to be attributable to the Bt trait.

Conclusions

The Bt protein from GM plants may enter the soil environment where it retains its insecticidal properties. In view of the variable results on the persistence time of Bt proteins in soil, there is potential for prolonged exposure of the microbial and invertebrate communities in soils to these proteins. It has been shown that the proteins were ingested by various soil organisms, but only minor adverse effects (especially on nematodes) were reported. The results of the

impact of Bt proteins on soil processes seem to be small. Because of the wide range of methodological techniques used and because many aspects of the soil communities are still not sufficiently understood (Kowalchuk *et al.* 2003), it is difficult to extrapolate results of effects on special taxa or communities to whole ecosystem processes.

E.1   Overall assessment:

Based on the literature review, the following process was identified for expert consultation on potential long-term effects for Bt maize:

Cry traits may cause changes in soil fertility (e.g. nutrient cycling, organic matter decomposition, biological N-fixation) due to

 a) additive, synergistic or delayed effects on non-target organisms including symbionts

 b) altered nutritional composition of the plant and impact on decomposition

According to the published information, the likelihood of these processes for the currently used GM crops in the EU is

- **Low** for Bt maize, but with some **uncertainty**, and
- **Negligible** for all HT and SM crops.

E.2   Effects on biological control

According to Eilenberg *et al.* (2001) biological control means 'The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be'. Biological control in plant crops is placed among other possibilities in integrated pest management and provides an economically important service in pest suppression (Romeis *et al.* 2006b). There is concern that GM insecticidal crops may have direct or indirect detrimental effects on non-target invertebrate organisms serving as natural enemies of crop pests and thus affecting natural biological control of pests and weeds (Dutton *et al.* 2003, Lövei 2006). Direct effects on natural enemies may arise by the ingestion of the GM insecticidal proteins and result in increased mortality or sublethal effects leading to reduced fitness and reproduction rates. Indirect adverse effects on the beneficial organisms may occur due to changed occurrence, population density and nutrient quality of prey or host being susceptible to the insecticidal proteins (Ferry *et al.* 2007, Meissle *et al.* 2005, Riddick *et al.* 1998) (see also Section 3.D).

Lövei & Arpaia (2005) listed 32 species of natural enemies that have been examined for the effect of GM plants. Most of the parameters indicated neutral impact of GM plants, but nearly 30% for predators and 39% for parasitoids respectively of the quantified parameters showed a significantly negative impact on mortality, development time or body mass in particular for

genetically modified products such as protease inhibitor or lectins.

Another recent review summarizes the effects of Bt plants on natural enemies (Romeis *et al.* 2006b). Eleven laboratory or glasshouse studies have been identified that investigated the effect of Bt crops on predators in a tri-trophic approach (citations in Romeis *et al.* 2006a, b). Deleterious effects on the predators (mortality, growth patterns, development) have been reported in studies using lepidopteran larvae as prey that were reared on Bt plants. Because arthropod predators can be omnivorous, in eight studies pollen, silk and leaf tissue from Bt plants was offered to the predators (citations in Romeis *et al.* 2006b). In no case the Bt plant material had any adverse effect on the measured life-table parameters compared to non-Bt plant material. In ten studies the potential effects of Bt plants on hymenopteran parasitoids developing in herbivores reared on Bt crops have been investigated (citations in Romeis *et al.* 2006b). Similar to the predators, in all cases where the hosts were lepidopteran herbivores ingesting the Bt protein, adverse effects on mortality, development or growth patterns of the parasitoids were observed. In the same review more than 50 field studies investigating the effects of various Bt crops on natural enemies have been evaluated (Romeis *et al.* 2006b). In general, the bulk of the studies revealed only minor, transient and mostly inconsistent effects of Bt crops versus non-Bt controls. In three field studies reductions in the abundance of specialist natural enemies in Bt crops compared to non-Bt crops were observed (citations in Romeis *et al.* 2006b) due to absence of virtually all? prey or hosts after insecticide use in the control.

In a 6-year field study the effect of Bt cotton on the abundances of 22 taxa of foliar-dwelling arthropod natural enemies was assessed (Naranjo 2005a, b). Significant differences for 0-2 taxa in Bt cotton compared to non-Bt cotton were found each year, but no chronic effects of Bt cotton were observed over multiple generations. The author concludes that the observed reductions are not ecologically meaningful. Performing multi-year statistical analyses, a significant reduction in five arthropod taxa in Bt maize compared to non-Bt maize occurred. A 5-year companion study assessed the effects of Bt cotton on the natural enemy function (Naranjo 2005b). Despite of the minor reductions in density of several predator taxa in Bt cotton, no effect on the predator/prey ratios was observed compared to non-Bt cotton.

Conclusions

Detrimental effects of Bt proteins on agronomic beneficial organisms may occur, especially if their prey or host spectrum includes Bt susceptible species. To what extent the ecological function, i.e. the control of a pest, is affected by slightly decreased population densities of the natural enemies, remains unclear and may not be simply deduced from abundance frequencies of the natural enemy species.

Based on the literature review, the following process was identified for expert consultation on potential long-term effects for Bt maize:

GM traits may cause changes in biological control due to

- a) additive, synergistic or delayed changes in diversity and abundance of natural enemies (see D)

- b) additive, synergistic or delayed changes in tri-trophic interactions

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Low** for Bt maize with **uncertainties** remaining regarding beneficial arthropods, and

- **Negligible** for all HT and SM crops.

E.3   Are GM traits causing changes in pollination?

Pollination is perhaps the best-known ecosystem function performed by insects. Losey & Vaughan (2006) tried to calculate the economic value of pollination: They assumed that pollinators may be responsible for more than 3 billion dollars of fruits and vegetables produced in the USA. Pollinating organisms in the temperate regions are mostly insects, namely bees and wasps (Buchmann & Nabham 1996). Among them, honey bees (*Apis mellilifera* L.) are the most important pollinators of many agricultural crops worldwide. There is concern that widespread planting of GM insecticidal crops could harm honey bee populations and thus adversely impact pollination (Duan *et al.* 2008, Winston 2003). Bees and other pollinators are agents of pollen spread and can therefore be exposed to any genetically modified product expressed in pollen or nectar (Lövei 2001). Adult bees consume pollen during their first week after emergence and thus might be exposed to Bt proteins originating from GM Bt plants, also larvae ingest pollen, but to a lesser amount (Malone 2004).

Recently, a meta-analysis of 25 studies that assessed potential effects of Bt proteins on honey bee survival has been published (Duan *et al.* 2008). No adverse effect on bees was reported looking at studies performed with lepidopteran and coleopteran specific Bt protein. In contrast, other genetically modified products such as protease inhibitors have the potential to harm honey bees and bumble bees (Malone & Pham-Delègue 2001) (see also Section 3.D).

Besides direct effects due to ingestion of insecticidal proteins, indirect effects of GM plants on pollinators may occur if unexpected changes as a side-effect of the genetic modification would alter the phenotype of the plant, e.g. nectar quality or colour of petals (Malone 2004). In a study on insect-attractiveness of white or yellow coloured flowers of the self-incompatible species *Raphanus raphanistrum, it* was shown that white coloured petals attracted lesser insects than yellow coloured did (Stanton *et al.* 1986) Similar results were reported from pollinator studies on hybrids of *R. sativus* and *R. raphanistrum*: White coloured hybrids were significantly lesser visited by pollinators than the wild yellow coloured parental plants. Consequently, in a majority of cases this resulted in lower seed production of the discriminated white coloured hybrids (Klinger & Ellstrand 1994) (see also Section 3.B).

The scent of plants emitted to the air by volatiles is important for the attraction of pollinators. Some volatiles are probably widespread among plants, whereas others are taxon-specific (Pichersky & Gershenzon 2002). Altered emission of volatiles because of potential side-effects of genetical modifications could affect pollination since the frequency of the visits of pollinators could be reduced (Pierre & Pham-Delègue 2000) (Section 3.B).

Conclusions

Adverse effects of GM Bt plants on the main pollinators, honey bees, have not been reported so far. Additionally, no reports are available regarding harmful effects on other NTOs involved in pollination. Some other genetically modified products such as protease-inhibitors have the potential to affect pollinators, in particular honey bees, but these are not on the market so far.

Potential less obvious changes in phenotype characteristics affecting pollination (like altered scent or colour) are factors which may affect pollination. Only minor effects have been reported for GM crops, not extending to ecosystem effects.

E.3   Overall assessment:

Based on the literature review, the following process was identified for expert consultation on potential long-term effects:

GM traits may cause changes in pollination due to

   a) additive, synergistic or delayed sublethal effects -> changes in diversity and abundance of the pollinator community (see 3.D)

   b) additive, synergistic or delayed altered attractiveness of flowers (see also section B)

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Low** for Bt maize, and
- **Negligible** for all HT and SM crops.

F. Impacts of the specific cultivation, management and harvesting techniques

As with the introduction of any new crops, the cultivation of pest resistant or herbicide tolerant crops may alter current management regimes and may introduce new cropping techniques (Champion *et al.* 2003, Hayes *et al.* 2004).

F.1 Altered use of agrochemicals

The use of glyphosate, the most frequently applied broad-spectrum herbicide in combination with HT crop cultivation, increased significantly from 2.5 to 30 million kg/yr in the United States as a consequence of the adoption of glyphosate-resistant soybean. The increasing use of glyphosate for weed control has also influenced the application timing of herbicides in soybean, with a growing tendency towards total post-emergence weed control. In fact, weed control can now be performed in the most important part of the growing season by a non-selective herbicide (Young 2006).

