BEETLE

Biological and **E**cological **E**valuation **t**owards **L**ong-term **E**ffects.

FINAL REPORT

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

Federal Office of
Consumer Protection
and Food Safety

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Executive Summary

and

Main Report

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List of Acronyms	
BAC	Bacterial Artificial Chromosomes
BEETLE	Biological and Ecological Evaluation Towards Long-term Effects
Bt	*Bacillus thuringiensis*
BVL	German Federal Office of Consumer Protection and Food Safety
CCSIS	Central Core DNA Sequences Information System
COM	European Commission
CREA	Creative
CRL	Community Reference Laboratory
CSW	Creative Space Workshop
DG ENV	Directorate General responsible for environment
DNA	Deoxyribonucleic acid
EC	European Community
ECB	European Corn Borer
EEP	Extended Expert Panel
ENV	Environment
EPSPS	5-enolpyruvylshikimate-3- phosphate synthase
EP	Expert Panel (subgroup of EEP)
EFSA	European Food Safety Authority
ERA	Environmental Risk Assessment
EU	European Union
FAO	Food and Agriculture Organization
FSE	Farm Scale Evaluations
GIS	Geographic Information System
GM	Genetically Modified
GMHT	Genetically Modified Herbicide Tolerant
HGT	Horizontal Gene Transfer
HT	Herbicide Tolerance
ICGEB	International Centre for Genetic Engineering and Biotechnology
INRA	Institut National de Recherché Agronomique
IR	Insect Resistance/Resistant
ISBR	International Society for Biosafety Research
NCB	Non-Crop Biodiversity
NGO	Non-Governmental Organisations
NT	Non-Target
NTO	Non-Target Organisms
OECD	Organization for Economic Cooperation and Development
OSE	Online Survey Environment
PCR	Polymerase Chain Reaction
PMEM	Post Market Environmental Monitoring
PRC	Peer Review Committee
SM	Starch Modification
T-DNA	DNA between the left and right border sequence of the Ti-plasmid of *Agrobacterium tumefaciens*
t-DNA	transgenic DNA = genetically modified DNA
TO	Target Organisms
WCR	Western Corn Rootworm
WHO	World Health Organization

0.   BEETLE Short Summary

1) In the BEETLE study, genetically modified (GM) crops with traits already on the market in the EU or possibly so in the near future were assessed with respect to potential long-term (10-20 years) adverse effects on environment and health. They included the following major crop/trait combinations:

  - **Maize:**   insect resistance (IR)   - **Oilseed rape:** herbicide tolerance (HT)
  - **Sugar beet:** herbicide tolerance (HT) - **Potato:**   starch modification (SM)

2) Information sources:

  - more than 700 scientific publications about GM crops and their potential effects on the environment including biodiversity, and more than 100 publications about GM crops and their potential effects on human and animal health, which were published mostly during the last decade, were analysed,

  - contributions to online surveys from 100 of 167 invited environment experts and 52 of 185 invited health experts, representing a wide range of expertise with focus on the EU were received,

  - potential ways forward for reducing uncertainty regarding environmental effects were discussed with 27 invited international experts in a CREA Space Workshop,

  - input and guidance was received from a Peer-Review Committee of international experts throughout the study.

3) In more than 20 years of experimental field releases and more than 10 years of commercial cultivation, adverse long-term effects reported in the scientific literature concern (i) the development of resistance in Bt crop target organisms and (ii) tolerance in weeds to complementary herbicides used in HT crops. No other adverse long-term effects have yet been established. However, other potential long-term effects are discussed in the relevant scientific literature and in scientific fora in general.

4) Resistance development in plant pests targeted by GM crops expressing protective Bt proteins, and tolerance in weeds to complementary herbicides used on HT crops, are long-term effects which were already anticipated from the risk assessments.

5) There is at least 10 years experience of cultivating GM crops worldwide and only few *established* long-term effects have yet been reported (insect resistance development in Bt crops, feralization of GMHT oilseed rape). Due to the nature of *potential* long-term effects, it is not yet possible to *quantify* the long-term risks associated with GM crops. However, the BEETLE study has identified a *qualitative* priorization concerning the processes linked to GM plants that could have long-term effects on the environment (including biodiversity) and health.

6) The following generic conclusions can be drawn:

- ▪ Potential adverse effects due to 'Cultivation and Management' issues:
  Long-term effects of GM crops are most likely to be caused indirectly through changes in cultivation and agricultural management of HT crops and consequently affecting wider biodiversity. The use of complementary herbicides can potentially change the management practice. These specific effects of such changes will depend on crop/trait combinations cultivated and possibly regional aspects. This process was clearly assigned with highest priority.

- ▪ Established adverse effects related to 'Resistance development' in pests targeted by IR crops, particularly Bt:
  Direct long-term effects of GM crops (mainly Bt maize) are likely to become apparent due to resistance development in Target Organisms (TO).

- ▪ Potential adverse effects on Non-Target Organisms[1] (NTO) related to 'Gene flow to wild relatives':
  Gene flow regarding GM traits from GM crop plants to wild relatives should be considered in cases of GM plants that have ancestors in the natural European flora, especially for crops related to the *Brassica* and *Beta* species, which have sexually compatible relatives. Although gene flow as such is not an adverse environmental effect, the long-term consequences for species conservation and biodiversity might be relevant.

- ▪ Potential adverse effects on 'NTO, ecological functions and the abiotic environment':
  Long-term effects of GM crops on NTO (e.g. in soil), ecological soil functions, and the abiotic soil environment should be considered due to uncertainty indicated by the fact that only a few scientific publications are available. Long-term effects are most likely to affect NTOs closely related to TO of Bt maize (beetles and butterflies).

7) Long-term effects on animal or human health linked to GM crops have not yet been identified. However, forthcoming generations of GM crops will include more complex genetic modifications, e.g. more stacked events (several GM traits in the same crop variety) which could increase exposure to potential allergens and thus the potential for allergies to develop.

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[1] A NTO is an organism which is affected by an interaction for which it was not the intended recipient. This includes gene flow to wild relatives.

8) A tool for providing pre-market information[2] on GMO characteristics is a database including novel bioinformatic applications guiding assessment of potential interaction between different genetic modifications, e.g. synergistic effects of stacked events (intended or unintended). Possible synergistic effects of proteins from intended and unintended combination of different GMOs should be considered during the ERA (according to Annex II of Directive 2001/18/EC) to improve the prognostic power of the long-term effect assessment.

9) The BEETLE report concludes that research studies, modelling and monitoring are appropriate tools to investigate long-term environmental effects during GMO cultivation close to practice[3]. It proposes the development of indicators and databases for an appropriate EU-wide surveillance of long-term effects on soil and other biodiversity resulting from GM crop cultivation and management. Potential indicators should be further developed over time by risk assessors and risk managers. The indicators for environmental monitoring should be selected in accordance with the crop/trait combination and the receiving environment

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2 Pre-market information must be in line with the requirements of Directive 2001/18/EC, where an ERA should always be carried out prior to placing on the market on a case-by-case basis. It should also take due account of potential cumulative long-term effects associated with the interaction with other GMOs and the environment.

3 'Close to practice' means here the initial cultivation phase after first consent for placing on the market is given. It is in many cases a priori (epistemically) not possible to experimentally study long-term effects related to large-scale cultivation.

1. Executive summary

The European Commission (COM) is in the process of collecting all scientific, technical and economic elements concerning long-term effects of GM crops on health and the environment (including biodiversity). A project team under the lead of the German Federal Office of Consumer Protection and Food Safety (BVL)[4] carried out a study project called 'Biological and Ecological Evaluation Towards Long-term Effects' (BEETLE) to support the COM initiative in relation to health and environmental aspects, but without consideration of coexistence issues.

The key objectives of this study were:

(1)   to collect and collate information on established or potential long-term effects of GM crops on health, biodiversity and the environment after more than ten years of worldwide experimental field research and commercial cultivation (data arising from both pre-marketing and post-marketing results);

(2)   to prioritize the potential risks[5] of the main crop-trait combinations currently approved worldwide but with respect to their relevance to the EU;

(3)   to identify the areas of greatest scientific uncertainty (and their origin) and

(4)   to identify indicators for measuring the development of long-term impacts on the environment over time.

A long-term effect in the context of the BEETLE study is defined as a process or scenario that becomes apparent directly or indirectly on a characteristic time-scale of the order of 10 to 100 generations; meaning for most annual organisms a time span of 10 to 20 years.

The study used wide scientific assessment, enabling transparency, and attempted to gain an almost complete overview concerning possible (realistic) long-term effects. To do this, the BEETLE study

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[4] The BEETLE project team was completed by BLaU Environment Consultancy (BLaU, Göttingen) and Genius Science & Communication (Genius, Darmstadt). Prof. Gerhard Flachowsky (Friedrich Loeffler Institute, Braunschweig) and Prof. Karl-Heinz Engel (Technical University of Munich) supported as subcontractors for human and animal health aspects the BEETLE project.

5 The term 'risk' has two major elements (see also definition in section 2.2.1 of this report): (1) magnitude of adverse effect and (2) probability of occurrence of effect. For (1), the BEETLE study identified a number of plausible processes causing adverse effects linked with GM plants. It is in the nature of - not established - long-term effects that no information is yet available on their quantitative magnitude. For similar reasons, the probability (2) is not yet quantifiable in detail, but can be elaborated via expert assessment of available information and transformed into the likelihood categories 'high', 'medium', 'low', and 'negligible'. Only three of these four categories were offered to experts within each assessment questionnaire. The category 'medium' was used as intermediate category if 'negligible' could already be excluded (see Annex 2 Tables 1, 2, and 3). However, prioritization in the BEETLE study led finally to classification of the potential adverse effects into the categories 'most likely', 'likely', and 'not likely'.

- analysed more than 700 scientific publications about GM crops and their potential effects on the environment including biodiversity and more than 100 publications about GM crops and their potential effects on human and animal health, which were published mostly during the last decade,

- received contributions to online surveys from 100 of 167 invited environment experts and 52 of 185 health experts, representing a wide range of knowledge with focus on the EU,

- discussed potential ways forward for reducing the uncertainties regarding environmental effects with 27 invited experts in a CREA Space Workshop (CSW), and

- received input and guidance from a Peer-Review Committee of international experts throughout the study.

It is not yet possible to quantify the long-term risks associated with GM plants as experience is lacking. However, in more than 20 years of experimental field releases and more than 10 years of commercial cultivation, adverse long-term effects reported in the literature have concerned the development of resistance in Bt crop target organisms and tolerance in weeds against complementary herbicides used in HT crops. No other adverse long-term effects have yet been established. In addition, several other potential long-term effects are discussed in the scientific literature and in scientific fora in general. Within the legal framework, applications requesting authorization of the cultivation of a GM plant in the EU must contain an Environmental Risk Assessment (ERA) in accordance with the objectives, elements, principles and methodology laid down in Annex II to Directive 2001/18/EC.

1.1   Prioritisation of long-term effects on the environment

A total number of 26 processes[6] and 62 scenarios[7] of potential long-term effects on the environment were identified in the Literature Review "Environment" (see section 5 and Annex 1).

Based on the analysis of the literature review, the possible influence of regional aspects in the EU was examined within 24 of these 62 scenarios. Equally, 11 of these 62 scenarios were assessed for their relevance concerning stacked events[8]. The BEETLE study assessed four crops from which GM varieties are on or close to the EU market for the purpose of culti-

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[6] In the context of this BEETLE study a 'process' is defined as a (biological) <u>mechanism</u> that potentially could lead to long-term effects under certain conditions (see Figure 1 x-axis).

[7] In the context of this BEETLE study a 'scenario' is defined as a <u>chain of events</u> in which a specific GM plant/trait combination could induce long-term effects.

[8] For the purpose of this project "stacked" events are defined as a GMO containing multiple transformation events combined by conventional breeding; see also: Guidance Document for the risk assessment of genetically modified plants containing stacked transformation events by the Scientific Panel on Genetically Modified Organisms (GMO) http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753824_1178623591786.htm

vation and/or import and use: maize, oilseed rape, sugar beet, and potato. Additionally, four crops from which also GM varieties are cultivated 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 relevant genetic modifications within the EU.

As a result of the Literature Review and the Online Survey (see section 5 and Annex 1 and 2), ten processes or scenarios (see Table 1) were identified by the consulted experts with highest potential for causing adverse long-term effects on the environment. The selected processes and scenarios are derived from section 5 and 6 of the BEETLE study. The processes and scenarios with priority regarding potential long-term effects are grouped by the BEETLE expert team into four cases as follows:

- Potential adverse effects due to 'Cultivation and Management' issues:
  Long-term effects of GM crops are most likely to be caused indirectly through changes in cultivation and agricultural management of HT crops and consequently affect wider biodiversity. The use of complementary herbicides can change the management practice. The specific effects of such changes will depend on crop/trait combinations cultivated and possibly regional aspects. This process/scenario was clearly assigned with highest priority.

- Established adverse effects related to 'Resistance development' in pests targeted by IR crops, particularly Bt:
  Direct long-term effects of GM crops (mainly Bt maize) are likely to become apparent due to resistance development in TO. Resistance development in plant pests targeted by GM crops expressing Bt proteins for their protection is a long-term effect already anticipated from the risk assessment.

- Potential adverse effects on NTO related to 'Gene flow to wild relatives':
  Gene flow regarding GM traits from GM crop plants to wild relatives should be considered in cases of GM plants that have ancestors in the natural European flora, especially for crops related to the Brassica and Beta Species, which have sexually compatible relatives. Although gene flow as such is not an adverse environmental effect, the long-term consequences for species conservation and biodiversity might be relevant.

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- <u>Potential adverse effects on 'NTO, ecological functions and the abiotic environment'</u>: Long-term effects of GM crops on NTO (e.g. soil), ecological soil functions, and the abiotic soil environment should be considered, due to uncertainty indicated by the fact that only a few scientific publications are available.

The third case covers potential long-term effects on NTOs concerning interactions between wild relatives and crop plants. The fourth case summarizes long-term effects on NT insects related to target organisms in Bt maize, on NT herbivores in starch-modified potato, and on NT soil micro-organisms in Bt maize or HT crops. However, an extensive body of research data has been assembled, e.g. on NT above-ground organism impacts of IR maize. The majority of laboratory studies and all the field studies reviewed (literature) did not reveal any unexpected adverse or long-lasting effect on NTOs. One important lesson is that even if negative effects were observed in the laboratory (e.g. under worst-case conditions) no similar quantitative or qualitative adverse effects were necessarily detected in the field. Further, regional variation in the potential appearance of adverse environmental and biodiversity related effects can be expected. Thus regional differences should be taken into account in the assessment of long-term effects with regard to the cultivation of GM crops.

Gene stacking in general was identified as having the potential to modulate possible long-term environmental effects (see EFSA 2007). Prioritization concerning categories and scenarios of GM plants with stacked events is not possible because few such GM plants have been placed on the market. From the results of the CREA Space Workshop it can be concluded, that - for the assessment of potential long-term effects of stacked events - the initial ERA according to Annex II of Directive 2001/18/EC (ERA) should already consider the occurrence of unintended stacks. The risk assessment for new events is required to take into account "the potential receiving environment" which would include other GM crop events incorporated into sexually compatible species. Synergistic effects on the level of proteins coded by the stacked events and other interactions on the level of gene regulation (e.g. via siRNA or microRNA) could lead to long-term effects. Stability versus instability of DNA inserted into GM plants is under ongoing discussion in the broader scientific community. Integration of transforming DNA into the plant genome is frequently accompanied by rearrangements and associated with the process of transformation (insertion). There are no signs of instability or increased recombination regarding inserted transforming DNA or its site of integration after integration. The integrated transforming DNA remains structurally intact in the GM plant in distant descendents of the originally transformed plant cell and becomes a fixed element of the plant genome.

Table 1: Processes/scenarios causing potential [p] or established [e] long-term effects and potential ways forward to increase knowledge regarding the analysed crop/trait combinations. The information is derived from different sources in the BEETLE study: literature review [LR], expert survey [ES], CSW, and BEETLE team evaluation [BE]).

Process / Scenario (LR, ES)	Priority for long-term effects (LR, ES, CSW, BE)	Way forward [CSW, BE]	Potential actions to increase knowledge [LR, CSW, BE]
HT crops: Indirect effects of altered cultivation and management	[p] Highest priority; Environmental effects depending on crop/herbicide combinations and potential further regional dependency.	Monitoring and Modelling in pre-selected agricultural areas	Data collection and monitoring on farm scale with studies on indicators for biodiversity or on fertilizer use.
HT crops and feralization	[e] High priority, specifically with feralizing crops (*Brassica* and *Beta* Species)	Monitoring	Data collection on persistence and invasiveness of HT oilseed rape and sugar beet
Bt crops: Resistance development of target organisms	[e] High priority, as resistance development already anticipated from the risk assessment	Monitoring	Case-specific monitoring
Bt crops: Effect of Bt proteins on NTO soil micro-organisms and on soil function	[p] High priority due to uncertainty indicated by the fact that only a few scientific publications are available	Monitoring and Research	Performing studies under "worst-case" conditions in different soils to reduce the effort for regional ERA aspects. Monitoring the Bt proteins as indicator. Selection of appropriate indicators for soil functions.
Bt crops: Effect of Bt crops on NTO closely related to TO	[p] High priority due to expected susceptibility to Bt-proteins because of close relation to TO	Monitoring	Selection of appropriate organisms indicating food –web-related effects.
SM crops: Effects on herbivores	[p] High priority due to uncertainty indicated by the fact that only a few scientific publications are available	Monitoring	Herbivore surveys with selected indicator species
Gene flow from HT crops to wild relatives (as NTO) and its consequences	[p] High priority, specifically with GM plants that have ancestors in the natural European flora (*Brassica* and *Beta* Species)	Monitoring	Data collection on HT hybrids of oilseed rape and sugar beet
Effects of Bt protein root exudation and of herbicides on symbiotic organisms like mycorrhiza	[p] Lower priority	Monitoring	Soil fertility – crop yield surveys as indirect indicators (altered management).
HT crops and altered susceptibility to pathogens	[p] Lower priority	Monitoring	Pathogen surveys
HT crops and altered landscape structure	[p] Lower priority	Modelling and Monitoring	Analysis of landscape structure using Geographic Information Systems (GIS) in combination with GMO cultivation registers
Allergenicity development (Health)	[p] Lower priority	Monitoring	Routine surveillance systems

1.2   Prioritisation of long-term effects on human and animal health

One potential long-term effect of GM crops on human and animal health has been identified thus far, namely a potential increase in allergenicity due to new or increased exposure to proteins with allergenic potential (see section 5 and Annex 4 and 5). The risk assessment established in the EU assesses potential adverse effects on human and animal health including allergenicity. For the currently-approved crop/trait combinations considered in this study, no evidence has emerged to challenge the assessment that there would be no adverse effects on health. However, future generations of GM crops will include more complex and/or greater use of stacked events of several GM traits into a single crop variety, thus increasing the potential for new or increased exposure to proteins with the potential to cause allergenicity.

1.3   Ways forward

The aim of the CREA Space Workshop was to identify ways forward to decrease uncertainties and to close knowledge gaps concerning potential long-term effects.

1.3.1   Selection of tools

The BEETLE report concludes that

- research studies[9]

- modelling[10], and

- monitoring[11]

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[9] It is a regulatory question as to who is responsible for carrying out such research studies. According to Regulation (EC) No 1829/2003 on genetically modified food and feed and to Directive 2001/18/EC on the deliberate release of genetically modified organisms into the environment, GM plants and their derived food and feed products are subjected to a risk assessment (RA) prior to their placement on the European market. In this risk assessment, the potential [long-term] effects that the commercialisation of a GMO might have on human or animal health and the environment are assessed based to a large extent on the information delivered by applicants. Where and when further research studies are necessary depends on the particular application. It is in many cases *a priori* (epistemically) not possible to experimentally study long-term effects related to scale before – market-approved – cultivation, or it would be practically disproportional to demand continuous years/decades of small-scale field testing, e.g. of crop varieties that would later be outdated in yield performance due to ongoing progress in conventional breeding. However, in reasoned exceptions it might also be possible to study long-term effects in more depth in a pre-market phase with 'close-to-practice' conditions (e.g. larger scale field releases to test for effects that are expected due to known accumulative effects, and which can be carried out within a reasonable time frame).

[10] A comprehensive overview about modelling as a tool was evaluated recently by EFSA (2008)

are appropriate tools which can be applied to gather further information with the aim to increase knowledge and to reduce uncertainties regarding adverse long-term effects of GM plants. The selection of tools can be based on the evaluation of the biological process behind potential long-term effects, e.g. as demonstrated by this study.

1.3.2   Environmental aspects

Predicting and assessing long-term effects requires information about the GMO and the status of the receiving environment, both in terms of baseline conditions and temporal changes following GMO introduction. A prerequisite for assessing potential long-term effects of GM crop cultivation is its comparison with conventional cultivation practices (consistent with the "baseline concept").

A workable definition of 'long-term' is important, and relevant endpoints (e.g. protection goals) for long-term effects need to be defined in order to allow for systematic long-term investigations (Sanvido *et al.* 2006). Then, appropriately defined indicators (e.g. general biodiversity indicators, soil species or soil functionality parameters, see Bühler 2006) should be selected for monitoring (Table 1 and section 7).

Monitoring and/or research to prove the conclusions of the ERA on long-term effects should be performed in defined typical 'hot spot' regions. Such hot spot regions are areas where potential long-term effects are more likely to occur, e.g. crop seed production areas in the vicinity of wild relative populations in mild climate areas (see section 8). For detecting, e.g. regionally differing Bt effects on NTOs, participants of the CREA Space Workshop recommended choosing insect groups related to ecological functions and representative of selected EU regions. The identification of typical soil insect guilds could help to cover ecological functions that vary from region to region.

Modelling can be used, e.g. to quantify effects such as gene flow and estimate their consequences at a landscape scale (Colbach et al. 2009, EFSA 2008, Sester et al. 2008). This 'upscaling' would allow predictions of impacts to be made if GM crop production increases over time and space. However, no appropriate model is currently available that can be routinely applied to risk assessment (including of long-term effects). Further research is required to develop new models or to explore the use of related models.

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[11] In this approach, monitoring is considered to confirm in reasoned cases the initial assumption of the ERA.

Monitoring is an efficient and available tool to recognise long-term effects if it is focussed on the nature of the potential long-term effects (e.g. identified processes and scenarios). The results could be compared with those obtained using the other abovementioned tools in order to test their reliability.

1.3.3   Health aspects

Concerning long-term health aspects, the BEETLE study recommends further developing the ERA methodology and the monitoring. The following specific conclusions can be drawn from the Literature Review "Health" and Online Survey "Health":

- If health-related differences were to be detected between food or feed derived from a GM crop and its most closely related non-GM comparator, more conventional crop varieties should be used in feeding trials in order to cover the natural biological range of the measured parameters.

- In relation to toxicology assessment, a range of comparators will help to assess whether any statistical differences detected between the GM plant and its non-GM counterparts are biologically relevant.

1.3.4   Stacked events

For potential impacts caused by 'Stacked Events', testing of synergistic effects e.g. of gene products coded by stacked events (intended and unintended stacks) has been prioritised in this study as the most important way to identify potential adverse effects. Possible synergistic effects of proteins from intended and unintended stacks should be identified during the ERA according to Directive 2001/18/EC to improve the prognostic power of the long-term effect assessment. Taking into account the growing knowledge of mechanisms of gene regulation, basic research in this field will reduce uncertainty. Consideration should be given to whether the knowledge gained over recent years in this area can be applied and developed in order to create tools for an improved risk assessment. In this context the BEETLE team discussed the potential value of gathering information on GM plants cultivated commercially worldwide including the nucleotide sequences of their genetic modification (see Annex 6).

1.3.5   Uncertainties

Recognising that an ERA is only as good as the current state of scientific knowledge, ERA can be based on limited scientific information, leaving some uncertainty. Therefore, under current EU legislation, it is recommended to describe these scientific uncertainties, and to clarify assumptions, extrapolations and predictions made. One uncertainty relates to long-

term impacts due to the large-scale exposure of different environments to GM crops when they are grown at a larger scale over long periods of time. Although further research might not always overcome the shortcomings identified in the ERA, environmental post-market monitoring will play an essential role in the detection and possibly the prevention of cumulative long-term adverse effects to the environment that could occur during and after the cultivation of GM crops (EFSA 2008).

The BEETLE study did not examine whether the identified potential long-term effects could have further additive or synergistic effects (e.g. unintended interaction between different Bt proteins with effects on a wider range of NTOs).

1.4   Conclusion

In conclusion, through its prioritization of potential long-term effects of GM crops and the ways forward, the BEETLE study has identified 11 potential actions, listed in Table 1, to increase knowledge and thus to decrease uncertainties. It proposes the development of both indicators and databases for an appropriate EU-wide surveillance of long-term environmental effects on soil and the wider biodiversity resulting from GM crop cultivation and management. The potential indicators listed in Table 1 should be further developed over time by risk assessors and risk managers. The indicators for environmental monitoring should be selected in accordance with the crop/trait combination and the receiving environment (for suggestions see section 7).

Further improvement of the risk assessment methodology for health (human and animal) accompanied by surveillance (e.g. of allergenicity) is proposed as the way forward to identify potential long-term effects of GM plants (section 6).

2. Introduction to the BEETLE Project

2.1. GM plant risk assessment

Article 4(3) of Directive 2001/18/EC (EC 2001) on the deliberate release into the environment of genetically modified organisms (GMO) requires Member States and, where appropriate, the Commission to ensure that potential adverse effects on human health and the environment are accurately assessed on a case-by-case basis as part of each notification for the deliberate release of a GMO into the environment. Importantly, this requirement applies both to applications for research purposes and for placing on the market.

An ERA must be carried out in accordance with the objectives, elements, principles and methodology laid down in Annex II to Directive 2001/18/EC. Several supporting documents have been prepared to assist the applicant. For example, Commission Decision 2002/623/EC (EC 2002a) of 24 July 2002 establishes guidance notes on the objective, elements, general principles and methodology of the ERA referred to in Annex II to Directive 2001/18/EC. Council Decision 2002/811/EC (EC 2002b) establishes guidance notes supplementing Annex VII to the Directive, describing the objectives and general principles to be followed to design the monitoring plan. Council Decision 2002/812/EC (EC 2002c) establishes the summary information format. In March 2003, a 'Guidance document for the risk assessment of GM plant and derived food and feed' was published by the Joint Working Group on Novel Foods and GMOs (EC 2003), and this was later replaced by the EFSA 'Guidance document for the risk assessment of genetically modified plants and derived food and feed' (EFSA 2006).

The objective of the ERA is, on a case–by-case basis, to identify and evaluate potential adverse effects of the GMO, whether direct or indirect, immediate or delayed, on human health and the environment which the deliberate release of the GMO may have.

A general principle for ERA is that an analysis of the "*cumulative long-term effects relevant to the release and the placing on the market is to be carried out*". "Cumulative long-term effects" refers to the accumulated effects of consents on human and animal health and the environment, including *inter alia* flora and fauna, soil fertility, soil degradation of organic material, the feed/food chain, biological diversity, animal health and resistance problems in relation to antibiotics.

According to Commission Decision 2002/623/EC (EC, 2002a), the ERA "*may not always re-sult in definitive answers to all the questions considered because of lack of data. For poten-tial long-term effects, in particular, the availability of data may be very low. In these cases in particular appropriate risk management (safeguards) has to be considered in accordance with the precautionary principle in order to prevent adverse effects on human health and the environment*".

