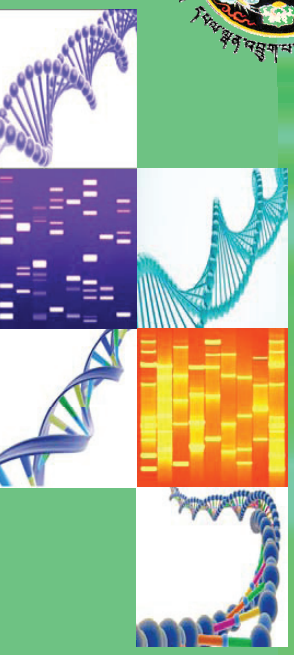

Guideline on Environmental Risk Assessment of Genetically Modified Plants

BHUTAN AGRICULTURE AND FOOD REGULATORY AUTHORITY (BAFRA)
MINISTRY OF AGRICULTURE AND FORESTS

FOREWORD

This document provides guidance for the environmental risk assessment (ERA) of genetically modified (GM) plant products submitted for import for food and feed or processing. It considers the unintended release of the imported plant products into the environment but not their cultivation. It does not include viable plants or plant parts introduced for propagation, cultivation or GM plants or plant products used for pharmaceutical or medicinal purposes.

The Guideline on Environmental Risk Assessment (ERA) of GM plants has been prepared with reference to the EFSA guidance documents on ERA (EFSA 2010, 2006 a, b) and Environmental Risk Management (ERM) and Post Market Environmental Management (PMEM) (EFSA 2006, 2011) which are in accordance with current international legislation associated with international agreements on GMOs, Organisation for Economic Co-operation and Development (OECD), guidelines and based on experiences of other countries.

This document provides guidance for assessing potential effects of GM plant products on the environment and the rationales for the data requirements for a comprehensive ERA of GM plant products. The ERA should be carried out on a case-by-case basis following a step-by-step assessment approach, meaning that the required information may vary depending on the type of the GM plants, their products and the trait(s) concerned, their intended use(s), and the potential receiving environment(s).

In addition, the guideline describes strategies and methods for monitoring imports and distribution of viable plant products for both anticipated risks identified in the risk assessment and for unanticipated effects. The guideline also contains consideration of several cross-cutting considerations (e.g. choice of comparator, the environmental protection goals of the receiving environment(s), general statistical principle (s) that needs to be considered in the ERA and ERM).

This document has been developed with support from the National Biosafety Framework Project implemented by Bhutan Agriculture and Food Regulatory Authority with the financial and technical support from the Global Environment Facility (GEF) and United Nations Environment Program (UNEP).

Karma Dorji
Director General

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ACRONYMS

ACRE	Advisory Committee on Releases to the Environment guidance
BAFRA	Bhutan Agriculture and Food Regulatory Authority
CAC	Codex Alimentarius Commission
DNA	Deoxyribonucleic Acid
EC	European Commission
EFSA	European Food Safety Authority
ERA	Environmental Risk Assessment
FAO	Food and Agriculture Organisation
GEF	Global Environment Facility
UNEP	United Nations Environment Program
GM	Genetically modified
GMO	Genetically Modified Organism
NTO	Non-Target Organism
OECD	Organisation for Economic Co-operation and Development
r-DNA	Recombinant Deoxyribonucleic Acid
PMEM	Post-market Environmental Management and Monitoring
WHO	World Health Organisation

GLOSSARY

Assessment endpoint: a natural resource or natural resource service that needs protection. It is the valued attribute of a natural resource worth of protection (Suter, 2000).

Baseline: is defined as a point of reference against which future changes can be compared (EC, 2002).

Biogeographical region or zone: is defined as spatial scale of Earth's surface containing related biotic (e.g. fauna and flora) and abiotic (e.g. climate, soil, or elevation) conditions.

Case-by-case: is defined as the approach by which the required information may vary depending on the type of the GMOs concerned, their intended use and potential receiving environment, taking into account i.e. GMOs already in the environment (EC, 2001).

Deliberate release: is defined as any intentional introduction into the environment of a GMO or a combination of GMOs for which no specific containment measures are used to limit their contact with and to provide a high level of safety for the general population and the environment (EC, 2001).

Desk study: is defined as an investigation of relevant available information, often before starting practical study of a problem.

Ecosystem services: include all services provided by ecosystems, e.g. production of food, fuel, fibre and medicines, regulation of water, air and climate, maintenance of soil fertility, cycling of nutrients. Ecosystems services are distinct from ecosystem functions by virtue of the fact that humans, rather than other species, benefit directly from these natural assets and processes (Millennium Ecosystem Assessment, 2005).

Effects:

- **Adverse effects:** are defined as a harmful and undesired effects consisting of measurable changes of protected entities (e.g. change in a natural resource or measurable impairment of a natural resource service) beyond accepted ranges.

- **Unintended effects:** are defined as consistent differences between the GM plant and its conventional counterpart, which go beyond the primary intended effect(s) introducing the target gene(s).

- **Direct effects:** are defined as primary effects on human health or the environment which are a result of the GMO itself and which do not occur through a causal chain of events (EC, 2001).

- **Indirect effects:** are defined as to effects on human health or the environment occurring through a causal chain of events, through mechanisms such as interactions with other organisms, transfer of genetic material, or changes in use or management (EC, 2001).

- **Immediate effects:** are defined as effects on human health or the environment which are observed during the period of the release of the GMO. Immediate effects may be direct or indirect (EC, 2001).

- **Delayed effects:** are defined as effects on human health or the environment which may not be observed during the period of the release of the GMO, but become apparent as a direct or indirect effects either at a later stage or after termination of the release (EC, 2001).

- **Cumulative long-term effects:** are defined as the accumulated effects of consents on human health and the environment, including 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 (EC, 2001).

Environmental harm: is defined as a measurable adverse change in a natural resource or measurable impairment of a natural resource service which may occur directly or indirectly (EC, 2004).

Environmental risk assessment: is defined as the evaluation of risks to human health and the environment, whether direct or indirect, immediate or delayed, which the deliberate release or the placing on the market of GMOs may pose and carried out in accordance with Annex II (EC, 2001).

Fitness: is defined as the number of seeds (or propagules) produced per seed sown, and includes the whole life cycle of the plant (Crawley et al., 1993). Enhanced fitness can be defined as a characteristic of an individual or sub-population of individuals that consistently contribute more offspring to the subsequent generation (Wilkinson and Tepfer, 2009).

Functional groups: are defined as non-phylogenetic, aggregated units of species sharing an important ecological characteristic and playing an equivalent role in the community (Cummins, 1974, Smith, 1997, Steneck, 2001, Blondel, 2003).