Based on this example, it can be assumed that pest resistant or herbicide tolerant crops may cause a change in the use of agrochemicals during cultivation, e.g. applying additional broad-spectrum herbicides to control HT weeds or HT volunteers (Owen & Zelaya 2005, Shaner 2000). It has been reported that the composition of weed communities may change towards species being naturally tolerant or having evolved tolerance to non-specific herbicides (to differing levels), especially if HT-tolerant crops would be grown continuously (Shaner 2000). New weeding strategies could be necessary like altered spraying frequencies or rates for controlling HT volunteers.

In addition, there might be the need to combine a given HT crop with the cultivation of HT crops tolerant to other broad-spectrum herbicidal agents to avoid increasing weed problems. Such new herbicide and crop rotation may lead to various effects on field organisms and ecological functions (Devos *et al.* 2004, Hayes *et al.* 2004).

Shifts in the weed composition to those species that are naturally resistant or the evolution of herbicide resistance in weed species were reported to be advanced in HT crop cultivation (Owen & Zelaya 2005, Puricelli & Tuesca 2005, Culpepper 2006, Cerdeira & Duke 2007, Norsworthy 2008, Powles 2008, Heap 2008). Shaner (2000) found natural tolerance to glyphosate e.g. in nutsedge species (*Cyperus* spp.) and in horseweed (*Conyza canadensis*). Nandula et al. (2005) reported of natural tolerance of birdsfoot trefoil (*Lotus corniculatus*) and lambsquarters (*Chenopodium album*). Evolvement of herbicide tolerance as consequence of

continuous cultivation of HT crops was reported by Heap (2008) e.g. for *Conyza canadensis*, *Lolium multiflorum* and *Plantago lanceolata*. However, it is anticipated that shifts towards naturally tolerant weed species or biotypes could occur more rapidly than the evolution of resistance due to the fact that the evolution of resistance to glyphosate proceeds very slowly (Shaner 2000).

The cultivation of GM HT crops caused significant primary effects on the abundance and biomass of weeds according to the UK Farm scale evaluation experiments. In two of the GM HT crops (sugar beet and spring oilseed rape), reductions of 60 to 80% in weed biomass were observed at the end of the growing season, reflecting increased weed control in these crops. In contrast, an increase of 82% in weed biomass was found in GM HT maize compared to the conventional maize crop (Freckleton *et al.* 2003). The reason for the increase of weed densities in GM HT maize was mainly due to the application of the highly effective maize herbicide atrazine used in the conventional variety (Perry et al. 2004).

Furthermore, a number of effects were reported on the growth and characteristics of weeds during crop development. In case GM HT crops were not sprayed with a pre-emergence herbicide, weed densities were initially much higher than in conventional beet and oilseed rape. After application of the broad-spectrum herbicide, these weeds were strongly depressed, typically before they were able to set seeds. Consequently, by the end of the growing season fewer weeds remained in GM oilseed rape and beet fields and tended to produce fewer seeds per plant than those surviving in the equivalent conventional crop. Thus, short-term increases in weed biomass before spraying could be outweighed by longer term declines in weed numbers since the broad-spectrum herbicide was applied before weed seed ripening (Freckleton *et al.* 2003).

Following GM HT maize cultivation dicot and total weed seedbank densities were significantly higher compared to conventional maize in the farm-scale evaluation trials, but weed seed numbers increased following the cultivation of conventional beet crops in contrast to the HT beet crop (Perry et al. 2004, Firbank *et al.* 2006). Heard *et al.* (2005) reported generally that most species declined even though the HT crops were grown in rotations and also seedbank densities were generally lower for HT than for conventional rotations.

On the other hand more beneficial expectations concerning the environmental impact of GM crops are that the use of hardly degradable conventional herbicides might decline and weed control could improve (Kleter *et al.* 2007, 2008). From this point of view the replacement of conventional crop varieties by GM HT varieties might have a net environmental benefit (Andow 2003). Concerns for GM crops are that populations of naturally or selection pressure driven resistant species might increase in HT crop cultivation if the crops are grown

continuously, leading to higher application rates of non-selective herbicides or to the necessity of using other herbicides (Shaner 2000, Foresman & Glasgow 2008).

An additional aspect to be taken into account is the increasing reduction of summer fallow acreage when HT varieties are planted (Graef *et al.* 2007). Also the applied tilling system caused different weed species densities by reason that higher weed species richness was found in systems with long-term no-tillage compared to tilled or short term no-tillage fields (Mulugeta *et al.* 2001). In glyphosate tolerant soybean the application of reduced or no-tillage field management systems have enormously increased from lesser than 0,1 million hectares in 1996 to about 15 million hectares in 2001 (Cerdeira & Duke 2006). This resulted in an average of 1.8 lesser till-passes per year in soybean fields.

In the EU only Bt maize is cultivated as a GM pest resistant crop. The emergence of secondary pests could prove to be a major problem, in case an altered pesticide spraying system is used during the cultivation of insect resistant crops (e.g. cotton and rice, Men *et al.* 2005, Wang *et al.* 2006a). Such problems have not been reported for Bt maize and they are unrealistic since there are no secondary pests known in maize so far.

Conclusions

HT plant cultivation [management] will lead to applications of non-selective herbicides later in the growing season. Such timing of broad-spectrum herbicide applications may either lead to a reduction in weed biomass and abundance and/or lead to shifts in weed composition due to selection of naturally tolerant species or varieties or the evolution of secondary tolerant weed species or varieties. This may cause in shifts of weed populations resulting in increased/altered use of slowly degradable additional agrochemicals (e.g. pesticides) controlling existing or evolved herbicide tolerant weeds or persistent GM crops (volunteers) in crop rotations with adverse effects on ecological functions. On the other hand applications after weeds seeding could enhance weed species abundance. Also reduced- or no-tillage field management systems could potentially result in benefits in comparison to the conventional weed control systems.

F.1   Overall assessment:

According to the published information, the likelihood of predominantly detrimental or beneficial effects of the several management options for the currently used GM crops in the EU is

- **Low - High** for HT crops (with **uncertainties** according to unknown changes in EU cultivation management systems)
- **Low** for Bt maize, and

- **Negligible** for SM potato.

F.2   Indirect changes in susceptibility of crops against plant pathogens

The susceptibility of crops to plant pathogens may theoretically change and result in altered interactions between plant and pathogens (Hilbeck 2000). Low doses of glyphosate are able to render pathogen-resistant cultivars susceptible to plant diseases (Brammall & Higgins 1988). On the other hand, this herbicide is also known to be toxic to specific microorganisms, including plant pathogens, and was reported to inhibit or reduce the growth of different plant pathogens, e.g. rust diseases (Feng *et al.* 2005, Cerdeira & Duke 2006).

However, increased susceptibility of HT soybean to plant pathogens was reported for *Sclerotinia sclerotiorum* by Michigan farmers (Lee *et al.* 2003a). Furthermore, an interaction between soybean cyst nematode and glyphosate was also observed (Yang et al 2002). Colonization of GM soybean roots with fungi was found to be significantly increased after application of glyphosate but not after application of conventional post-emergence herbicides (Sanogo *et al.* 2001, Njiti *et al.* 2003, Kremer *et al.* 2005). Following application of glyphosate to crops, plant root exudates may potentially provide a special advantage to certain *Fusarium* strains relative to other fungi commonly found in soils (Benbrook 2005). It is reported that glyphosate in root exudates stimulated growth of selected rhizosphere fungi, possibly by providing a selective C and N source combined with high levels of soluble carbohydrates and amino acids associated with glyphosate treatment of soybean plants. Increased rhizosphere populations of the fungi *Fusarium spp*. and the bacteria *Pseudomonas* spp. developing under glyphosate treatment of GM soybean may adversely affect plant growth and biological processes in the soil and rhizosphere (Kremer *et al.* 2005).

Conclusions

The application especially of glyphosate in HT crops may potentially affect soil microbial activity and/or soil microbial abundance through exudation of the herbicide into the rhizosphere by HT crops. As a consequence HT crops may show increased susceptibility to fungal attacks. These changes could result in increased use of fungicides. As a consequence additional adverse effects on soil microbial activity might occur.

However, published results on increased susceptibility of HT crops to fungi are still rare and broad-spectrum herbicides also affect pathogenic microorganisms.

F.2   Overall assessment:

The following process was identified for potential long-term effects due to (indirectly) altered susceptibility to pathogens:

GM plant [management] of HT crops may cause indirect changes in susceptibility of crops to plant pathogens (mainly fungi) with adverse effects on non-target microorganisms due to increased use of additional pesticides. Beneficial side-effects on pathogenic microorganisms may also occur.

According to the published information, the likelihood of adverse effects for the currently used GM crops in the EU is

- **Low** for HT crops, but with remaining **uncertainty** (due to rare reports on adverse effects), and
- **Negligible** for Bt maize and SM potato.

F.3 Adverse effects on agro-biodiversity

On a worldwide scale, biodiversity loss has occurred across all terrestrial ecosystems for various reasons. Many of its drivers are generally associated with the intensification of agriculture (Hails 2002, Green *et al.* 2005). Attention has been drawn to the concern that GM crop cultivation might have specific impacts on biodiversity and the environment different from conventional cultivation practices.