The ERA is based initially on information on the GM plant [e.g. compositional analysis] grown in field trials during multiple growth seasons and/or in various geographic locations. The po-tential for long-term effects is assessed on the basis of knowledge and experience of non-GM crops, near equivalents and GM crops. Where the ERA identifies a specific manageable risk (e.g. development of resistance to Bt in target insects) or significant uncertainty (e.g. missing exposure information of GM-crop-produced toxins) **case-specific monitoring** and/or **further experimental studies** may be required to determine impacts due to scale and/or time. Even when the degree of uncertainty in the ERA according to Annex II of Directive 2001/18/EC is negligible, unanticipated long-term effects or large-scale effects on human health and the environment remain an important component of the **general post-marketing surveillance** which must be part of GM plant cultivation in the EU.

It is important to collate findings on potential long-term effects and to analyse the underlying causal chain of effects, e.g. related to the character of the GM crop, application of plant pro-tection chemicals to tolerant GM crop, or HGT (e.g. antibiotic resistance genes).

2.1.1. Environment (including biodiversity)

Intensive techniques of agricultural production in industrialized countries have resulted in increasing non-crop species extinction and become part of public debate. The term "biodi-versity" was introduced in these discussions on the causes of increasing environmental damage in the '70s and '80s of the last century. The term is used to describe variations of biological organisational structures; usually distinguishing between three organisational lev-els: the genetic, the species, and the ecosystem level (Chapin 2000; Kevin and Spicer 2004).

In public debate the term 'biodiversity' is often still used in both positive and negative refer-ences, positively as an indicator of environmental health, negatively - in terms of loss of bio-diversity - as an indicator of natural species erosion. Although it might be useful to differenti-ate between effects on biodiversity and effects on environment, the effects are commonly discussed under the headline 'environment'. The ERA is concerned with potential adverse effects resulting from GM PLANT cultivation. Since species of similar taxonomic groups will be predominantly affected in a similar way, e.g. by GM crops expressing Bt protein, the focus

of biodiversity-oriented effect considerations is mainly directed to single species with additional effects on dependent food webs (see below). Generally, impacts on biodiversity are related to a specific crop/trait combination. Potential threats to non-crop biodiversity (NCB) due to the agricultural cultivation of GM crops have to be observed carefully to determine effects on, e.g. the abundance of plant dwelling insects and their predators and parasitoids (beneficial organisms) and weed communities (Ammann 2005). The evaluation of adverse effects on biodiversity resulting from GM crop cultivation needs comparative long-term approaches using conventional agriculture as the comparator (Dolezel *et al.* 2006), since this also has effects on NCB.

Potential negative effects on the environment, including biodiversity, caused by changes in agricultural practices will not always occur directly or immediately but frequently indirectly or after a delay. New management practices used in agriculture such as altered crop rotation or soil tillage often affect biological systems (species and their functions) in ways which become apparent after a certain time span. Such effects mainly result in decreased vitality or decreased abundance over time. However, impacts on vitality and abundance may also potentially affect specific functional aspects (e.g. pollination, predation) related to the affected species. The most adequate tools for evaluating such temporal negative effects are research studies and monitoring using sufficient indicators if applicable. Indicators for revealing negative environmental, including biodiversity-related, impacts could include direct abundance measurements of important affected species or studies on the fate of toxins used or studies on potentially adversely affected ecological functions (Arndt *et al.* 1987, Schubert 1985, OECD 1993).

2.1.2 Health (animal and human health)

Within the ERA, an assessment is required of the possible immediate and/or delayed effects on human health resulting from potential direct and indirect interactions of the GM plant and persons working with, coming into contact with, or in the vicinity of the GM plant release(s). In addition, an assessment is required of the possible immediate and/or delayed effects on animal health and consequences for the feed/food chain resulting from exposure to or consumption of the GM plant and any products derived from it, if it is intended to be used as animal feed. GM crops destined for human or animal consumption usually have been meticulously studied for their impacts on human and animal health.

Screening for long-term effects on health has in the past included studies of nutritional value, toxicity, horizontal gene transfer (HGT) in gastrointestinal tracts of humans and animals, and allergenicity (Einspanier *et al.* 2004, Kuiper *et al.* 2001, Flachowsky and Aulrich 2001, Neth-

erwood *et al.* 2004, Goldstein *et al.* 2005). To cover potential toxicological and nutritional long-term aspects it is necessary to look not only at food use but also at animal feed use of GM crops. Toxicological studies are usually performed with animals. Livestock are likely to take a diet with high content of GM crops or with a high content of feedstuff produced from GM crops. In contrast, humans in developed countries usually take much more diverse foodstuffs and are unlikely to consume food with a high proportion of ingredients derived from single GM crops. Therefore, it is much more likely that potential toxicological and nutritional long-term effects on health will be detected in connection with feed use of GM crops than with food use, e. g. data on nutritional trials with quails are available, with the birds being fed on a diet containing 50% GM crops for 20 generations (Flachowsky 2007).

2.2 BEETLE Project objectives

The key objectives of the BEETLE study were:

- to collect and collate information on established or potential long-term effects of GM crops on health and the environment including biodiversity after more than ten years of worldwide experimental field research and commercial cultivation (data arising from both pre-marketing and post-marketing results);
- to prioritize the potential risks of the main crop-trait combinations currently approved worldwide but with respect to their relevance to the EU;
- to identify the areas of greatest scientific uncertainty (and their origin) and
- to identify indicators for measuring the development of long-term impacts on the environment over time.

The BEETLE project performed a ranking of identified potential long-term effects of the main crop/trait combinations approved worldwide according to likelihood (see Annex 1-4, and section 5). Areas of greatest scientific uncertainty regarding long-term effects were investigated by analysing notifications for placing on the market of GM crops in the EU and by screening the scientific literature. Identifying areas of greater scientific uncertainty regarding long-term environmental and health effects of GM crop requires taking into account the development of the GM technology and the scientific knowledge.

2.3 Terminology used in the BEETLE study

The terminology aims to be as precise as possible. The BEETLE study uses a number of definitions listed below.

2.3.1 Environmental Risk Assessment (ERA) and Monitoring

According to Harwood and Stokes (2003), risk is an inevitable consequence of the uncertainties that are inherent in our knowledge of ecological systems, and ecologists currently develop rigorous methods for evaluating these uncertainties. The first step in quantifying risk is to identify the sources of uncertainty. Within the BEETLE study, the following terminology is used:

- **Long-term effect**: a process or scenario that becomes apparent directly or indirectly on a characteristic time-scale of the order of 10 to 100 generations. Thus for most annual organisms long-term effects should emerge after a minimum of 10 to 20 years (see also section 2.3.2 of this report).

    o *Established* long-term effects of GM plants are those which have already become apparent and can be found in the literature or in expert reports

    o *Potential* long-term effects of GM plants have not been observed so far but can be hypothesised from analogous examples and knowledge (e.g. from non-GM plant experience or from GM plant short-term studies) derived from experience or literature studies

- **Uncertainty:** incomplete information about a particular process or scenario. Uncertainty applies to predictions of future events both on the 'probability' (temporal dimension) and the 'adverse effect' (spatial dimension) in the risk assessment (see also figure 1).

- **Risk:** is a consequence of uncertainty. It can be defined as an equation = $\int$ (1) magnitude of adverse effect x (2) likelihood of occurrence of effect. If there is no uncertainty, the concept of risk is irrelevant because the probability of the outcome is 1 or 0. Due to the lack of quantifiable information, the risk prioritization in the BEETLE study led finally to qualitative classification into the categories 'most likely', 'likely', and 'not likely'.

- **Likelihood:** the probability that an effect (process or scenario) will happen. There is no probability if all conditions are known in a deterministic environment. However, the

complexity of interactions leads to probabilistic descriptions such as 'negligible', 'low', or 'high' which are more qualitative than quantitative.

There have so far been no reports of *un*anticipated long-term effects caused by GM plants. However, potential [anticipated] long-term effects are discussed in the relevant scientific literature and on scientific forums.

One of the key elements in every aspect of GMO risk assessment is consideration of potential long-term effects. Scientific experts, in particular, consider the possibility of adverse long-term effects on humans/animals consuming food/feed derived from GM plants and the cultivation of GM plants in the environment.

2.3.2   Long-term effects

A pragmatic definition of long-term effects is given by Crawley (1994): a process (or effect) is long-term if its characteristic time-scale is of the order of 10 to 100 generations. Thus for most annual organisms long-term effects should emerge after a minimum of 10 to 20 years. A more detailed view was added by Den Nijs and Bartsch (2004) particularly in relation to the complexity of affected ecological system structures versus system processes (Figure 1). For the purpose of this project the BEETLE study concentrated effort on long-term effects of GM crops represented by the 'grey shaded area', i.e. 'medium' or 'slow' responses of complex biological systems to disturbances in time. However, human input may accelerate system responses.

The evaluation of potential long-term effects is subject to controversial discussion. One side argues that methods used in the current risk assessment are not sufficient to address long-term effects adequately; others claim that long-term effects have not been possible to predict (EFSA 2008). However, since the first introduction of GMOs in 1996, the global area of GM plants has grown continually to more than 114 Mio ha in 2007 (James 2008). Therefore, more than ten years of expanding experience and evaluation of adverse effects on health and environment (including biodiversity) should be available. Additionally, the experience of an increasing number of experimental releases of GMOs with new traits, results of various scientific programmes on biosafety research all over the world, and published theoretical considerations (e.g. Crawley 1994, Dutton *et al.* 2003, Andow and Hilbeck 2004, Zwahlen and Andow 2005) should provide a good basis for identifying established and potential long-term effects.

COMPLEXITY OF SYSTEM STRUCTURE (SPATIAL DIMENSION)

- Increasing importance of the phenomenon's history
- Growing influence of environmental parameters on system behaviour
- Decreasing prognostic power of deterministic description

Landscape unit

Ecosystem, community

Population

Organism

EXTREMELY SLOW > 1.000yrs

VERY SLOW < 1.000 yrs

SLOW < 100yrs

MEDIUM < 10 yrs

VERY FAST < 0.1yr

FAST < 1 yrs

COMPLEXITY OF SYSTEM PROCESSES (TEMPORAL DIMENSION)

Individual physico-chemical process (cause-effect reaction)

Feedback reaction (interdependent process)

System adaptation (change of parameters)

System self-organisation and re-establishment (change of system structure)

System evolution (emergence / incorporation of new system components)

Figure 1: Velocity of environmental effects in relation to environmental structure and process. The grey shaded area represents the effect in relation to expected time (short to medium) of potential occurrence (= defined long-term effect in the BEETLE study) – after Den Nijs and Bartsch (2004).

2.4 BEETLE Project organisation and implementation

2.4.1 Project approach

The approach to meeting the project objectives was to start with a literature review and then to involve external experts in analysing and assessing outputs from the review. The steps are described in detail in section 3. An online survey and a workshop were organised to facilitate input from a sizeable panel of experts (the Extended Expert Panel - EEP) and a sub-group (the Expert Panel, EP), respectively. A Peer Review Committee (PRC) was established which provided expert input throughout the duration of the study.

2.4.2. Management and participation

2.4.2.1 Coordination

The BEETLE project involved a consortium of partners coordinated by the BVL (Figure 2). The coordinator was responsible for the communication between DG ENV and all the partners. This comprised regular correspondence, planning and participation in several meetings with the EU Commission (Directorate General Environment) in Brussels, regular progress reports, an interim report after 3 months and a draft final report after 6 months.
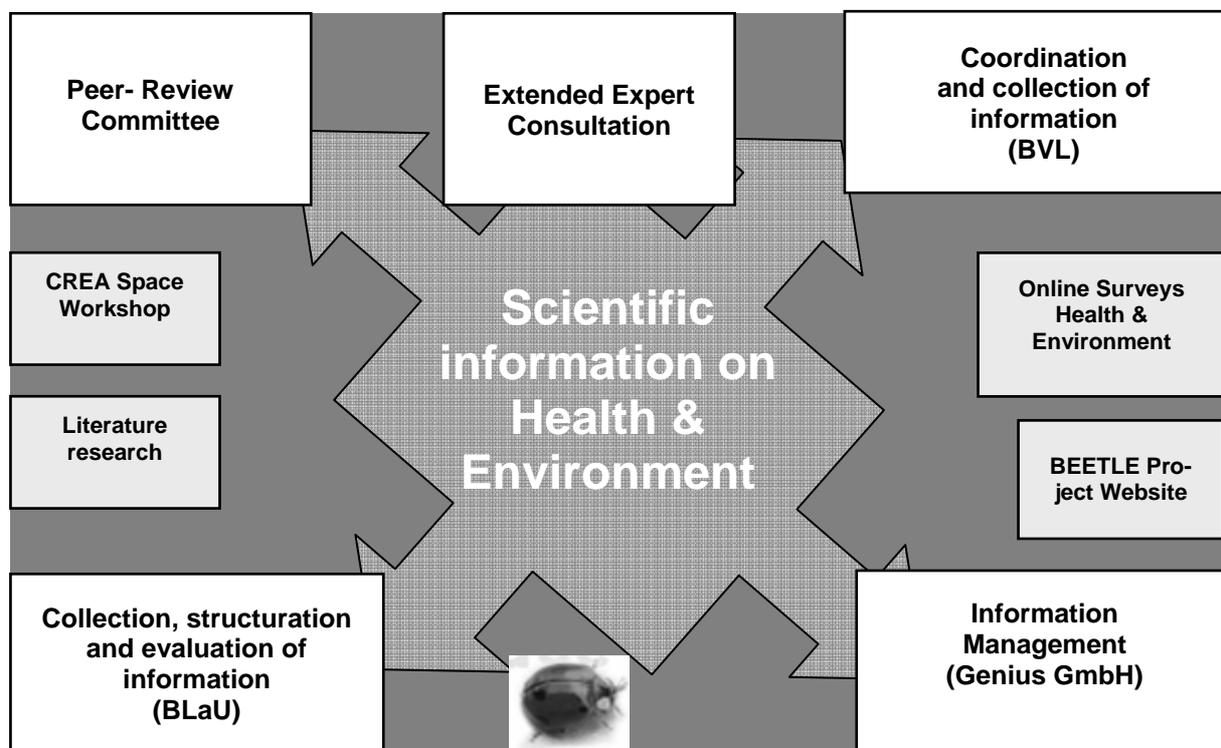
Figure 2: Structure of the BEETLE project (Biological and Environmental Evaluation Towards Long-term Effects).

The BVL established and managed the PRC. The coordinator was also responsible for the operating schedule and the coordination of activities between the consortium partners and the PRC. BVL, together with Genius GmbH organised the CREA Space Workshop.

2.4.2.2 Peer-Review Committee (PRC)

The PRC supervised the project and was actively involved in the main steps of the project. A list of members is presented in Table 2.

Table 2: Members of the peer-review committee

No.	Name	Organisation
1	Dr. Kakoli Ghosh	FAO
2	Dr. Peter Kearns	OECD
3.	Prof. Dr. Volkmar Wolters	Ecological Society of Germany, Switzerland and Austria and German Research Foundation
4	Dr. Henri Darmency	INRA Dijon, France
5	Prof. Dr. Bernd Müller-Röber	Berlin-Brandenburgische Academy of Science
6.	Prof. Dr. Jozsef Kiss	University of Gödöllö, Hungary
7	Dr. Brian Johnson	Ecology Consultant, UK

2.4.2.3 External expert panel

In order to achieve the main objectives of the project – particularly the prioritisation of potential effects of GM plants, as well as the effective identification of areas of scientific uncertainty – the BEETLE study consulted an extended expert panel (EEP) and an expert panel (EP) (a subgroup of the EEP), as well as the PRC. The EEP participants were involved in the Online Surveys with foci on (i) environment (including biodiversity) and (ii) health issues; the EP participants were involved in the CREA Space Workshop with focus on environment (including biodiversity) issues.

A broad spectrum of representatives was selected for the EEP/EP with accredited expertise in the field of biosafety issues (see below and Appendix 2 [Survey Environment] and Appendix 4 [Survey Health]).

The majority of the EEP and EP participants were scientists from research institutions and universities and representatives of the international biosafety community. In the early '90s the first large, publicly financed biosafety research programmes started in the EU as well as in many other countries worldwide. This period marked the beginning of the biosafety research community. A continuous and intensive knowledge exchange was started with the

first national and international meetings such as the International Biosafety Symposium of the ISBR (International Society for Biosafety Research).

The EEP and EP participants were selected based on three major criteria: (a) known expertise substantiated by relevant scientific publications cited in the ICGEB database, (b) specialist expertise for the requested fields in the BEETLE project (due to the area of specific competence) and (c) known representativeness for important stakeholder groups (for details see A2-8 and A2 Table 4).

In addition, some experts were selected based on single publications in a field that was not sufficiently covered by the above-mentioned criteria. Membership of the European Commission Monitoring Working Group, or participation in EU-funded research projects highly relevant to BEETLE or national Biosafety Commissions were other selection criteria.

For completeness, representatives of three further important stakeholder groups were also invited to participate in the online survey and the CSW: (1) Companies developing GM plant applications at the EU level, (2) non-governmental organisations (NGO) contributing scientifically to the GMO debate and (3) regulators working in governmental bodies. Known experts were chosen representing the major companies. Members of NGOs were selected based on recommendations of the PRC. Similar numbers of industry and NGO representatives were invited to try to ensure balance.

In addition experts from countries with a long experience of GM cultivation were invited to participate in the online surveys from outside the EU. The numbers of invited and participating experts for the online survey are presented in Tables 3 and 4.

Table 3: EEP participants in the Online Survey "Environment". Presented are numbers of invited and participating experts for each stakeholder group and their relative proportion.

Stakeholder	Number of participants		Percentage	
	invited	participating	invited	participating
Research institution	114	59	68.3%	59 %
Regulation	24	16	14.4 %	16 %
Industry	15	16	9.0 %	16 %
NGO	14	4	8.4 %	4 %
Other[12]	-	5	-	5%
Sum	167	100	100 %	100%

An additional selection criterion for the EP participants in the CSW was fundamental contribution to the online survey. For further discussions in the CSW it was essential that experts

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[12] Differences between the 'invited' and 'participating' stakeholder affiliation are caused by the fact that experts relocated themselves to other stakeholder groups after registration. It is unknown to the BEETLE team why and where invited experts changed their affiliation.

were familiar with the content of the online survey. The goal was to get a similar distribution of experts in the EP from the different stakeholder groups comparable to the online EEP survey "Environment" (see A2 tab. 4). Therefore 45 representatives of all above-mentioned stakeholder groups were invited. However, it was not possible for all invited experts to participate for several reasons. Altogether 27 experts participated in the CSW, complemented by three members of the PRC and one representative of DG Environment (Table 5).

Table 4: EEP participants in the Online Survey "Health". Presented are number of invited and participating experts for each stakeholder group and the relative proportion.

Stakeholder	Number of participants		Percentage	
	invited	participating	invited	participating
Research institution	108	28	58.4 %	53.9 %
Regulation	33	8	17.8 %	15,4 %
Industry	26	10	14.1 %	19.2 %
NGO	18	1	9.7 %	1.9 %
Other[22]	-	5	-	9.6 %
Sum	185	52	100 %	100%

It was noted that none of the invited representatives from NGOs was able to participate in the online survey or the CSW.

Table 5: EP participants of the CREA Space Workshop. Presented are numbers of invited and participating experts for each stakeholder group and the relative proportion.

Stakeholder	Number of participants		Percentage	
	invited	participating	invited	participating
Research institution	27	15	52 %	55 %
Regulation	8	4	15 %	15 %
Industry	5	4	9 %	15 %
NGO	5	-	9 %	-
Others[13]	8	4	15 %	15 %
Sum	53	27	100 %	100%

2.4.2.4 Information management

Effective communication was crucial to enable continuous and efficient internal and external information flow, bringing together all project partners and the PRC and encompassing the implementation of the methodologically professional and effective external expert assessment (Figure 2). Genius Science and Communication was responsible for information and procedure management during the project. The collection and collation of information was carried out by BVL in collaboration with BLaU Umweltstudien.

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[13] In addition the members of the PRC and DG Environment were invited to participate on the CSW.

3. Methodology

The study used wide-ranging scientific assessment, enabling transparency, and attempting to gain a complete overview of [realistic] long-term effects. The focal points of BEETLE were the most frequently cultivated GM crops: soybean, cotton, maize, and oil seed rape with HT and/or insecticide-resistance traits. The 'type' of processes are taken from the complexity ordinate of system processes presented in Figure 1 as starting point, and these processes were further identified in separate steps (Step 2 and 4, see below) of the BEETLE study (see section 5 and 6). The upcoming challenge to assess multiple trait combinations and the likely occurrence of unintended trait combinations was assessed as a 'stacked events' focal point. However, any assessment of potential long-term effects is a multidimensional task (Figure 3).

BEETLE - Multidimensionality

CROP
• potato
• maize
• oilseed rape
• sugar beet
• soybean

TRAIT
• insect-resistance (Bt)
• herbicide tolerance (HT)
• starch modif. (SM)

EFFECT - Environment - Health

PROCESS (MECHANISM)
• cause-effect reactions
• feedback reactions
• structural system change
• new system components
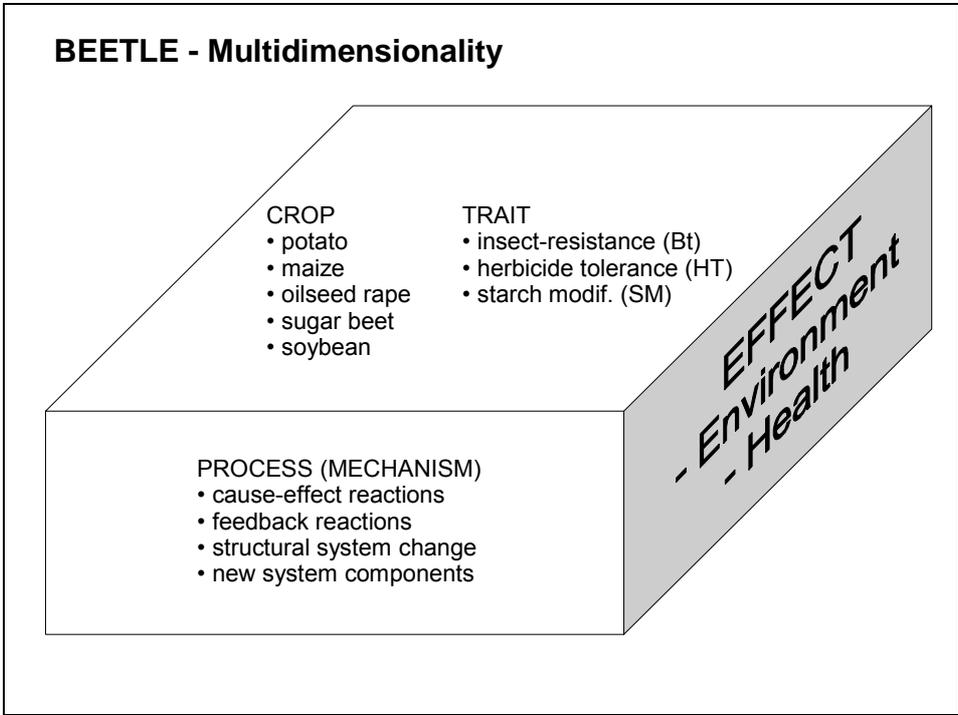
Figure 3: Addressing multidimensionality in BEETLE: The study started with 'Effect Categories', followed by assignment to 'Processes', and then – as main work carried out - identified and prioritized 'Scenarios' on crop/trait combinations derived from literature review and expert consultation.

Additional GM crops of relevance for the European market such as potatoes, rice, sugar beet and wheat were taken into consideration as well as new GM traits which are under development such as disease resistance, altered compositions (e.g. modified starch content, increased amino acids or proteins), new food and feed constituents (e.g. vitamins), pharmaceutical substances or improved plant resistance against plant stressors like drought, salt, or

heat. However, the BEETLE study concentrates on the crop/trait combinations listed in Figure 3.

The BEETLE study approached the assessment of long-term effects from the 'process side': General categories and processes were identified first, and then the relevance for crop/trait combinations and effects on health and the environment (including biodiversity) were evaluated by assessment of published results (literature review) and expert consultation based on scenarios (Figure 4).

A number of potential long-term processes assigned to health and environment (including biodiversity) were identified and explored in detail.

The BEETLE study

- analysed more than 700 scientific publications about GMOs and their potential effects on environment including biodiversity and more than 100 publications about GMOs and their potential effects on human and animal health, which were published mostly during the last decade,
- received contributions to online surveys from 100 of 167 invited environment experts and 52 of 185 health experts representing a wide range of knowledge with a focus on the EU,
- discussed potential ways forward for reducing uncertainty regarding environmental effects with 27 invited experts in a CREA Space Workshop.

The BEETLE study used a multi-step approach to achieve the project goals: the prioritisation of potential long-term effects of GM crops on environment and health and the identification of areas of greatest scientific uncertainty (Figure 4 and 5). All steps of the evaluation and decision process were part of an assessment by the International Peer Review Committee (PRC), extended expert consultation, and the BEETLE team as illustrated in Figure 5.
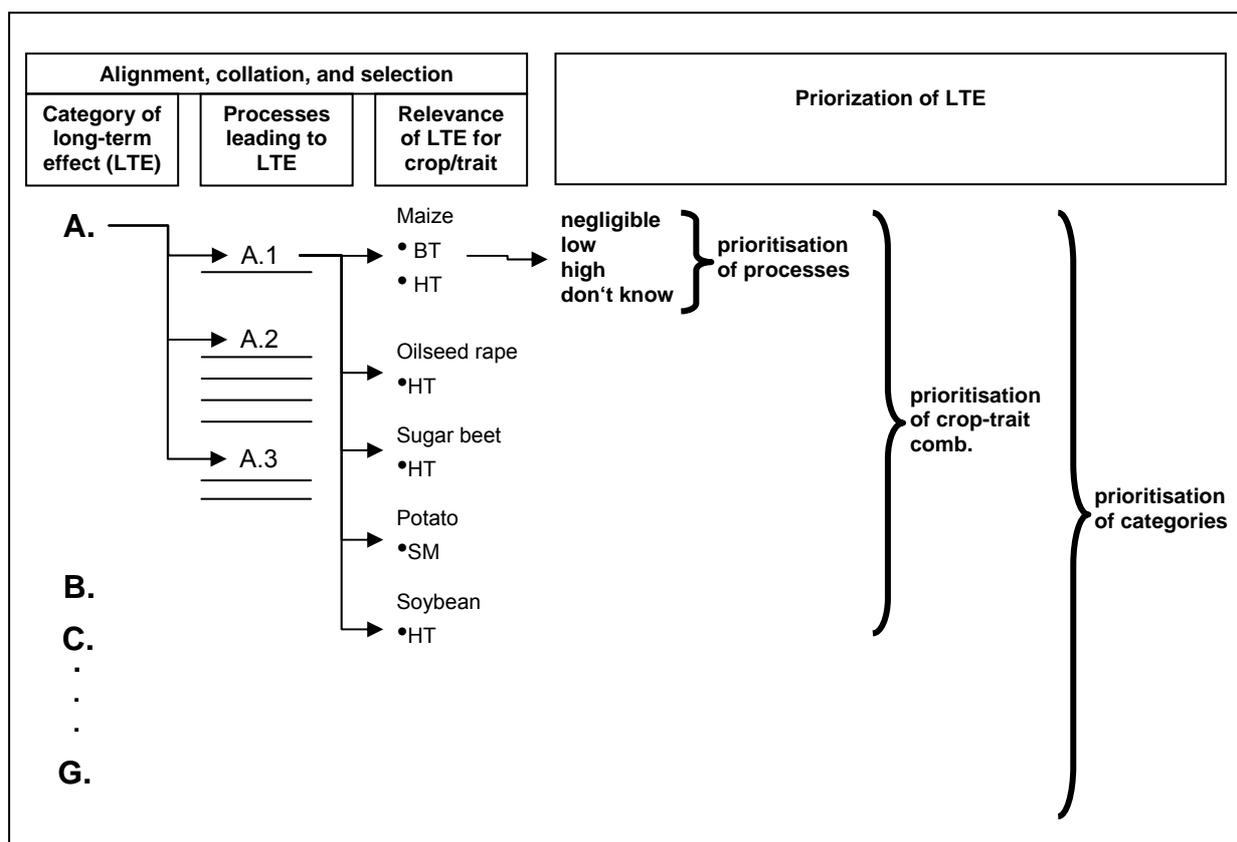
Figure 4: Process of prioritisation as performed in Step 2, 3, and 4 (see Fig. 5) on different levels: Firstly, categories, processes and crop-trait combinations (scenarios), were identified in literature surveys; secondly, these were assessed in detail with the help of expert (EEP, EP) surveys and PRC consultation.