Genetically modified organism (GMO): is defined as an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination (EC, 2001).

Hazard (harmful characteristics): is defined as the potential of an organism to cause harm to or adverse effects on human health and/or the environment (EC, 2002).

Limits of concern: are defined as the minimum ecological effects that are deemed biologically relevant and that are deemed of sufficient magnitude to cause harm. These limit, limits of concern are set for each assessment endpoint in the problem formulation.

Measurement endpoint: is defined as a quantifiable indicator of change in the assessment endpoint, and constitute measures of hazard and exposure. Examples include: fitness, growth, behaviour, development.

Problem formulation: is defined as the process including the identification of characteristics of the GM plant capable of causing potential adverse effects to the environment (hazards) of the nature of these effects, and of pathways of exposure

through which the GM plant may adversely affect the environment (hazard identification). It also includes defining the assessment endpoints and setting of specific hypothesis to guide the generation and evaluation of data in the next risk assessment steps (hazard and exposure characterisation).

Production system: is defined as the specific use of the GM plant, the context in which the GM plant is grown, its cultivation (including crop rotation), harvesting and management, and the genetic background in which the transgenic trait has been introduced.

Protection goals: are defined as natural resources (e.g. arthropod natural enemies, bees) or natural resource services (e.g. regulation of arthropod pest populations, pollination) that are to be protected as set out by EU legislations.

Risk: is defined as the combination of the magnitude of the consequences of a hazard, if it occurs, and the likelihood that the consequences occur (EC, 2002).

Receiving environment: is defined as the environment into which the GM plant(s) will be released and into which the transgene(s) may spread.

Stacked events: are GM plants in which two or more single events have been combined by conventional crossing.

Step-by-step approach: is used in this ERA guideline to describe the six assessment steps for the ERA:

1. Problem formulation;
2. Hazard characterisation;
3. Exposure characterisation;
4. Risk characterisation;
5. Risk management strategies and
6. Overall risk evaluation and conclusions.

This assessment approach is different from the Stepwise approach defined hereunder.

Stepwise Approach: is defined as all the steps (used in the sense of 'containment-level') beginning with experiments in the contained use system through temporarily and spatially restricted deliberate release up to placing on the market, where data should be collected stepwise as early as possible during the procedure (EC, 2002).

Stressor: the GM plant itself, the transgene(s) in this organismal context and its products, are all considered as potential stressor.

Weight-of-evidence approach: is defined as the use of scientific evidence from various data sources to support assessment conclusions.

1. INTRODUCTION

This document provides guidance for the ERA of GM plants submitted within the framework of the Cartagena Protocol on the import of GMOs. It covers the ERA of imported non-viable GM Plant products as food and feed containing or consisting of GM plants or produced from GM plants, and GM plant products used for non-food purposes such as industrial uses. It does not include GM plants or plant products used for pharmaceutical or medicinal purposes.

It considers intended and unintended release of GM plant products into the environment but does not include assessment of the propagation and cultivation of GM Plants within Bhutan for experimental or production purposes. However, it does consider the possibility of unavoidable low level admixture of GM seeds and viable materials in imported grain, seeds and other plant products and the possibility of unintended and adventitious spread across borders into Bhutan by the activities of man and by natural dissemination of seeds and pollens. Bhutan is bordered by India and China and these countries cultivate and develop GM crops (e.g. cotton, brinjal, rice) and other plants (e.g. GM poplar).

This document also considers the establishment of post-market environmental management and monitoring (PMEM) of risks and uncertainties identified in the ERA, and also the establishment of environmental monitoring of unintentional introduction of viable GM plant products.

This ERA guideline provides detailed guidance for those conducting risk assessments in support of applications to introduce non-viable GM plant products into Bhutan and for Bhutan Agriculture and Food Regulatory Authority in Bhutan to assess these applications. The ERA guideline addresses the environmental issues, while all molecular characterisation issues and food and feed safety issues (such as toxicology, allergenicity, nutritional aspects) are addressed in the Guideline for Risk Assessment of Food and Feed products derived from GM plants.

This ERA guideline discusses traceability and labelling in relation to PMEM of imported and transported GM plants, but not in relation to production, processing and supply chains and co-existence. Socio-economic, ethical and risk/benefit issues are also outside the scope of this guideline.

This guideline does not cover GM organisms which are not plants and therefore excludes consideration of GM micro-organisms and GM animals.

Cross-references between these two Guidelines are made wherever necessary.

2. STRATEGIES FOR ERA OF IMPORTED GM PLANT PRODUCTS

The purpose of the ERA is to assess the potential adverse effects on the environment through the introduction of GM plant products.. The ERA of GM plant products involves generating, collecting and assessing information on a GM plant in order to determine its potential adverse impact relative to its non-GM plant comparator, and thus assessing its comparative safety. The underlying assumption of the comparative assessment for GM plant products is that the biology of traditionally cultivated plants from which the GM plants have been derived, and the appropriate comparators is well known. To this end the concept of familiarity was developed by the OECD (OECD, 1993).

In the ERA, it is appropriate to draw on previous knowledge and experiences and to use the appropriate comparator in order to highlight differences associated with the GM plant in the receiving environment(s). The ERA for GM plant products containing events combined by conventional breeding (stacked events) may also involve comparison with GM events as well as appropriate comparators (Appendix 4).

The ERA should be carried out in a scientifically sound and transparent manner. The ERA should include any relevant data (e.g. research data, scientific publications, monitoring reports) obtained prior to and/or during the risk assessment process. The purpose of the performed studies, the data and their interpretation, as well as the assumptions made during the ERA, should be clearly described. In addition, the use of models could provide further information useful for ERA. The final risk evaluation should result in qualitative and if possible quantitative conclusions on risk that inform risk managers and allow decision-making. Any uncertainties associated with the identified risks should be outlined.

The ERA should be carried out on a case-by-case basis, meaning that the required information may vary depending on the species of GM plants concerned, the introduced genes, their intended use(s) and the potential receiving environment(s), taking into account specific cultivation requirements and the presence of other GM plants in the environment.

2.1. Comparative safety assessment as a general principle for the risk assessment of GM plants

The risk assessment strategy for GM plant products seeks to use appropriate methods to compare the GM plant and derived products with their appropriate comparator (Appendix 1). Thus non-GM plants and their products serve as comparators for the ERA of GM plants. The comparative safety assessment is being followed in order to identify differences caused by either intended or unintended effects.

Comparative safety assessment includes molecular characterisation, the agronomic and phenotypic characteristics of the GM plant in question, as well as its compositional analysis (OECD, 1993, FAO/WHO, 1996).