The Farm Scale Evaluation (FSE) in the UK was designed to test the hypothesis that GM crops and conventional crops do not differ in their impact on biodiversity. The study was carried out over 3 years at 60 field sites across England and Scotland. Fields were divided into two sections; one half was sown with a conventional crop, the other with a GM crop (Firbank *et al.* 2003a). The crops grown were sugar beet (including fodder beet), maize, and winter and spring oilseed rape. To record biodiversity, the abundance of weeds and invertebrates was assessed. The data were published in a series of eight papers in the *Philosophical Transactions of the Royal Society of London* (Brooks *et al.* 2003, Champion *et al.* 2003, Heard *et al.* 2003a, Heard *et al.* 2003b, Haughton *et al.* 2003, Hawes *et al.* 2003, Roy *et al.* 2003, Squire *et al.* 2003).

The FSE results revealed large negative (indirect) impacts of growing GM HT crops on weeds in fields with cultivated sugar beet, smaller but consistent negative effects on weeds in fields with cultivated oilseed rape, and positive effects on weeds in maize fields. However, the effect found in maize fields is presumably due to the application of the very effective but environmentally harmful herbicide atrazine as conventional comparator (Perry *et al.* 2004).

The change in timing of herbicide applications leads to shifts in invertebrate resource abundance during the growing season and the invertebrate response to this change (Freckleton *et al.* 2003). Reduced weed densities may result in fewer weed seeds being available as food for wintering birds (Watkinson *et al.* 2000, Gibbons *et al.* 2006) and may

lead to a reduction in the number of invertebrates feeding on weeds and subsequently to a reduced occurrence of their predators (ACRE 1999; Beringer 2000). Other research suggests that the use of GM HT varieties might benefit farmland biodiversity during the growing season, because such crops facilitate delayed applications of herbicide compared to conventional weed treatments. Thus, weeds may be allowed to persist for a longer time span than in conventional varieties, providing food resources and habitat structure for animals during the breeding season (Buckelew *et al.* 2000; Strandberg *et al.* 2005). The overall balance of these potential positive and negative effects of GM HT crops on farmland biodiversity remains uncertain (Firbank & Forcella 2000): despite the large areas used to grow GM HT crops world-wide commercially, there remains a global paucity of appropriate large-scale experiments and relevant research on field plantings (Firbank *et al.* 2003b).

Secondary effects of GM cultivation tend to mirror the effects on weeds. As other plants, weeds have important functions for species like arthropods, e.g. as food for arthropod herbivores, reproduction sites, hiding places, and for birds, which e.g. feed on the weed seed associated arthropods (Watkinson *et al.* 2000, Marshall *et al.* 2003). It was demonstrated in the FSE that the herbicide regimes associated with spring-sown GM HT beet, maize and oilseed rape had - apart from the direct effects on weeds (Heard *et al.* 2003a, b) - knock-on indirect effects on invertebrate abundance and diversity (Brooks *et al.* 2003; Haughton *et al.* 2003; Hawes *et al.* 2003; Roy *et al.* 2003). It was discovered that detrital food webs were affected with regard to late-season fungal-feeding springtails and springtail predators (Brooks 2003, Haughton *et al.* 2003). The authors suggested that the delayed use of herbicides in the GM HT crops resulted in more dead-weed biomass and greater detrital activity. In addition, densities of carabid beetles feeding on weed seeds tended to be higher in conventional beet and oilseed rape, as well as in GM maize, because of the greater weed seed production. Other trophic groups (pollinators, herbivores, and their natural enemies) showed similar shifts in abundance relative to effects on the abundance of their resources (Freckleton *et al.* 2003).

Three generalisations could be drawn from the FSE in terms of food resources that are likely to be exploited by birds: (i) weed abundance was higher in GM HT crops than in conventional crops prior to herbicide treatment to the GM HT crop; (ii) in general weed abundance and seed resources were higher in conventional than in GM HT spring rape and sugar beet but lower in maize after application of a broad-spectrum herbicide to the GM HT crop (Heard *et al.* 2003a, Bohan *et al.* 2005, Firbank *et al.* 2006); (iii) similar patterns were apparent for invertebrate abundance, but differences were less clear-cut than for weeds (Brooks *et al.* 2003, Haughton *et al.* 2003, Roy *et al.* 2003, Bohan *et al.* 2005) and often involved species that rarely feature in the diet of birds, e.g. bees (Wilson *et al.* 1999, Holland *et al.* 2006).

Whether the introduction of GM HT crops will cause severe impacts on the bird community will depend on the applied management strategies as they might lead to an intensification of agriculture. However, the introduction of GM HT crops may have additional potential environmental benefits in that fewer herbicide sprayings may encourage minimum tillage systems (Cunningham *et al.* 2004, Holland 2004), spring sowing and delayed herbicide application (Freckleton *et al.* 2004). But GM HT crop management potentially causes effects on both invertebrate and weed seed food resources for birds. Removal of weed plants could have important implications for granivorous birds as many rely on weed seeds in winter and associated invertebrates in summer (Watkinson *et al.*, 2000). On the other hand, it was reported that no significant differences were detected in spring oilseed rape and in any crop prior to herbicide application. In winter, granivores were more abundant on bare plough following conventional sugar beet treatment than following GM HT treatment. Some bird species were more abundant on maize stubbles following GM HT treatment. These differences were in accord with likely differences in food availability ascertained under the FSE (Chamberlain *et al.* 2007). Butler *et al.* (2007) developed a trait-based risk assessment framework capable of predicting the impact of environmental change on biodiversity and ecosystem services using farmland birds as a model system. Risk score calculation has its basis in the assumption that species with broader niches will be less vulnerable to the effects of agricultural change than species with narrower niches. Overall, it appears that replacing equivalent conventional crops in the current agricultural landscape with GM HT crops would only have a limited effect on farmland birds (Butler *et al.* 2007).

Furthermore, it is well known that the type of ploughing and crop rotation can modify soil microarthropod communities, usually by decreasing the density of sensitive taxa (Cortet *et al.* 2002). With reduced tillage due to cultivation of HT maize, soil microarthropods show a greater abundance compared to HT maize fields with conventional tillage (Cortet *et al.* 2007). In contrast, a model for a Bt maize cropping system did not find any effects on functional groups of soil fauna (Debeljak *et al.* 2007). Only a small effect on soil nematodes and other species of the soil community was reported for Bt maize (Griffith *et al.* 2005, 2006, 2007b) or HT maize (Griffith et al 2007a), which was accredited to normal variation in the agricultural system. Although the FSE represents a very comprehensive and costly study, conclusions on long-term effects of cultivation and management regime impact are difficult to draw (but see also Qi et al. 2008).

F.3   Overall assessment:

The following process was identified for potential long-term effects due to altered crop management:

GM plant management may cause indirect changes and potential adverse effects on agro-biodiversity due to the knock-on-effect of additive, synergistic or delayed effects in cropping systems; effects may differ with regard to specific agricultural landscapes and management systems.

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Low - High** for HT crops but strongly depending on herbicide management, and
- **Negligible** for Bt maize or SM potato.

F.4   Potential changes in fertilizer use

Since HT soybean is resistant to glyphosate, glyphosate is transported to the rhizosphere through the plants. Thus application of glyphosate could decrease nodule formation, nodule biomass, nitrogen fixation and nitrogen accumulation (Moorman *et al.* 1992, King *et al.* 2001, Powell *et al.* 2007, Zablotowicz & Reddy 2007). It has been reported that symbiotic nitrogen fixation can be affected by herbicides due to direct effects on the rhizobial symbiont as well as due to indirect effects on the physiology of the host plant (Moorman 1989). Glyphosate transported into the roots of HT plants lead to the accumulation of growth inhibiting benzoic acids.

Glyphosate reduces the nitrogenase activity of *Bradyrhizobium japonicum* bacteroids with the inhibition being proportional to the *in vitro* sensitivity of these strains under culture conditions (Hernandez *et al.* 1999). The symbiont is known to possess a glyphosate susceptible enolpyryvate-shikimate-synthase (EPSPS) protein (King *et al.* 2001). Consequently, soybean plants in untreated plots had higher nodule biomass compared to soybean plants in glyphosate-treated plots that were relatively weed-free (Zablotowicz & Reddy 2007). In addition, a reduction of the N_2 fixation potential could have long-term effects on sustainable soil nitrogen pools (King *et al.* 2001, Zablotowicz & Reddy 2004). Soils may lose natural fertility and may lack sufficient nitrogen to produce optimal soybean yields in fields where nitrogen fixation is impaired. This will cause increasing usage of fertilizers to maintain the yield levels (Benbrook 2005).

Another aspect of potential changes in fertilizer use connected with use of the non-selective glyphosate is reported by Eker *et al.* (2006). In greenhouse experiments the authors sprayed non-glyphosate-tolerant sunflowers (*Helianthus annuus*) with low glyphosate doses (~ 6% of recommended dosage) to simulate effects of glyphosate drifts on non-target plants. In root uptake experiments Fe and Mn uptake were significantly reduced 12 and 24 hours after glyphosate application. This effect may be caused by the formation of poorly soluble

glyphosate-metal complexes in plant tissues and or in the rhizosphere as the most important nutrient source since glyphosate is known as an effective chelating agent. Due to the chelating effect poorly soluble glyphosate-metal complexes may be formed in the rhizosphere reducing the availability of these two nutrients.

Conclusions

The cultivation of HT soybean may affect the nitrogenase activity of symbiotic Rhizobia leading to reduced effectiveness of nitrogen fixation. Reduced nitrogen fixation will then be counteracted by application of additional nitrogen fertilizer. In addition, the chelating effect of glyphosate affects the availability of Mn and Fe for GM crop roots. Also these fertilizer micronutrients are already recommended to be supplemented in soybean cultivation stewardship programmes depending on soil quality. However, in both cases potential negative effects for general soil nutrient mineralization processes are still unclear.