In **Step 1** of the BEETLE study a broad Literature Review of published peer-reviewed data and internal documents was performed to collect and collate information on established and potential long-term effects. Based on this initial collection, the BEETLE team identified in **Step 2** about 26 general mechanisms ('processes') and assigned them to seven categories (A-G). Among the 'processes' about 63 more detailed scenarios for specific crop/trait combinations were elaborated that potentially could lead to long-term environmental effects (see Table 6). The processes and scenarios selected were cross-checked with the Peer-Review Committee. These results were taken as the basis for **Step 3**, the development of questionnaires sent to an extended expert panel (EEP) via online surveys. Further, based on the results of the online surveys, long-term effects were prioritized and areas of uncertainty were identified (**Step 4**). In the following **Step 5** – the CREA Space Workshop "Environment" – the identified areas of scientific uncertainty were specified after intensive discussions with a subgroup of the EEP on environment, the expert panel (EP). The final prioritization of long-term effects – **Step 6** - was performed by the BEETLE team and the PRC together with recommendations for methods and ways forward to reduce uncertainty, and topics for future research.

In the BEETLE project both the environmental and the health aspects of GM plants were analysed and assessed by literature reviews and online surveys. However, it was decided to address exclusively the environmental uncertainties in the CREA Space Workshop due to the primary focus of this project on environmental issues.
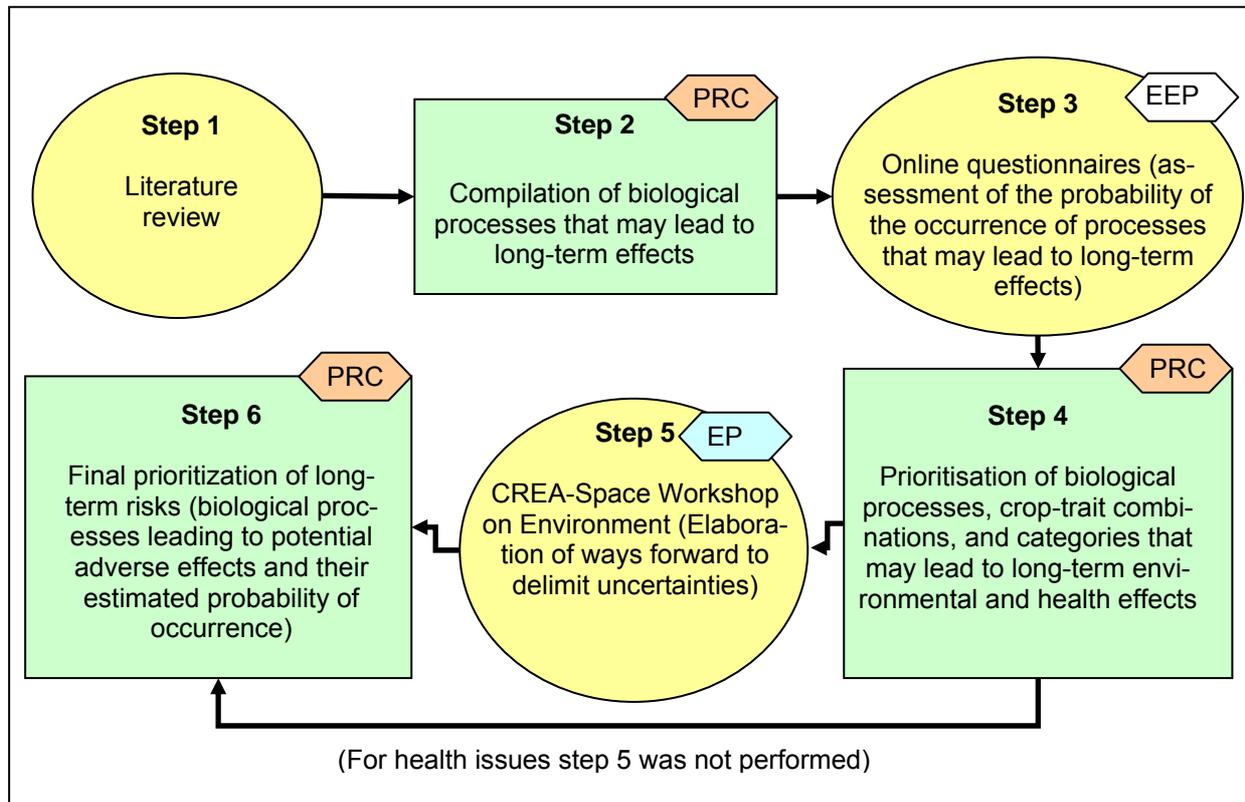
Figure 5: The sequential steps used in BEETLE for the procedure of prioritisation. Circled steps were designed to collect available knowledge (to decrease uncertainty arising from missing information). Rectangles represent steps undertaken to prepare decision making on prioritization. The involvement of experts (EEP, EP and PRC) in assisting decision making by the BEETLE team is indicated in the hexagons.

3.1 Structured literature research

The first step in the project was to prepare a structured Literature Review to collect and collate information on established or potential long-term effects of GM crops on the environment, including biodiversity and on health. This review was the basis of the prioritisation work and the evaluation procedures. Starting with the existing expertise within the BEETLE team and the library of the BVL housing more than 5.600 papers relating to GMOs, a broad spectrum of further sources of information was searched such as internet-based information, library catalogues (e.g. Central catalogue of German libraries, International Centre for Genetic Engineering and Biotechnology (ICGEB)), and online literature databases (e.g. ISI Web of Science, Medline). Additionally, unpublished data (e.g. confidential but reviewed reports from pre-marketing experiments, personal communications with experts from science and regula-

tion) were analysed. In particular, resources in countries with a long experience of GMO cultivation (USA, Canada, and Australia) were explored. All collected data were assigned to the relevant categories and processes, evaluated for their scientific relevance by internal review, and preliminarily ranked and prioritized by the BEETLE Team. These results were fed into the following evaluation procedures (questionnaire and workshop). Details of the methodology are provided in Annex 1 (Environment) and Annex 4 (Health).

3.2 Expert consultation

The procedures (Steps 4 and 6) of prioritisation of potential adverse effects and identification of areas of greater scientific uncertainty were performed with the help of expert consultation, including information collection from experts (Steps 3 and 5). The BEETLE study used different communication methods incorporating broad expert know-how (Figure 5).

Involving both the PRC and the extended expert panel (EEP) [14], the prioritisation of potential effects and the identification of areas of greatest scientific uncertainty were realised by different methods. This procedure included the detailed Online Surveys 'Health' (Annex 4-5) and 'Environment'(Annex 1-2) including statistical analysis tools and the CREA Space Workshop focussing on environmental issues with the expert panel (EP) (see Annex 3).

To facilitate the information exchange between all partners and to accelerate communication between project team, PRC and the EEP, BEETLE used the establishment of an "internet-based information management system" (a project intranet). All tools were designed to be simple to use and to be functionally adequate. The project website was built on several components, including:

- An internal forum for the exchange of information between all partners.

- Different folders for result papers (e.g. deliverables).

- A platform offering the Online Surveys for the EEP and PRC. The surveys were not public and only accessible *to* participants via a login procedure. BEETLE made use of the website www.gmo-safety.eu[15] as a technical platform.

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[14] The names of the EEP participants are not included in this document for reasons of personal data protection.

[15] www.gmo-safety.eu is the counterpart of www.biosicherheit.de in English language. This Site is promoted by the German Federal Ministry of Education and Research. It is meanwhile the best-known information platform in the German speaking area on environmental biosafety research on GMO with approximately 100.000 visitors per month. The EU granted information platform **BIOSAFENET** (6th Framework Programme) is a further partner website covering international biosafety research.

3.2.1 Online Survey

Initial and preliminary compilations of potential adverse effects of GMOs and areas of greater scientific uncertainty were made by the BEETLE Team (see Figure 5) for the development of the Online Surveys 'Health' and 'Environment'. The surveys

- addressed the ranking of potential effects in the areas of environment and health and
- requested information on attitudes, research ideas, and other important aspects.

The response to the Online Survey 'Environment' was surprisingly high given the workload to fill in the survey.

In the case of the survey 'Environment', a total of 167 experts were invited to participate. At least 100 experts registered for the Online Survey and an average of 53 experts (29.7%) responded to each question. In the case of the survey 'Health' a total of 185 experts were invited to participate. At least 52 experts registered for the Online Survey and an average of 27 experts (14.4%) responded to each question.

3.2.2 CREA Space Workshop 'Environment'

CREA Space is a method for the development of creative potential in teams and in larger groups. This tool is methodologically derived from organisational development procedures and mainly serves to provide a framework for the achievement of sociological whole group arguments and findings. The methodology can also be used outside of organisational development procedures and is most favourable when aimed at gaining a structured picture of a group's opinions and ideas out of a broad variety of political, professional or regional provenance. These procedures offer the crucial advantage of gaining a manageable outcome in a relatively short period of time.

In the context of the BEETLE project, the CREA Space methodology was used to effectively check and validate issues derived from first findings of the literature review and the online survey in order to find out whether there are additional items, and whether there is clarity and consensus within the group. The aim of the CREA Space method within this project was:

- to employ the creative potential of an expert group
- to conduct structured discussions within expert groups
- to benefit from sharing knowledge and experience
- to amplify information in a transparent manner

Details of the methodology and the results are provided in Annex 3.

3.3   Sources of uncertainty

According to Commission Decision 2002/623/EC (EC, 2002a), ERA has to take into account uncertainty at various levels, and scientific uncertainty results usually from five characteristics of the scientific method:

(i)   the variable chosen,

(ii)   the measurements made,

(iii)   the samples taken,

(iv)   the models used and the

(v)   causal relationships employed.

Scientific uncertainty may also arise from

(vi)   a controversy on existing knowledge or

(vii)   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 in the level of uncertainty; the notifier of an application for GM plant authorization needs to provide information that will allow assessment of uncertainty (resulting from e.g.lack of data, knowledge gaps, standard deviation, complexity, etc.) in comparison with the scientific uncertainties in current practice. In relation to the BEETLE study, the characteristics of uncertainty resulted exclusively from sources (v), (vi), and (vii). Due to lack of experience about long-term effects, in particular, the availability of data (category vii source of uncertainty) is generally very low. A more detailed analysis about the origin of uncertainties in relation to the prioritization steps is provided in section 8.

4. Molecular characterization and stability of transforming DNA

4.1 Introduction

GM plants are the result of plant transformation, a set of procedures applied to introduce a genetic modification construct (transforming DNA) into a recipient plant genome. For the commercial production of GM plants this is carried out by

- either *Agrobacterium* mediated transformation: infecting plant cells with disarmed *Agrobacterium tumefaciens* harbouring the transforming DNA;

- or particle acceleration.

The molecular mechanisms by which transforming DNA is inserted into the host genome are not well understood.

4.1.1 Transformation-mediated genetic alterations

Insertion of transforming DNA usually involves a tissue culture procedure. Tissue culture induces dedifferentiation of plant tissues and allows selection and regeneration of a plant from a single GM cell having incorporated the transforming DNA. However, the procedure is known to be associated with various types of mutations. Stable mutations in plants induced by passages through tissue culture are known as somaclonal variation (Sala et al. 2000) Induction of somaclonal variation is a method used in conventional plant breeding and has been patented for that purpose (European Patent EP0606759, 2001).

Insertion of superfluous DNA is also a common feature of Agrobacterium-mediated transformation. This superfluous DNA may consist of complete or partial copies of the intended transforming DNA, other vector DNA, or filler DNA newly created at DNA-DNA junctions. Due to the small numbers of events analysed and reported, conclusions regarding particle acceleration transformation events are provisional. But it seems that integration of transforming DNA is usually accompanied by insertion of superfluous DNA. Mutations induced by transformation can be separated in:

- those introduced at the site of insertion of the transforming DNA (insertion-site mutations), and

- those introduced at other random locations (genome-wide mutations), as summarized by Latham et al. (2006).

Genome-wide mutations are not necessarily genetically linked to the insertion site of the transforming DNA. In a limited number of studies using DNA polymorphism analysis to compare the genomic DNA of GM plants with that of non-transformed controls it was attempted to quantify mutations introduced throughout the plant genome during plant cell transformation. The results suggested that plant transformation procedures typically introduce many genome-wide mutations into the DNA of the resulting transformed plant. These studies suggested that genome-wide mutations are mainly caused by passages through tissue culture, as both GM plants and non-GM controls were altered during tissue culture in the Latham et al. (2006) studies.

It is common knowledge that large numbers of individual transformed plants must be produced in order to obtain one or a few plants that express the desired trait in an otherwise normal plant. To avoid the phenotypic consequences arising from tissue culture induced mutations, plants are selected for further development that are as identical to their parent as possible. Furthermore, to develop a commercial variety several back crosses are normally done to combine the introduced transforming DNA with the desired germplasm of a certain variety. By this conventional breeding procedure a high percentage of the genetic background of the original transformant is eliminated and replaced by the genome of a successful commercial variety. Thus, even undetected genome-wide mutations resulting from somaclonal variation are drastically reduced during variety development.

The integration mechanism of transforming DNA into a plant chromosome is poorly understood, both in the case of Agrobacterium-mediated transformation and direct DNA transfer like particle acceleration. The position of integration and the structure of the integration locus can vary considerably among independent transformants. A number of studies provide evidence that integration of the insert involves illegitimate recombination of DNA mediated by a double-stranded DNA break and join mechanism.

DNA junctions resulting from integration linked to this process are characterised by the following properties:

- Deletion of nucleotides at one or both of the recombining ends, and/or,

- Presence of additional DNA at the junction of the recombined ends, and/or

- Rearrangements in the inserting and/or the receiving DNA

In fact, both Agrobacterium-mediated transformation (Deroles and Gardner, 1988) and particle acceleration (Register et al., 1994) may result in such effects.

4.1.2 Particle-acceleration-mediated transformation

Particle acceleration is the predominant direct method to transfer transforming DNA into the genome of cereals, but can also be applied to other plants. It involves the acceleration of DNA-coated metal particles through the cell wall and membrane. The integration mechanism does not appear to be sequence-dependent, nor is there a preference for chromosomal sites with special properties on a chromosome. Particle acceleration often generates multiple and high-copy-number integration of transforming DNA. The insert structure varies from a single intact copy to complex configurations comprised of intact copies, tandem or inverted repeats, concatemers, truncated and rearranged sequences, and interspersed DNA. The analysis of junctions within the integrated transforming DNA and between transforming DNA and the host DNA at integration sites generated by particle acceleration suggests that integration occurs by illegitimate recombination. Characteristics of illegitimate recombination include regions of microhomology at the recombining junctions (i.e. 4-8 nucleotides in common between the recombining partners), the presence of filler DNA (short sequences that are not recognizable as belonging to either recombination partner), small deletions probably reflecting nuclear "nibbling" of the transforming DNA prior to joining, and DNA sequence motifs with similarity to known binding/cleavage sites for the enzyme topoisomerase I, that has the function to introduce single-strand cuts in double-strand DNA molecules and to rejoin the ends of such nicks. Following penetration of the cells by the metal particles, the plant's wound response induces DNA repair enzyme activity (by nucleases, topoisomerases, and ligases) which together with large amounts of exogenous DNA would support both degradation of the incoming DNA and joining of free ends. This would generate variable arrays containing both intact and rearranged transforming DNA. Such arrays would then act as the substrate for integration. Integration is proposed to occur at chromosome breaks (summarized by Kohli et al. 2003).

4.1.3 *Agrobacterium*-mediated transformation

The soil micro-organism Agrobacterium tumefaciens can transfer a small segment of DNA (known as T-DNA) from its resident Ti plasmid into a plant chromosome. It has a broad host range of dicotyledonous plants. Monocotyledonous plants like cereals are not natural hosts of Agrobacterium species and only recently has it been possible to achieve transformation of cereals by this method. Early studies on dicotyledonous plants suggested that T-DNA integrates randomly in the genome of the recipient. But preferential integration into transcription units has also been reported. The distribution of DNA inserts in the model plant Arabidopsis is essentially random whereas in rice, T-DNA integration events occur predominantly in the region known as "gene space". Generally Agrobacterium-mediated transformation gives rise

to lower copy number on integrated transforming DNA compared to direct transformation methods like particle acceleration. Studies have shown that the structure and complexity of the insert may depend on the Agrobacterium strain used and other factors. Transfer of transforming DNA by Agrobacterium was initially considered as a transformation system by which only sequences between the two T-DNA borders were introduced into the plant genome. But it was shown that many transformants contain in addition back bone sequences of the Ti plasmid linked to the T-DNA insert. In a recent study by Ülker et al. (2008) evidence was presented that T-DNA of Agrobacterium can also mediate the transfer of fragments of its chromosomal DNA into plants. As with particle acceleration, rearrangement of transforming DNA after Agrobacterium-mediated transfer occurs during integration of the transforming DNA into a plant chromosome. The prevalence of rearrangement is lower for the transforming DNA transferred by Agrobacterium probably because this DNA is protected from degradation by its association with Agrobacterium proteins. Researchers have investigated the structure of junctions between the integrated transforming DNA and the chromosomal DNA at the integration site. They have concluded that integration occurs by illegitimate recombination as it does for particle acceleration (e.g. Gheysen et al. 1991; Gorbunova and Levy 1997; Salomon and Puchta 1998, summarized by Kohli et al. 2003).

4.2   Characteristics of GM maize MON810 and GM soybean 40-3-2

Accumulated scientific information is available on GM crops which have been cultivated on large acreage over ten or more years. These GM crops have a history of repeated characterisation of the transformation event. Two of them, soybean 40-3-2 and maize MON810, are of particular relevance to the BEETLE report.

4.2.1   GM soybean 40-3-2

A technical dossier containing all available information necessary to evaluate the safety of the GM soybean was submitted in order to obtain market authorization in the EU as well as in the United States (APHIS 2007, EC 2008). Padgette *et al.* (1995) described that the transforming DNA inserted into soybean 40-3-2 by particle acceleration includes

- a functional portion of the CaMV 35S promoter,

- a *Petunia hybrida* chloroplast transit peptide,

- the CP4 EPSPS coding sequence and

- the 3′ non-translated region of the nopaline synthase gene (nos terminator).

In a study by Windels *et al.* (2001) both junction regions of the DNA inserted were amplified using anchored PCR and adapter ligation PCR and their nucleotide sequence was deter-

mined subsequently. The results presented by Windels *et al.* (2001) establish that no major rearrangements occurred at the 35S border. In contrast, adjacent to the nos terminator region a 254 bp portion of truncated CP4 EPSPS coding sequence is present which was not reported previously by Padgette *et al.* (1995). The notifier confirmed that the molecular characterisation reported by Windels *et al.* (2001) is correct and applies to soybean 40-3-2 plant material that was used to produce data for the dossier submitted for market approval of soybean 40-3-2 in the EU (Lirette *et al.*, 2000). In addition, the study of Rang *et al.* (2005) also confirmed the presence of the additional 254 bp fragment reported by Windels *et al.* (2001). In summary, the studies indicate that the molecular structure of the transforming DNA inserted in the GM soybean remained stable over several years and generations.

4.2.2   GM maize MON810

The GM maize MON810 was generated by the particle acceleration method which resulted in the successful integration of a portion of plasmid PV-ZMBK07 into the maize genome. Plasmid PV-ZMBK07 consists of the following functional elements:

- The CaMV 35S promoter with duplicated enhancer region (e35S),

- an intron from the maize hsp70 (heat-shock protein) gene,

- the cry1Ab gene coding for the complete, functional Cry1Ab protein,

- a 3´ non-translated region of the nopaline synthase gene (nos terminator),

- the promoter and a partial sequence of the *E. coli* lacZ gene encoding the N-terminal portion of the ß-galactosidase protein,

- the bacterial origin of replication (ori-ColE1) of the bacterial plasmid ColE1,

- the nptII gene as an antibiotic resistance marker gene.

The first report on MON810 by Monsanto (see APHIS 2007) described the presence of the inserted transforming DNA as an element of 3.6 kb co-linear to PV-ZMBK07, corresponding to a translated cry1Ab gene. MON810 was characterised to have the transforming DNA of plasmid PV-ZMBK07 inserted at a single locus, comprising

- the enhanced CaMV 35S promoter (e35S),

- an intron from the maize hsp70 (heat-shock protein) gene, and

- a truncated cry1Ab coding sequence encoding a functional active Cry1Ab protein.

The initial molecular characterization of MON810 has been confirmed by the following studies:

1. Studies by Hernandez *et al.* (2003) confirmed the truncation of *cry1Ab.* Hernandez *et al.* (2003) described a 1222 bp 3´ junction fragment of MON810, consisting of 633 bp of the truncated *cry1Ab* gene and 598 bp of junction DNA with no significant homology to known plant DNA sequences.

2. Rosati *et al.* (2008) sequenced 345 additional bp downstream of the fragment described by Hernandez *et al.* (2003), with the aim of further characterising the 3´ flanking region of the insertion site in the genome of maize MON810. Bioinformatics analysis of the whole 3´ flanking sequence (943 bp) showed 99% homology with a maize chromosome 5 bacterial artifical chromosome (BAC) clone.

3. The 5' flanking region was sequenced by Holck *et al.* (2002). A 440 bp DNA stretch next to and upstream of the 5' junction displayed high similarity with the *Zea mays* chromosome 4 22 kD alpha zein-gene cluster region.

The BAC clone mentioned (Rosati *et al.* 2008) did not display sequence homology with the 5´annotated flanking region. The characterisation of the 3´ flanking region in MON810 suggests that the transformation process resulted in deletion of maize genomic DNA and in a partial loss of plasmid DNA used for transformation.

Recently, a report by Singh *et al.* (2007) also revealed both the absence of the nos terminator and the truncation of the cry1Ab gene at the characterised insertion site in MON810 maize. In this study and contrary to the claims of Monsanto the presence of both nptII and T-nos in the genome of MON810 was reported based on observations from PCR assays. The authors concluded from this that a relocation of T-nos from its original position elsewhere into the genome occurred. However, the findings by Singh *et al.* (2007) regarding the presence of T-nos and nptII are to be questioned for several reasons:

- Identity and purity of the maize used in the study,

- no verification of the identity of the obtained PCR fragments by sequencing, and

- the fact that nptII gene contaminations are widespread leading to false positive PCR results which were not tested for.

In fact, incorrect information in a subsequently withdrawn patent application on the MON810 insert may have been misleading for the authors. It also has to be noted that the report by Singh *et al.* (2007) was submitted to an online peer-reviewed journal but under a non peer-reviewed section (Protocols Network).

In a recent study the intactness of the entire MON810 integration (transforming DNA and flanking sequences) has been analysed in 26 varieties of MON810 (Aguilera *et al.* 2008a). In a combined strategy the restriction fragment length polymorphism patterns of the transforming DNA were compared and the Cry1Ab protein expression levels were determined. The same set of 26 varieties was used to assess the copy number of transforming DNA (Aguilera *et al.* 2008b). The results for 24 out of 26 varieties revealed a stable integration of one copy of the transformed DNA as described above. For two varieties ARISTIS BT and CGS4540 the calculated copy number of the transforming DNA typical for MON810 was zero. Both varieties were negative when tested specifically for the whole transforming DNA typical for MON810. But variety CGS4540 was positive in screening test for GM plants detecting the 35S promoter as well as for Cry1Ab expression test. The authors of the reports speculated that in varieties ARISTIS BT and CGS4045 the transforming DNA typical for MON810 was altered, thus indicating instability. For ARISTIS BT they discussed that the absence of the transforming DNA could be an example of a variety having lost the transforming DNA. Without any speculation the results clearly demonstrated that the material analysed as ARISTIS BT was not the transformant-line MON810. There is no further evidence presented that their sample had lost the transforming DNA or that ARISTIS BT is unstable in that sense. It is not reasonable to market a variety as a derivative of MON810 if the Cry1Ab protein is not expressed and the coding gene not even present in the genome. CGS4045 was tested negative for the MON810 event-specific test but showed the necessary genetic elements (35S promotor and Cry1Ab) required for a phenotype like MON810. The authors reported that no theoretical and technical information on this variety was available and that this variety was never submitted and approved for marketing in the EU. It can be concluded from the results hat the material analysed as CGS4045 was not the transformant –line MON810. But the result could indicate a different but similar transformation event like Bt11, developed by the same company as the variety CGS4045. The authors have not tested CGS4045 specifically for the transformation event Bt11, nor provided further evidence that its transforming DNA has been rearranged after transformation and therefore indicate instability.

In summary, the transforming DNA inserted into maize MON810 was originally described by Kania *et al.* (1995) as in the dossier submitted to a regulatory body in the EU. Independent studies (Holck *et al.*, 2002; Herandez *et al.* 2003; Rosati *et al.* 2008, v. d. Eede 2008) revealed a more detailed molecular characterisation but confirmed the structure and stability of transforming DNA inserted into the genome of maize MON810.

4.3   Stability of the CaMV 35S promoter

In a study designed to analyse rearrangements of the transforming genetic modification construct in rice, 12 multicopy GM rice lines derived from particle acceleration were analysed (Kohli *et al.*, 1999). The plants were transformed with the vector pWRG2426, carrying the bar, hpt and uidA genes each driven by a separate CaMV 35S promoter.

From analysed sequences of novel junctions in the plasmid DNA after transformation the authors concluded that the transforming plasmid DNA had undergone rearrangements involving illegitimate recombination. Kohli *et al.* (1999) discovered that recombination events were clustered within the CaMV 35S promoter. It was established that the 3' end of the CaMV 35S promoter, an imperfect palindrome of 19 bp, in conjunction with specific flanking sequences derived from the transforming plasmid DNA, may have acted as a hotspot for recombination during the transformation process.

The authors discussed intramolecular cruciform structures that could have linked CaMV 35S sequences at various loci. Illegitimate recombination involving hairpin structures has also been demonstrated for vertebrate cells (Stary and Sarasin 1992; Osterholm *et al.* 1996).

In summary, there is no indication that the CaMV 35S promoter is subject to molecular instability. Although the CaMV 35S promoter has been described as a hotspot of recombination in the transforming DNA of GM plants as well as in the Cauliflower Mosaic Virus itself under experimental conditions, no genetic rearrangements after the initial transformation took place.