Any type of genetic modification of plants may also result in unintended effects. The ERA is focused on the identification and characterisation of both intended and unintended effects with respect to possible adverse impacts on the environment. Effects can be direct and indirect, immediate and delayed, including cumulative long-term effects.

Intended effects are those that are designed to occur and which fulfil the original objectives of the genetic modification. Alterations in the phenotype may be identified through a comparative analysis of growth performance, yield, pest and disease resistance, etc. Intended alterations in the composition of a GM plant compared to its appropriate comparator, may be identified by measurements of single compounds.

Unintended effects of the genetic modification are considered to be consistent (non-transient) differences between the GM plant and its appropriate comparator, which go beyond the primary intended effect(s) of introducing the transgene(s). Since these unintended effects are event-specific, applicants must supply data on the specific event.

Sources of data that may reveal such effects are:

- **Molecular characterisation**
  A starting point in the identification of potential unintended effects is to analyse the DNA construct and insertion site to establish whether the insertion is likely to have potential effects other than the intent of the original genetic modification (e.g. unintended effect(s) could be due to loss of function of an endogenous gene at the insertion site).

- **Compositional analysis**
  Unintended effects may be detected through the comparison of the compositional characteristics of the GM plant with its appropriate comparator (e.g. unintended effect(s) could potentially be linked to metabolic perturbations).

- **Agronomic and phenotypic characterisation**
  Unintended effects may also be detected through the comparison of the phenotypic and agronomic characteristics of the GM plant with its appropriate comparator (e.g. unintended effects could be linked to morphological alterations).

- **GM plant-environment interactions**
  Unintended effects may be detected through comparisons of biotic and abiotic interactions of the GM plant and its appropriate comparator with components of their receiving environment(s). *In planta* data are the fundamental source of information (e.g. unintended effects could be linked to changes in the interaction of the GM plant on functionality of NTO guilds).

Statistically significant differences between the GM plant and its appropriate comparators, which are not due to the intended modification, may indicate the occurrence of unintended effects, and should be assessed specifically with respect to their biological relevance and potentially hazardous environmental implications. The outcome of the comparative safety assessment allows the determination of those "identified" characteristics that need to be assessed for their potential adverse effects in the environment, regardless of whether they were intended or unintended, and will thus further structure the ERA.

The level and routes of environmental exposure to the imported GM plant products shall be taken into account. Comparisons should be made between the GM plant and its appropriate comparators (Appendix 1), wherever applicable, grown in relevant production systems and similar environments.

2.2. Objectives of the different steps of the environmental risk assessment

The objective of the ERA is on a case-by-case basis to identify and evaluate potential adverse effects of the GM plant product, direct and indirect, immediate or delayed (including cumulative long-term effects) on the receiving environment(s) where the GM plant product will be released.

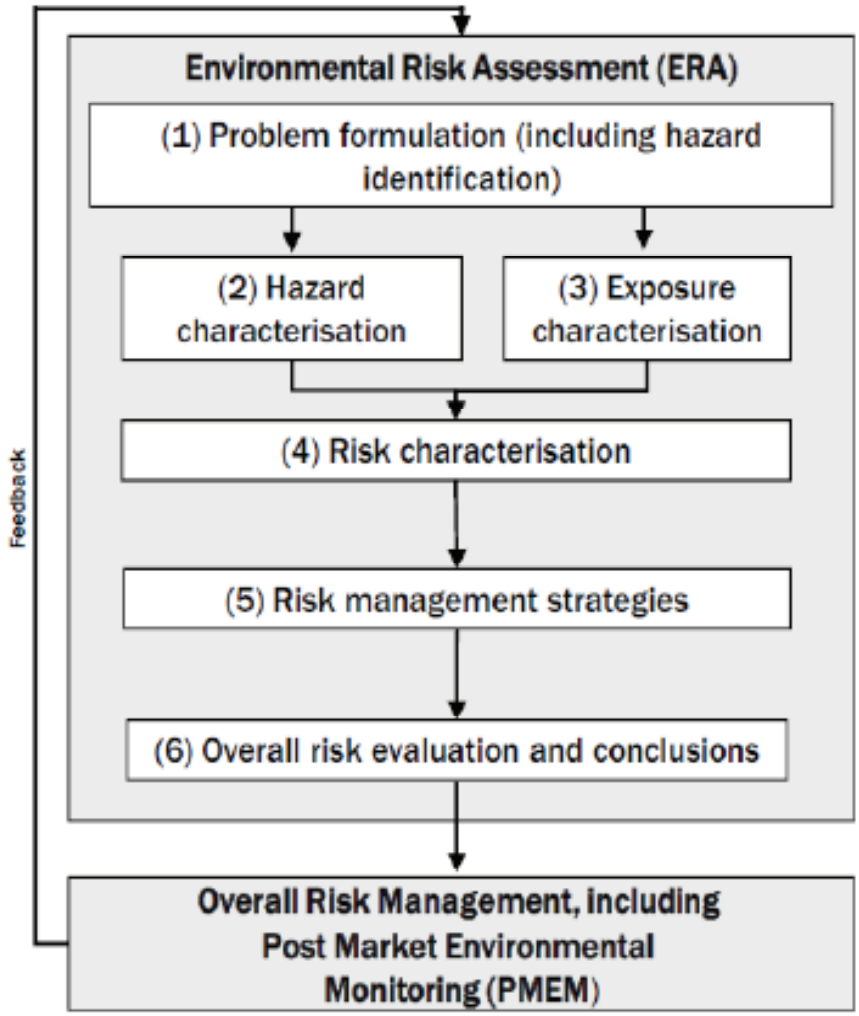

Figure 2: Six steps within the environmental risk assessment (ERA) and relationship to risk management and monitoring.

Source: EFSA 2010

The ERA consists of the six steps:

1. Problem formulation including hazard identification
2. Hazard characterisation
3. Exposure characterisation
4. Risk characterisation
5. Risk management strategies
6. Overall risk evaluation and conclusions

The ERA is conducted starting with Step 1 and moving towards Step 6; Step 2 and 3 can, however, be carried out in parallel (Figure 2). If no hazards are identified in Step 1 then no further risk evaluation is required. Any uncertainty inherent to the different steps of the ERA should be highlighted and quantified as much as possible.

2.2.1. Step 1: Problem formulation: including hazard identification

The risk assessment begins with problem formulation in which all important questions for the risk characterisation are identified. Problem formulation helps to make the risk assessment process transparent by explicitly stating the assumptions underlying the risk assessment.

In this document, problem formulation starts with the identification of whether the GM plant product contains or consists of any viable material from which plants could be generated. The characteristics of any establishing GM plant capable of causing potential adverse effects to the environment (hazards), of the nature of these effects, and of pathways of exposure through which the GM plant may adversely affect the environment are considered. It also includes defining assessment endpoints and setting of specific hypotheses to guide the generation and evaluation of data in the next risk assessment steps (hazard and exposure characterisation). In this process, both existing scientific knowledge and knowledge gaps are considered.