F.4 Overall assessment:

The following process was identified for potential long-term effects due to altered fertilizer use:

GM plant management may cause indirect changes in fertilizer use with adverse effects on nitrogen availability or symbiotic NTOs and uptake of cationic nutrients resulting in potential adverse effects on ecological functions.

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Low – High** for HT soybean but with remaining **uncertainty** due to little experience of cultivation under EU soil conditions,
- **Low** for HT oilseed rape and HT sugar beet, and
- **Negligible** for Bt maize or SM potato.

F.5 Potential changes in landscape structure

Due to market-orientated and modern production processes the intensity of farming in many regions of the world is currently increasing. On global scale mainly deforestation for receiving additional agricultural area causes changes in the landscape structure (Begon *et al.* 2005). In Argentina, the increase in surface area dedicated to GM soybean cultivation has been at the expense of other crops and caused marginalisation of cattle and dairy farming. In addition, the rate of deforestation and the soybean acreage nearly doubled from 1995 to 2004 (Trigo & Cap 2003, Benbrook 2005, Joensen *et al.* 2005).

In Europe intensification of agricultural production is driven by increasing food and feed demands after World War II when human population started to increase again. The additional rise of production costs led to significant changes in landscape structure especially during the last four decades. These changes were often initiated and supported by political programs for supporting agricultural practices in underprivileged regions. However, the intensity of landscape changes has differed enormously from region to region; often depending on the type of political system ruling or the specific aims of a supporting program.

Coexistence measures combined with cultivation of GM crops in Europe may increase the requirement of acreage even further and consequently having impacts on future landscape structure. Farmers who are willing to cultivate GM crops have to fulfill requirements for isolation distances. Fulfillment of these prerequisites may lead to further enlargement of cropping fields in order to minimize isolation conflicts to neighbours. This might alter field sizes and habitats typical for agricultural landscapes like groups of trees or bushes or border structures as hedges, ditches or edges. Especially these landscape structurizing habitats are the most important habitats for maintaining biodiversity in agricultural landscapes (Hendrickx *et al.* 2007). The connectivity and consequently any potential biological exchange will be negatively affected if these habitats decrease in size and numbers.

However, today there are still no clear tendencies identified in Europe for significant landscape changes due to varying national coexistence measures and their potential consequences.

F.5   Overall assessment:

The following process was identified for potential long-term effects due to altered landscape structure:

GM plant management may cause indirect changes in landscape structure e.g. due to coexistence measures (e.g. larger fields, larger distances between specific crops), resulting in loss of habitat connectivity and reduced local biodiversity.

According to the little information available, the likelihood of this process for the currently used GM crops in the EU is

- **Low** for all GM crops, but with remaining **uncertainties** (due to varying coexistence measures and their potential consequences in Europe).

G. Effects on the abiotic environment

According to the EFSA guidance document (EFSA 2004), examples of possible interactions between the GM plant and its abiotic environment are:

- alteration of climatic conditions (e.g. altered production of greenhouse gases),

- altered sensitivity to, or tolerance of, climatic conditions (e.g. cold, heat, humidity),

- altered sensitivity to, or tolerance of, abiotic fractions of soil (e.g. salinity, mineral nutrients, mineral toxins),

- altered sensitivity to, or tolerance of, gases (e.g. CO_2, oxygen, NH_3),

- alteration of mineralisation (e.g. root exudates changing the soil pH).

Based on the available information concerning the major GM crops actually important for the EU, three items were identified that merited more intensive literature screening. Changes in the abiotic environment caused by GM plants may have impacts on the biotic environment as well, and there is some overlap to issues discussed in previous Sections (3.A - 3.F).

G.1 Increased production of greenhouse gases

"Global change" encompasses changes in atmospheric composition, climate, land cover and land use. These changes and their interactive effects on biological systems are on a worldwide scale (Scherm *et al.* 2000). Any living population in terrestrial or limnic environment in future including cultivated GM crops will continue to be exposed to the complex environmental alterations. One of most important causes for global change scenarios is increasing CO_2. Beside light, water, nutrients and temperature CO_2.represents one of the most important factors for plant biomass production (Begon *et al.* 2005). There are meanwhile a number of studies on the impact of green house gases on crops including several GM crops (e.g. Ryle & Powell 1992, Traore *et al.* 2000, Pritchard *et al.* 2007, Wan *et al.* 2007, Lobell *et al.* 2008, Taub *et al.* 2008). *Vice versa*, the impact of plants on the production of green house gases is in its infancy. Of particular interest is whether GM plants may enlarge the problems following global change. Indirect effects might be the increased production of green house gases by use of GM crops.

According to Brookes & Barfoot (2005) GM crops contributed to significantly reduced greenhouse gas emissions from agricultural practices. This reduction resulted from decreased fuel use, about 1.8 billion litres in the years 1996-2004, and additional soil carbon sequestration because of reduced ploughing or improved conservation tillage associated with biotech crops. According to the authors, this reduction was equivalent to eliminating more than 10 billion kg of carbon dioxide from the atmosphere in 2004. An increased adoption of

soil conservation practices was also reported for the USA by Fernandez-Cornejo & Caswell (2006). In contrast, concerns were expressed that the production of HT soybean leads to environmental problems such as deforestation and soil degradation (Benbrook 2005, Pengue 2005).

In general, intensification of agriculture will influence the use of fossil energy sources for agricultural practice and thus may lead to increased CO_2 emissions. Similarly, intensive high-yield agriculture is dependent on addition of fertilizers, especially synthetic N produced through a fossil fuel-consuming industrial process that converts abundant atmospheric N to available form for plants (Matson *et al.* 1997).

<u>Conclusions regarding GM plants and impact on climate change</u>

Literature data are very limited with respect to long-term impacts of GM crops on climate change. Theoretically, an intensification of agriculture solely related to GM crops could potentially be connected with higher use of fossil energy resources, global deforestation and decline of the organic soil fraction, which might finally increase carbon dioxide release into the atmosphere. The following process was therefore identified from the literature:

<u>G.1   Overall assessment:</u>

Cultivation of GM plants might cause adverse effects on the abiotic environment due to increased production of green house gases and thus might have an impact on climate change.

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Negligible** for all GM crops, but with remaining uncertainties.

G.2   Increased mineral nutrient erosion and fertilizer leaching

Concerns were raised about potential detrimental effects on soil quality and function with respect to the increasing area of GM crops being cultivated worldwide (Motavalli *et al.* 2004). On the one hand soils could potentially be impacted by root exudates originating from the genetic modifications; on the other hand larger amounts of toxic compounds (e.g. Bt proteins) might be incorporated into soil after harvest, as GM plants produce recombinant metabolites throughout the season. In addition, changes in compositional character of plant tissue could affect composition of plant necromass (Raubuch *et al.* 2007).

Regarding interactions with soil organisms the GM plants that received most attraction in the literature are those with Bt expression. Potentially, Bt could exhibit side-effects on potentially susceptible soil insects or soil microbes being involved in organic matter degradation.

Decomposition and mineralization of organic matter are the main natural nitrogen and phosphorus sources in soils.

Baumgarte & Tebbe (2005) found differences in microbial community structures in the rhizosphere of Bt protein expressing maize compared to control treatments. However, the extent of Bt depending alterations of microbial community structure was less than those determined by biological (e.g. age of plants) or physical factors (field heterogeneity). No differences were reported by Griffith *et al.* (2006) in soil microorganism communities by comparing conventional and Bt maize. Some differences were observed for protozoa depending on the year and experimental sites.

The phenomenon of increased lignin contents in Bt maize residues could be of importance for potential effects on nitrogen mineralization (Saxena & Stotzky 2001c). The authors found 33 to 97% higher lignin content in several Bt maize varieties. Additionally, Masoero *et al.* (1999) determined that two Bt maize varieties exhibited higher starch, higher lignin but lesser protein and nitrogen content. A theoretical chain of events – higher C : N ratio of Bt maize resulting in delayed N mineralization would increase nitrogen fertilizer demand – can be generated from the data reported by Flores *et al.* (2005). Any necessity for additional applications of fast degradable organic or mineral fertilizer increases the likelihood of enhanced mineral fertilizer input into groundwater or of losses of fertilizer by surface runoff to water streams in vicinity of the fields.

Higher lignin concentrations of Bt maize residues could lead to an enrichment of slowly decomposing Bt containing organic matter in soils. This scenario is more important if Bt maize would be cultivated continuously for several years on the same fields. Under such conditions losses of mineral fertilizers from soils would – contrary to the paragraph above - be remarkably reduced.

Direct input of pollen and other parts of Bt maize plants into headwater streams nearby to maize fields cultivated with Bt maize in the Midwest of USA was investigated by Rosi-Marshall *et al.* (2007). They found evidence for transport of the Bt containing maize residues downstream in the water bodies. However, with respect to breakdown rates of Bt containing plant litter no differences were found between Bt and non-Bt containing litter. In laboratory feeding trials with aquatic insects (*Helicopsyche borealis*) decreased growth rates and in one case an increased mortality was observed with unusually high amounts of Bt pollen. The conclusions of the Rosi-Marshall study are thus not supported by their data[8].