4.4   Stability at the genomic insertion site

In both *Agrobacterium*-mediated DNA delivery and particle acceleration DNA transfer into plant cells, a wound respond is elicited involving the activation of nucleases and DNA repair enzymes that maintain the integrity of the host genome. As a consequence, exogenous DNA may be degraded, but some of the DNA may be used as a substrate for DNA repair, resulting in its incorporation into the genome, including DNA rearrangement. The similarities between recombination junctions generated by particle acceleration and those generated by other transformation methods strongly suggest that the underlying mechanisms controlling rearrangement of transforming DNA and its integration into the plant genome are likely to be the same (Kohli *et al.*, 1999).

In summary, these and other studies show that rearrangements are always linked with plant transformation processes in the model plant *Arabidopsis thaliana* and crops (Kohli *et al.* 2003; Windels *et al.* 2003). Hence, these rearrangements very likely result as a consequence

of the transformation process itself. Less data are available about post transformation stability of the transforming DNA and its flanking regions at the insertion site.

The papaya sequencing project has presented an opportunity to critically analyse insertion sites of transforming DNA. Five of the six flanking sequences of the three insertions are nuclear DNA copies of papaya chloroplast DNA sequences (Ming, R. *et al.* 2008). This indicates that the sequence flanking the inserted transforming DNA is very similar to those around "natural" DNA integration events, such as occasional integration of chloroplast DNA into the papaya genome (Kohli and Christou 2008).

4.5 Antibiotic resistance marker genes

In the context of stability of the inserted transforming DNA at the genomic insertion site questions have also been raised about the risk of transfer from parts of the inserted DNA, e.g. antibiotic resistance marker genes to soil bacteria with possible consequences for the spread of antibiotic resistance to soil and clinical strains of bacteria. In a recent study (Demanèche *et al.* 2008) culture-dependent and -independent approaches were combined to study the prevalence of bla genes in soil bacteria. The bla genes encode resistance to the beta-lactam antibiotics like ampicillin and the nucleotide sequence of the blaTEM gene is present as part of the inserted transforming DNA in the GM maize Bt176, which has been grown commercially for more than 10 years. This study includes the potential impact that a 10-successive-year culture of GM maize Bt176 could have on the prevalence of bla genes in soil bacteria. Like others before, Demanèche *et al.* (2008) did not detect any cellular or molecular evidence that the blaTEM gene from the GM maize Bt176 was transferred to bacteria. The authors discuss that their results may be partly due to the low frequency at which transfer events happen and that the detection of such events is limited mainly because bla genes are already present in soil bacteria. Bacteria that would have acquired a blaTEM gene from the plant would not have a specific selective advantage relative to other resistant bacteria.

4.6 Stability in breeding generations of a model plant

Papazova *et al.* (2008) studied the impact of propagation through generations, the influence of gene stacking and of photo oxidative stress caused by high light intensity on the stability of the flanking regions in the model plant *Arabidopsis thaliana*. Their results show that also in the fourth generation of self-pollinated GM lines, the proximal flanking regions remain unchanged up to 2 kb upstream and downstream of the transforming DNA. Also cross-pollination, done in order to stack two or more transformation events in one genome, did not have an effect on the stability of the stacked events. No sequence alterations in the transforming DNA of the combined events were observed as a result of interaction at DNA level.

Three GM lines were grown under photo oxidative stress conditions and screened. No evidence for the appearance of point mutations in the flanking regions of the transforming DNA was obtained. Therefore, it is unlikely that high light intensity will substantially increase the mutation frequency. Similar observations were made in a previous study (Papazova *et al.* 2006) in which the stability of the junctions of the transforming DNA with the host DNA was investigated for the same events after tissue culture stress.

In summary, by screening of seven GM *Arabidopsis thaliana* lines no evidence for occurrence of mutations were found, implying that the nucleotide sequence of the flanking regions of the studied events is unlikely to be unstable (Papazova *et al.* 2008).

4.6   Discussion and conclusions for the BEETLE study

In general, data from primary scientific literature provide a consistent picture regarding

- Rearrangements of the inserted transforming DNA,
- Rearrangements (including deletion and addition of DNA at the insertion site, and
- Stability of the transforming DNA after insertion.

However, there is still controversy in secondary scientific literature regarding the interpretation of the data, e.g.:

a) The insert in every commercial approved GM line has undergone rearrangement. The cauliflower mosaic virus promoter plays a mayor role (Ho 2003);

b) The statement made with regards to instability of GM crops: Transgenic DNA may also get into human cells and insert into the human genome, and a large body of evidence from so called gene therapy experiments have amply demonstrated that this occurs (Ho 2008a),

c) Presence of the nos terminator and the nptII gene in the genome of GM maize MON810 (Ho 2003; 2008b).

Many characterisitics of GM plants are considerably different from those of GMOs used as or contained in medicinal products (gene therapy products) for clinical trials (gene therapy experiments).

In GM plants all cells of the plant carry the genetic modification which is stably integrated into the genome by covalent linkage to the host DNA and protected in the same way as the host DNA in the genome. Starting from a single cell that has integrated the transforming DNA in its genome a whole plant has been generated and multiplied.

In contrast, gene therapy products are not intended to target germ-line cells. They are designed to target somatic cells of a patient. In general, viral vectors are used to transport

therapeutic DNA to somatic target cells in the body of the patient. These viral vectors carrying the therapeutic nucleic acid (DNA or RNA) inserted into their own nucleic acid can at least at certain stage of their life cycle be regarded as GM organisms (gene therapy product). Vectors in use as gene therapy products are based on defective viruses, e.g. on retroviruses (RV), adenoviruses (ADV), adenovirus-associated viruses (AAV), or modified vaccinia virus Ankara (MVA). RV vectors have the ability to integrate into the genome of the host cell but they can be excised again by the same mechanism. ADV and AAV vectors do not integrate into chromosomes of the host cell and require a specific viral helper functions for their replication. MVA vectors are able to replicate their DNA, but do not integrate into host chromosomes. They are not able to from infectious particles and the targeted cell carrying the replicating viral DNA will die after a while. The viral vectors used for gene therapy trials today are designed to be replication defective and thus to be maintained in the patient only transiently. They have an intrinsic instability also to ensure safety for third parties and the environment.

The arguments stressed by Ho (2003, 2008a, 2008b) have to be considered taking into account that:

- re a): The communication by Ho (2003) does not distinguish between rearrangements taking place during the process of insertion of the transforming DNA and the situation after the event of insertion. This may create the misunderstanding that the inserted transforming DNA is unstable at all stages in the development and multiplication in GM crops.

- re b): The underlying hypothesis that the likelihood of recombination (homologous or illegitimate) involving the inserted transforming DNA in GM plants is similar to that in mammalian cells (e.g. so called gene therapy experiments) is not supported by the scientific literature (Puchta 2002, Puchta and Hohn 2005, Chen *et al.* 2008). The statement (Ho 2008a) as such is correct, that transforming DNA may get into human cells. In gene therapy studies (experiments) it is intended to introduce certain DNA (or RNA) for therapeutics purposes into human cells. In such studies the transferred nucleid acid either integrates into the genome of the human cell or stays in an episomal condition, depending on the chosen method of transfer. But in the context of the instability of GM plants as the statement was presented it may lead to misunderstandings, e. g. that the transforming DNA of GM plants might be taken up by human cells as easily as in gene therapy studies. There is limited scientific evidence on the presence of ingested DNA in the tissues of animals (first reported by Schubbert *at al.* 1994, see also Annex 4 Literature Report Helath, Chapter 4.4). As DNA is a natural component of plant and animal tissue, DNA is regularly taken in with feeds and foods.

The fate of DNA from GM plants ingested as feeds and foods does not differ from the fate of DNA of non-GM-plants; both are subjected to digestion in the same manner. Similarly, in the few cases were foreign DNA derived from ingested feed has been detected in animal tissue no difference in uptake or effect of the foreign DNA is observed regardless of whether the foreign DNA is GM or not.

- re c): The communication by Ho (2008b) is addressed in section 4.2.2 and gives no reason for concern.

Integration of transforming DNA into the plant genome is frequently accompanied by small or large rearrangements of DNA at the insertion site and of the DNA introduced for transformation. This phenomenon has been observed in different plant species including crop plants. Under experimental conditions the CaMV 35S promoter has been described as a hotspot of recombination in the transforming DNA of GM plants during the process of insertion.

According to the relevant scientific literature rearrangements of the transforming DNA and at the site of its insertion into the plant genome are associated with the process of transformation (insertion) as such. Where the CaMV 35S promoter provides a hotspot of recombination this recombination occurs associated with the transformation process. Increased recombination involving the CaMV 35S promoter has not yet been reported post transformation following further propagation of GM plants.

Commercialised GM crop plants harbouring the CaMV promoter in their transforming DNA and grown at large acreage over several years, like GM soybean 40-3-2 and GM maize MON810, have not shown signs of instability or increased recombination regarding their inherited transforming DNA. This observation is in line with experimental data observed from studies of post-transformation stability of the transforming DNA inserted and its flanking regions. It is further supported by the papaya sequencing project (Ming, *et al.* 2008), which provides molecular evidence against in situ rearrangements of integrated transforming DNA. The observation that the integrated transforming DNA remains structurally and functionally intact in distant descendents of the original integration event is proof that integrated transforming DNA generally becomes a fixed element of the plant genome (Kohli and Christou 2008). There is no reason to assume that papaya DNA differs from other [higher] plant DNA, e.g. in terms of genetic stability.

In conclusion, there is no indication from the molecular characterization of GM plants that the insertion of GM DNA as such poses a long-term risk due to new mechanisms of genetic instability or re-arrangements. Therefore the BEETLE study does not consider DNA instability or re-arrangements as an issue of risk prioritization for the environment or human and animal health.

5. Results on Environment

In a first step, the BEETLE study carried out a structured Literature Review Environment of published peer-reviewed data and internal documents (see Annex 1). The results of the literature review were summarized and classified into seven categories (A-G, see Table 6):

Table 6: Environmental categories and processes with potentially adverse long-term effects identified in the BEETLE study.

Categories affected			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 (e.g. male sterility)
		B.2	Altered flower phenology
		B.3	Altered sexual compatibility reducing or favouring outcrossing
		B.4	Altered fecundity increasing seed (gene) flow
		B.5	Increased frequency of effective horizontal gene transfer (HGT)
C	Effects on Target Organisms	C.1	Effects on target pests and pathogens
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 causing changes in soil function
		E.2	Effects on biological control
		E.3	GM traits causing functional changes due to effects on pollinators
F	Cultivation and Management	F.1	Altered use of agrochemicals
		F.2	Indirect changes in susceptibility of crops to 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 greenhouse gases
		G.2	Increased mineral nutrient erosion and fertilizer leaching
		G.3	Altered chemical attributes of soil fractions
Independent of categories, 'Gene Stacking' and 'Regional Aspects' were identified as having the potential for modulating long-term effects.			

In a second step, from each category specific 'processes' and 'scenarios' were identified and selected, based on their importance for potential long-term effects. In total, a number of 26 processes[16] (see Table 6) and 62 scenarios[17] of potential long-term effects on the environment were compiled in the Literature Review "Environment" (see Annex 1).

Then, in a third step, international experts and representatives of international organisations forming the EEP were consulted to confirm and assess the relevance of the selected effects in an Online Survey (see Annex 2 for Environment and Annex 4 for Health). After assessment of the Online Survey by the BEETLE team, in a fourth step, the potential long-term effects were tentatively ranked and prioritizised. The assessment of the responses resulted in the identification of four main fields of uncertainty regarding potential long-term effects due to GM plant cultivation (see section 5.2). In a fifths step, clarification of potential ways forward was requested. For further clarifications an expert audience was invited to participate in a CREA Space Workshop (see Annex 3), where remaining areas of uncertainties were detected, methods for reducing uncertainty were developed, and indicators to address uncertainties were derived (see chapter 7). Finally, the BEETLE team performed a sixth step: the prioritization of identified long-term effects.

5.1   Potential long-term effects on the environment

The potential impacts of GM crops for the environment, especially for biodiversity, have been extensively assessed worldwide over the past 10 years of commercial cultivation of GM crops (Gressel 2005, Romeis *et al.* 2008). Substantial scientific data on environmental effects of currently commercialized GM crops are now available, and more will be obtained from several research programmes underway in a number of countries.

An ERA according to Annex II of Directive 2001/18/EC must be performed before marketing and cultivation of a GM crop. The results may not always give definitive answers to all the questions considered because of lack of data or the relatively short duration of field experiments. But in particular for potential long-term effects experience and information from 10 years of cultivation worldwide are available. However, in Europe effects of e.g. altered cultivation and management practices with regard to GMOs are limited. One aim of the BEETLE project is to summarize the latest available information on potential long-term effects of cultivation of GM crops.

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[16] In the context of this BEETLE study a 'process' is defined as a (biological) <u>mechanism</u> that potentially could lead to long-term effects under certain conditions (see Figure 1 x-axis).

[17] In the context of this BEETLE study a 'scenario' is defined as a <u>chain of events</u> in which a specific GM plant/trait combination could induce long-term effects.

5.1.1 Effects on persistence and invasiveness (Category A)

There is potential for intraspecific and interspecific hybridization, ferality and gene flow as pathways for seed transport and environmental spread of GM crops. 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 with sexually compatible wild plants or of potential feralized GM crop plants. Feral crop plants (in contrast to crops) can reproduce and persist on their own, without being dependent on managed cultivation. They can also evolve into persistent ecotypes in agricultural landscapes. Feral crop plants are more likely to evolve from volunteer crop species (crop plants germinating in subsequent seasons after a crop had been cultivated) than from crops producing seeds before or during harvest.

Where persistence and invasiveness is considered, 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 of a given GM crop species. Gene flow (including seed flow, flowering and pollination) and the formation of hybrids is a prerequisite for any transfer of a trait to NT crops of the same species or to potential hybrids.

In general, spontaneous (interspecific) hybridization between vascular plants has been documented worldwide for decades. Gene flow seems to be the rule rather than the exception for specific crop species; a lot of information is available providing evidence for gene flow between the crop and its wild relatives (Darmency *et al.* 1998, Ellstrand *et al.* 1999, Ellstrand 2001). 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 parents. Under environmental conditions all these parameters may affect hybridization between different parents. Two crop species are of particular interest in the EU. Oilseed rape feral populations are common weeds in fields as well as elements of disturbed habitats including fallow land. The same applies to sugar beet e.g. in southwestern France, northern Italy, and in UK. 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 (like *Brassica rapa, Brassica oleracea, Raphanus raphanistrum* or *Hirschfeldia incana* for oilseed rape and *Beta vulgaris* ssp. *maritima* for sugar beet). Furthermore, for both sugar beet and oilseed rape Europe is part of their centre of origin. The five crop species (oilseed rape, sugar beet, potato, maize, soybean) considered in the BEETLE project have

different potential for outcrossing and hybridisation; for maize, potato and soybean no sexually compatible wild relatives exist in Europe.

5.1.1.1 Increased fitness of the GM cultivar

Data in the literature are scarce with respect to long-term effects on increased fitness (resulting in higher persistence) of GM crops or GM hybrids. Currently, information for the ERA needs to be derived from analogous data on the behaviour of conventional crop varieties selected e.g. for salinity or drought resistance. Biotic stress tolerance has been studied more intensely in the past. According to the often cited case of fecundity enhancement of Bt 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. Each hybrid is likely to have different biology and ecology.

In some areas, oilseed rape (France, UK, and Germany) and sugar beet (South-western France, northern Italy) can naturally establish feral populations outside of cropping fields. According to literature this tendency seems to be - for oilseed rape - more striking for northern, western (atlantically oriented), and western Mediterranean areas. HT genes will only enhance fitness in cropping systems or in disturbed habitats where the complementary herbicide is applied, and this rarely, if ever, occurs. IR in maize will not enhance fitness as maize does not tolerate EU winter conditions and is not therefore invasive. In addition, since the target pests are newly introduced in many European regions, the cultivation of Bt maize did not change the situation in comparison to the time before occurrence of the new pests.

Recombinant new fitness enhancing traits may lead to increased fitness of the GM cultivar, but this phenomenon is relative to a specific habitat. 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 more likely in situations where the GM plant has (i) increased stress tolerance (e.g. towards temperature, water, salinity), (ii) increased fecundity and survival of progeny, (iii) decreased pathogen susceptibility, (iv) increased pest tolerance/resistance, and/or (v) increased tolerance against herbicides in cultivated areas where the specific herbicide is used.

Based on the 31 publications evaluated, the likelihood for increased fitness for the currently used GM crops in the EU is:

- **High** for HT oilseed rape or HT sugar beet in complementary herbicide crop rotations and in non-agricultural habitats being applied with the herbicide, and

- **Negligible** for HT maize, HT Soybean, SM potato, or Bt maize (see Annex 1, Category A, chapter A.1, p. A1-12-A1-18).

Responses to the OSE confirmed these results from the literature survey: potential adverse effects due to an increasing number of volunteers or the occurrence of ferals are only relevant for HT crops (see Annex 2, 3.2, p. A2-10). Potential long-term effects are most likely for oilseed rape and sugar beet due to their biology and the existence of cross compatible wild relatives. This aspect is of higher (if wild relatives are present) or lesser importance (if wild relatives are absent) depending on the specific EU regions in question. The Online Survey results confirm that potential for persistence and invasiveness is of negligible or low relevance for the other crop/trait combinations examined in the BEETLE project.

5.1.1.2 Outbreeding depression after hybridization with wild relatives

Data on the mechanisms of "outbreeding depression" are rarely found in the GM crop literature, as these are 'natural' phenomena of in crop breeding (see Annex 1, Category A, chapter A.2, A1-18 - A1-23). Outbreeding depression is often found in hybrids of crossings between a domesticated crop plant and a wild relative. Due to missing homology the hybrids often are of lesser vigour than the wild ancestors if the new trait does not offer additional selective advantage (Hails and Morley 2005). This phenomenon occurs where any genotype that exhibits an outbreeding depression in crossings with other crop lines will be eliminated from breeding programs. 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 in the extreme case have already gone 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 not yet led to any population decline. 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 may 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 abiotic stress tolerance (e.g. towards temperature, water, salinity), (ii) decreased number of progeny, (iii) increased pathogen susceptibility, and/or (iv) decreased pest tolerance/resistance.

Based on the 30 evaluated papers, 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 (see Annex 1, Category A, chapter A.2, p. A1-18 – A1-23).

Responses to the OSE confirmed that outbreeding depression is of general interest, but not of important relevance for the selected crop/trait combinations (see Annex 2, 3.2, p. A2-10). Phenotypic effects influencing gene flow (flower and fecundity biology) are addressed sufficiently in the ERA according to Annex II of Directive 2001/18/EC. For most of the experts, adverse long-term effects caused by outbreeding depression are 'negligible' in general and 'low' for the listed crop/trait combinations.

5.1.1.3 GM crop/feral/wild hybrid persistence

So far no clear rules can be derived concerning outcrossing between related species and the fate of a transferred GM trait (see Annex 1, Category A, chapter A.3, p. A1-23 - A1-26). Introgression and persistence of transferred genes in a recipient population are dependent on a number of random factors. The chance for dissemination of new traits in wild populations should increase if the hybrids still exhibit sufficient weedy characteristics. Under these conditions GM hybrids may persist in and outside fields. In case the new GM trait causes increased fitness after gene flow and introgression into wild relatives (hybrids), GM hybrids might become invasive through time or 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, pests, pathogens), (ii) increased number of progeny, (iii) decreased pathogen susceptibility, and/or (iv) increased pest tolerance/resistance.

Based on the 20 publications evaluated, the likelihood of hybrid persistence for the currently used GM crops in the EU is:

- **High** for HT oilseed rape and HT sugar beet in crop rotations 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.

The OSE results confirmed in general that persistence is of importance for long-term effects. Potential adverse effects due to hybrids persisting outside of fields were assessed ambiguously by the experts. Regarding oilseed rape and its potential hybrids, the answers were characterized by an equally distributed response ranging from low to high. For sugar beet and its potential hybrids the majority of experts vote for low; but about a third voted for medium.

5.1.1.4 Conclusion

Gene flow to wild relatives should be considered in cases of GM plants that have ancestors in the natural European flora. Consequently, this effect can be excluded for maize, potato and soybean. Outcrossing to wild relatives will potentially be of importance for oilseed rape (*Brassica napus*) and its wild relatives like *Brassica rapa, Brassica oleracea, Raphanus raphanistrum* or *Hirschfeldia incana*. A similar situation exists for cultivated beets, which cross readily with feral or weedy forms of *Beta vulgaris* ssp. *vulgaris* as well as wild sea beet (*Beta vulgaris* ssp. *maritima*). A crucial question is whether the GM trait offers any fitness advantage. However, in no case fitness enhancement as such is decisive for founding persistent populations of oilseed rape. In the future, GM crops with genes conferring resistance against abiotic stress may give higher potential for adverse long-term effects on the environment, especially from GM and conventionally bred plants expressing salinity or drought resistance genes.

5.1.2 Altered gene transfer (Category B)

The potential for altered gene transfer to the same or other sexually compatible plant species under conditions of cultivating the GM plant was detected as a further category for assessing long-term effects by the BEETLE project.

5.1.2.1 Potential reduction of pollination

Specific genetic modifications might alter the chemical composition of flower scents, most importantly for the insect pollinated oilseed rape among the relevant GM crops in the EU. Theoretically, the potential reduction of pollination could be the consequence of decreased flower attractiveness for pollinators (altered colour, altered scent). Based on the literature and field trial database review none of the important crop/trait combinations currently used in the EU are likely to reduce pollination. Therefore, based on the 6 evaluated publications (see Annex 1, Category B, chapter B.1, p. A1-27 – A1-28) the likelihood of this process for currently used GM crops in the EU is

- **Negligible** for all HT, Bt and SM[18] crops.

However, the reduction of pollination was selected for expert consultation due to the overall importance. The OSE confirmed the results of the literature survey. 60% of the experts were of the opinion that phenotypic effects influencing gene flow (flower and fecundation biology) were addressed sufficiently in the ERA according to Annex II of Directive 2001/18/EC (see Annex 2, 3.3, p. A2-10).

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[18] SM = Starch Modified

5.1.2.2 Altered flower phenology

GM traits may theoretically cause altered flower phenology, which could lead to genetic isolation of the crop plants, or, after gene introgression, of wild relatives (see Annex 1, Category B, chapter B.2, p. A1-28 – A1-30). 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. Based on the available literature and unpublished data sources available to BEETLE, which is not very comprehensive, the likelihood of this process for Bt maize could be assessed as being 'low'. However, this effect is not important for environmental effects since altered flower phenology does not lead to altered yields of Bt maize for cultivation practise. Thus the altered flower phenology is assessed for all currently used GM crops in the EU as

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

In general, the OSE results confirmed the results of the literature survey. 60% of the experts were of the opinion that phenotypic effects influencing gene flow (flower and fecundation biology) were addressed sufficiently in the ERA according to Annex II of Directive 2001/18/EC (see 5.1.2.1).

5.1.2.3 Altered compatibility reducing or favouring outcrossing

Male sterility - a characteristic mainly used for breeding purposes - 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. Based on the seven evaluated publications (see Annex 1, Category B, chapter B.3, p. A1-30 – A1-31) the likelihood of this process for the GM crops currently being applied for cultivation in the EU is

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

Again, the OSE confirmed the results of the literature survey. 60% of the experts were of the opinion that phenotypic effects influencing gene flow (flower and fecundation biology) were addressed sufficiently in the ERA according to Annex II of Directive 2001/18/EC (see 5.1.2.1).

5.1.2.4 Altered fecundity increasing seed (gene) flow

New traits conferring stress tolerance e.g. against herbivory (the case discussed in the literature) may alter fecundity by altering the number of seeds produced, which may cause increased seed (gene) flow from Bt crops to wild habitats if there is a fitness advantage due to escape from herbivory. Studies with non-GM interspecific hybrids revealed increasing hybrid seed numbers when the stressor was still present. However, Bt, HT and SM traits are not likely to produce such effects in the major crops studied in this review. Especially, according to the case discussed in literature, this effect can be excluded for Bt maize since this crop has no wild relatives in Europe. For HT crops with wild relatives in our flora increasing fecundity could only occur if the herbicides would be applied outside of fields. However, there are no reports published on such a phenomenon. For potato the wild relatives are not cross-compatible. Based on the two papers of sufficient quality available (see Annex 1, Category B, chapter B.4, p. A1-31 – A1-32) the likelihood of this process for the currently used GM crops in the EU is

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

Again, responses from the OSE confirmed the results of the literature survey. 60% of the experts were of the opinion that phenotypic effects influencing gene flow (flower and fecundation biology) were addressed sufficiently in the ERA according to Annex II of Directive 2001/18/EC (see 5.1.2.1).

5.1.2.5 Increased frequency of horizontal gene transfer (HGT)

A GM trait may theoretically increase the frequency of HGT from plants to populations of micro-organisms, thereby introducing new traits into microbial communities. However, since a relatively high homology between plant DNA and bacterial genes is the prerequisite for increasing frequency of HGT in reality this effect was not observed in the environment so far. Bt, HT and SM genes are not likely to increase the HGT for the major crops studied in this review, due to sequence adaptation of the introduced DNA (Bt, HT) to plant specific codon sequences. Based on the 22 papers evaluated on this aspect (see Annex 1, Category B, chapter B.5, p. A1-32 – A1-36), the likelihood of this process for the currently used GM crops in the EU is

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

In the OSE, the question of whether HGT (HGT) from crops to micro-organisms should be assessed during the ERA was answered by 51% of the experts with 'no', whereas only 28%

of the experts would recommend taking HGT into consideration (see Annex 2, 3.3, p. A2-10). However, the EU regulation clearly requires considering HGT within ERA.

When all of the available information is taken into account, the BEETLE study concludes that the probability of functional gene transfer from plants into micro-organisms is extremely low and of negligible relevance for long-term effects of the currently used GM crops in the EU.

5.1.2.6 Conclusions

Altered gene transfer does not seem to play an important role for the GM crops being currently in the pipeline for cultivation in the EU. The available tools in ERA are regarded as sufficient to address potential long-term effects.

5.1.3   Effects on Target Organisms (Category C)

Target Organisms are defined as the pests (insects) or pathogens (fungi, bacteria, viruses, protozoa, nematodes) harming the crops which are targets of plant protection measures. For example GM Bt-plants expressing Cry1Ab protein (measure) kill corn borer larvae (target organism). The situation of weeds growing in fields cultivated with HT crops differs from the above-mentioned 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).

5.1.3.1 Resistance development of pests

Resistance development of pest or pathogens against plant protection measures is well documented. For example, about 500 arthropod species and 100 pathogens have developed resistance against pesticides over the last forty years (Eckert 1988, Whalon *et al.* 2008). It is likely that in the long-term pests or pathogens will also develop resistance against GM-crops designed to protect against pests and pathogens.

Summarizing the available literature, resistance development by lepidopteran species against Bt-protein was not observed in Europe until 2007. So far, the IR management applied seems to have been successful for delaying or preventing resistance during seven years of large scale cultivation. Additionally, knowledge about the potential for resistance development was gained due to extended studies about basis susceptibility and frequency of resis-

tance alleles[19] in Europe. Based on the 34 publications evaluated (see Annex 1, Category C, p. A1-37 – A1-40), 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.

The wide span of assessment of resistance development for Bt maize is caused by published results indicating that the risk of pest resistance development is related to specific management issues.