Problem formulation starts with the identification of the hazards of the GM plant products and their use. A comparison of the characteristics of the GM plant with those of its appropriate comparator (plant) enables the identification of differences in the GM plant that may lead to harm (Chapter 2.1). These differences are identified in the problem formulation process in order to focus the ERA on the potential environmental consequences of these differences. While some differences may be deemed irrelevant to the assessment, others will need to be assessed for their potential to cause harm. More detailed guidance for applicants on how to apply problem formulation on specific areas of risk to be addressed in the ERA is provided in Chapter 3 of this document.

After identifying the hazards and potential adverse effects that warrant further consideration, problem formulation considers the available information on exposure through which the GM plant product may interact with the environment. The intended uses of a GM plant product, such as processing, food, feed, and the pathways and levels of exposure of the GM plant product to the environment will vary. The problem formulation will consider exposure:

1) Via the accidental release into the environment of viable propagules, such as seeds, of the GM plant during transportation and processing potentially leading to sporadic feral GM plants and;
2) Indirect exposure, for example, through manure and faeces from the gastrointestinal tracts mainly of animals fed the GM plant products, and/or;
3) Organic plant matter either imported as a fertiliser or soil amendment or derived from other bioproducts of industrial processes.

A crucial step in problem formulation is to identify the aspects of the environment that need to be protected from harm according to the environmental protection goals of Bhutan. Because protection goals are often general concepts, they should be translated into measurable assessment endpoints (Suter, 2000, Romeis et al., 2008, Sanvido O, 2009,

EFSA, 2010f, Wolt et al., 2010). Defining assessment endpoints is necessary to focus the risk assessment on assessable/measurable aspects of the environment – a natural resource (e.g. natural enemies) or natural resource service (e.g. biological control functions of pest populations performed by natural enemies) that could adversely be affected by the GM plant and that require protection from harm.

Subsequently, within the problem formulation, the identified potential adverse effects need to be linked to assessment endpoints in order to derive testable hypotheses that allow quantitative evaluation of the harm posed to those assessment endpoints. The hypotheses are of importance as they will further guide the setting up of a methodological approach on how to evaluate the magnitude of harm. Through hypothesis, assessment endpoints are translated into quantitatively measurable endpoints, termed measurement endpoints (such as measurements of mortality, reproduction, abundance). A measurement endpoint can be regarded as an indicator of change in the assessment endpoint, and constitutes measures of hazard (Chapter 2.2.2) and exposure (Chapter 2.2.3).

Finally, for each measurement endpoint, the level of environmental protection to be preserved is expressed through the setting of 'limits of concern' which may take one of two forms. For studies in the environment(s) that are controlled (Chapter 3.4) the limits of concern will usually be trigger values which, if exceeded, will either lead to conclusions on risks or the need for further assessment in receiving environment(s). For field studies, the limits of concern will reflect more directly the minimum effect that is considered to potentially lead to harm (also see Appendix 2). If these limits are exceeded, then detailed quantitative modelling of exposure may be required to scale up effects at the field level both temporally and spatially. Limits of concern can be defined by literature data, modelling, existing knowledge and policy goals.

The information considered in problem formulation can take many forms, including published scientific literature, scientific and expert opinions, and/or research data. Available data from analyses performed to characterise the GM plant, including molecular, compositional, agronomic/phenotypic analysis and plant-environment interactions, shall also address the occurrence of unintended effects. The relevance of data generated outside Bhutan on the GM plant should be related to the environment and conditions in Bhutan.

Problem formulation is always on a case-specific basis:

- Identify characteristics of the GM plant product and, where appropriate, the associated production and management systems capable of causing potential adverse effects to the environment;
- Identify the potential adverse effects linked to those harmful characteristics;
- Identify exposure pathways through which the GM plant products may adversely affect the environment;
- Define assessment endpoints being representative of the aspects of the environment that need to be protected from harm according to protection goals set out by Bhutan and their translation into national policies, and describe criteria used for the selection of assessment endpoints (e.g. relevance, practicality);
- Define measurement endpoints that can be used to assess the potential harm to assessment endpoints defined;

- Formulate testable hypotheses that are clearly phrased and easily transferable to data to be generated or evaluated;
- Set the limits of concern for each measurement endpoint;
- Consider knowledge gaps (such as scientific uncertainties).

2.2.2. Step 2: Hazard characterisation

Hazard characterisation in this guideline is defined as the qualitative and/or quantitative evaluation of environmental harm associated with the hazard as set out in one or more hypotheses derived from problem formulation.

The magnitude of each potential adverse environmental effect should, if possible, be expressed in quantitative rather than qualitative terms. Ordered categorical descriptions such as "high", "moderate", "low" or "negligible", where the ordering is from 'high' at one end to 'negligible' at the other (Liu and Agresti, 2005), may be used to place identified hazard on a scale of severity. If at all possible, these terms should themselves be defined in quantitative terms, as precisely as possible. If the expression of magnitude is not made in quantitative term, but solely using the "ordered categorical description", a justification for this categorisation is necessary and should be provided.

The following classifications are extracted from the European Commission Decision 2002/623/EC (EC, 2002) and are suggested as illustrative and qualitative examples in a very broad sense. They are not intended to be definitive or exclusive, but to give an indication of the considerations that might be taken into account when weighing up the consequences:

- **"High level consequences"** might be significant changes in the numbers of one or more species of other organisms, including endangered and beneficial species in the short or long-term. Such changes might include a reduction in or complete eradication of a species leading to a negative effect on the functioning of the ecosystem and/or other connected ecosystems. Such changes would probably not be readily reversible and any recovery of the ecosystem that did take place would probably be slow;

- **"Moderate consequences"** might be significant changes in population densities of other organisms, but not a change which could result in the total eradication of a species or any significant effect on endangered or beneficial species. Transient and substantial changes in populations might be included if likely to be reversible. There could be long-term effects, provided there are no serious negative effects on the functioning of the ecosystem;

- **"Low level consequences"** might be non-significant changes in population densities of other organisms, which do not result in the total eradication of any population or species of other organisms and have no negative effects on functioning of the ecosystem. The only organisms that might be affected would be non-endangered, non-beneficial species in the short or long-term;

- **"Negligible consequences"** would mean that no significant changes had been caused in any of the populations in the environment or in any ecosystems.

2.2.3. Step 3: Exposure characterization

This step is to evaluate the exposure, i.e. likelihood of adverse effects occurring, and to estimate the exposure quantitatively.