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[8] Analysis by the EFSA GMO Panel of Rosi-Marshall et al. 2007 under item 12.2 of the 37th Plenary Minutes: http://www.efsa.europa.eu/EFSA/Event_Meeting/GMO_Minutes_37th_plenmeet,3.pdf

Aquatic ecosystems were also studied by Douville *et al.* (2007). The group spiked surface water and sediment of a surface water body in Canada with genomic maize DNA containing the cry1Ab gene. At different times of a season samples from water and sediment were collected and tested on cry1Ab residues. The gene was still detected 40 days after introduction in clay and sand-rich sediment. Persistence of the genes was significantly higher in sediments than in the open water. Investigations of potential effects were not carried out.

Effects on the abiotic environment from the cultivation of herbicide tolerant crops will potentially result from the application of the non-selective herbicides. The widespread proteins (e.g. EPSPS in microorganisms and plants; pat or bar in microorganisms (Busse *et al.* 2001)) expressed by the herbicide tolerant plants are not known to act different from the natural ones. However, potential side-effects of the herbicide itself are still controversially discussed (Haney *et al.* 2000, 2002). Depending on the time from the applications of the non-selective herbicides soils will be more or less bare of weeds. The likelihood for losses of nutrients by surface run-off will increase with decreasing weed coverage. Such situation would potentially pose a risk for eutrophication of waterstreams in agricultural landscapes. This kind of risk would not depend on the crop but on the herbicide application management.

Another potentially adverse aspect of glyphosate usage on nutrient availability is reported by Eker *et al.* (2006). Glyphosate applications in low doses (~ 6% of recommended dosage) to non-target (glyphosate-sensitive) sunflowers (*Helianthus annuus*) resulted in significantly reduced root uptake of Fe and Mn. Also root-to-shoot translocation of the cationic minerals was almost completely inhibited potentially leading to severe impairments in Fe and Mn nutrition of non-target plants. Due to the chelating effect of glyphosate poorly soluble glyphosate-metal complexes may be formed in plants as well as in the rhizosphere reducing the availability of these two nutrients. US farmers are recommended to add Mn to fields cultivated with glyphosate-tolerant crops.

Theoretically, starch modified potato would impact nutrient losses from soils if these varieties would have additional need for mineral fertilization, but no evidence was published in the literature.

Conclusions regarding GM plants and soil mineral nutrients

Literature data are limited with respect to long-term impacts of GM crops on soil mineral nutrients. Indirect effects due to an intensification of agriculture with higher use of fertilizers and reduction in natural nitrogen fixation (toxic herbicide effects) might be a chain of impacts, but no conclusive evidence has been reported that GM crops indirectly affect soil nutrient transformations. However, an indirect effect of glyphosate as a chelating agent of Fe and Mn

availability is reported that potentially might cause increasing nutrient deficits. The following process was identified:

G.2   Overall assessment:

The cultivation of GM plants may cause adverse effects on the abiotic environment due to increased mineral nutrient erosion, and/or fertilizer leaching into water bodies affecting water quality.

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Low** for HT crops with remaining **uncertainty**, and
- **Negligible** for Bt maize, SM potato.

G.3   Altered chemical attributes of soil fractions

The fate and behaviour of Bt proteins in soils is a field of intensive research and discussions. Recently Icoz & Stotzky (2008) have published an extensive review on this issue. The authors are reporting very different results with respect to the persistence of Cry-proteins in soils. Half-lives of cry1Ab protein range from 1,6 days (Sims & Holden 1996) up to 34 days (Wang *et al.* 2006b). Also long-term persistence of cry proteins in soils is variable. Cry1Ab proteins in low concentrations were detected up to 56 days (Donegan *et al.* 1995) or up to 234 days (Tapp & Stotzky 1998) or up to 180 to 350 days in residues of Bt maize (Saxena & Stotzky 2002b).

The reported differences regarding half-life as well as persistence of Bt proteins in soil may potentially be a result of the specific chemical and physical conditions in the soils. Of special importance are pH, clay content and type and electrokinetic charge of external clay surfaces (Pagel-Wieder *et al.* 2007, Blackwood & Buyer 2004). Icoz & Stotzky (2007) found differences in Cry3Bb1 persistence in soils depending on the type of the predominant clay minerals. The protein was found for a short period of 21 days in the presence of montmorillonite. If kaolinite was amended, the protein was detectable for 40 days. However, also with kaolinite the protein only was found up to 20 days if pH was adjusted to 7.

Pagel-Wieder *et al.* (2007) have chosen an approach to elucidate the interdependence between chemical and physical soil properties and Cry protein persistence. The authors tested the adsorption of Cry1Ab protein at Na-montmorillonite and were able to perform adsorption kinetics of Cry protein and clay. The best adsorption per unit weight of the protein was detected with high protein and low but highly dispersed clay concentrations. With higher clay concentrations the minerals clumped leading to lower surface charge or binding sites exhibiting relatively decreased adsorption capacity. Additionally, with lower soil pH binding of

Cry 1Ab to clay surfaces was improved. About 10% of the bound Cry proteins could be recovered by washing. A variability of shape confirmations is known for Cry proteins (e.g. folded - unfolded shapes, oligomerized – non-oligomerized forms) according to Schnepf *et al.* (1998), Bravo *et al.* (2004) and Rausell *et al.* (2004). It is yet unclear whether there is a relationship between protein shape and soil adsorption.

Also the active ingredients of non-selective herbicides are at least partially bound to soil particles. For glyphosate a rapid adsorption in soils is reported (Goldsborough & Brown, 1993). Nevertheless, some publications emphasize transport processes of the negatively charged glyphosate in soils. Those transports are depending on structural and chemical soil characteristics like clay content or iron vice versa phosphate availability (Gimsing & Borggard 2002, Borggard & Gimsing 2008). Mobility of glyphosate is increased to a small extent if pH is high. Glufosinate will also be bound by soil particles; however the efficiency of binding is moisture dependent (Gallina & Stephenson 1992).

However, binding to the surfaces of soil minerals of GM plant (directly or indirectly) related metabolites would lead to a reduction of the exchange capacity of soils. Cation exchange capacity (CEC) of soils is to an important extent responsible for preventing the risk of at least cationic nutrients being transported through the root zone into lower soil horizons or into the groundwater. The beneficial effect of nutrient retaining for plants would be decreased by binding huge amounts of Bt proteins at this soil particle sites. Additionally, the clay negatively charged surfaces play an important role for binding H^+-cations. If the H^+-binding would be anticipated through covering the exchange sites by Bt proteins buffering and neutralizing of high H^+-concentration in soil would be altered, and finally affect cultivated plants by lower pH.

Conclusions regarding GM plants and impact on chemical soil attributes

Available literature data are limited with respect to long-term impacts of GM crops on chemical soil attributes.

G.3   Overall assessment:

Based on the available literature, the following process was identified:

Cultivation of GM plants may cause adverse effects on the abiotic environment if soil particle exchange sites are covered e.g. due to an increased release of ionic proteins.

According to the published information, the likelihood of this process for the currently used GM crops in the EU is

- **Low** for Bt maize and HT crops, and
- **Negligible** for SM crops.

H. Stacked Events

Where GM events have been approved under Regulation (EC) No 1829/2003 or Directive 2001/18/EC, genotypes produced by crossing plants containing these events with non-GM plants are not required to undergo further risk assessment. However, where applications involve the crossing of plants to stack GM events, a risk assessment is required in the European Union. The stacking of approved events can arise from intentional crosses as well as unintentional crosses.

Stacked events have become more important lasting recent years. The first cultivation started in 1997-1999 with a stacked event of insect resistance (IR) and herbicide tolerance (HT) in cotton and maize. Today, the most common stacked events are a combination of (i) different IR genes or (ii) of an IR and a HT gene, obtained by the crossing of single trait paternal lines. An increasing number of stacked events are submitted for cultivation in the EU. This raised the question if the safety of stacked events has to be assessed differently from single trait plants (EFSA 2007; de Schrijver et al, 2007).

Based on the EFSA Guidance (EFSA 2007) the ERA should take into account the evaluation of the individual events and additional data from molecular characterisation and comparative compositional analysis of the stacked events when determining potential interactions between genes or between gene products. As far as the BEETLE team is aware, no detrimental negative interaction has been observed between stacked genes in GM crops so far.

The ERA should evaluate any interactions between the stacked events which could result in modified environmental effects of the GM plant. In particular the combination of transgenes may result in changes in expression levels which may lead to a significant biological impact that may need to be assessed. However, it should be noted that expression levels may vary significantly also in the individual events. The EFSA guidance (2007) set out certain minimum requirements for the provision of information. If possible adverse effects have been identified through experimentation or if there are scientific reasons to believe they might exist then further data should be provided or information given. The EFSA guidance (2007) put particular emphasis on GM traits with altered efficacy of biocidal gene products to target organisms as well as NTO. Stacked biocidal events may have different effects on non-target organisms when compared with the individual events. Therefore EFSA (2007) saw in particular a need to focus on changes in sensitivity of non target organisms and/or specificity of biocidal gene products.

Although stacked events are cultivated for about 10 years, very few articles are published and investigations addressing potential long-term effects are lacking. McCann *et al.* (2007)

and Taylor *et al.* (2003) reported that the combination of several trait proteins could have synergistic effects but might also change the toxic or allergenic properties. However, first studies in the nutrient composition of double resistant maize and its impact on feeding of chickens did not show any significant differences.

Stacked events expressing different IR genes against the same target organism might help to prevent the development of resistance (Bates *et al.* 2005, Gonzalez-Cabrera *et al.* 2006).