The OSE confirmed the results of the literature review. Most of the experts were of the opinion that this issue is of 'low' (52%) to 'high' relevance (26%) whereas 20% of the experts stated a 'negligible' potential (see Annex 2, 3.4, p.A2-10 – A2-11).

5.1.3.2 Conclusions

Direct long-term effects of GM crops (mainly Bt maize) expressing proteins conferring resistance against the lepidopteran maize pest European Corn Borer (ECB, *Ostrinia nubilalis*) or against the beetle maize pest Western Corn Rootworm (WCR, *Diabrotica virgifera virgifera*) on target organisms are likely to become apparent due to resistance development over time.

5.1.4 Effects on NTO (Category D)

The potential impact of GM traits on NTO is a substantial part of any ERA of GM crops, and this issue was discussed in the literature long before the first commercial use in Europe. Today, an assessment is required of the possible immediate and/or delayed environmental impact resulting from direct and indirect interactions of the GM plant with NTO, including impact on population levels of competitors, herbivores, symbionts (where applicable), predators, parasites and pathogens. Tests on NTO along bi- and tri-trophic interactions including direct and indirect effects are widely applied in risk assessment and results are regularly and extensively published in the literature.

An extensive body of research data has been assembled e.g. on NT organism impacts of IR maize. The majority of laboratory studies and all the field studies reviewed did not reveal any unexpected adverse or long-lasting effect on NTO. One important lesson is that even if negative effects were observed in the laboratory (e.g. under worst-case conditions) no similar quantitative or qualitative adverse were necessarily detected in the field.

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[19] An allele refers to one member of a pair (or any of the series) of genes occupying a specific spot on a chromosome (called locus) that controls the same trait e.g. resistance against toxic compounds.

A tiered process of toxicity testing is generally used to assess the NT effects posed by traditional insecticides because it is suitable for assisting the decision-making process in an effective and rigorous way. The application of tiered approaches is widely accepted, but there is some debate about how to use the results of information for decision making. There is considerable disagreement about the most appropriate framework to use ecological approaches and a major difference between the approaches is in accordance with confidence and certainty within the tiered framework (EFSA 2008). In conclusion, 'tiering' provides a very useful concept to group the published literature into the following five categories within the BEETLE project:

- 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 Real field studies with GM insecticidal plants (open environment)

5.1.4.1 Direct toxic effects on plant-associated NTO

It can be concluded from the publications evaluated (see Annex 1, Category D, chapter D.1, p. A1-41 – A1-47) that toxic effects of crops expressing Bt protein found within short term Tier 1 and Tier 2 studies are rarely – if at all – also found in Tier 3 and Tier 4 studies. Therefore, an inherent uncertainty remains to extrapolate from short term ecotoxicological experiments on long-term environmental effects. In particular, the observed sublethal effect could have the intrinsic potential to affect NTO in the long run. In contrast, there is no indication for direct adverse effects on NTO caused by HT crops. Based on the literature evaluated (107 publications), 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.

The OSE results confirmed the results of the literature review (see Annex 2, 3.5, P. A2-11). In most cases the majority of the experts considered that the listed processes were 'negligible' regarding potential long-term effects. Exceptions were potential long-term effects on ECB or WCR resistant Bt-crops to NTO with close relation to target organisms since a similar mode of action of the Bt protein can be expected.

5.1.4.2 Effects on NTO due to altered nutritional composition of the GM plant

Nutritional composition of GM crops is potentially altered by newly expressed proteins (e.g. 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 (alteration of amylose or amylopectine content) of potato tubers. In general, very few studies presently support any assumption for herbivorous insects favouring GM in contrast to non-GM plants (see also Annex 1, Category D, chapter D.2, p. A1-47 – A1-49). Consequently, data regarding altered herbivore attractiveness of GM crops with changed nutritional composition are scarce. The available studies report no adverse effects or negligible effects being within the normal variation. Altogether, there is a lack of experience so that the knowledge of potential long-term effects remains poor, which results in identified uncertainty. Based on the available literature (13 citations), 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.

The OSE confirmed the results of the literature review. In most cases the majority of the experts considered that the listed processes were 'negligible' regarding potential long-term effects. However, in several cases more than 10% of experts emphasized that the available data basis is insufficient, in the section specific to herbivores and starch modified potatoes.

5.1.4.3 Tritrophic interactions on NTO

GM protein susceptible herbivores (2nd trophic level organism) feeding on GM protein-expressing host plants (1st trophic level organism) have the potential to experience reduced nutritional value leading to a reduced fitness of predators or parasitoids. Long-term adverse effects might be likely on populations or diversity of predators or parasitoids feeding on larvae with altered nutritional value. On the other hand, the majority of available studies report no adverse effects or negligible effects of predators or parasitoids being within the normal variation. Based on the available literature (see Annex 1, Category D, chapter D.3, p. A1-49 – A1-51, 28 citations), the likelihood of this process for the GM crops currently being applied for cultivation in the EU is

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

The OSE confirmed in general the results of the literature review. In most cases the majority of the experts (ECB: negl. 41%, low 35%, high 12, insuff 6; WCR: negl. 39, low 35, high 8,

insuff 10%) considered that the listed processes were 'negligible' regarding potential long-term effects. Exceptions were potential long-term effects on ECB or WCR resistant Bt-crops to NTO with close relation to target organisms since a similar mode of action of the Bt protein can be expected (see Annex 2, D. 4, S.A2-26).

5.1.4.4 Effects on NTO due to accumulation of toxic compounds

Bt proteins may theoretically accumulate in some host species. Short-term studies showed so far that fate of Bt proteins in the soil is not fully understood in the low concentration range. The time-spans of identifying residues of Bt proteins are varying enormously. Bt-protein concentrations being measured by using standard ELISA-tests in soil or water are very low indicating that direct toxic effects to soil or water organisms are presumed to be unlikely. However, it is still unclear whether soil persistence processes could be more important and could lead to long-term effects on soil organisms and soil ecofunction. Based on the available literature (see Annex 1, Category D, chapter D.4, p. A1-51 – A1-57, 12 publications), 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 on same fields,
- **Negligible** for all HT and SM crops.

The OSE confirmed in general the results of the literature review. The majority of the experts considered that the listed processes were 'negligible' regarding potential long-term effects.

5.1.4.5 Effects on rhizosphere microbiota

Decaying plant material or root exudates containing GM products may affect population size and activity of the rhizosphere organisms. Field experiments led to the conclusion that GM products in particular Bt proteins could have a significant small transient negative effect on soil protozoa and micro-organisms. However, data are only available from short-term experiments and predictions of potential long-term effect are difficult to make. Based on the available literature (see Annex 1, Category D, chapter D.5, p. A1-54 – A1-58, 20 citations), the likelihood of this process for the currently GM crops being cultivated in EU is

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

The OSE confirmed in general the results of the literature review. In most cases the majority of the experts considered that the listed processes were 'negligible' regarding potential long-term effects. However, more than 10% of the experts emphasized that the available data

basis is insufficient, in particular for the issue of rhizosphere organisms (17%) or mycorrhiza (17%). This is an important uncertainty.

5.1.4.6 Effects on symbiotic NTO

Adverse long-term effects might be observed on symbiotic activity of mycorrhizal fungi in Bt-maize plants if these varieties were continuously cultivated on one field over several years (which is normal practice in several areas of the EU), expressing lepidopteran specific or coleopteran specific proteins. Based on the literature evaluated (see Annex 1, Category D, chapter D.6, p. A1-58), the likelihood of this process for the currently in the EU used GM crops is

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

The OSE confirmed the results of the literature review. In most cases the majority of the experts considered that the listed processes were 'negligible' regarding potential long-term effects. However, in several cases more than 10% of the experts emphasized that the available data basis is 'insufficient', for the issue of mycorrhiza 17% of respondents were concerned about insufficient data.

5.1.4.7 Conclusions

Long-term effects on NTO (except soil microorganims) were not identified as a priority issue due to the fact that, in the view of the BEETLE study, an extensive body of research data has been assembled on NT impacts of insect resistance (IR) in maize. One important lesson is that minor negative effects observed in the laboratory do not necessarily predict impacts in the field, where many other factors could affect the impact on NT species (including climate, food availability and predation). The majority of studies reviewed do not show any unexpected negative or long-lasting effects on NT insects. However, some uncertainty was identified concerning indirect effects of Bt proteins, mostly on soil organisms (see also section 5.1.5).

5.1.5 Effects on ecological functions (Category E)

Some species or groups of species potentially affected by GM plants participate in ecological processes; some may be key species for fundamental ecological functions like soil fertility maintenance, biological control of pests, and pollination. In addition to their broad ecological relevance, these functions are useful or necessary for agricultural production. However, stud-

ies relating potential long-term effects on single species or species groups to ecosystem functions are rare.

5.1.5.1 GM traits may cause changes on soil functions

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 the 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 are ingested by various soil organisms, but only minor adverse effects (especially on nematodes) were reported. From these limited studies, the impact of Bt proteins on soil processes seems to be small. Because of the wide range of methodological techniques used and because many aspects regulating soil communities are still not sufficiently understood, it is difficult to extrapolate results of effects on special taxa or communities to whole ecosystem processes, and even more difficult to make predictions about long-term impacts. This is an important uncertainty. Based on the available literature (see Annex 1, Category E, chapter E.1, p. A1-59 – A1-61, 46 publications), the likelihood of these processes for the currently cultivated GM crops in the EU is

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

The OSE confirmed the results of the literature review (see Annex 2, 3.6, p. A2-11). None of the experts demanded additional processes or ecological functions to be considered. Most of the experts assessed potential long-term effects on soil functions as 'negligible' for the listed crop/trait combinations (Category E, chapter E.1 54%, Category E, chapter E.2 50%, Annex 2, A2-30). With regard to the potential effects of increased lignin content in Bt-maize 12% of the experts were of the opinion that the data basis is insufficient.

5.1.5.2 Effects on biological control

Detrimental effects of Bt proteins on beneficial organisms may occur, especially if their prey or host spectrums include 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 available literature (see Annex 1, Category E, chapter E.2, p. A1-62 – A1-64 10 publications), 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.

The OSE confirmed the results of the literature review. None of the experts demanded additional processes or ecological functions to be considered. Most of the experts (Category E. chapter 2.1: 62%, Category E. chapter 2.2: 54%; Annex 2, p. A2-31) assessed potential long-term effects on ecological functions as 'negligible' for the listed crop/trait combinations. With regard to the potential effects of sublethal toxic effects to natural enemies 15% of the experts were of opinion that the data basis is insufficient to be able to come to a conclusion.

5.1.5.3 GM traits may cause changes in pollination

Adverse effects of GM Bt plants on the main pollinators, honey bees, have not been reported so far. Additionally, no reports are available on harmful effects to NTOs which are involved in pollination. Future GM products like protease-inhibitors could have the potential to affect pollinators in particular honey bees, but these are not yet on the market. Potentially less obvious changes in phenotype characteristics (like altered scent or colour) are factors which may affect pollination. Only minor effects have been reported for GM crops without causing ecological effects. Based on the available literature (see Annex 1, Category E, chapter E.3, p. A1-64 – A1-65, 11 publications), the likelihood of this process for the currently cultivated GM crops in the EU is

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

The OSE confirmed the results of the literature review. None of the experts demanded additional processes or ecological functions to be considered. Most of the experts (Category E, 3.1: 69%, Category E, 3.2: 73%; Annex 2, p. A2-31) assessed potential long-term effects on pollination as 'negligible' for the listed crop/trait combinations.

5.1.5.4 Conclusions

Long-term effects of GM crops on ecological functions should be initially considered in the ERA, but finally be addressed in monitoring due to some uncertainty based on the lack of literature data, specifically regarding effects on soil functions like Bt proteins potentially affecting soil micro-organisms, or non-selective herbicides being transferred in herbicide tolerant crops into roots potentially affecting mycorrhizal fungi or nitrogen fixation by symbiotic rhizobia. Bt proteins in soil may alter susceptibility against pathogens, or affect beneficial organisms causing alterations in biological control mechanisms. However, this potential impact is of minor relevance since the persistent Bt concentrations measured in soils are very low (see section E.1, Annex1, p. A1-56 – A1-58). Glyphosate usually is known to be degraded rapidly when entering the soil. However, some publications emphasise 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 and Borggard 2002, Borggard and Gimsing 2008).

5.1.6. Impacts of the cultivation, management and harvesting techniques (Cat. F)

As in case of the introduction of other new crops, the cultivation of pest resistant or herbicide tolerant crops may alter current management regimes and may introduce new cropping techniques.

5.1.6.1 Altered use of agrochemicals

Under the current EU legislation for the introduction of GM plants into the environment (Directive 2001/18/EC regarding the deliberate release of GMOs into the environment) (EC, 2001), there is a requirement to assess the environmental impacts of GM crops and also to assess the environmental impacts of the specific cultivation and management of GM crops. In the case of genetically modified herbicide tolerant (GMHT) plants, this means evaluating the overall environmental impact of the specific herbicide programmes associated with these GMHT crops, as well as the environmental impacts directly associated with the GM plant itself. In the current legislation governing pesticide registration in Europe (Directive 91/414/EEC) (EC, 1991), the ERA of pesticides includes an assessment of impacts on certain NTO and studies of residual activities in soil and water. However, a pesticide ERA does not include studies of impacts on biodiversity within crops and changes in agro-ecosystems, which are required in relation to GM crops. Thus a herbicide used on a GMHT crop is assessed differently from the same herbicide used on non-GMHT crops (e.g. naturally imidazolinone- and atrazine-tolerant crops) and conventional crops. The EFSA GMO Panel issued a working document[20] in order to propose an approach to be followed in the frame of the ERA of GMHT crops, specifically in relation to assessing the environmental impacts of the specific cultivation practices (i.e. treatments of broad-spectrum herbicides after HT crop emergence) associated with these crops. In specific situations, ERAs of GMHT crops may conclude that the herbicide programmes applied to GMHT crops may cause reductions in biodiversity compared with conventional weed management systems. However, since it is primarily the herbicide management programmes eventually combined with the tillage system that determine the environmental impact, it is proposed that applicants and the appropriate competent authorities in EU Member States establish and implement herbicide manage-

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[20] Working Document of the GMO Panel on the interplay between Directive 2001/18/EC (GMOs) and Directive 91/414/EEC (Plant Protection Products). http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902125247.htm

ment systems for GMHT crops that do no more put environmental harm than conventional systems and are consistent with the environmental protection goals and biodiversity action plans in each EU Member State. The EFSA GMO Panel recommends that monitoring of the herbicides is conducted as part of the stewardship of the herbicides by the agrochemical companies involved, under the auspices of the pesticide regulatory systems operating in EU Member States, in order to record compliance with the approved uses of the herbicides on GMHT crops, levels of weed control and development of resistant weeds. Monitoring will play an important role for potential long-term effects: Case-Specific Monitoring of the GMHT crops will depend on the specific conclusions of the ERA and so no generalised advice for GMHT crops can be given by EFSA as the case-by-case approach applies. In addition to the monitoring of GMHT crops for unanticipated adverse environmental effects (as part of the General Surveillance activities), EFSA proposes that the applicant should describe which herbicide management strategy could be used to reduce potential adverse unanticipated environmental effects caused by the HT system.

GM plant cultivation and management could potentially cause increased/altered use of agrochemicals controlling herbicide tolerant weeds, and persistent GM crops (volunteers) with adverse effects on NTO and/or ecological functions. Based on the 20 publications evaluated (see Annex 1, Category F, chapter F.1, p. A1-66 – A1-67), the likelihood of this process for the currently used GM crops in the EU is

- **Low - High** for HT crops (with some uncertainty due to management aspects),
- **Low for** Bt maize, and
- **Negligible** for SM potato.

The OSE confirmed in general the results of the literature review (see Annex 2, 3.7, p. A2-12). The expert contributions were characterized by a high number of answers in the assessment option 'insufficient data' or 'no expert' in general. One reason could be that the data basis for the assessment is deficient. At the same time a high number of the consulted 'generalists' among the experts felt potentially a specific lack of personal expertise. High uncertainty was expressed in particular for cases regarding the use of HT-GM plants with the complementary herbicides. Many experts believed that weed communities might be affected on a low level through changes in tillage (55%)[21] or herbicide drift (64%)[22]. Questions regarding increasing number of volunteers, increasing development of tolerant weeds or consequences of changes in weed communities causing changes in ecological functions were an-

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[21] Sum of answers 'negligible' + 'low'

[22] Sum of answers 'negligible' + 'low'

swered ambiguously. In contrast, all other aspects were assessed by the majority of those experts, who did not answer 'don't know', as 'negligible'.

In context with Bt-maize cultivation 34% of the experts were of the opinion that the potential of secondary pest's development is low, whereas 36% assess the potential as negligible (see Annex 2, F.1.9, p. A2-34). However, this expert assessment is partly not in line with current knowledge. Other insect pest organisms than lepidopteran species do not economically affect maize production systems.

5.1.6.2 Indirect changes in susceptibility of crops against plant pathogens

An altered use of pesticides might result from the new cultivation management and – in consequence – adverse effects on NTO might occur. GM plant [management] of HT crops may cause indirect changes in susceptibility of crops against plant pathogens with adverse effects on NTO due to increased use of other pesticides. Based on the available literature (see Annex 1, Category F, chapter F.2, p. A1-68 – A1-69), the likelihood of this process for the currently used GM crops in the EU is

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

The OSE confirmed in general the results of the literature review. The expert contributions were characterized by a high number of answers in the assessment option 'insufficient data' or 'no expert' in general (see 5.1.6.1 above). This indicates a high level of uncertainty in this area.

5.1.6.3 Adverse effects on agro-biodiversity

The major results are based on the Farm Scale Evaluations (FSE) performed in the UK. The FSE results revealed significant negative (indirect) impacts of growing GM HT crops on non-crop biodiversity (NCB) in fields with cultivated sugar beet, smaller but consistent negative effects on NCB in fields with cultivated oilseed rape, and positive effects on NCB in maize fields. The FSE represents a very comprehensive study, and conclusions on long-term effects of cultivation and management regime impact can be drawn (e.g. seed bank declines in some crop rotations, see also Qi *et al.* 2008). In general, GM plant management may cause indirect changes and potential adverse effects on agro-biodiversity due to knock-on-effects of additive, synergistic or delayed effects in cropping systems; effects may differ with regard to specific agricultural landscapes. Based on the available literature (see Annex 1, category F,

chapter F.3, p. A1-69 – A1-72), 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.

The OSE confirmed in general the results of the literature review. The expert contributions were characterized by a high number of answers in the assessment option 'insufficient data' or 'no expert' in general (see 5.1.6.1 above). So there is still some uncertainty about this area.

5.1.6.4 Indirect changes in fertilizer use

Based on the literature review (see Annex 1, Category F, chapter F.4, P. A1-72 – A1-73), GM plant management may cause indirect changes in fertilizer use with adverse effects on uptake of cationic nutrients or symbiontic NTOs and/or ecological functions caused by blocking of ion exchange sites. The likelihood of this process for the currently used GM crops in the EU is

- **Low - high** for HT soybean but with remaining uncertainty,
- **Low** for HT oilseed rape, HT sugar beet, HT maize, and
- **Negligible** for Bt maize or SM potato.

The OSE confirmed in general the results of the literature review. The expert contributions were characterized by a high number of answers in the assessment option 'insufficient data' (14%, both 4.1.and 4.2)) or 'no expert' (38% and 42% respectively) in general (see 5.1.6.1 above; Annex 2, F. 4.1 and 4.2, p. A2-35).

5.1.6.5 Potential changes in landscape structure

GM plant management may cause indirect changes in landscape structure due to coexistence measures (e.g. larger fields, larger distances between specific crops), resulting in loss of habitat connectivity and reduced local biodiversity. Based on the available literature (see Annex 1, Category F, chapter F.5, P. A1-73, the likelihood of this process for the currently used GM crops in the EU is

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

The OSE confirmed in general the results of the literature review. The expert contributions were characterized by a high number of answers in the assessment option 'insufficient data' or 'no expert' in general (see 5.1.6.1 above).

5.1.6.6 Conclusions

Long-term effects are most likely to be caused indirectly through changes in cultivation management and the consequences for the wider biodiversity by the use of herbicide tolerant crops. The frequency of tillage can also be reduced due to lesser densities of weed populations, as weeds could be controlled more efficiently in-season with broad-spectrum herbicides. However, efficient weed control potentially resulting in "bare soils" is in conflict to the EU targets for protection of wider plant diversity if broad-spectrum herbicide would be applied intensively (reduction of weed populations, reduction of insect populations hosting on weeds, potential decrease of bird feed, reduction of weed seed banks). It should be considered that current conventional weed control (including herbicide mixtures) also aims to broadly decrease of weed abundance as well. On the other hand the greater variability in timing of applications could offer the opportunity of applications oriented at 'weed damage thresholds' if the farmers will observe weed emergence on fields cultivated with HT crops. Such measures could potentially lead to a higher level of weeds being able for seed production than under traditional herbicide regimes. In addition, some uncertainty was identified whether altered pesticide applications might indirectly affect mycorrhizal fungi, nitrogen fixation capacity or plant pathogens.

5.1.7 Effects on the abiotic environment (Category G)

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 and fundamental scientific research. 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.

5.1.7.1 Increased production of green house gases

Literature data are very limited with respect to long-term impacts of GM crops on climate change. Theoretically, intensification of agriculture related to GM crops could potentially be connected with higher use of fossil energy resources, global deforestation and decline of organic soil fraction, which might increase carbon dioxide release into the atmosphere. Based on the available literature (see Annex 1, Category G, chapter G.1, P. A1-74 – A1-75),

the likelihood of increased production of greenhouse gases for the currently used GM crops in the EU is

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

The OSE confirmed in general the results of the literature review (see Annex 2, 3.8, p. A2-12). The majority of the experts assessed potential long-term effects on the abiotic environment as 'negligible'.

5.1.7.2 Increased mineral nutrient erosion and fertilizer leaching

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 was reported that GM crops indirectly affect soil nutrient transformations. However, an indirect effect of glyphosate as chelating agent on Fe and Mn availability is reported that potentially might cause increasing nutrient deficits. Based on the available literature (see Annex 1, Category G, chapter G.2, p. A1-75 – A1-78), 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.

The OSE confirmed in general the results of the literature review. The majority of the experts assessed potential long-term effects on the abiotic environment as 'negligible'. However, a noteworthy number of experts (29%) felt that they did not have sufficient expertise to answer the questions in this category.

5.1.7.3 Altered chemical attributes of soil fractions

Bt proteins as well as the molecules of the non-selective herbicides may affect soil attributes if they would be released to high amounts combined with high persistence. Due to their charge to some extent these molecules may then affect the surfaces of exchange minerals or interfere with ionic substrates like iron. Available literature data are limited with respect to long-term impacts of GM crops on chemical soil attributes. Based on the available literature (see Annex 1, Category G, chapter G.3, p. A1-78 – A1-80), 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.

The OSE confirmed in general the results of the literature review. The majority of the experts assessed potential long-term effects on the abiotic environment as 'negligible'. However, a noteworthy number of experts (31%) felt that they did not have the expertise to answer the questions in this category.

5.1.7.4 Conclusions

Long-term effects of GM crops on the abiotic ecological functions should initially be considered in ERA, but addressed more specifically in monitoring. There is some uncertainty based on the lack of literature data.

5.1.8 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 from unintentional crosses. Stacked events have become more important during the last few years. The first cultivation started in 1997-1999 in the US with a stacked event of insect resistance (IR) and 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. 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.

5.1.8.1 Assessment within the sections 5.1.1 - 5.1.7

To our knowledge, no detrimental negative interaction has been observed between stacked genes in GM crops so far. Although stacked events have been cultivated for about 10 years, very little research has been published and investigations addressing potential long-term effects are missing. However, first studies on the nutrient composition of double resistant maize and its impact on feeding of chickens did not show any significant differences.

In the OSE, the experts were asked for their view on stacked events and a potential differentiation with regard to intended or unintended stacks for each category mentioned (see Annex

2, 3.10, p. A2-13). For none of the processes the majority of the experts (56 - 68%; see Annex 2, A2-15 - A2-38) tended to make a difference in assessment between intended or unintended stacks. About 10% of the experts were of the opinion that the data basis is insufficient for the processes concerning interactions with NTO, effects on ecological functions and effects on cultivation and management.

5.1.8.2 Conclusions

For the assessment of long-term effects resulting from stacked events, the initial ERA according to Annex II of Directive 2001/18/EC should already consider the occurrence of unintended stacks (Annex 3, 3.3, p.A3-10).

5.1.9   Regional aspects

Regional aspects regarding environmental adverse aspects of GMO cultivation did not play a major role in the literature survey since data comparing the behaviour of GM crops under different prevailing environmental conditions are scarce. This issue is most important in studies taking into account wild plant relatives of GM crops, the occurrence of target and NTO, or local agricultural practice (see section 5.1.1, 5.1.3, 5.1.4, and 5.1.6).

5.1.9.1 Assessment within the sections 5.1.1 - 5.1.7

For each group of processes mentioned in the sections above, the experts were asked in the OSE whether the assessment needs differentiation concerning geographical regions in Europe (see Annex 2, 3.9, p. A2-12 - A2-13). Within eight of 23 cases, the majority (>50%) of experts answered 'yes'. In 10 cases the responses were ambiguous. Accordingly, there seems to be a need for more regional approaches within the assessment; since the expert majority agreed or felt uncertain. This is mainly true for invasiveness, persistence, seed survival, and hybridization issues. Regional aspects played a relative minor role in the assessment of nutritional composition, toxic compounds, interactions between GM crops and mycorrhiza and bacteria, fitness change due to root exudation, effects on pollinations, effects due to fertilizer use and all aspects taken into account in category G (Potential interactions with the abiotic environment).

5.1.9.2 Conclusions

Specific long-term effects will vary regionally, e.g. due to differing climate peculiarities potentially being responsible for wild relatives growth in the receiving environment or influencing survival of reproductive units. In addition, locally adopted cultivation and management techniques may have regionally dependent impacts.

5.2 Prioritization with the help of Online Surveys

The level of relevance for bearing potential long-term effects is directly linked to the level of uncertainty due to missing knowledge. This result is supported due to the fact that highest uncertainty is revealed concerning indirect effects resulting from altered cultivation and management primarily combined with cultivation of HT crops (e.g. regarding agrochemical use). Some lesser uncertainty was identified concerning indirect effects mostly on soil aspects like side-effects of Bt proteins or the non-selective herbicides on microbial soil communities (at least for HT GM crops). Any specific prioritization for a given process was performed within BEETLE based on:

(i)   missing or insufficient scientific data due to results of the literature review,

(ii)   clear proportion of 'low' to 'high' options chosen by the experts for a specific scenario,

(iii)   clear proportion of consulted experts that were not able to assess this long-term process due to their choice "insufficient data", and

(iv)   unclear tendency in the Online Survey due to the inconsistency of the experts in choosing a distinct level of likelihood (negligible, low, medium, high) for a long-term effect (see Annex 2).

Within the BEETLE project, uncertainty became apparent where:

a)   the expert assessment was associated with an ambiguous responding or a high proportion of answers 'insufficient data,

b)   the Literature Review in specific areas did not reveal a significant number of qualitative and quantitative information, and

c)   Data and opinions from the Literature Review were conflicting.