For each hazard identified and characterised, it may not be possible to estimate the exposure (likelihood) precisely. Likelihood of exposure can be expressed either qualitatively using an ordered categorical description (such as "high", "moderate", "low" or "negligible") or quantitatively as a relative measure of probability (from zero to one, where zero represents impossibility and one certainty). However, if qualitative terms are used to express such likelihoods, then the link between likelihood and probability should be accounted for. Thus, whatever term is chosen, an indication should be given of the range, within a numeric scale of 0 to 1, to which the term is intended to refer. For example, "the likelihood of exposure of a non-target lepidopteran species to Bt toxin (Cry1Ab protein) in field margins was estimated to be moderate, where 'moderate' in this context means within the range 0.1 to 0.4".

2.2.3.1. Receiving environments

The receiving environments are the environments into which the GM plant products may be released and into which any viable GM plant and transgene(s) may spread.

A receiving environment for a GM plant is determined by three components:

- The characteristics of GM plant (e.g. plant species and hybridising relatives, genetic modification(s));
- The geographical zones (e.g. the climate, altitude, soil, water, flora, fauna, habitats...);
- The management systems (e.g. points of import, transport routes and processing systems).

The three components listed above result in biotic and abiotic interactions that shall be considered by applicants when establishing representative scenarios considering receiving environment(s) for carrying out the ERA of unintended release of GM plants (Table 2). A broad range of environments in terms of fauna and flora, climatic conditions, habitat composition and ecosystem functions and human interventions occurs in Bhutan. Accordingly, GM plants will differ in how they potentially interact with those differing environments.

The ERA shall be carried out on a case-by-case basis, meaning that the required information varies depending on the types of the GM plant products and trait(s) concerned, their intended use(s), and the potential receiving environment(s). There may be a broad range of environmental characteristics (regional specific) to be taken into account. To support a case-by-case assessment, it may be useful to classify regional data, reflecting aspects of the receiving environment(s) relevant to the GM plant (e.g. botanical data on the occurrence of compatible relatives of GM plants in different agricultural or (semi) natural habitats of Bhutan).
Relevant baseline(s) of the receiving environment(s), including production systems, indigenous biota and their interactions, should be established to identify any potentially (harmful) characteristics of the GM plant. Relevant baselines refer to current production

systems for which generally published literature is available. These baseline(s) serve as a point of reference against which future changes can be compared. The baseline(s) will depend to a considerable extent on the receiving environment(s), including biotic and abiotic factors (for example, natural preserved habitats, agricultural farmland or contaminated land).

Both the plant and the transgenic trait(s) determine where the GM plant will most likely establish (Table 2). GM plants will generally only survive in geographical zones where their non-GM counterparts occur unless they have specific new GM traits that allow them to occur in other regions.

Transgenic traits such as biotic (e.g. pest resistance) and abiotic stress tolerance (e.g. drought and salt) will also indicate whether GM plants are likely to survive in different environments from their progenitors. Therefore, these elements should be taken into account when defining the potential receiving environment(s) for the ERA of the unintended release of each GM plant.

Applicants should initially consider representative scenarios for the unintended release of viable GM plant products, including a worst-case scenario where the exposure and impact are expected to be the highest.

Applicants shall describe:

- The characteristics of the receiving environments where the GM plant is likely to establish, specifically considering the transgenic trait(s);
- The relevant management systems of the introduced plant (e.g. use of the plant, transport, distribution, production systems);
- The range of relevant biotic interactions (e.g. the interactions between plants and other organisms) likely to occur in the receiving environment(s) taking into consideration the range of natural environmental conditions, protection goals (including those related to species differences across Bhutan) and production systems. Where appropriate, the presence of cross-compatible wild/weedy relatives nearby, the ability of the GM plant to form feral populations and hence the potential impacts on the receiving environment should be considered.

Based on the criteria listed above, applicants shall provide evidence that data generated are representative of the range of receiving environment(s) where the GM plant is likely to be dispersed in Bhutan.

2.2.4. Step 4: Risk characterisation

Risk is characterised by combining the magnitude of the consequences of a hazard and the likelihood that the consequences occur (EC, 2002). It is described in this guideline as the quantitative or semi-quantitative estimate, of the probability of occurrence and severity of harmful effect(s) based on problem formulation, hazard and exposure characterisation. It is important that the values obtained for each measurement endpoint are related to the limits of concern to test whether the observed effect falls within those limits and, thereby, to aid in the assessment of the biological relevance of the observed effect (Appendix 2).

On the basis of the conclusions reached in Steps 2 and 3, an estimate of the risk of adverse effects should be made for each hazard identified in Step 1. If a hazard has more than one

adverse effect, the magnitude and likelihood of each individual adverse effect should be assessed. Where precise quantitative evaluation of risk is not possible, terms should be defined where possible.

The evaluation for each risk should consider:

- The magnitude of the consequences of the hazard ("high", "moderate", "low" or "negligible", with an explanation of what is meant by these terms);
- The likelihood of the consequences related to hazard occurring ("high", "moderate", "low" or "negligible", with quantified definitions of terms, using ranges of probability) in the receiving environment(s).

The risk is characterised by combining the magnitude of the consequence of the hazard and its likelihood.

The uncertainty for each identified risk should be described where relevant, possibly including documentation relating to:

- Assumptions and extrapolations made at various levels in the ERA;
- Different scientific assessments;
- Specified uncertainties (also see Appendix 3);
- Conclusions that can be derived from the data.

The risk characterisation should indicate whether the problem formulation (including hazard identification), hazard characterisation and exposure characterisation are complete.

2.2.5. Step 5: Risk management strategies

When the risk characterisation (Step 4) identifies risks that viable GM plants could be released, then applicants should propose measures to manage them. These risk management strategies should aim to reduce the identified risks associated with the GM plant product to a level of no concern and should consider defined areas of uncertainty.

Applicants should describe the risk management in terms of reducing hazard and/or exposure, and the consequent reduction in risk should be quantified (when possible). Where applicants have identified risk management characteristics (e.g. reduced fertility) in the GM plant which can reduce these risks, then the reliability and efficacy of these characteristics should be assessed. In addition, if applicants place restrictions or conditions on the release of a GM plant product in order to reduce risks, then the efficacy and reliability of these measures should be assessed.

In cases where the risk assessment has identified that viable GM plants could be released, applicants should also state the measures they will put in place post-commercialisation in order to monitor and verify the efficacy of the risk management measures and to allow changes in risk management strategies in case circumstances change, or new data become available which require changes to the risk management (also see PMEM plan in Chapter 4).

2.2.6. Step 6: Overall risk evaluation and conclusions

An evaluation of the overall risk of the GM plant products should be made taking into account the results of the ERA and associated levels of uncertainty, the weight of evidence and the risk management strategies proposed (Step 5) in the receiving environment(s).