According to Sisterson *et al.* (2007), varieties of genetically modified crops with multiple Bt proteins or novel toxins might be more harmful to non-target arthropods. The development and persistence of double HT populations, which were documented in unintended stacks in oilseed rape in Canada and Japan (Hall *et al.* 2000; Aono *et al.* 2006; Warwick *et al.* 2007) might well have an impact on cultivation practices.

In view of the available data and currently little experience, the questionnaire developed for the BEETLE expert assessment was amended for each of the sections A-G with a general question as to whether stacked events should be assessed differently with regard to the identified process.

4. Conclusions for Beetle expert questionnaires

Based on the reviewed data one or more processes were identified for each of the seven major fields of risk classification, which may potentially have adverse long-term effects on the environment.

The Literature review is not a concluding analysis resulting in a prioritization and identification of uncertainties.

For the next step of the BEETLE project, the expert assessment via online survey, scenarios were developed taking into account the identified processes and the most frequently cultivated GM-crops/trait combinations in Europe. These scenarios focused on maize, oil seed rape, potato, sugar beet and soybean with herbicide tolerance and/or insect resistance or altered composition.

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OECD (2003): Consensus document on compositional considerations for new varieties of bread wheat (*Triticum aestivum*): Key food and feed nutrients, anti-nutrients and toxicants. Series on Safety of Novel Foods and Feeds 7.

OECD (2004): Consensus document on compositional considerations for new varieties of cotton (*Gossypium hirsutum* and *Gossypium barbadense*): Key food and feed nutrients and anti-nutrients. Series on Safety of Novel Foods and Feeds 11.

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Annex L2: Tier ranking table (to Section 3.D)

201 studies on effects of GM insecticidal plants on non-target organisms were evaluated and tabled according to tier level. For each study, only one level was assigned. In the case of different tier level approaches in the same study, only the highest rank was attributed.

Tier	Definition	Number of studies
0	Literature reviews or modelling approaches	42
1	Laboratory studies with purified insecticidal protein	19
2	Laboratory or glasshouse studies with GM insecticidal plants (or parts of plants)	55
3	Semi-field studies (contained environment) with GM insecticidal plants	8
4	Real field studies with GM insecticidal plants (open environment)	65

Tier 0	Tier 1	Tier 2	Tier 3	Tier 4
Andow et al. 2006b	Burgess et al. 2002	Ahmad et al. 2006b	Bailey et al. 2005	Ahmad et al. 2005
Andow & Zwahlen 2006	Carter et al. 2004	Alvarez et al. 2005	Büchs et al. 2005	Andersen et al. 2007
Birch et al. 2007	Dechaume-Moncharmont et al. 2005	Ammitzbøll & Jørgensen 2006	Lutz et al. 2006	Aono et al. 2006
Bohanec et al. 2007	Deml et al. 1999	Babendreier et al. 2005	Raubuch et al. 2007	Baumgarte & Tebbe 2005
Butler et al. 2007	Kramarz et al. 2007a	Bai et al. 2005	Romeis et al. 2007	Bhatti et al. 2005
Clark et al. 2005	Malone et al. 2001	Bakonyi et al. 2006	Sanden et al. 2006	Bitzer et al. 2005
Debeljak et al. 2007	Malone et al. 1999	Birch et al. 1999	de Vaufleury et al. 2007	Bourguet et al. 2002
Dolezel et al. 2005	Malone et al. 2004	Castaldini et al. 2005	Zwahlen et al. 2007	Candolfi et al. 2004
Doull et al. 2007	Marroquin et al. 2000	Clark & Coats 2006		Champion et al. 2003
Duan et al. 2008	van Munster et al. 2007	Clark et al. 2006		Cortet et al. 2006
Dutton et al. 2003a	Ramirez-Romero et al. 2007	Down et al. 2003		Cortet et al. 2007
Eilenberg et al. 2001	Ramirez-Romero et al. 2005	Escher et al. 2000		Cowgill et al. 2004
Evans 2002	Raybould et al. 2007	Faria et al. 2007		Crawley et al. 2001
FAO 2007	Rodrigo-Simón et al. 2006	Felke et al. 2002		Darmency et al. 2007
Felke & Langenbruch 2005	Romeis et al. 2003	Ferry et al. 2007		Desplanque et al. 1999
Greenpeace 2006	Romeis et al. 2004	Griffiths et al. 2006		Devare et al. 2007
Greenpeace 2007	Schmidt et al. 2004	Griffiths et al. 2007b		Dively et al. 2004
Hails & Raymond 2004	Vandenberg 1990	Halfhill et al. 2002		Dively 2005
Halpin et al. 2007	Wei et al. 2003	Hammond et al. 2006		Duan et al. 2006
Hilbeck et al. 2000		Hanley et al. 2003		Dunfield & Germida 2003
Hilbeck & Schmidt 2006		Harwood & Obrycki 2006		Eckert et al. 2006
Icoz & Stotzky 2008		Heckmann et al. 2006		Einspanier et al. 2004
Krogh & Griffiths 2007		Hellmich et al. 2001		Eizaguirre M. et al. 2006
Lövei & Arpaia 2005		Hilbeck et al. 1998b		Farinós et al. 2008
Malone 2004		Kramarz et al. 2007b		Firbank et al. 2006
Malone & Pham-Delègue 2001		Lang & Vojtech 2006		Floate et al. 2007
O'Callaghan et al. 2005		Liu et al. 2005		Funk et al. 2006

Tier 0	Tier 1	Tier 2	Tier 3	Tier 4
Poppy & Sutherland 2004		Losey et al. 1999		Gathmann et al. 2006a
Romeis 2006		Ludy & Lang 2006b		Gathmann et al. 2006b
Romeis et al. 2008		Lundgren et al. 2005		Griffiths et al. 2007a
Romeis et al. 2006b		Lundgren & Wiedemann 2002		Griffiths et al. 2005
Rose 2007		Meissle et al. 2005		Habuštová et al. 2006
Sanvido et al. 2007		Mullin et al. 2005		Harwood et al. 2005
Schmitz et al. 2003		Naef et al. 2006		Hoheisel & Fleischer 2007
Schuler 2006		Obrist et al. 2006		Jasinski et al. 2003
Sears et al. 2001		Obrist et al. 2005		Kjaer et al. 2005
Séralini et al. 2007		Ponsard et al. 2002		Kreutzweiser et al. 1994
Sisterson et al. 2007		Pont & Nentwig 2005		Krogh et al. 2007
Stotzky 2004		Prasifka et al. 2007		Leslie et al. 2007
Widmer 2007		Prütz et al. 2004		Ludy & Lang 2006a
Winston 2003		Prütz & Dettner 2004		Lumbieres et al. 2004
Wolt et al. 2003		Rasche et al. 2006a		Lutman et al. 2005
		Rose et al. 2007		Manachini et al. 2004
		Rosi-Marshall et al. 2007		Marvier et al. 2007
		Sagstad et al. 2007		Meissle & Lang 2005
		Sanders et al. 2007		Mohr & Tebbe 2007
		Saxena & Stotzky 2001a		Musser & Sehlton 2003
		Schuler et al. 2003		Naranjo 2005b
		Vercesi et al. 2006		Naranjo 2005a
		Vojtech et al. 2005		Obrist et al. 2006
		Wandeler et al. 2002		Pilcher et al. 2005
		Wei et al. 2008		Pons et al. 2005
		Zemková-Rovenská et al. 2005		Pons & Starý 2003
		Zwahlen et al. 2003b		de la Poza et al. 2005
		Zwahlen et al. 2000		Rauschen et al. 2004
				Riddick et al. 1998
				Rose & Dively 2007
				Schorling & Freier 2006
				Sisterson et al. 2004
				Stanley-Horn et al. 2001
				Toschki et al. 2007
				Wiedemann et al. 2007
				Wold et al. 2001
				Wraight et al. 2000
				Zwahlen & Andow 2005