At the end of the survey the experts were asked to which field of research regarding cultivation of GMOs in EU the highest priority for financial support should be given. This question gave additional hints to important areas of uncertainty. The relative majority of experts (see Annex 2, H, p. A2-58 – A2-63) recommended investment into the research areas of:

- cultivation and management (17),

followed by the issues:

- NTO (9),
- gene flow (persistence and invasiveness), and (7)
- ecological functions (7).

All other issues were of minor interest.

The consulted experts confirmed that (i) the potential processes causing potential long-term effects were complete and (ii) the same was true for the preliminary prioritisation by the BEETLE study in most cases.

The most important areas of uncertainty were

- impact of cultivation and management,

- impact on soil organisms,

- regional aspects in the assessment and

- the evaluation of stacked events.

As a result of the Literature Review and the Online Survey (see section 5 and Annex 1 and 2), ten processes (see Table 1 or 8) were identified with relevance for bearing potential long-term effects on the environment and grouped into the following five major points:

- Potential adverse effects due to 'Cultivation and Management' issues

  The most likely long-term effects are those caused indirectly through changes in cultivation management and the consequences for the wider biodiversity by the use of HT crops.

- Potential adverse effects related to 'Resistance development' in pests targeted by IR crops, particularly Bt.

  Direct long-term effects of GM crops (mainly Bt maize) are likely to become apparent due to resistance development over time.

- Potential adverse effects related to 'Gene flow to wild relatives'

  Gene flow to wild relatives should be considered most likely in cases of GM plants that have ancestors in the natural European flora, especially for crops related to Brassica and Beta species.

- Potential adverse effects on 'Ecological functions and the Abiotic environment'

  Long-term effects of GM crops on ecological functions and the abiotic soil environment should be considered due to some uncertainty based on potential adverse effects of recombinant proteins, especially on soil functions.

- <u>Regional variation of potential adverse environmental and biodiversity related effects</u>

    Regional differences should be taken into account in the assessment of long-term effects with regard to the cultivation of specific GM crops in the EU.

These five points were further elaborated by the BEETLE team based on the results of the Crea Space Workshop, and final conclusions on prioritization are provided in section 8 grouped into four cases.

5.3   CREA Space Workshop

Within the CREA Space Workshop (see Annex 3) it was possible to gain in a relatively short period of time helpful information on the third and last project goal: the identification of ways and methods to delimit uncertainties in case of potential long-term effects. In the case of the four workshop issues (i) potential impacts in relation to cultivation and management (in particular of HT crops), (ii) potential impacts on soil, (iii) potential impacts caused by stacked versus single events, and (iv) potential impacts caused by regional aspects a set of methods and ways forward was identified and prioritized.

In the case of potential impacts in relation to cultivation and management of HT crops modelling was selected as a most helpful tool to address long-term effects. An experimental approach like FSE was considered to be useful to assess impacts such as changes in pesticide use, but it was controversially discussed whether these investigations should be performed pre- or post marketing. Due to the high prioritization by the participants both the significance of baselines and reference points and the definition of agricultural protection goals (e.g. agricultural biodiversity) are essential prerequisites to assess long-term effects of GMOs in comparison to conventional crops. In addition guidelines for "good agricultural practice" have been prioritized as a useful tool to avoid potential adverse effects of GMOs, but this does not directly address targets.

In case of potential adverse impacts of GMOs on soil, experts prioritized soil functionality, especially soil fertility as the most relevant monitoring subject.

In general, the development of adequate indicators was emphasized. Such indicators should preferably focus on functional groups or ecosystem functions related to protection goals. As long as such protection goals are not defined, a precise list of such indicators can hardly be achieved (see section 7 of this study).

In case of potential impacts caused by stacked events, tests of synergies between different proteins (from intended and unintended stacks) have been prioritized as the most important

way to address potential adverse effects. Possible synergy effects of proteins from intended and unintended stacks should be regarded during ERA.

The majority of the workshop experts considered the issue "regional aspects" as important. However it was discussed controversially whether pre-market ERA according to Annex II of Directive 2001/18/EC or Post Market Environmental Monitoring, possibly oriented to a pre-selection of specific site according to a 'hot spot' principle, is better suited to target regional long-term effects. Research on identifying agro-ecological regions was prioritized.

The participants got the opportunity to add points to the discussion which were not addressed under the selected subjects and processes. The participants stressed that economic/environmental and health benefits of GMOs should be considered in the assessment of potential long-term effects as well. In addition the participants stressed that "adverse" in the context of evolving and dynamic ecosystems has still to be defined. In addition, a clear definition of "adverse" in terms of biodiversity and environment is still missing.

Overall, the CREA Space Workshop achieved the intended goals: The areas of scientific uncertainty were specified, some ways forward were more clearly mapped, and topics for future research were identified (see section 8).

6. Results on Human and Animal Health

The potential impact of GM crops on animal and human health has been intensively assessed worldwide over the past 10 years of commercial cultivation of GM crops. Strategies for the safety assessment of GM crops have been jointly developed by various international bodies, such as the Organization for Economic Cooperation and Development (OECD), the United Nations World Health Organization (WHO) and the Food and Agriculture Organization (FAO). However, the evaluation of potential long-term effects is discussed controversially. One side argues that currently used methods in risk assessment are not sufficient to address long-term effects adequately; others claim that long-term effects could not be generally predicted. The aim of the BEETLE project is to summarize the available information on potential long-term effects of GM food and feed (Annex 4).

The identified processes and scenarios of potential long-term human and animal health effects were grouped into the following four categories:

    A. Nutritional assessment

    B. Toxicity

    C. Horizontal gene transfer

    D. Allergenicity

The expertise of the BEETLE team and PRC was complemented by experts, who participated in the Online Survey Health (Annex 5). The experts were also asked whether and how the risk assessment procedure might be improved in relation to animal and human health.

The results of the Online Survey Health should be interpreted carefully due to a low number of participating experts (52 experts registered, mean number of answers = 27 per question).

6.1 Nutritional assessment

Feed and food from GM crops with input traits[23] are mostly nutritionally equivalent to their isogenic counterpart. Neither biologically relevant differences in digestibility or effects on animal or human health, nor any unintended effects on the performance of animals or the composition of food of animal origin have been described in the literature so far (see Annex 4, 3.2, p. A4-5 and 4.2, p. A4-10 – A4-11). Chemical analyses and animal studies reveal no

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[23] Input traits help are intended to lower the cost of production, e.g. GMHT or Bt plants

significant differences between GM plant feeds and their near-isogenic counterparts and hence strongly support their substantial equivalence to non-GM feeds. This has been documented in more than 100 studies published in the literature. Altogether more than 50 GM crops have been approved worldwide, and it can be concluded that the foods and feeds derived from these GM crops are "as safe as those derived from traditional crops".

The potential occurrence of unintended effects is not a phenomenon specific to genetic engineering. It is well known that traditional plant breeding methods may also result in unexpected changes (Cronk et al. 1974, Cellini et al. 2004). It should also be emphasized that the potential for unexpected and unintended compositional changes will arise with all forms of genetic modification, but to date, no adverse health effects from the consumption of GM foods have been documented in the human population.

The conclusions of the Literature Review Health (Annex 4) were confirmed by the Online Survey Health (Annex 5). The majority of the experts were of the opinion that adverse long-term effects on animal or human health due to altered nutritional values of GM crops are negligible.

The question of whether genetic modification of plants might result in unintended changes in the spectrum of their metabolites was answered ambiguously. Even though the spectrum of metabolites is tested in the risk assessment (test of substantial equivalence) 45% of the experts voted that increasing modifications could result in unpredictable changes in metabolites. The answers to the question on additive, synergistic or antagonistic effects of gene products showed no clear picture (38% [yes] vs. 38% [no]).

Potential long-term effects due to the consumption of food or feed mixtures containing different GM crop/trait combinations were assessed as low. 47 % of the experts decided for the response option 'negligible', but 23% decided for 'low' and 13% for 'high' respectively. Furthermore 13% of the experts believe that the data basis is still insufficient for an assessment.

There is partly a disagreement between the conclusion from the Literature Review Health and the Online Survey Health in relation to potential long-term effects of unintended changes and the consumption of food or feed mixtures. The assessment in the Online Survey Health was characterized by ambiguous voting and a high uncertainty. In contrast there is a high number of scientific publications on short- and medium term studies and experience with currently deregulated GM crops which do not report adverse (including long-term) effects. It is true that tests on long-term effects are rare, but the available publications do not indicate adverse effects so far. Therefore the BEETLE study concluded that this aspect is of minor relevance.

6.2 Toxicity

The toxicity of GM plants and derived products is tested extensively in the risk assessment procedure. So far no toxic effects have been observed after consumption of Bt crops or derived products. In some studies single parameters were altered such as enzyme activities or the triglyceride concentration observed in quails fed Bt-maize in the 13th generation of a long-term feeding experiment; and feed intake, growth rate and some physiological functions in a 90-day rat feeding trail. These results were discussed controversially. However the observation of single altered parameters is common in toxicology tests. They are only relevant if a set of parameters describing a physiological function reacts in the same direction. In none of the studies assessed by the BEETLE study was such an effect observed.

In contrast, Bt-maize contains mycotoxin levels mostly lower than conventionally-produced maize. As mycotoxins may have adverse impact on livestock, long-term benefits are more likely than risks.

Based on the Literature Review Health (see Annex 4, 3.3, p. A4-7 – A4-8) no toxicological long-term effects are likely. Discussion is more focused on the quality of the risk assessment. Here, the experts were asked whether and how risk assessment might be improved. This aspect is discussed in section 6.5.

6.3 Horizontal gene transfer

The consumption of feed or food from GM plants results in the intake of genetically modified DNA and proteins. Therefore the fate of DNA and proteins within the gastrointestinal tract of animals, and the potential for t-DNA or its products to be incorporated into animal tissues or transferred to micro-organisms, could theoretically pose a risk to animal or human health. In general, t-DNA would be processed in the gut in the same manner as from conventional feed ingested genetic material. No data exists showing that t-DNA is characterized by unique behaviour compared to native plant-DNA during feed treatment or in the animals. Neither intact t-DNA genes nor their minimal functional units have been detected in livestock tissues.

Furthermore no HGT to micro-organisms has so far been observed in the gastrointestinal tract. From the experimental experience, the occurrence of such an event is extremely unlikely. This assessment was confirmed by the majority of the experts. Some experts (13%) were of the opinion that the data base is not sufficient for drawing a conclusion on this point.

Based on the available information, HGT of functional genes of GM plants to animals, humans or micro-organisms has not been observed so far and is extremely unlikely (see Annex

4, p. A4-19). In conclusion, a potential long-term effect due to HGT is negligible for GM crops currently cultivated in the EU.

6.4 Allergenicity

Adverse long-time effects could arise from the introduction of a GMO which newly expresses proteins with high allergenic potential. Discovering the features that make proteins allergenic has led to an allergy assessment strategy that characterizes the potential allergenicity of biotechnology products prior to their commercialization. This testing strategy appears to be effective as shown by the fact that there have been no clinically documented food allergic reactions to any of the biotechnology proteins introduced into food crops. However, the increasing use of GM crops in staple foods will result in an increase of the consumption of novel proteins or proteins from previously not or seldom-consumed crops. So far, it is hard to predict if newly introduced proteins will become new allergens. For any evaluation of results, allergenicity studies need to consider different sensitivities to allergens e.g. of people from different regions. Furthermore, the next generation of GM crops will probably contain more complex traits and/or increasing use of the stacking of more GM traits into the same crop, thus increasing the possibility of unintended effects. To face this, it might become essential to examine the overall allergenic potential of the transformed food crop in addition to examining features of the introduced proteins themselves.

The allergenic potential due to new proteins in crops was assessed as negligible to low by the experts in the Online Survey Health (43% (negligible) vs. 35% (low); see Annex 5, 3.5, p. A-8). The same opinion was expressed in response to the question of whether the development of allergic reactions may arise due to the increased exposure of consumers.

A question was posed as to whether adverse effects will arise due to increased exposure of consumers to a higher expression of natural endogenous allergens. The experts assessed this scenario to be negligible or low (33 % (negligible) vs. 38 % (low)). Contrary to the preceding question, a higher proportion of the experts assessed the probability of this scenario as high (17 %).

In conclusion, allergenicity seems to have a low relevance for potential long-term effects on health. However, the next generation of GM crops with more or stacked traits may have an increased potential to allergenicity. Therefore an improvement of the risk assessment may help to minimize unexpected development of allergenicity (see following section)

6.5   Improvement of risk assessment

There is no evidence for potential long-term effects for most issues concerning animal and human health. However the quality of risk assessment is under discussion, specifically in relation to nutritional value and toxicity. Therefore, the BEETLE study asked the health experts whether and how the risk assessment procedure should be improved. The aim of these questions was to determine how more clarification could be reached for potential ways forward. The majority of experts saw a need to improve experimental design and statistical methods. In addition, most experts agreed that additional control groups are useful to demonstrate the biological range of measured parameters. In contrast, the experts did not clearly vote for longer exposure tests (e.g. experiments covering whole lifespan or several generations of target animals).

Furthermore, most health experts expressed the opinion that toxicity tests are also helpful to assess the potential risk to NT vertebrates.

The health experts were additionally asked for their view on stacked events in each category. The majority of the experts would make no difference in assessment between single or stacked events (range of agreement: 55% to 71%).

These results are in agreement with the evaluation of the BEETLE study using the available literature. Feeds from GM plants have been used for various species/categories of target animals/food-producing animals. Some studies were done over the whole lifespan of the animals (e.g. ≈ 35 days in broilers), but the majority of studies were carried out over a limited period not covering the whole lifespan (esp. in long-living animals such as laying hens, dairy cows etc.). A further weakness of most animal-feeding studies is the experimental design. Most authors compared only feeds from GM plants with their near-isogenic counterpart and did not include commercial varieties to get an impression of the biological range of the investigated parameters. Most feeding studies with food-producing animals were done according to the national rules for such experiments (e.g. to measure the digestibility or the feed value, to measure the feed conversion, the animal yield etc.). Despite some shortcomings mentioned above the following conclusions can be drawn:

- It is recommended to use – if differences are detected between a GM crop and its most closely related non-GM comparator - an adequate number of commercial crop varieties in feeding trials in order to cover the biological range of the measured parameters.

- In relation to toxicology assessment, more comparators will help to assess any statistical differences observed between the GM plant and its counterparts with respect to the biological relevance.

- A general screening frame for all newly developed plant varieties on the base of scientific criteria should be developed.

In the future more GM crops with output traits[24] are likely to be placed the market. Therefore more long-term feeding studies with target animals are recommended with feeds from GM plants with these output traits of the so called 2nd GM plant generation.

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[24] Output traits are intended to enhance the quality of the food and fiber in the products for consumers.

7.   Indicator definition and identification

According to the EU Environmental Agency[25], an environmental indicator is a parameter or a value derived from parameters that describe the state of the environment and its impact on human beings, ecosystems and materials, the pressures on the environment, the driving forces and/or the responses steering that system. An indicator [should] have gone through a selection and/or aggregation process to enable it to steer action. Indicators for long-term environmental effects should reveal the proposed effects of GM plants, e.g. derived from their cultivation. However, as long as protection goals are not defined, a precise list of GM-plant-specific indicators can hardly be achieved.

The most important tool for detecting adverse effects will be long-term monitoring. Historically, a broad set of methods was developed already in the 70´s and 80`s of the last century to detect the adverse biological effects of environmental pollutants, especially for long-term monitoring of specific airborne or water contaminants (mostly with regard to chemicals). Specific plants, insects, or their communities were used as tools for indicating hazardous biological effects of the contaminants (Arndt *et al.* 1987, Schubert 1985).

An important set of environmental indicators are so called 'bioindicators'. They are selected for qualitative or semi-quantitative measurement to detect alterations e.g. in species community composition or to measure disturbance in ecological functions such as pollination or litter decomposition. The cause for such alterations could be stressors e.g. natural pollutants or anthropogenic impacts. Accordingly, specific sensitive organisms, sets of organisms or biological systems used to indicate the causes and effects of stressors are called 'bioindicators'.

Bioindicators are differentiated with respect to their specific performance as 'responding' or 'accumulating' indicators. Responding indicators show specific negative symptoms (morphological damage, alteration of colour, growth retardation, alteration of behaviour), which are caused by adverse metabolic effects when exposed to the stressor. Accordingly, they are highly sensitive. Accumulating indicators collect more or less high amounts of the pollutant but often don't show (negative) symptoms. Responding indicators can be used under laboratory or under field conditions, whereas accumulating indicators will only be used in the field. For indicating potential adverse effects of GM crop cultivation bioindicators should be selected according to their sensitivity for indicating specific GMO-related adverse effects. According to the definition for bioindicators they have to be qualified as 'responding indicators'.

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[25] http://glossary.eea.europa.eu/EEAGlossary/E/environmental_indicator

According to the 'Criteria for selecting environmental indicators' (OECD 1993) any bioindicator has to fulfil the following basic criteria: (i) relevance, (ii) utility for users, (iii) analytical soundness, and (iv) measurability. Accordingly, environmental indicators should

- provide a representative picture of environmental conditions and of the pressures affecting the environment;
- be simple, easy to interpret and able to show trends over time;
- be responsive to changes in the environment and related human activities;
- be applicable to significant regional environmental issues;
- have a threshold or reference value against which to compare it, so that users can assess the significance of the values associated with it;
- be based on international standards
- be readily available or made available at a reasonable cost/benefit ratio;
- be adequately documented and of known quality.

Delos *et al.* (2006, 2007) reported on the French experience with a national biological survey program, the so-called 'biovigilance'. The program started in 1999 and is mainly focussing on early detection of significant modifications in agriculture. Important parameters being monitored are the evolution of crop pests, effects due to changing agricultural practices, application of new pesticides or effects due to cultivation of new plant cultivars. Surveys are done annually. e.g. weed flora has been monitored nation-wide on nearly 900 fields of different crops in rotation since 2002; also the spread of the main crops in specific territories is monitored. Bird abundances and the abundance of native lepidopteron species has been monitored since 2005.

From a study investigating biodiversity-related aspects at the European scale, Billeter *et al.* (2008) conclude that specific taxa taken as indicators for the whole of Europe are unlikely to provide an effective means for predicting effects on biodiversity at such a large spatial scale. The main reason for ineffectiveness of specific taxa is the large variation in species richness in the differing biogeographical regions of the EU. As a useful approach for assessing biodiversity issues at the landscape level the authors recommend investigating only some specific landscape parameters such as the area covered by semi-natural habitats as an indicator for species richness in an agricultural landscape. Such ecosystems are known to be important habitats for species and their community members.

Consistent with the conclusions of the BEETLE study, a range of biological processes should be monitored with respect to the list of processes and scenarios selected as being potentially relevant for future long-term effects. There are indicators necessary for indicating very specific processes acting at small scales (e.g. soil organisms), but also indicators for indicating potential large-scale alterations at the landscape scale. In general, all of them should be ap-

plicable for measuring the development of long-term impacts on the environment over time. The following indicators will be applicable for detecting the potential long-term effects as mapped in Table 1:

- Monitoring the **habitat composition** could reveal changes in biodiversity at the field and landscape scale (landscape structure); e.g. such information could be gained with the help of geographic information systems,
- Information on **fertilizer and pesticide use** could reveal alterations in agricultural practices at the farmscale level,
- Changes in **soil treatment measures** could reveal information with respect to HT crop cultivation,
- Measurements of **Bt protein** in the case of continuous cultivation of Bt crops in soil could clarify potential adverse effects due to accumulation or not of Bt proteins in soils,
- The consequences of increased use of broad-spectrum herbicides could be revealed by using **weed community composition,**
- Side-effects related to HT or Bt crop cultivation could be monitored through measuring changes in **soil fertility,**
- Changes in the **composition of functional groups of organisms** could provide information on potential adverse effects of Bt crop cultivation,
- **Survey of pest development** could provide insight into potential changes in the occurrence of pathogens or pests, and
- **Allergenicity** surveillance is a tool for identifying potential long-term health effects.

According to the results gained in the study performed by Billeter *et al.* (2008), it is to be expected that the appropriate indicators will generally be suited to one or few specific environments. Accordingly, if the environment changes potentially new (appropriate) indicators could become necessary.

8.   Major conclusions and recommendations for the way forward

Controversy often arises from different interpretation of scientific data. This is because scientific data are always associated with uncertainties, making prediction of potential long-term or cumulative effects difficult. Uncertainties can either be related to the circumstance that there is not yet a sufficient data basis available for an assessment of consequences (the "unknown", Sanvido *et al.* 2007), or to the fact that the questions to solve are out of reach of scientific methodology (the "unknowable", Sanvido *et al.* 2007).

Recognising that an ERA is only as good as the current state of scientific knowledge, ERA can be based on limited scientific information, leaving some uncertainty. Therefore, under current EU legislation, it is recommended to describe these scientific uncertainties, and to clarify assumptions, extrapolations and predictions made. One of these uncertainties relates to potential long-term impacts due to the large-scale exposure of different environments to GM crops when they are grown EU-wide over long periods of time. Although further research might not always overcome the shortcomings identified in the ERA (and further research is likely to be short-term research as well), environmental post-market monitoring - as demanded according to Annex VII of EU Directive 2001/18/EC - will play an essential role in the detection and possibly the prevention of long-term adverse effects to the environment that could occur during and after the cultivation of GM crops (EFSA 2008).

However, the major sources of uncertainty identified during the BEETLE study were related mostly to the following three characteristics:

- causal relationships employed (= derived from compilation of processes from the literature – Step 2 of the BEETLE study),

- controversy on existing knowledge (= derived from unambiguous voting from EEP and EP participants in Steps 3 and 4 of the BEETLE study), and

- lack of some relevant data (= derived from results of literature review in Step 1 of the BEETLE study).

For potential long-term effects, in particular, the availability of literature data is generally very limited due to the relatively short time period of experience of cultivating GM plants. As a result of the Literature Review and the Online Survey, the identified processes were ranked according to their potential to cause long-term effects (see Table 7), taking into account the results of the CSW and the three major sources of uncertainty in the BEETLE study as defined in section 3.3).

Table 7: Estimation of likelihood and major sources of uncertainty for long-term effects[26]. The information is derived from different sources in the BEETLE study: literature review [LR], expert survey [ES], CSW, and BEETLE team evaluation [BE]).

Occurrence of long-term effect	Estimation of likelihood following LR, ES, CSW			Evaluation of uncertainty[27] derived from different sources in the BEETLE study		
Processes/scenarios potentially causing long-term effects	LR	ES	CSW	Causal relationships employed[28] [ES]	Controversy on existing knowledge [ES, CSW]	Lack of some relevant data [LR, ES, CSW]
HT crops: Indirect effects of altered cultivation and management	**high**	low - high	**high**	low	**unambiguos expert voting**	**yes, but minor**
HT crops (sugar beet and oilseed rape) and feralization	**high**	low - high	n.e[29].	low	**unambiguos expert voting**	negligible
Bt crops: Resistance development of TO	**low-high**	low	n.e.	low	negligible	negligible
Bt maize: Effect of Bt proteins on NTO soil micro-organisms and on soil function	low	low	**low - high**	low	negligible	**yes**
Bt maize: Effect of Bt crops on NTO closely related to TO	low	low - negligible	n.e.	low	low	**yes**
SM potato effect on herbivores	low	low - negligible	n.e.	low	**high 'don't know' expert voting**	**yes**
Gene flow from HT sugar beet and oilseed rape to wild relatives (as NTO) and its consequences	**high**	low - negligible	n.e.	low	**unambiguos expert voting**	negligible
Effects of Bt protein root exudation and of herbicides on (symbiotic organisms like mycorrhiza)	low	negligible	**low - high**	low	negligible	**Yes, but minor**
HT crops and altered susceptibility against pathogens	low	low - negligible	n.e.	low	negligible	**yes**
HT crops and altered landscape structure	low	low - negligible	n.e.	low	negligible	**yes, but minor**
Allergenicity development	low - negligible	low	n.e.	low	negligible	**yes, but minor**
All other processes compiled in BEETLE step 2	low - negligible	low - negligible	n.e.	low	negligible	Negligible

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[26] The highlighted assessment fields (**red bold** font) indicate what fields were most important for the BEETLE team to weight a process being of higher relevance in the final prioritization (see Step 6 in Figure 5)

[27] Sources of uncertainty are identified based on the criteria listed in section 3.3 and applied to sections 5 and 6

[28] There is the intrinsic uncertainty that the causal relationships compiled by the BEETLE team are incomplete. However, most of the EEP participants were of the opinion that the listed processes in the different categories were complete (ranging from 70% to 82%). The degree of remaining uncertainty was therefore assigned as 'low'.

[29] n.e. = not estimated; It was only possible to discuss questions with highest degree of uncertainty during the CSW.

The processes and scenarios with priority regarding potential long-term effects are grouped by the BEETLE expert team into four cases as follows:

- Potential adverse effects due to 'Cultivation and Management' issues:
  Long-term effects of GM crops are most likely to be caused indirectly through changes in cultivation and agricultural management of HT crops and consequently affecting wider biodiversity. The use of complementary herbicides can change the management practice. These specific effects of such changes will depend on crop/trait combinations cultivated and possibly regional aspects. This process/scenario was clearly assigned with highest priority.

- Established adverse effects related to 'Resistance development' in pests targeted by IR crops, particularly Bt:
  Direct long-term effects of GM crops (mainly Bt maize) are likely to become apparent due to resistance development in TO. Resistance development in plant pests targeted by GM crops expressing Bt proteins for their protection is a long-term effect already anticipated from the risk assessment.

- Potential adverse effects on NTO related to 'Gene flow to wild relatives':
  Gene flow regarding GM traits from GM crop plants to wild relatives should be considered likely in cases of GM plants that have ancestors in the natural European flora, especially for crops related to the *Brassica* and *Beta* Species, which have sexually compatible relatives. Although gene flow as such is not an adverse environmental effect, the long-term consequences for species conservation and biodiversity might be important.

- Potential adverse effects on "NTO", ecological functions and the abiotic environment':
  Long-term effects of GM crops on NTO (e.g. in soil), ecological soil functions, and the abiotic soil environment should be considered, due to uncertainty indicated by the fact that only a few scientific publications are available.

8.1   Selecting the approach to reduce uncertainty

The BEETLE project identified a number of ways forward to decrease the uncertainties and knowledge gaps concerning potential long-term effects.

It is not yet possible to quantify the long-term risks associated with GM plants as experience is lacking. However, in more than 20 years of experimental field releases and more than 10 years of commercial cultivation, adverse long-term effects reported in the literature have

concerned the development of resistance in Bt crop target organisms and development of ferality in HT oilseedrape. No other adverse long-term effects have yet been established. However, several other potential long-term effects are discussed in the scientific literature and in scientific forums in general. So far, adverse long-term effects under the given limited conditions (e.g. few GM plants authorized for cultivation, low percentage of GM plant area in EU) seem to be rare events. Therefore, it is unlikely that further research on the level of short-term field trials (1-3 years) can fill substantial knowledge gaps on potential long-term effects of GM plants. This is likely to hold true at least for GM plants which have passed the risk assessment in conjunction with the approval procedure in the EU.

An experimental approach like the farm-scale evaluations (FSE)[30] might be useful to assess some processes before market approval. However, even such an intensive experiment can only address a limited number of scientific questions. The FSE example from the UK demonstrated that - with respect to the cultivation of HT crops - the herbicide management and not the GM crop as such triggers wider biodiversity effects in agricultural landscapes. However, the BEETLE expert consultation did not unambiguously support such an FSE approach since it was a very general and broad approach. Instead, specific methods and tools for reducing uncertainty should be selected (case-by-case and tool-by-tool approach) based on the identified main fields of uncertainty.