The overall risk evaluation should result in informed qualitative and, if possible, quantitative guidance to risk managers. The applicants should explain clearly what assumptions have been made during the ERA and what is the nature and magnitude of uncertainties associated with the risk(s). When risks are identified in the overall risk evaluation, applicants should indicate why certain levels of risk might be acceptable.

The overall risk evaluation, including risk management strategies, may give indications for the requirement of specific activities within environmental monitoring (EM) of unintended release of GM plants. ERA and environmental monitoring are closely linked. The ERA provides the basis for the monitoring plans, which focus on detecting any adverse effects on human health and the environment in the receiving environment(s). EM may provide data on long-term, potentially adverse effects of GM plants. Monitoring results may confirm the assumptions of the ERA or may lead to its re-evaluation (Chapter 4).

ERA is an iterative process. If new information on the GM plant and its effects on human health or the environment becomes available, the ERA may need to be re-addressed in order to (1) determine whether the risk characterisation has changed; and (2) determine whether it is necessary to amend the risk management.

2.3. Cross-cutting considerations:

See Appendices for information on comparators, statistical analysis, stacked events and long term effects.

3. SPECIFIC AREAS OF RISK TO BE ADDRESSED IN THE ERA

The import of GM plant products should be risk assessed by initially addressing the 4 specific areas of risk:

1) Persistence and invasiveness of any GM plants that are unintentionally released, or its compatible relatives, following gene flow;
2) Plant-to-micro-organism gene transfer;
3) Interaction of the unintentionally released GM plant with non-target organisms,
4) Effects on human and animal health (through non-food/feed exposure).

For each specific area of risk, applicants are requested to provide information in a clear and concise way, following systematically the first 5 Steps of the ERA as described below and in Chapter 2.2.

- Step 1: Problem formulation (Chapter 2.2.1);
- Step 2: Hazard characterisation (Chapter 2.2.2);
- Step 3: Exposure characterisation (Chapter 2.2.3);

- Step 4: Risk characterisation (Chapter 2.2.4);
- Step 5: Risk management strategies (Chapter 2.2.5);
- Step 6: Overall risk evaluation and conclusions (Chapter 2.2.6).

For each specific area of risk (Step 1 to 5), applicants should conclude by summarising the assessment, the assumptions taken, the available information and identified gaps, the data produced, the estimated uncertainty, the characterisation of the risk(s) and the need, or not, for risk management strategies.

At Step 6, applicants are requested to consider the overall evaluation performed and to provide overall conclusions and recommendations of the ERA. The overall conclusions and recommendations should provide the frame for the risk management strategies including the PMEM and therefore, a link to Chapter 4 should be made.

3.1. Fitness of unintentionally released GM plants and associated gene flow

This section relates to the unavoidable importation of viable GM plant products in admixture with the non-viable GM plant products leading to the unintended introduction of GM plants into the environment. If the problem formulation determines that no adventitious presence of viable material will occur then applicants do not need to consider this part of the risk assessment and can go to Section 3.2.

3.1.1. Step 1: Problem formulation

Some environmental concerns about GM plants relate to the fitness of the GM plant in terms of potential persistence or invasiveness of the plant itself, or of its compatible relatives, as a result of vertical gene flow within either agricultural or other production systems, or semi-natural and natural habitats. Enhanced fitness can be defined as a characteristic of an individual or sub-population of individuals that consistently contribute more offspring to the subsequent generation (Wilkinson and Tepfer, 2009).

Fitness will vary depending upon the environmental context (including anthropogenic influences like mowing), particularly upon the presence of inter and intra-specific competitors, herbivores and pathogens, and the abiotic environmental conditions. Fitness can be measured by the number of seeds (or propagules) produced in relation to the numbers of seeds sown, as this is a reflection of how the life cycle of the plant is responding to the environment in which it is growing (Crawley et al., 1993).

In some studies, other components of fitness are measured – frequently this is fecundity as it reflects seed numbers (Snow et al., 2003). If other vital rates (e.g. establishment, persistence, vigour, competitiveness) are unchanged (which is an assumption that should be substantiated), an increase in fecundity will often lead to an increase in fitness. Variations in fitness according to biotic and abiotic conditions are often referred to as the 'genotype by Environment' interactions and so, it is important that the appropriate range of environmental conditions are considered when assessing fitness changes and impacts.

The potential adverse effects associated with changes in fitness of the GM plant are of two main types:

1) First, enhanced fitness of the GM plant or of transgenic (introgressed) wild relatives may make them more persistent, allowing them to establish in agricultural land and become problematic weeds.
2) Second, enhanced fitness of transgenic feral plants or of transgenic (introgressed) wild relatives in semi-natural or natural habitats may reduce the diversity/abundance of valued flora and fauna. For instance, native plant species may be displaced, which in turn might affect species that use those plants as food, shelter, etc.

Alternatively, and depending on which plant and which transgenes are involved, gene flow to wild relatives may decrease the fitness of hybrid offspring. Therefore, problem formulation should focus on the potential of a GM plant to be more persistent or invasive than conventional counterparts, and on the potential for gene flow to compatible relatives whose hybrid offspring may become more weedy or invasive, or may suffer from outbreeding depression.

To cover all relevant receiving environments of the GM plant and its compatible relatives, problem formulation should address not only the conditions of the production systems under which the GM plant will be grown, but also the relevant semi-natural and natural habitats. It should consider viable GM plant seeds or propagules spilled during import, transportation, storage, handling and processing that can lead to feral plants that colonize and invade agricultural, ruderal, semi-natural and natural habitats.

A staged approach describing how the presence of an introduced trait may exacerbate weed problems in a production system, or cause environmental harm within the wider environment is proposed as outlined in Figure 4. The purpose of the staged approach is to ensure that relevant case-specific information is supplied to test hypotheses formulated in the problem formulation process, and that information requirements remain proportionate to the potential risk.

Questions 1 to 10 in Figure 4 outline the issues to be addressed to estimate the likelihood of occurrence of adverse effects in agricultural, ruderal, semi-natural and natural environments. These questions are divided into different stages. Whether information is required for all stages or only for specific stages will depend upon the trait(s), plant species, the intended use, receiving environments under consideration, and the conclusions drawn from lower stages.