Annex L3: Number of references according to the BEETLE categories

Category A: Persistence and invasiveness

A General	A 1 Increased fitness	A 2 Outbreeding depression	A 3 Persistence
54 citations	31 citations	30 citations	20 citations
Abbott et al. 2003	Ahmad et al. 2007	Al Mouemar &Darmency 2004	Bartsch et al. 2003
Allainguillaume et al. 2006	Arnold &Hodges 1995	Allainguillaume et al. 2006	Chapman &Burke 2006
Ammitzbøll &Jørgensen 2006	Bartsch et al. 1996	Ammitzbøll &Jørgensen 2006	Crawley &Brown 2004
Bartsch et al. 2003	Bartsch et al. 2003	Arnold &Hodges 1995	Crawley et al. 1993
Caviness 1966	Begon et al. 2005	Bartsch et al. 1996	Crawley et al. 2001
Chapman &Burke 2006	Burke &Rieseberg 2003	Bartsch et al. 2003	Darmency et al. 2007
Chèvre et al. 2004	Chapman &Burke 2006	Bergelson 1994	Ellstrand 2003a
Conner 1997	EFSA 2006	Campbell et al. 2006	Ellstrand 2003b
Crawley &Brown 1995	Foolad 2004	Chapman &Burke 2006	Ellstrand et al. 1999
Cureton et al. 2006	Hails &Morley 2005	Chèvre et al. 1997	Ellstrand &Schierenbeck 2000
Darmency et al. 1998	Heap 2008	Chèvre et al. 2004	FitzJohn et al. 2007
Darmency et al. 2007	Huang et al. 2000	Coley et al. 1985	Hails &Morley 2005
Devos et al. 2005	Karim et al. 2007	Darmency et al. 1998	Hansen et al. 2003
van Dijk 2004	Koger et al. 2004	Ellstrand 2001	Johannessen et al. 2006a
van Dijk et al. 1997	Koziel et al. 1993	Ellstrand et al. 1999	Johannessen et al. 2006b
Eastham &Sweet 2002	Nováková et al. 2006	Ford et al. 2006	Knispel et al. 2008
Eijlander &Stiekema 1994	Powles 2008	Guéritaine et al. 2002	Pivard et al. 2007
Ellstrand 2001	Powles et al. 1998	Hails &Morley 2005	Stewart et al. 1997
Ellstrand 2003a	Rasche et al. 2006a	Halfhill et al. 2004	Sukopp et al. 2005
Ellstrand 2003b	Rasche et al. 2006b	Halfhill et al. 2005	Warwick et al. 2007
Ellstrand et al. 1999	Snow et al. 2003	Hauser et al. 1998a	
Emberlin et al. 1999	Snow et al. 2005	Hauser et al. 1998b	
FAOSTAT 2005	Stewart et al. 1997	Lee &Snow 1998	
Fénart et al. 2007	Soukup et al. 2006	Mikkelsen et al. 1996	
Ford-Lloyd &Williams 1975	Vacher et al. 2004	Pertl et al. 2002	
Gressel 2005	Warwick et al. 2007	Pertoldi et al. 2007	
Gould 1968	Wetzel et al. 1999	Rhymer &Simberloff 1996	
Hails &Morley 2005	Wu et al. 2005	Snow et al. 1999	
Hall et al. 2005	Wu et al. 2008	Strauss et al. 2002	
Henry et al. 2003	Xue et al. 2007	Vacher et al. 2004	
Knispel et al. 2008	Yeo et al. 2000		
Luna et al. 2001			
Ma et al. 2004			
Jørgensen &Andersen 1994			
Lavigne et al. 2002			
Lu 2005			
McPartlan &Dale 1994			
OECD 1997			
OECD 2003			
Owen 2005			
Raybould &Gray 1994			
Rieger et al. 2002			
Saeglitz &Bartsch 2002			
Saeglitz et al. 2000			
Scott &Wilkinson 1998			
Simard et al. 2006			
Snow 2002b			
Snow et al. 2001			
Snow et al. 2005			
Sukopp et al. 2005			
Warwick et al. 2003			
Weber &Hanson 1961			
Wilkinson et al. 2003			
Zohary &Hopf 2000			

Category B: Altered gene transfer

B 1 Reduction of pollination	B 2 Altered flower phenology	B 3 Altered outcrossing capability	B 4 Altered fecundity	B 5 Horizontal gene transfer
6 citations	3 citations	7 citations	2 citations	22 citations
Klinger & Ellstrand 1994	Hoheisel & Fleischer 2007	Biancardi et al. 2005	Campbell & Snow 2007	Bennet et al. 2004
Lee & Snow 1998	Marques et al. 2007	Bisht et al. 2007	Snow et al. 2003	Davison 1999
Pichersky & Gershenzon 2002	Mestel 2000	Feil et al. 2003		Droge et al. 1998
Pierre & Pham-Delègue 2000		Poppy & Wilkinson 2005		EFSA 2007b
Stanton et al. 1986		Ribartis et al. 2007		Gay & Gillespie 2005
Turlings & Ton 2006		Sandhu et al. 2007		Gebhard & Smalla 1998
		Sleper & Poehlmann 2006		Gebhard & Smalla 1999
				Heuer & Smalla 2007a
				Kay et al. 2002
				Lorenz & Wackernagel 1994
				Mohr & Tebbe 2007
				Monier et al. 2007
				Nielsen et al. 1997
				Nielsen et al. 1998
				Nielsen et al. 2007
				Nielsen & Townsend 2004
				Tepfer et al. 2003
				de Vries et al. 2001
				de Vries et al. 2004
				de Vries & Wackernagel 2002
				de Vries & Wackernagel 2005
				Wellington et al. 1992

Category C: Effects on target organisms

C Target organisms
34 citations
AGBIOS 2008
Alves et al. 2006
Andow & Zwahlen 2006
Bates et al. 2005
Bolin et al. 1999
Bourguet 2004
Chaufaux et al. 2001
Downes et al. 2007
Eckert 1988
Eizaguirre et al. 2006
Fakrudin et al. 2003
Farinós et al. 2004
Fernandez-Cornejo & Caswell 2006
Ferré & van Rie 2002
Fox 2003
Gunning et al. 2005
Huang et al. 1997
Huang et al. 2007
Jurat-Fuentes et al. 2003
Lheureux et al. 2003
McGaughy 1985
Ranjekar et al. 2003
van Rensburg 2007
Saeglitz et al. 2006
Schuphan 2006
Shen et al. 1998
Siegfried et al. 2005
Stodola et al. 2006
Tabashnik et al. 1990
Tabashnik et al. 2003
Tepfer 2002
Whalon et al. 2008
Zhao et al. 1996
Zhao et al. 2000

Category D: Effects on non-target organisms (Remark: table is divided into two parts)

D General	D 1 Direct toxic effects	D 2 Altered nutritional composition	D 3 Tritrophic interactions	D 4 Accumulation of toxic compounds
20 citations	107 citations	13 citations	28 citations	12 citations
Andow et al. 2006	Ahmad et al. 2006a	Bourguet et al. 2002	Birch et al. 1999	Andersen et al. 2007
Andow &Zwahlen 2006	Ahmad et al. 2006b	Clark et al. 2005	Bourguet et al. 2002	Baumgarte &Tebbe 2005
Birch et al. 2007	Alvarez et al. 2005	Eckert et al. 2006	Chamberlain et al. 2007	Douville et al. 2007
Dolezel et al. 2005	Ashouri et al. 2001	EFSA 2006	Down et al. 2003	Hopkins &Gregorich 2003
EFSA 2004	Babendreier et al. 2005	Escher et al. 2000	Dutton et al. 2003b	Hopkins &Gregorich 2005
Ehler 1990	Bai et al. 2005	Halpin et al. 2007	Gibbons et al. 2006	Icoz &Stotzky 2007
Greenpeace 2007	Bailey et al. 2005	Jung &Sheaffer 2004	Faria et al. 2007	Nguyen &Jehle 2007
Hails &Raymond 2004	Bakonyi et al. 2006	Lumbierres et al. 2004	Firbank et al. 2003a	Obrist et al. 2006a
Hilbeck &Schmidt 2006	Bhatti et al. 2005	Poerschmann et al. 2005	Harwood et al. 2005	Obrist et al. 2006b
Lövei &Arpaia 2005	Bourguet et al. 2002	Saxena &Stotzky 2001b	Hilbeck &Schmidt 2006	Rauschen &Schuphan 2006
Marvier et al. 2007	Büchs et al. 2005	Wandeler et al. 2002	Lövei &Arpaia 2005	Saxena et al. 2002a
O'Callaghan et al. 2005	Burgess et al. 2002	Zemková-Rovenská et al. 2005	Malone &Pham-Delègue 2001	Zwahlen et al. 2003a
Romeis 2006	Candolfi et al. 2004	Zwahlen et al. 2003a	Obrist et al. 2006a	
Romeis et al. 2006a	Carter et al. 2004		Obrist et al. 2006b	
Romeis et al. 2008	Clark et al. 2006		Pilcher et al. 2005	
Rose 2007	Cortet et al. 2007		Prütz et al. 2004	
Sanvido et al. 2007	Deml et al. 1999		Prütz &Dettner 2004	
Schuler 2006	Dively 2005		Ramirez-Romero et al. 2007	
Widmer 2007	Dively et al. 2004		Riddick et al. 1998	
Woiwod &Schuler 2007	Douville et al. 2007		Rodrigo-Simón et al. 2006	
	Down et al. 2003		Romeis 2006	
	Duan et al. 2006		Romeis et al. 2003	
	Eckert et al. 2006		Romeis et al. 2004	
	Eizaguirre et al. 2006		Romeis et al. 2006b	
	Escher et al. 2000		Sanders et al. 2007	
	Evans 2002		Schuler et al. 2003	
	Felke et al. 2002		Zemková-Rovenská et al. 2005	
	Ferry et al. 2007		Zwahlen &Andow 2005	
	Gathmann et al. 2006a			
	Griffiths et al. 2005			
	Griffiths et al. 2006			
	Griffiths et al. 2007a			
	Griffiths et al. 2007b			
	Habuštová et al. 2006			
	Habuštová et al. 2007			
	Hanley et al. 2003			
	Harwood &Obrycki 2006			
	Harwood et al. 2005			
	Heckmann et al. 2006			
	Hellmich et al. 2001			
	Hilbeck et al. 1998a			
	Hilbeck et al. 1998b			
	Hilbeck &Schmidt 2006			
	Huang et al. 2004			
	Jasinski et al. 2003			
	Jurat-Fuentes et al. 2003			
	Kramarz et al. 2007a			
	Kramarz et al. 2007b			
	Kreutzweiser &Capell 1996			
	Kreutzweiser et al. 1992			
	Kreutzweiser et al. 1994			
	Krogh et al. 2007			
	Lang 2004			
	Lang et al. 2006			
	Lang &Vojtech 2006			
	Liu et al. 2005			
	Losey et al. 1999			
	Lövei &Arpaia 2005			