From the results of this project, the BEETLE study recommends using **studies**, **modelling** and **monitoring** as tools to be applied after market approval of GMOs.

Research studies close to practice[31], and **studies within PMEM**[32] can be performed in defined EU-typical "hot spot" regions where potential long-term effects are more likely to occur

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[30] The detailed results of the extraordinary Farm Scale Evaluations (FSEs), the environmental impact study of herbicide tolerant GM crops) performed in the United Kingdom, are available at http://www.defra.gov.uk/environment/gm/fse/

[31] 'Close to practice' means the initial cultivation phase after first consent for placing on the market is given. However, in reasoned cases it might also be possible to study long-term effects in more depth in a pre-market phase with 'close to practice' conditions (e.g. large scale field releases). The 'need' to study potential impacts in relation to cultivation, the coverage of different European regions and experiments conducted on farm scale level, were controversially discussed among CSW experts with respect to timing either within pre- or post marketing (e.g. see Annex 3, pages A3-8, A3-9, and A3-17).

[32] It is a regulatory question as to who is responsible for carrying out such research studies. According to Regulation (EC) No 1829/2003 on genetically modified food and feed and to Directive 2001/18/EC on the deliberate release of genetically modified organisms into the environment, GM plants and their derived food and feed products are subjected to a risk assessment (RA) prior to their placement on the European market. In this risk assessment, the potential [long-term] effects that the commercialisation of a GMO might have on human or animal health and the environment are assessed based to a large extent on the information delivered by applicants. Where and when further research studies are necessary depends on the particular application. It is in many cases *a*

e.g. crop seed production areas in the vicinity of wild relative populations in mild climate areas or areas with a high proportion of GM crops to test the conclusions of the ERA (see Annex 3, 3.4, p. A3-11 – A3-12).

Modelling can be used, for example, to quantify effects such as gene flow and estimate their consequences at a landscape scale (EFSA 2008). This 'upscaling' would allow predictions of impacts to be made if GM crop production increased with time and scale. However no appropriate model is currently available that can be routinely applied to risk assessment (including long-term effects). Further research is required to develop these models or to explore the use of related models (EFSA 2008). However, there is already implicit upscaling in risk assessments and efforts should be made to consider how this process can be formalised. Discussions and consultation should take place to consider the process by which upscaling is initiated and conducted in an ERA.

Monitoring is – based on the BEETLE study and compared to other methods listed before – probably the most efficient and realistic tool to address long-term effects. According to Annex II of 2001/18/EC, the assessment of potential long-term effects is one of the fundamental tasks within risk assessment. Applicants are required to provide adequate data to allow the assessment of the potential long-term adverse effects on both the human/animal health and environmental aspects of a GMO as part of their applications. In addition, post-market environmental monitoring will play an important role in determining long-term effects of GM plants and in testing model predictions. Specifically General Surveillance can be improved for identifying, e.g. regional aspects in a case-by-case way.

It was concluded that the scientific knowledge and experiences gained from monitoring GM crops will in turn inform the risk assessment process which can be updated in the light of any new knowledge on long-term effects. The BEETLE team does not recommend carrying out research studies parallel to cultivation *in order to* demonstrate safety. Instead, the BEETLE team considers post market research studies as a tool to confirm the initial assumption of the ERA in selected cases and therefore to decrease the inherent uncertainty that underlies every ERA. It is a matter of discussion who should carry out such post-market research studies and where.

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priori (epistemically) not possible to experimentally study long-term effects related to scale before – market-approved – cultivation, or it would be practically disproportional to demand continuous years/decades of small-scale field testing, e.g. of crop varieties that would later be outdated in yield performance due to ongoing progress in conventional breeding. However, in reasoned exceptions it might also be possible to study long-term effects in more depth in a pre-market phase with 'close-to-practice' conditions (e.g. larger scale field releases to test for effects that are expected due to known accumulative effects, and which can be carried out within a reasonable time frame).

In the case of potential impacts caused by 'Stacked Events', testing of synergistic effects e.g. of gene products coded by stacked events (intended and unintended stacks) has been prioritised as the most important way to identify potential adverse effects. Possible synergistic effects of proteins from intended and unintended stacks should be identified during the ERA according to Directive 2001/18/EC to improve the prognostic power of the long-term effect assessment. Taking into account the growing knowledge of mechanisms of gene regulation, basic research in this field will reduce uncertainty. Consideration should be given to whether the knowledge gained over recent years in this area can be applied and developed in order to create tools for an improved risk assessment. In this context it was discussed by the BEETLE team to collect information about GM plants cultivated world-wide including the GM nucleotide sequences (see Annex 6). Such a data-base could provide data required to develop and test bioinformatics tools and principles with the aim of predicting possible interaction between stacked events at the level of nucleic acids including gene regulation.

It is important to **define endpoints of long-term effects,** e.g. protection goals, first in order to select appropriate indicators (e.g. soil species or soil functionality parameters). Then, the best management measures should be selected and appropriate indicators to monitor the potential impact should be chosen. The BEETLE study defined **indicators** for measuring the development of long-term impacts on the environment over time as listed in section 7. According to the CREA Space Workshop, a reasonable tool for selecting networks that carry out the monitoring might be qualified independent extension services. Good agricultural practice may include a type of stewardship based on a kind of 'driver's licence', but stewardship programs may also contain information on how farmers should make decisions.

Additional **analyses at socio-economic levels** could be helpful to estimate the demand of the market and the opinion of farmers. Monitoring the abundance of wild relatives and gene introgression from GM crops is a way to identify regionally different aspects of persistence, invasiveness and hybridization. Case-Specific Monitoring of resistance development in target insects will also need regional monitoring tools.

Recently, the EU Joint Research Center in Ispra has established the Central Core DNA Sequences Information System (CCSIS). The CCSIS is a database where GMO-related DNA sequence data are stored to run homology searches in order to assess the specificity of the proposed GMO detection method as required by Commission Regulation (EC) No. 641/2004. The main focus of CCSIS is on GMOs notified according to European legislation and it has been set up in order to give support to the Commission's Biotechnology Unit and its Community Reference Laboratory for GM Food and Feed (CRL). However, at present it is not clear if

CCSIS will be made accessible to the Competent Authorities or how far information on GMOs outside the EU will be incorporated.

Another tool is the **Molecular Register (MR)** planned by the German BVL for operation from 2009/2010 (see Annex 6). The MR is intended as a web-application integrating regulatory, phenotypic and molecular information on GMOs, and allowing the usage of currently established bioinformatics tools for supporting the Competent Authorities in their responsibilities according to Directive 2001/18/EC and Regulation (EC) No. 1829/2003. The MR might be a very helpful instrument for identifying the possibility of unintended stacks and resulting possible synergstic effects to be taken into account during the ERA.

Concerning health effects, the issue of allergenicity was regarded to have the highest potential for long-term effects. On the other hand, the risk of anticipated potential effects on health other than allergenicity can be regarded as negligible for the currently approved GM crops in the EU, as they have been adequately addressed in the ERAs.

8.2   Selecting the baselines

Any assessment of long-term effects of GM crop cultivation should include comparison with conventional cultivation techniques. Baselines are needed mainly for monitoring and not for risk assessment since comparators are used in (ERA) experimental settings. The dynamics of agriculture should be recognized in baseline choice. Due to market demands, agriculture is often adapted to changing needs on very short time-scales. These adaptations could also affect health or the environment including biodiversity, in particular over the long-term. Analogies for possible long-term effects can be seen in conventional agriculture, e.g. the effects of herbicide use on weed flora, insecticide use on beneficial organisms or changes in soil cultivation in the context of conservation programmes. However, baselines are difficult to define in agro-ecosystems where the environment is constantly disturbed and where farmers orientate their cultivation plans to the needs of the market from year to year.

Predicting and assessing long-term effects will require information relating to the GMO, the status of the receiving environment, both in terms of baseline conditions and temporal changes following GMO introduction, and any potential receptors. A variety of sources, ranging from peer-reviewed data in scientific publications to anecdotal observation, could potentially provide beneficial information. Relevant sources according to EFSA (2008) are:

- Data in peer-reviewed journals,

- Scientific reports,

- Databases relating to conventional agricultural systems and the wider receiving environment (e.g. morphological studies of the hybrid zone),

- Monitoring reports of similar GMOs if previously or currently grown elsewhere,

- Data collected and presented by applicants,

- Consensus documents, such as those produced by the Organisation for Economic Co-operation and Development (OECD),

- Predictive models.

8.3   Recommendations for the way forward

Recommendations for the way forward in the area of environment were developed by analysing the overall results (sections 4 and 5) and the knowledge extracted from the CREA Space Workshop Environment (Annex 3).

In the case of potential impacts in relation to cultivation and management of HT crops, the 'modelling' tool was selected as the most promising tool based on the CSW[33] to address 'above-ground' long-term effects (e.g. on wider biodiversity of weeds and epigeic NTOs) before marketing. An experimental approach like FSE was considered to be useful to assess some impacts such as changes in pesticide use. However, cost-effectiveness and the more specific focus of selected activities needs to be considered in all approaches.

Biodiversity including weed abundance is regarded to be the most relevant indicator in this respect (see also Qi *et al.* 2008). Overall, monitoring of selected agricultural areas is potentially the most cost-effective way to address long-term effects. In any case, there is a need for baselines and for the definition of protection goals in agriculture. These are essential prerequisites to assess long-term effects of GMOs in comparison to conventional crops. In addition, guidelines for "good agricultural practice" can be a useful tool to avoid potential adverse effects of GMOs and to complement the potential actions listed in Table 8.

Based on the outcome of the CSW, two actions need more attention to reduce uncertainty regarding potential adverse impacts of GMOs in soil: research and monitoring. More research activities can be developed in defined typical 'hot spot' regions under worst-case conditions, which can reduce the effort for regional ERA aspects. 'Worst-case' sites could be

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[33] It should be noted that there is still inadequacy in current models (see also EFSA 2008). Useful modelling approaches for soil effects seem to be currently far from practical use.

selected e.g. according to representativeness regarding to agroecological conditions (e.g. Qi *et al.* 2008), occurrence of wild relatives of the GM plant, or presence of host plants for NTO-insects. Research on identifying agro-ecological regions was prioritized during the CSW (Annex 3). For post-market environmental monitoring, suggestions were developed to address uncertainty: Soil functionality, especially soil fertility, is one of the most relevant monitoring subjects. Here, further research can develop methods on combining information collection e.g. on herbicide application outside of fields, cultivation intensity and relative proportion of herbicide- treated non-field habitats. First attempts for selection of a limited number of bio-geographical areas have been made by Neemann *et al.* (2007). In such regions information selection and analysis may help to improve monitoring in a cost-effective way, e.g. for the HT crops of main concern (HT oilseed rape and HT sugar beet).

For detecting regionally differing Bt effects on NTOs, the use of functional insect groups representative of specific pre-selected EU regions is recommended. The identification of typical soil insect guilds or sum parameters (e.g. litter decomposition) could help to cover ecological functions that vary from region to region (see Annex 3, 3.2, p. A3-8 – A3-9). Long-term monitoring of the fate of Bt protein in parallel to commercialized Bt crop cultivation could help to test the Bt protein as an indicator for potential adverse effects on soil functions. However, this monitoring approach should be directed according to a 'worst-case-principle' in the selection of potential observation sites (Neemann *et al.* 2006). The BEETLE study did not examine whether the identified potential long-term effects could have further additive or synergistic effects (e.g. unintended interaction between different Bt proteins with effects on a wider range of NTO). This issue could be a task for future studies following BEETLE. There is currently no knowledge regarding whether additive or synergistic effects might occur. However, monitoring would also be in this case the best tool to identify such effects.

Monitoring is also the preferred method for identifying SM potato effects on herbivores, effects of HT crops on altered susceptibility to pathogens, and the potential impact of HT crops on landscape structure (potentially with the help of Geographic Information Systems - GIS). However, when sufficient experience has been gained, modelling might also help in the latter case.

Table 8[34]: Processes or scenarios causing potential long-term effects in the BEETLE study and potential ways forward to decrease uncertainty regarding the analysed crop/trait combinations in the EU

Process / Scenario	Priority[35]	Way forward	Potential actions to decrease uncertainty [36]
HT crops: Indirect effects of altered cultivation and management	1	Modelling and Monitoring of pre-selected agricultural areas	Data collection and monitoring on farm scale with studies on indicators for biodiversity or on fertilizer use.
HT crops and feralization	2	Monitoring	Data collection on persistence and invasiveness of HT oilseed rape and sugar beet
Bt crops: Resistance development of target organisms	2	Monitoring	Case-specific monitoring
Bt maize: Effect of Bt proteins on NTO soil micro-organisms and on soil function	2	Monitoring and Research	Performing studies under "worst-case" conditions in different soils to reduce the effort for regional ERA aspects. Monitoring the Bt proteins as indicator. Selection of appropriate indicators for soil functions.
Bt maize: Effect of Bt crops on NTO closely related to TO	2	Monitoring	Selection of appropriate organisms indicating food-web-related effects.
SM potato: Effects on herbivores	2	Monitoring	Herbivore surveys with selected indicators (e.g. Colorado beetle infestation).
Gene flow from HT crops to wild relatives (as NTO) and its consequences	2	Monitoring	Data collection on HT hybrids of oilseed rape and sugar beet
Effects of Bt protein root exudation and of herbicides on symbiotic organisms like mycorrhiza	3	Monitoring	Soil fertility – crop yield surveys as indirect indicators (altered management).
HT crops and altered susceptibility to pathogens	3	Monitoring	Pathogen surveys
HT crops and altered-landscape structure	3	Modelling and Monitoring	Geographic information (GIS) in combination with GMO cultivation registers
Allergenicity development (Health)	3	Monitoring	Routine surveillance systems

Concerning long-term health aspects, the BEETLE study recommends further improvement of the ERA methodology and the monitoring. The following specific conclusions can be drawn from the Literature Review "Health" and Online Survey "Health":

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[34] This table is in some parts identical with Table 1 in the executive summary.

[35] The BEETLE team suggests ranking according to the relative priority (1 = highest, 3 = lowest) based on Table 7

[36] The potential indicators are subject to further development over time.

- If health-related differences were to be detected between food or feed derived from a GM crop and its most closely related non-GM comparator, more conventional crop varieties should be used in feeding trials in order to cover the natural biological range of the measured parameters.

- In relation to the toxicology assessment, a range of comparators will help to assess whether any statistical differences detected between the GM plant and its non-GM counterparts are of biological relevance.

Through its prioritization of potential long-term effects of GM crops and the ways forward, the BEETLE study has identified 11 potential actions, listed in Table 1 and 8, to increase knowledge and thus to decrease uncertainties. The development of indicators for an appropriate EU-wide surveillance of long-term effects on soil and the wider biodiversity resulting from GM crop cultivation and management is proposed. The potential indicators listed in Table 1 are subject to further development over time by risk assessors and risk managers. These indicators for environmental monitoring are to be selected in accordance with the crop/trait combination and the receiving environment (for suggestions see chapter 6). Further improvement of the risk assessment methodology for health (human and animal) accompanied by surveillance (e.g. allergenicity) is proposed as the way forward to identify potential long-term effects of genetically modified plants (GM plants).

In conclusion, detectable long-term effects, whether anticipated pre-release or unanticipated, are likely to result from the accumulation of small effects over time (e.g. changes in population dynamics of competitor species) or the occurrence of new events at a later date (e.g. due to gene stacking or in response to external changes). Monitoring strategies in the EU could consist of both case-specific monitoring to assess the impact of identified adverse [including long-term] effects and general surveillance programmes to identify any unanticipated adverse effects. However, it is clearly not possible to monitor every eventuality so monitoring effort needs to be focused. Protection goals could potentially represent a useful set of targets for directing monitoring effort. For instance, priority could be given to the monitoring of protected species and habitats, wild relatives of the cultivated crop or the abiotic or biotic characteristics of soils or water, and agricultural development. It is also important to clearly identify which characteristic of the long-term effect is to be monitored and to ensure that the environmental significance of any measurements taken is understood. For instance, it is possible to detect subtle changes in soil microflora in the laboratory, but as yet hard to follow how this translates into environmental impact (EFSA 2008). Well-designed experimental approaches might be useful to assess suspected potential long-term processes before market approval. It was not within the remit of the BEETLE study to provide case-specific details on the design of such experiments. This issue could be another follow-up study beyond BEETLE.

9.   References

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10. Appendix 1:

Short summary of the evaluation of processes and scenarios in the BEETLE literature survey (a) and expert survey (b) together with the BEE-TLE team overall conclusion (c). The procedure and questions generated are presented in 'thesis' form together with a qualitative assessment[38]. Uncertainties in column a) are caused by contradictory data or small data basis. Uncertainties in column b) are caused by unambiguous voting by the experts or a high proportion of the answer 'don't know, insufficient data' (see also Annex 2 Tab. 3).

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[38] The assessment options (negligible, low, medium, high) and their interpretation are introduced in Annex 1 Table 3 and in Annex 2 Tables 1-3. Only three of these four categories were offered to experts within each assessment questionnaire. The category 'medium' was used as intermediate category if 'negligible' could already be excluded (see Annex 2 Tables 1, 2, and 3). However, prioritization in the BEETLE study led finally to classification of the potential adverse effects into the categories 'most likely', 'likely', and 'not likely'.

Category A: Persistence and Invasiveness

A.1		Increased fitness of the GM cultivar					
Process description		The new GM trait causes increased fitness of the GM cultivar. 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, (v) increased tolerance against herbicides.					
Evaluation		**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
HT Sugar beet	A.1	If complementary herbicide is applied in or outside of fields	high	A.1.4	Increasing number of volunteers occurring in GM HT cropping systems	low-medium	**high**
				A.1.5	The occurrence of feral HT plants at sites outside of fields (e.g. by seed spillage) establishing viable populations if the herbicide is applied (at sites like railway tracks or roadsides)	low-medium	**high**
HT Oilseed rape	A.1	If complementary herbicide is applied in or outside of fields	high	A.1.4	Increasing number of volunteers occurring in GM HT cropping systems	medium-high	**high**
				A.1.5	The occurrence of feral HT plants at sites outside of fields (e.g. by seed spillage) establishing viable populations if the herbicide is applied (at sites like railway tracks or roadsides)	medium-high	**high**

HT Soybean	A.1	If complementary herbicide is applied in or outside of fields	negligible	A.1.4	Increasing number of volunteers occurring in GM HT cropping systems	low	**negligible**
				A.1.5	The occurrence of feral HT plants at sites outside of fields (e.g. by seed spillage) establishing viable populations if the herbicide is applied (at sites like railway tracks or roadsides)	low	**negligible**
Bt maize	A.1	Bt expression could increase fitness	negligible	A.1.1	Increasing numbers of volunteers occur in GM Bt maize cropping system	low	**negligible**
				A.1.2	The occurrence of feral Bt maize plants at sites outside of fields (e.g. by seed spillage) establishing viable populations and consecutively increased invasiveness of Bt maize; possibly affecting nearby non-cultivated habitats	low	**negligible**
HT maize	A.1	If complementary herbicide is applied in or outside of fields	negligible	A.1.4	Increasing number of volunteers occurring in GM HT cropping systems	low	**negligible**
				A.1.5	The occurrence of feral HT plants at sites outside of fields (e.g. by seed spillage) establishing viable populations if the herbicide is applied (at sites like railway tracks or roadsides)	low	**negligible**
SM potato	A.1	SM could alter survival	negligible	A.1.8	Increasing number of potato volunteers occurring in GM cropping systems	negligible-low	**negligible**

Category A: Persistence and Invasiveness

A.2	Outbreeding depression after hybridization with wild relatives					
Process description	Outbreeding depression of the GM trait in wild relatives causes reduced GM hybrid fitness. With continuous gene swamping the recipient wild population has less and less genetic barrier, more GM hybrids are released, the wild species becomes less fit in natural or semi-natural habitats and the size of populations decreases. The process is favoured by (i) decreased stress tolerance (e.g. towards temperature, water, salinity), (ii) decreased number of progeny, (iii) increased pathogen susceptibility, (iv) decreased pest tolerance/resistance.					
Evaluation	**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
HT Sugar beet / Wild relative complex	A.2	EU: Wild relatives present	**low**	A.2.1	Due to outbreeding depression long-term gene introgression will decrease population sizes of wild relatives of GM HT crops (up to extinction)	low	**low**
HT Oilseed rape / Wild relative complex	A.2	EU: Wild relatives present	**low**	A.2.1	Due to outbreeding depression long-term gene introgression will decrease population sizes of wild relatives of GM HT crops (up to extinction)	low	**low**
HT Soybean	A.2	EU: No wild relatives present	**negligible**	A.2	For the considered crop/trait combinations no wild relatives are present. In consequence this issue was not asseed in the online survey.	**negligible**
Bt maize	A.2	EU: No wild relatives present	**negligible**			**negligible**
HT maize	A.2	EU: No wild relatives present	**negligible**			**negligible**
SM potato	A.2	EU: Wild relatives present; however, hybridization not successful	**negligible**			**negligible**

Category A: Persistence and Invasiveness

A.3	GM hybrids persist in and outside fields, and finally become invasive					
Process description	GM hybrids with HT traits will have selective advantage at sites where the non-selective herbicides are applied The outside fields persisting GM hybrids become invasive over time or changing environmental conditions, and finally affects 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					
Evaluation	**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
HT Sugar beet / Wild relative complex	A.3	EU: Wild relatives present If complementary herbicide is applied in or outside of fields	**high**	A.3.4	i) Out-competing natural geno-types of related cross compatible weedy relatives by HT-hybrids in GM HT cropping systems, ii) re-placing wild relatives of GM HT crops by gene swamping or by competition in habitats where her-bicides are occasionally used, iii) replacing other plant species in plant communities where herbi-cides are occasionally used	low	**high**[39]
HT Oilseed rape / Wild relative complex	A.3	EU: Wild relatives present If complementary herbicide is applied in or outside of fields	**high**	A.3.4	i) Out-competing natural geno-types of related cross compatible weedy relatives by HT-hybrids in GM HT cropping systems, ii) re-placing wild relatives of GM HT crops by gene swamping or by competition in habitats where her-bicides are occasionally used, iii) replacing other plant species in plant communities where herbi-cides are occasionally used	uncertain (due to unambi-guous voting)	**high**

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[39] Based on the unclear outcome of the online survey and the experience of the BEETLE team, the overall conclusion was set to 'high'

HT Soybean	**A.3**	EU: No wild relatives present	**negligible**	**A.3**	For the considered crop/trait combinations no wild relatives are present. In consequence this issue was not asseed in the online survey.	**negligible**
Bt maize	**A.3**		**negligible**			**negligible**
HT maize	**A.3**		**negligible**			**negligible**
SM potato	**A.3**		**negligible**			**negligible**

Category B: Altered Gene Transfer

B.1	**GM trait reduces pollination, e.g. due to a decreased attractiveness for pollinators** (Remark: In the Expert survey (questionnaire, part b) all cases (crop/trait combinations) were summarized into one general question, see below)						
Process description	Petal colours as factor for attracting pollinators as well as volatiles emitted from the flowers to ambient air play an important role for orientation of insects and plant pollination. Theoretically, some specific genetic modification might alter the chemical composition of flower scents. 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 can neither be expected nor are likely to produce such effects in the major crops studied in this review.						
Evaluation	**a) Literature Survey**			**b) Expert Survey**			**c) BEETLE Overall Conclusion**
HT Sugar beet / Wild relative complex	B.1	Not relevant since mainly wind-pollinated	negligible	B.1	Are phenotypic effects such as altered flower or fecundation biology sufficiently assessed during the GM approval procedure and variety registration currently applied in the EU?	yes[40]	negligible
HT Oilseed rape / Wild relative complex	B.1	For oilseed rape this effect potentially could occur; however no indications are existing	negligible				negligible
HT Soybean	B.1	No indication	negligible				negligible
Bt maize	B.1	Not relevant since mainly wind-pollinated	negligible				negligible
HT maize	B.1	Not relevant since mainly wind-pollinated	negligible				negligible
SM potato	B.1	No indication	negligible				negligible

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[40] Due to rare information available on such effects the BEETLE team concluded to summarize all potential cases (crop/trait combinations) into one general question. With respect to the generality of the questions the experts were asked to answer with "yes" or "no". The overall conclusion was given by the BEETLE team.

Category B: Altered Gene Transfer

B.2	**Altered flower phenology** (Remark: In the Expert Survey (questionnaire, part b) all cases (crop/trait combinations) were summarized into one general question, see below)						
Process description	GM traits may theoretically cause altered flower phenology, which would, 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.						
Evaluation	**a) Literature Survey**			**b) Expert Survey**			**c) BEETLE Overall Conclusion**
HT Sugar beet / Wild relative complex	B.2	No indication	**negligible**	B.2	Are phenotypic effects such as altered flower or fecundation biology sufficiently assessed during the GM approval procedure and variety registration currently applied in the EU?	Yes[41]	**negligible**
HT Oilseed rape / Wild relative complex	B.2	No indication	**negligible**				**negligible**
HT Soybean	B.2	No indication and no wild relatives in Europe	**negligible**				**negligible**
Bt maize	B.2	One indication reported, however no wild relatives do exist in Europe	**low**				**low**
HT maize	B.2	No indication and no wild relatives in Europe	**negligible**				**negligible**
SM potato	B.2	No indication and no cross-compatible wild relatives in Europe	**negligible**				**negligible**

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[41] Due to rare information available on such effects the BEETLE team concluded to summarize all potential cases (crop/trait combinations) into one general question. With respect to the generality of the questions the experts were asked to answer with "yes" or "no". The overall conclusion was given by the BEETLE team.

Category B: Altered Gene Transfer

B.3	**Altered compatibility reducing or favouring outcrossing**						
	(Remark: In the Expert Survey (questionnaire, part b) all cases (crop/trait combinations) were summarized into one general question, see below)						
Process description	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 can neither be expected nor are likely to produce such effects in the major crops studied in this review						
Evaluation	**a) Literature Survey**			**b) Expert Survey**			**c) BEETLE Overall Conclusion**
HT Sugar beet / Wild relative complex	**B.3**	No indication	**negligible**	**B.3**	Are phenotypic effects such as altered flower or fecundation biology sufficiently assessed during the GM approval procedure and variety registration currently applied in the EU?	yes[42]	**negligible**
HT Oilseed rape / Wild relative complex	**B.3**	No indication	**negligible**				**negligible**
HT Soybean	**B.3**	No indication and no wild relatives in Europe	**negligible**				**negligible**
Bt maize	**B.3**	No indication and no wild relatives in Europe	**negligible**				**negligible**
HT maize	**B.3**	No indication and no wild relatives in Europe	**negligible**				**negligible**
SM potato	**B.3**	No indication and no cross-compatible wild relatives in Europe	**negligible**				**negligible**

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[42] Due to rare information available on such effects the BEETLE team concluded to summarize all potential cases (crop/trait combinations) into one general question. With respect to the generality of the questions the experts were asked to answer with "yes" or "no". The overall conclusion was given by the BEETLE team.