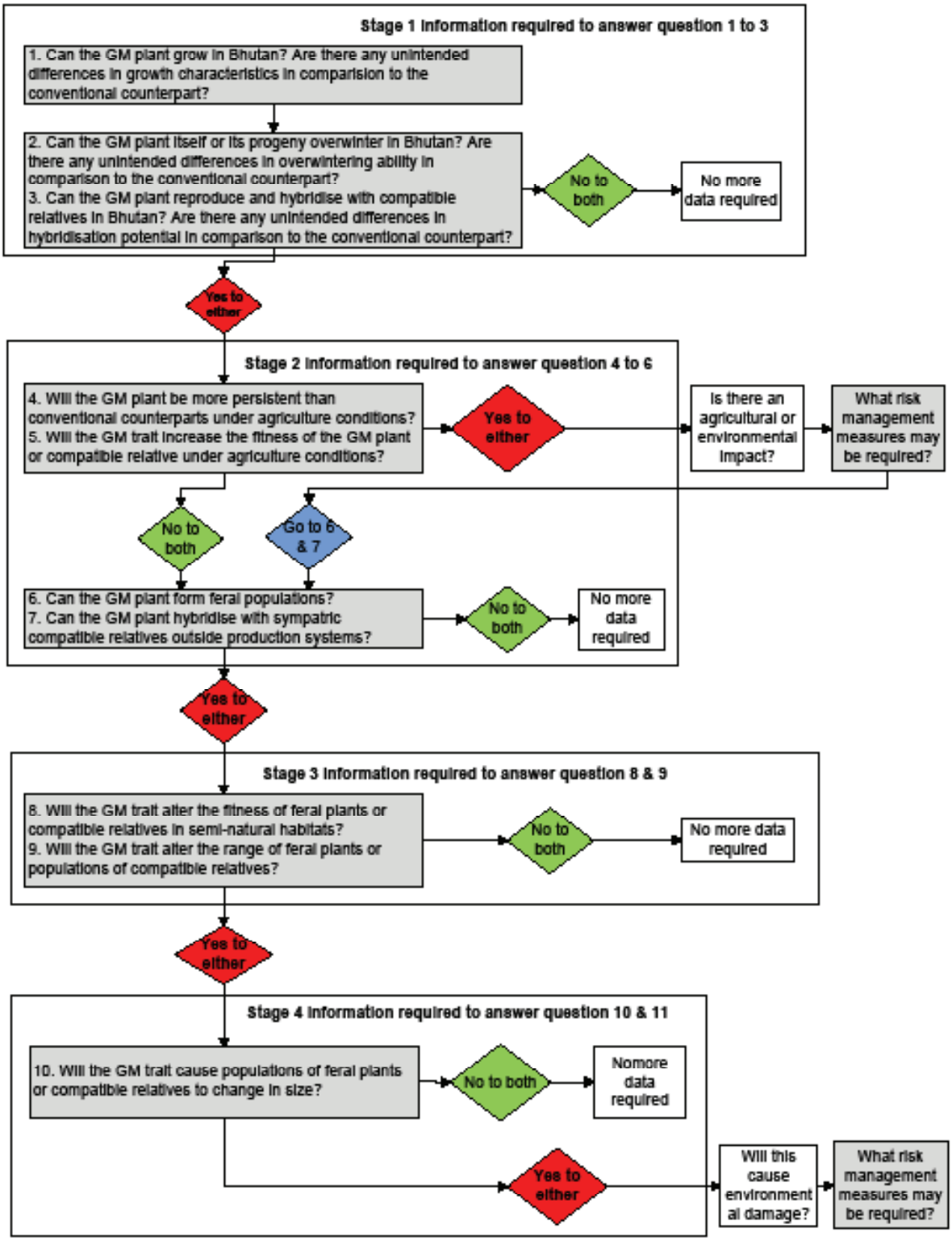

Figure 4: Questions defining the different stages of information required to test formulated hypotheses concerning persistence and invasiveness of a GM plant itself, or any of its introgressed relatives, as a result of vertical gene flow.

Information required for testing the hypotheses formulated in the problem formulation process can be species, trait- or event-specific. This information can be extracted from data generated by applicants, from the scientific literature, or from any other relevant sources. Some GM plants with the same traits or similar events may have been grown for a number of years at a large scale outside Bhutan such that field-generated data on fitness, persistence or invasiveness are available. Applicants should indicate the relevance of the data for the range of potential receiving environments in Bhutan.

Species-specific background information is required at the outset, describing the biology of the parental species including reproductive biology, survival, dispersal and cultivation characteristics in different environments. In addition, sexual compatibility with other cultivated or wild plants occurring in Bhutan, and the biology and ecology of these relatives should also be considered. National Biodiversity Centre has initiated the studies on Crop Wild Relatives and the information on the crop wild relatives is available from National Biodiversity Centre, Thimphu.

In considering the questions in Figure 4, the mechanisms and routes by which plants are exposed to the introduced trait should be taken into account.

For GM plant applications for food and feed uses, import and processing, the ERA on persistence and invasiveness is concerned mainly with the environmental consequences of accidental release of viable GM seeds or propagating material during import, transportation, storage, handling and processing. Therefore, the ERA needs to consider the scale of environmental exposure, and if this could ultimately lead to GM plants being established in receiving environments.

Stage 1 consists of providing event-specific information that enables the GM plant to be characterised, identifying intended and potential unintended differences between it and conventional counterparts. Information provided should be used to establish whether (1) the GM plant can grow, reproduce and overwinter in Bhutan, and if so (2) how its growth, reproduction and overwintering characteristics compare to its conventional counterpart. It is possible that GM traits may move to wild relatives through hybridisation within one growing season, even if the GM plant is unable to overwinter – consequently, it is important that the hybridisation potential described in the background information is considered before concluding on Stage 1 information requirements. It should thus be considered whether sexual compatibility with any relative species is altered since this may result in differences in the rate of gene flow and the establishment of transgenes in other species.

For plants that can either reproduce or overwinter in Bhutan, **Stage 2** should explore whether the GM trait will enhance the potential for the GM plant to contribute to weed and volunteer populations and persist in production systems, and if so, assess the potential environmental consequences.

Stage 2 will also establish whether the GM plant will be capable of forming feral populations outside production systems, or whether the transgene can be transmitted to any relatives independently of the existence of volunteers or ferals. Together, these considerations allow an assessment of whether the transgene is likely to remain confined to production systems.

If feral populations are likely and/or if hybridisation is plausible, then Stage 3 requires information to establish if GM traits will alter the fitness of feral plants, or of transgenic (introgressed) wild relatives. Since feral plants, or transgenic (introgressed) wild relatives may exhibit fitness differences across a wider range of environmental settings, stage 3 also consists of providing information that enables assessing the ability of these plants to occupy larger ecological niches than their conventional counterparts. It is possible that certain GM traits may enable the GM plant to expand its geographical range, and to grow in new areas close to wild relatives from which it was previously isolated, so the potential for this should be considered.