	Lozzia 1999			
	Ludy &Lang 2006a			
	Lundgren et al. 2005			
	Lundgren &Wiedemann 2002			
	Malone 2004			
	Malone et al. 1999			
	Malone et al. 2001			
	Manachini et al. 2004			
	Marroquin et al. 2000			
	Marvier et al. 2007			
	Meissle &Lang 2005			
	Meissle et al. 2005			
	Mullin et al. 2005			
	Naranjo 2005a			
	Oberhauser et al. 2001			
	Obrist et al. 2005			
	Obrist et al. 2006a			
	Obrist et al. 2006b			
	O'Callaghan et al. 2005			
	Otsu et al. 2003			
	Pleasants et al. 2001			
	Pons et al. 2005			
	Ponsard et al. 2002			
	de la Poza et al. 2005			
	Prasifka et al. 2007			
	Ramirez-Romero et al. 2005			
	Rauschen et al. 2004			
	Raybould et al. 2007			
	Richardsen &Perrin 1994			
	Riddick et al. 2000			
	Romeis et al. 2006b			
	Rose &Dively 2007			
	Rose et al. 2007			
	Rosi-Marshall et al. 2007			
	Saxena &Stotzky 2001a			
	Schmidt et al. 2004			
	Schmitz et al. 2003			
	Sears et al. 2001			
	Sisterson et al. 2004			
	Stanley-Horn et al. 2001			
	Toschki et al. 2007			
	de Vaufleury et al. 2007			
	Volkmar et al. 2003			
	Wandeler et al. 2002			
	Wei et al. 2003			
	Wold et al. 2001			
	Wraight et al. 2000			
	Zwahlen &Andow 2005			
	Zwahlen et al. 2003b			

Category D: (continuing)

D 5 Effects on rhizosphere	D 6 Effects on symbiontic NTO
20 citations	**4 citations**
Andersen et al. 2007	Castaldini et al. 2005
Baumgarte &Tebbe 2005	Leyval et al. 2002
Brusetti et al. 2004	Turrini et al. 2004
Birch et al. 2007	de Vaufleury et al. 2007
Bohanec et al. 2007	
Cortet et al. 2006	
Devare et al. 2007	
Griffiths et al. 2005	
Griffiths et al. 2006	
Griffiths et al. 2007a	
Griffiths et al. 2007b	
Kremer et al. 2005	
Krogh &Griffiths 2007	
Lilley et al. 2006	
Njiti et al. 2003	
O'Callaghan et al. 2005	
Saxena et al. 2002b	
Schuler 2006	
de Vaufleury et al. 2007	
Widmer 2007	

Category E: Effects on ecological functions

E General	E 1 Soil functions	E 2 Biological control	E 3 Pollination
1 citation	46 citations	10 citations	11 citations
Lövei 2001	Ahmad et al. 2005	Dutton et al. 2003b	Buchmann &Nabham 1996
	Baumgarte &Tebbe 2005	Eilenberg et al. 2001	Duan et al. 2008
	Blackwood &Buyer 2004	Ferry et al. 2007	Klinger &Ellstrand 1994
	Bruinsma et al. 2003	Lövei 2006	Losey &Vaughan 2006
	Carpenter 2001	Lövei &Arpaia 2005	Lövei 2001
	Cortet et al. 2006	Meissle et al. 2005	Malone 2004
	Crecchio &Stotzky 2001	Naranjo 2005°	Malone &Pham-Delègue 2001
	Devare et al. 2004	Naranjo 2005b	Pichersky &Gershenzon 2002
	Devare et al. 2007	Riddick et al. 1998	Pierre &Pham-Delègue 2000
	Dolezel et al. 2005	Romeis et al. 2006b	Stanton et al. 1986
	Donegan et al. 1995		Winston 2003
	Dubelman et al. 2005		
	Einspanier et al. 2004		
	Flores et al. 2005		
	Griffiths et al. 2007b		
	Harwood &Obrycki 2006		
	Head et al. 2002		
	Hopkins &Gregorich 2005		
	Icoz &Stotzky 2007		
	Icoz &Stotzky 2008		
	Icoz et al. 2008		
	Jung &Sheaffer 2004		
	Kowalchuk et al. 2003		
	Losey et al. 1999		
	Motavalli et al. 2004		
	Mulder et al. 2006		
	Mungai et al. 2005		
	Obrycki et al. 2001		
	Pagel-Wieder et al. 2007		
	Poerschmann et al. 2005		
	Pont &Nentwig 2005		
	Raubuch et al. 2007		
	Rauschen &Schuphan 2006		
	Saxena et al. 2004		
	Saxena et al. 2002b		
	Saxena &Stotzky 2001a		
	Saxena &Stotzky 2001b		
	Saxena &Stotzky 2001c		
	Sims &Holden 1996		
	Stotzky 2004		
	Tapp &Stotzky 1998		
	Taylor et al. 1989		
	de Vaufleury et al. 2007		
	Wolfenbarger &Phifer 2000		
	Zwahlen et al. 2003a		
	Zwahlen et al. 2007		

Category F: Impacts of the specific cultivation, management and harvesting techniques (Remark: table is divided into two parts)

F General	F 1 Altered use og agrochemicals	F 2 Susceptibility changes against pathogens	F 3 Effects on agro-biodiversity	F 4 Changes in fertilizer use
2 citations	**20 citations**	**10 citations**	**38 citations**	**9 citations**
Champion et al. 2003	Andow 2003	Benbrook 2005	ACRE 1998	Benbrook 2005
Hayes et al. 2004	Cerdeira &Duke 2007	Cerdeira &Duke 2006	Beringer 2000	Eker et al. 2006
	Culpepper 2006	Feng et al. 2005	Bohan et al. 2005	Hernandez et al. 1999
	Devos et al. 2004	Hilbeck et al. 2000	Brooks et al. 2003	King et al. 2001
	Firbank et al. 2006	Kremer et al. 2005	Buckelew et al. 2000	Moorman 1989
	Foresman &Glasgow 2008	Lee et al. 2003a	Butler et al. 2007	Moorman et al 1992
	Freckleton et al. 2003	Moorman et al. 1992	Chamberlain et al. 2007	Powell et al. 2007
	Graef et al. 2007	Njiti et al. 2003	Champion et al. 2003	Zablotowicz &Reddy 2004
	Heard et al. 2005	Sanogo et al. 2001	Cortet et al. 2002	Zablotowicz &Reddy 2007
	Kleter et al. 2007	Yang et al. 2002	Cortet et al. 2007	
	Kleter et al. 2008		Cunningham et al. 2004	
	Men et al. 2005		Debeljak et al. 2007	
	Mulugeta et al. 2001		Firbank &Forcella 2000	
	Norsworthy 2008		Firbank et al. 2003a	
	Owen &Zelaya 2005		Firbank et al. 2003b	
	Powles 2008		Firbank et al. 2006	
	Puricelli &Tuesca 2005		Freckleton et al. 2003	
	Shaner 2000		Freckleton et al. 2004	
	Wang et al. 2006a		Gibbons et al. 2006	
	Young 2006		Green et al. 2005	
			Griffiths et al. 2007a	
			Griffiths et al. 2005	
			Griffiths et al. 2006	
			Griffiths et al. 2007b	
			Hails 2002	
			Haughton et al. 2003	
			Hawes et al. 2003	
			Heard et al. 2003a	
			Heard et al. 2003b	
			Holland 2004	
			Holland et al. 2006	
			Marshall et al. 2003	
			Perry et al. 2004	
			Roy et al. 2003	
			Squire et al. 2003	
			Strandberg et al. 2005	
			Watkinson et al. 2000	
			Wilson et al. 1999	

Category F: (continuing)

F 5 Changes in landscape structure
3 citations
Benbrook 2005
Joensen et al. 2005
Trigo &Cap 2003

Category G: Effects on the abiotic environment

G General	G 1 Production of green house gasses	G 2 Mineral nutrient erosion, fertilizer leaching	G 3 Altered chemical attributes of soil
1 citation	12 citations	13 citations	15 citations
EFSA 2004	Benbrook 2005	Baumgarte &Tebbe 2005	Blackwood &Buyer 2004
	Brookes &Barfoot 2005	Busse et al. 2001	Bravo et al. 2004
	Fernandez-Cornejo & Caswell 2006	Douville et al. 2007	Donegan et al. 1995
	Lobell &Field 2008	Eker et al. 2006	Gallina &Stephenson 1992
	Matson et al. 1997	Flores et al. 2005	Goldsborough &Brown 1993
	Pengue 2005	Griffiths et al. 2006	Icoz &Stotzky 2007
	Pritchard et al. 2007	Haney et al. 2000	Icoz &Stotzky 2008
	Ryle &Powell 1992	Haney et al. 2002	Pagel-Wieder et al. 2007
	Scherm et al. 2000	Masoero et al. 1999	Rausell et al. 2004
	Taub et al. 2008	Motavalli et al. 2004	Saxena &Stotzky 2002
	Traore et al. 2000	Raubuch et al. 2007	Saxena et al. 2002
	Wan et al. 2007	Rosi-Marshall et al. 2007	Schnepf et al. 1998
		Saxena &Stotzky 2001c	Sims &Holden 1996
			Tapp &Stotzky 1998
			Wang et al. 2006b

Category H: Stacked events

H Stacked events
10 citations
Aono et al. 2006
Bates et al. 2005
EFSA 2007
Gonzalez-Cabrera et al. 2006
Hall et al. 2000
McCann et al. 2007
de Schrijver et al. 2007
Sisterson et al. 2007
Taylor et al. 2003
Warwick et al. 2007