Category B: Altered Gene Transfer

B.4	Altered fecundity increasing seed (gene) flow						
	(in the Expert Survey (questionnaire, part b) all cases (crop/trait combinations) are summarized into one general question, see below)						
Process description	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 can neither be expected nor are likely to produce such effects in the major crops studied in this review.						
Evaluation	**a) Literature Survey**			**b) Expert Survey**			**c) BEETLE Overall Conclusion**
HT Sugar beet / Wild relative complex	**B.4**	No indication	**negligible**	**B.4**	Are phenotypic effects such as altered flower or fecundation biology sufficiently assessed during the GM approval procedure and variety registration currently applied in the EU?	yes[43]	**negligible**
HT Oilseed rape / Wild relative complex	**B.4**	No indication	**negligible**				**negligible**
HT Soybean	**B.4**	No indication and no wild relatives in Europe	**negligible**				**negligible**
Bt maize	**B.4**	No indication and no wild relatives in Europe	**negligible**				**negligible**
HT maize	**B.4**	No indication and no wild relatives in Europe	**negligible**				**negligible**
SM potato	**B.4**	No indication and no cross-compatible wild relatives in Europe	**negligible**				**negligible**

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[43] Due to rare information available on such effects the BEETLE team concluded to summarize all potential cases (crop/trait combinations) into one general question. With respect to the generality of the questions the experts were asked to answer with "yes" or "no". The overall conclusion was given by the BEETLE team.

Category B: Altered Gene Transfer

B.5	**Increased frequency of horizontal gene transfer (HGT)**				
	(Remark: In the Expert Survey (questionnaire, part b) all cases (crop/trait combinations) are summarized into one general question, see below)				
Process description	A GM trait may theoretically increase the frequency of HGT from plants to populations of micro-organisms, 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 HGT. When all available information is taken into account the probability of functional gene transfer from plants into micro-organisms is extremely low and of negligible relevance for long-term effects.				
Evaluation	**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
HT genes	**B.5**	No indication	**negligible**	**B.5**	Should frequency of gene transfer from GM plants to micro-organisms be assessed during the approval process?	**negligible**
Bt genes	**B.5**	No indication	**negligible**		No[44]	**negligible**
SM genes	**B.5**	No indication	**negligible**			**negligible**

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[44] Due to rare information available on such effects the BEETLE team concluded to summarize all potential cases (crop/trait combinations) into one general question. With respect to the generality of the questions the experts were asked to answer with "yes" or "no". The overall conclusion was given by the BEETLE team.

Category B: Altered Gene Transfer

B.6		Specific Effects regarding interspecific hybrids or hybrids between GM crop and wild relatives						
Process description		Effects like altered flower phenology or altered fecundation biology are not restricted to the GM crop itself. Potentially also interspecific hybrids or hybrids between the GM crop and wild relatives could be affected						
Evaluation		**a) Literature Survey**			**b) Expert Survey**			**c) BEETLE Overall Conclusion**
HT Sugar beet / Wild relative complex	**B.6**	See B.1, B.2, B.3, B.4	**negligible**					**negligible**
HT Oilseed rape / Wild relative complex	**B.6**	See B.1, B.2, B.3, B.4	**negligible**					**negligible**
HT Soybean	**B.6**	See B.1, B.2, B.3, B.4	**negligible**	**B.6**	No question asked as outcome of literature survey was unambigious			**negligible**
Bt maize	**B.6**	See B.1, B.2, B.3, B.4	**low**					**low**
HT maize	**B.6**	See B.1, B.2, B.3, B.4	**negligible**					**negligible**
SM potato	**B.6**	See B.1, B.2, B.3, B.4	**negligible**					**negligible**

Category C: Interactions of the GM plant with Target Organisms

C.1		Effects on target pests					
Process description		Target organisms are defined as pests (mostly insects) which are targets of plant protection measures. GM traits lead over time to development of resistance in target organisms (pests), which results in a loss of environmentally desired plant protection tools.					
Evaluation		**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
Bt maize	**C.1**	Resistance development against target insects has not been detected in EU so far. However, in South Africa resistance development of a target lepidopteran species has been reported.	**low - high**[45]	**C.1.1**	Adverse effects affecting Bt maize crops will arise due to resitance development of target insects	**low**	**low – high**[47]

C.2		Effects on target pathogens				
Process description		Plant pathogens (bacteria and viruses) are targets of plant protection measures. GM traits lead over time to development of resistance in target organisms (pathogens), which results in a loss of environmentally desired plant protection tools. No report was found demonstrating development of pathogen resistance against GM crops. Whether viral pathogens are able to develop resistant against GM-crops is discussed controversially. However from the experience with other plant protection measures long-term resistance development cannot be precluded. In EU currently no GM crops - targeting pathogens - are on the market.				
Evaluation		**a) Literature Survey**			**b) Expert Survey**	**c) BEETLE Overall Conclusion**
	C.2	Not relevant since no pathogen related GM traits on the EU market	**negligible**		This issue was not adressed in the Online Survey since in EU no pathogen resistant GM crops are in the pipeline	**negligible**

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[45] Resistance development of target insects is depending on the availability and treatment of refuge sites cultivated with non-Bt-maize; in the South African case the reason for resistance development was that conventional maize cultivated at refuge site was not irrigated during drought

Category D: Interactions of the GM plant with Non-Target Organisms (NTO)

D.1		Direct adverse effects on plant-associated NTO				
Process description		Bt proteins act as toxins causing adverse effects on plant- associated NTO due to sublethal toxicity (chronic exposure) by consumption of pollen and plant tissue (e.g. on herbivores, pollinators, soil organisms, predators, parasitoids). Bt proteins are known to be highly specific to the target species groups. Due to expression of *lepidopteran* or *coleopteran* specific proteins in all parts of GM Bt plants (including pollen and roots) during the whole vegetation period, not just European Corn Borer (ECB) or Western Corn Rootworm (WCR) but also the larvae of NT butterflies or beetles may be affected.				
Evaluation		**a) Literature Survey**		**b) Expert Survey**		**c) BEETLE Overall Conclusion**
Bt maize	D.1	Bt maize lines expressing high levels of Cry1Ab protein in pollen	**low**	D.1.1	Adverse long-term effects expected for ECB resistant maize on lepidopteran species	negligible - low	**low**
Bt maize	D.1	Bt maize lines expressing Cry3Bb1 protein in entire plant including roots	**low**[46] (Ch. E.1)	D.1.2	Adverse long-term effects expected for WCR resistant maize on coleopteran species	negligible - low	**low**
Bt maize	D.1	Bt maize lines expressing Cry1Ab or Cry3Bb1 protein may affect bees	**negligible**[48] (Ch. E.3)	D.1.3	Adverse long-term effects expected for both traits (ECB resist.*or* WCR resistance) on bees	negligible	**negligible**
Bt maize	D.1	Bt maize lines expressing Cry1Ab or Cry3Bb1 protein may affect other insects	**low**[48] (Ch. D.2)	D.1.4	Adverse long-term effects expected for both traits (ECB resistance *or* WCR resistance) on other insects	negligible - low	**low**
Bt maize	D.1	Bt maize lines expressing Cry1Ab or Cry3Bb1 protein may affect soil micro-organisms by exudating the proteins or through decomposition	**low**[48] (Ch. E.1)	D.1.5	Adverse long-term effects expected for both traits (ECB resistance *or* WCR resistance) on micro-organisms	negligible - low	**low**
HT sugar beet HT oilseed rape HT soybean HT maize SM potato	D.1	not relevant	**negligible**	D.1	not relevant		**negligible**

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[46] This effect is discussed in the referenced chapter

Category D: Interactions of the GM plant with NTO

D.2	Adverse effects on NTO due to altered nutritional composition of the GM plant					
Process description	1. Nutritional composition of GM crops is altered by newly expressed proteins (e.g. Bt). 2. Altered starch composition caused by the genetic modification (amylose or amylopectine content) of potato tubers could alter fitness of plant associated (non-pest) herbivores or composers. Only NTOs feeding on tubers should be taken into consideration because altered starch composition occurs only in the tubers due to tuber specific promoter and knock down of granule bound starch synthase.					
Evaluation	**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
Bt maize	D.2	Bt maize lines expressing Cry1Ab or Cry3Bb1 protein	**low**	D.2.1	Long-term adverse effects are expected on herbivore or decomposer populations feeding on Bt protein expressing crops due to altered nutritional composition	negligible **(with uncertainty)**	**low (with uncertainty)**
SM potato	D.2	SM potatoes expressing starch with altered composition	**low (with uncertainty)**	D.2.2	Long-term effects are expected on herbivore populations feeding on altered starch expressing potato tubers	negligible - low **(with uncertainty)**	**low (with uncertainty)**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize**	D.2	not relevant	**negligible**	D.2	not relevant		**negligible**

Category D: Interactions of the GM plant with NTO

D.3		Effect of GM plants on tritrophic interactions					
Process		Bt-susceptible herbivores (2nd tropic level organism) feeding on Bt-toxin expressing host plants (1st trophic level organism) show a reduced nutritional value leading to a reduced fitness of predators or parasitoids due to altered nutritional value of host plants					
Evaluation	**a) Literature Survey**			**b) Expert Survey**			**c) BEETLE Overall Conclusion**
Bt maize	D.3	Bt maize lines expressing Cry1Ab protein	**low**	D.3.1	Long-term adverse effects are expected on populations or diversity of predators or parasitoids feeding on larvae with altered nutritional value.	negligible - low	**low**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize** **SM potato**	D.3	not relevant	**negligible**	D.3	not relevant		**negligible**

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Category D: Interactions of the GM plant with NTO

D.4	GM traits cause accumulation of toxic compounds in various environmental compartments						
Process description	Bt-proteins will either accumulate in decomposed substrate from Bt protein expressing crops or will be bound and accumulate in soil particles from decomposed Bt plant residues						
Evaluation	**a) Literature Survey**			**b) Expert Survey**			**c) BEETLE Overall Conclusion**
Bt maize	**D.4**	Bt maize lines expressing Cry1Ab or Cry3Bb1protein	**low (with uncertainty)**	**D.4.1**	Long-term adverse effects are expected on decomposing populations feeding on plant residues from Bt expressing crops or feeding as saprophytes on dead organic substrate in soils.	negligible - low	**low**
				D.4.2	Long-term adverse effects are expected on aquatic organisms due to accumulation of Bt proteins into water bodies being leached from soils or from Bt maize pollen or detritus being transported into waters	negligible - low	**negligible - low**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize** **SM potato**	**D.4**	**not relevant**	**negligible**	**D.4**	not relevant		**negligible**

Category D: Interactions of the GM plant with NTO

D.5		GM traits cause adverse effects on rhizosphere (plant-associated) bacteria and mycorrhizal fungi					
Process description		Exudates containing Bt protein may affect population size and activity of the rhizosphere organisms. There are no studies available showing the presence of transgene products in exudates of HT crops. Therefore only Bt-maize is taken into consideration					
Evaluation		**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
Bt maize	D.5	Bt maize lines expressing Cry1Ab or Cry3Bb1protein	**low** (with uncertainty regarding mycorrhiza)	D.5.1	Adverse long-term effects on abundance and diversity are expected on rhizosphere micro-organisms (except Rhizobia) of Bt-crops expressing lepidopteran specific or coleopteran specific proteins	uncertain (high proportion of answers 'in-suff. data')	**low** (with uncertainty)[47]
				D.5.2	Adverse long-term effects on abundance and diversity are expected on saprophytic or pathogenic fungi of the rhizosphere expressing lepidopteran specific or coleopteran specific proteins	negligible	**low - negligible**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize** **SM potato**	D.5	not relevant	**negligible**	D.5	This issue was not adressed in the Online Survey since there are no studies available showing the presence of transgene products in exudates of HT cropsor SM potato.		**negligible**

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[47] Uncertainties are related to the long-term importance of disturbances found for development of rhizosphere organisms (e.g. mykorrhiza) of Bt maize roots

Category D: Interactions of the GM plant with NTO

D.6		GM trait specific root exudations could lead to changes in interactions with symbiotic organisms					
Process description	Bt protein exudations into the rhizosphere may affect abundance, diversity and activity of symbiotic organisms. Studies on altered root exudates are not available for HT plants. Only Bt-maize is taken into considerations since it is the only Bt crop cultivated in EU. For maize N-fixation processes as in nodules of rhizobia are not relevant, too. Consequently, only potential interactions between Bt-maize and mycorrhizal fungi are taken into consideration						
Evaluation	**a) Literature Survey**			**b) Expert Survey**			**c) BEETLE Overall Conclusion**
Bt maize	D.6	Bt maize lines expressing Cry1Ab or Cry3Bb1protein	**low (with uncertainties)**	D.6.1	Adverse long-term effects on abundance and diversity are expected on rhizosphere micro-organisms (except Rhizobia) of Bt-crops expressing lepidopteran specific or coleopteran specific proteins	negligible	**– negligible – low (with uncertainties)**
HT Sugar beet, HT Oilseed rape HT Soybean HT maize SM potato	D.6	not relevant	**negligible**	D.6	This issue was not adressed in the Online Survey since there are no studies available showing the presence of transgene products in exudates of HT cropsor SM potato.		**negligible**

Category E: Effects on Ecological Functions

E.1		GM traits cause changes in soil functions					
Process description	GM traits cause changes in soil fertility (e.g. nutrient cycling, organic matter decomposition, biological N-fixation) due to (i) additive, synergistic or delayed effects on NTO including symbionts and (ii) altered nutritional composition of the plant and impact on decomposition						
Evaluation	**a) Literature Survey**			**b) Expert Survey**			**c) BEETLE Overall Conclusion**
Bt maize	E.1	Bt maize lines expressing Cry1Ab or Cry3Bb1protein	**low** (withuncertainty)	E.1.1	Bt toxins produced by Bt maize will partially be accumulated in necro-mass of maize residues and will be incorporated into soil organic matter. Additionally, the Bt toxins will be adsorbed at soil minerals like clay. Bt residues in soil will have adverse long-term effects on decomposition of soil organic matter	negligible - low	**low** (with uncertainty)
Bt maize	E.1	Bt maize lines expressing Cry1Ab or Cry3Bb1protein	**low**[48] (Ch. D.2)	E.1.2	In Bt maize a significant increase of lignin content in tissues was reported which could result in retardations of Bt residue decomposition. Distur-bances in decomposition processes as a result of increased lignin con-tent in Bt maize residues will occur in the long-term	negligible - low	**low**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize** **SM potato**	E.1	not relevant	**negligible**	E.1	This issue was not adressed in the Online Survey since there are no studies available showingsuch effects for HT cropsor SM potato.		**negligible**

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[48] This effect is addressed in the referenced chapter

Category E: Effects on Ecological Functions

E.2		GM traits cause changes in biological control					
Process description	GM traits cause changes in biological control due to (i) additive, synergistic or delayed changes in diversity and abundance of natural enemies and (ii) additive, synergistic or delayed changes in tritrophic interactions						
Evaluation	**a) Literature Survey**			**a) Expert Survey**			**c) BEETLE Overall Conclusion**
Bt maize	E.2	Bt maize lines expressing Cry1Ab or Cry3Bb1protein	low	E.2.1	Adverse long-term effects are expected for Bt maize, specifically on natural enemies due to sublethal toxic effects and in consequence for a successful biological control of pests, too	negligible	**low - negligible**
				E.2.2	Adverse long-term effects are expected for Bt maize, specifically on natural enemies due to a decreasing number of prey/hosts and in consequence for a successful biological control of pests, too	negligible - low	**low**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize** **SM potato**	E.2	not relevant	**negligible**	E.2	This issue was not adressed in the Online Survey since there are no studies available showing such effects for HT cropsor SM potato.		**negligible**

Category E: Effects on Ecological Functions

E.3		GM traits cause changes in pollination					
Process description		GM traits cause changes in pollination due to (i) additive, synergistic or delayed sublethal effects -> changes in diversity and abundance of the pollinator community and (ii) additive, synergistic or delayed altered attractiveness of flowers					
Evaluation		**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
Bt maize	**E.3**	Bt maize lines expressing Cry1Ab or Cry3Bb1protein	**low**	**E.3.1**	Bt proteins as produced by Bt maize are expressed in pollen. Long-term adverse effects on pollinators such as honey bees will affect pollination of crops	negligible	**low**
				B.2 / E.3.2	Bt proteins will cause an overall diminished attractiveness of plants for pollinators	negligible	**negligible**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize** **SM potato**	**E.3**	not relevant	**negligible**	**E.3**	This issue was not adressed in the Online Survey since there are no studies available showing such effects for HT cropsor SM potato.		**negligible**

Category F: Impacts of the specific Cultivation, Management and Harvesting Techniques

F.1		GM plant [management] causes increased/altered use of agrochemicals					
Process description		GM plant [management] causes increased/altered use of agrochemicals (e.g. pesticides) controlling herbicide tolerant weeds, persistent GM crops (volunteers) with adverse effects on NTO and/or ecological functions					
Evaluation		**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize**	**F.1**	HT crops expressing glyphosate or glufosinate tolerance proteins resulting in repeated use of non-selective herbicides	**low – high (with uncertainty)**	**F.1.1**	In comparison to conventional herbicide application and management long-term effects are expected due to increasing number of GM crop volunteers occurring in fields	uncertain (due to unambiguous voting)	**low – high**[49] (with uncertainty)
				F.1.2	In comparison to conventional herbicide application and management long-term effects are expected due to increased number of weeds being tolerant to non-specific herbicides	uncertain (due to unambiguous voting)	**low – high**[51] (with uncertainty)
				F.1.3	In comparison to conventional herbicide application and management long-term effects are expected due to a shift in weed communities causing changes in ecological functions (e.g. refuge for predators important for biocontrol)	low - high	**low – high**[51] (with uncertainty)
				F.1.4	In comparison to conventional herbicide application and management long-term effects are expected due to increasing use of minimal tillage system favouring changes of weed communities which leads to a decrease in ecological functions (e.g. refuge for predators important for biocontrol	negligible – low (with uncertainty)	**low – high**[51] (with uncertainty)

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[49] Potential adverse effects on NTOs or ecological functions due to development of herbicide tolerant weeds or volunteers are highly depending on the mode of herbicide management (date, frequency of applications, etc)

	F.1	HT crops expressing glyphosate or glu-fosinate tolerance proteins resulting in repeated use of non-selective herbicides		**F.1.5**	In comparison to conventional herbicide application and management long-term effects are expected due to decreasing non-crop plant biodiversity and thus feed resources for herbivores and associated taxa like birds	uncertain (due to unambiguous voting)	**low – high**[50] (with uncertainty)
				F.1.6	In comparison to conventional herbicide application and management long-term effects are expected due to later application date of herbicides (in higher crop and weed stands) leads to increasing drift of non-selective herbicides into field margins	uncertain uncertain (due to unambiguous voting)	**low – high**[52] (with uncertaintiy)
				F.1.7	In comparison to conventional herbicide application and management long-term effects are expected due to the use of additional chemical additives in herbicide formulations (e.g. due to late-season herbicide management), which cause additional environmental problems	uncertain (due to unambiguous voting)	**low – high**[52] (with uncertainty)
Bt maize	**F.1**	Bt maize lines expressing Cry1Ab or Cry3Bb1protein	**low**	**F.1.9**	In comparison to conventional herbicide application and management long-term effects are expected due to the development of secondary pests leading to new insecticide application (e.g. with adverse effects on NTO)	negligible - low	**low**
SM potato	**F.1**	not relevant	**negligible**	**F.1**	not relevant	negligible	**negligible**

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[50] Potential adverse effects on NTOs or ecological functions due to development of herbicide tolerant weeds or volunteers are highly depending on the mode of herbicide management (date, frequency of applications, etc)

Category F: Impacts of the specific Cultivation, Management and Harvesting Techniques

F.2		GM plant [management] causes indirect changes in susceptibility of crops against plant pathogens					
Process description		GM plant [management] causes indirect changes in susceptibility of crops against plant pathogens with adverse effects on NTO due to increased use of other pesticides					
Evaluation		**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize**	**F.2**	HT crops expressing glyphosate or glufosinate tolerance proteins resulting in repeated use of non-selective herbicides	**low (with uncertainty)**	**F.2.1**	In view of non-selective herbicide application long-term effects are expected due to altered farming practices e.g. low-till farming, altered weed management etc. This effect will cause increased susceptibility of the crops to plant pathogens with adverse effects on NTOs due to increased use of other pesticides	negligible **(with uncertainty)**	**low** **(with uncertainty**
				F.2.2	In view of non-selective herbicide application long-term effects are expected due to reduction of weed populations which will lower the attractiveness of insect pests for natural predators. This effect could enhance insect infestations with adverse effects on NTOs due to increased use of other pesticides	negligible – low **(with uncertainty)**	**low - high** **(with uncertainty)**
Bt maize	**F.2**	not relevant	**negligible**	**F.2**	not relevant		**negligible**
SM potato	**F.2**	not relevant	**negligible**	**F.2**	not relevant		**negligible**

Category F: Impacts of the specific Cultivation, Management and Harvesting Techniques

F.3		GM plant [management] causes indirect changes and adverse effects on agro-biodiversity						
Process description		GM plant [management] causes indirect changes and adverse effects on agro-biodiversity due to knock-on-effect of additive, synergistic or delayed effects cropping systems in agricultural landscape						
Evaluation		**a) Literature Survey**			**b) Expert Survey**			**c) BEETLE Overall Conclusion**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize**	**F.3**	HT crops expressing glyphosate or glufosinate tolerance proteins resulting in repeated use of non-selective herbicides	**low - high[51]** (depending on management)	**F.3.1[52]**	In comparison to conventional management practices long-term effects are expected due to decrease in biodiversity via loss of habitat niches	negligible – low	**low - high[53]**	
				F.3.2[54]	In comparison to conventional management practices long-term effects are expected due to increased fertilizer use in GM cropping systems affecting NTO abundance or community structure	negligible - low **(with uncertainty)**	**low - high**	
Bt maize	**F.3**	not relevant	**negligble**				**negligible**	
SM potato	**F.3**	no data available, but no indiction in the risk assessment	**negligible (with uncertainty)**				**negligible – low (with uncertainty)**	

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[51] Intensity of adverse effects on biodiversity are depending on the mode of management
[52] In the expert survey the questions were not differentiated between the crop/trait combinations
[53] Based on the online survey and the experience of the BEETLE team, the overall conclusion was set to 'low-medium'. However, this issue is covered as higher priority in the recommendations (see case 1 in the executive summary)

Category F: Impacts of the specific Cultivation, Management and Harvesting Techniques

F.4		GM plant [management] causes indirect changes in fertilizer use					
Process description		GM plant [management] causes indirect changes in fertilizer use with adverse effects on NTO and/or ecological functions					
Evaluation		**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize**	F.4	HT crops expressing glyphosate or glufosinate tolerance proteins resulting in repeated use of non-selective herbicides	**low - high** (for HT Soybean) **low** (for HT Sugar beet, HT Oilseed rape, HT maize)	F.4.1	Long-term adverse effects are expected by toxic effects of non-selective herbicides on rhizosphere organisms affecting mineralization or N-fixation, leading to adverse effects on ecological functions and increased fertilizer use (with impact on eutrophication of soils and water bodies)	negligible – low **(with uncertainty)**	**low - high** (for HT Soybean) **(with uncertainty)** **low** (for HT Sugar beet, HT Oilseed rape. HT maize) **(with uncertainty)**
				F.4.2	Long-term adverse effects are expected by increased phosphorus application in GM cropping systems leading to adverse effects on mycorrhiza fungi and ecological functions	negligible – low **(with uncertainty)**	**low (with uncertainty)**
Bt maize	F.4	not relevant	**negligible**		not relevant		**negligible**
SM potato	F.4	not relevant	**negligible**		not relevant		**negligible**

Category F: Impacts of the specific Cultivation, Management and Harvesting Techniques

F.5			GM plant [management] causes indirect changes in landscape structure				
Process description	GM crop cultivation will lead to an altered cultivation practice due to the coexistence requirements (e.g. following minimum distance needs: e.g. larger fields, larger distances between specific crops), resulting in loss of connectivity and reduced local biodiversity						
Evaluation	**a) Literature Survey**			**b) Expert Survey**			**c) BEETLE Overall Conclusion**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize** **Bt maize** **SM potato**	**F.5**	HT crops expressing glyphosate or glufosinate tolerance proteins resulting in repeated use of non-selective herbicides or Bt crops expressing Cry-proteins or SM potatoes with altered starch composition	**low** (withuncertainty)	**F.5.1**	Long-term adverse effects are expected by enlargement of fields connected with increasing habitat fragmentation and increasing isolation of plant and animal populations	negligible - low	**low** (with uncertainty)

Category G: Potential Interactions with the Abiotic Environment

G.1	Use of GM plants and climate change					
Process description	Use of GM plant causes adverse effect on the abiotic environment due to increased production of green house gases by GM plants [climate change]					
Evaluation	**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize** **Bt maize** **SM potato**	G.1	HT crops expressing glyphosate or glufosinate tolerance proteins resulting in repeated use of non-selective herbicides or Bt crops expressing Cry-proteins or SM potatoes with altered starch composition (uncertainties due to few publications)	**negligible (with uncertainty)**	G.1.1	Adverse effects on abiotic environment are expected for cultivation of Bt maize, HT oilseed rape, HT sugar beet, HT soybean and starch modified potato due to an intensification of agriculture solely related to the GM crops. Its cultivation will be connected with higher use of fossil energy resources, global deforestation and decline of organic soil fraction resulting in additional release of carbon dioxide	negligible	**negligible (with uncertainty)**

Category G: Potential Interactions with the Abiotic Environment

G.2	GM plants and mineral nutrient erosion					
Process description	Use of GM plant causes adverse effect on the abiotic environment due to increased mineral nutrient erosion and fertilizer leaching into water bodies affecting water quality					
Evaluation	**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
HT Sugar beet **HT Oilseed rape** **HT Soybean** **HT maize** **Bt maize** **SM potato**	**G.2**	HT crops expressing glyphosate or glufosinate tolerance proteins resulting in repeated use of non-selective herbicides or Bt crops expressing Cry-proteins or SM potatoes with altered starch composition	**low (with uncertainty)** (HT crops) **negligible** (Bt maize, SM potato)	**G.2.1**	Adverse effects on abiotic environment are expected for Bt maize, HT oilseed rape, HT sugar beet, HT soybean and starch modified potato, due to an intensification of agriculture with higher use of fertilizers and reduction in natural nitrogen fixation (toxic herbicide effects) resulting in an increase of mineral nutrient erosion and leaching of fertilizer into water bodies affecting water quality	negligible-low	**low (with uncertainty)** (HT crops) **negligible** (Bt maize and SM potato)

Category G: Potential Interactions with the Abiotic Environment

G.3	HT or IR GM plants and soil exchange capacity					
Process description	Use of HT or IR GM plant causes adverse effects on the abiotic environment by covering soil particle exchange sites e.g. due to more release of Bt protein or of herbicide molecules including their metabolites into soils					
Evaluation	**a) Literature Survey**			**b) Expert Survey**		**c) BEETLE Overall Conclusion**
HT Sugar beet HT Oilseed rape HT Soybean HT maize Bt maize SM potato	G.3	HT crops expressing glyphosate or glufosinate tolerance proteins resulting in repeated use of non-selective herbicides Bt crops expressing Cry-proteins SM potatoes with altered starch composition	**low** (Bt maize, HT crops) **negligible** (SM potato)	G.3.1	The use of GM plants causes adverse effects on the abiotic environment by covering soil particle exchange sites e.g. due to enhanced release of ionic protein and enhanced release of molecules of the non-selective herbicides into soils. The reduced soil exchange capacity will negatively affect ecological functions of soil (pH-buffer, mineral nutrient availability)	negligible	**low** (Bt maize and HT crops) **negligible** (SM potato)

11. Annexes