Finally, if altered fitness or the ability to occupy new niches are demonstrated, Stage 4 information is needed to establish whether this will allow populations to increase and invade new communities or, alternatively if this will lead to populations of wild relatives to decline or become extinct. In both cases, the potential environmental consequences should be assessed.

Trait-specific information will be appropriate to address questions of changed fitness in Stages 2 to 4, provided that potential unintended effects, resulting from the transformation process, have been shown not to alter the fitness of the GM plant compared to its conventional counterpart in Stage 1.

3.1.2. Step 2: Hazard characterisation

Step 2 of the ERA consists of characterising any hazards, identified during the problem formulation process, which might lead to adverse effects as a consequence of altered fitness, persistence and invasiveness in agricultural, disturbed (e.g. ruderal), semi-natural or natural environments.

3.1.2.1. Background information requirements

Applications for import and processing of GM plant material, which include adventitious presence of viable GM material, should provide general background information describing the parental species. Species-specific information on the following characteristics should be given in order to summarise existing knowledge of that species.

a) ***Reproductive biology***
The reproductive biology of the parental species, including their mode(s) of reproduction, dissemination and survivability are important, as plants have different reproduction strategies. Since genetic material can move spatially and temporally via the transfer of pollen, seeds, or vegetative propagules, this description should consider relevant avenues and vectors for gene flow, together with factors that affect the probability of these processes.

b) ***Characteristics associated with weediness and invasiveness***
Characteristics associated with weediness or invasiveness have been bred out of many crops during domestication (Warwick and Stewart, 2005), though the degree of domestication varies by crop. While most crops share a similar suite of domestication characteristics, some species may still contain weedy or invasive characteristics (such as seed dormancy, discontinuous germination, rapid seedling growth, phenotypic plasticity, asynchronous flowering, propagule shattering, seed dispersal mechanisms, strong competitive ability: Warwick et al., 2009). It is

therefore considered useful to describe characteristics of the parental plant species that may favour weediness or invasiveness. In this respect, the history of cultivation of the parental species can be examined for confirmatory evidence of whether these plants have become a weed or invasive elsewhere. Historic data from a region may be a valuable indicator of the potential for persistence or invasiveness of the GM plant itself.

c) ***Factors limiting persistence and invasiveness***
Many abiotic and biotic factors limit the ability of plants to form self-sustaining populations under either cultivated or uncultivated conditions. It is therefore relevant to describe factors that may restrict or limit the niche of the plant to certain habitats, or that may control its population size, according to the current state of knowledge.

d) ***Hybridisation and introgression potential with any sympatric compatible relatives***
Sexual compatibility with other cultivated or wild plants occurring in Bhutan is to be considered in general terms. The potential for a plant to hybridise with a wild relative is highly dependent on their sexual compatibility and relatedness (Eastham and Sweet, 2002, Ellstrand, 2003, Jenczewski et al., 2003, Fitz John et al., 2007, Jorgensen et al., 2009). Some level of genetic and structural relatedness between genomes of both species is needed to produce viable and fertile plant crossed with wild relative hybrids that stably express the transgene. Also, both species must occur in their respective distribution range of viable pollen, which requires at least partial overlap in flowering in time and space, and common pollinators (if insect-pollinated). For the stabilisation of the transgene into the genome of the recipient (introgression), genes must be transmitted through successive backcross generations or selfing. (Ellstrand et al. 2013)

Therefore, the risk characterisation should consider features such as the proximity of and flowering synchrony of wild relatives, and the viability, fertility, genetic compatibility and fitness of hybrid and backcross plants.

3.1.2.2. *Stage 1 information requirements:*

Applications for import and processing of GM plant material, which include adventitious presence of viable GM material, should provide information to answer all questions in Stage 1 of Figure 4. The purpose of this information is to answer whether the GM plant and its progeny can grow, overwinter, reproduce and hybridise in Bhutan, and if so, how the phenotypic growth and reproduction characteristics compare to conventional counterparts.

Stage 1 information should include whether there are any unintended differences between the GM plant and its conventional counterpart in growth, reproduction or hybridisation. To answer these questions, event-specific information on the following characteristics should be collated and assessed, and compared with those of the conventional counterparts. For stacked events, applicants should consider whether the combination of events may lead to enhanced persistence or invasiveness that is more than the expected from the simple product of the single traits. Additional field data may be required if changes are observed in phenotype or growth characteristics (e.g. such as behaviour, fitness, reproduction, survivability or dissemination).

a) ***Seed germination characteristics***
Growth chamber experiments or information collected during field trials enable assessment of seed germination characteristics of the GM plant under various conditions. The comparison of germination characteristics between the GM plant and its conventional counterpart might identify potential unintended changes, resulting from the transformation process, in the GM plants that require further analysis.

b) ***Phenotype under agronomic conditions***
The general phenotypic and agronomic characteristics of the GM plant should be assessed in multi-location field trials representative of the different environments where the GM plant may be grown in order to establish intended or potential unintended differences between the GM plant and its conventional counterpart (e.g. Horak et al., 2007, Garcia-Alonso, 2009, Raybould et al., 2009). Characteristics under consideration include plant establishment and vigour, time to flowering and maturity, growth, plant height and dry matter production, seed and yield characteristics, vernalisation requirement, attractiveness to pollinators, and pollen shed, viability, compatibility and morphology.

In addition to plant growth, development and reproduction observations, any visually observable response to naturally occurring insects, diseases and/or abiotic stressors (such as heat, drought, and excess of water) should be recorded during the growing season, as these observations provide indications of biotic and abiotic stress responses and thus susceptibility/adaption to stresses. The comparison of phenotypic and agronomic characteristics between the GM plant and its conventional counterpart might identify potential unintended changes, resulting from the transformation process, in the GM plants that require further analysis.

c) ***Reproductive biology***
When considering the potential impact of gene transfer from GM plants, it is important to assess whether the GM plant has any different capacity for gene transfer than its conventional counterpart. The gene(s) inserted may modify the potential for plant to plant gene transfer due to altered flower biology (e.g. altered flowering period), attractiveness to pollinators, fertility, or changed pollen viability and compatibility.

d) ***Seed persistence leading to volunteer and weed occurrence***
Measurements or observations such as volunteer number in subsequent crops/plantations indicate the potential for seeds and vegetative propagules from a GM plant to give rise to volunteer populations. Post-harvest field inspection data in which volunteer numbers are reported can serve as an information source and provide indications on the overwintering potential of the GM plant seeds. Seed burial experiments can also give indications of changes in dormancy and seed persistence (e.g. Hails et al., 1997).

3.1.2.3. *Stage 2 information requirements*

Stage 2 information will be required for plants that could overwinter in some parts of Bhutan under production system (e.g. agricultural) conditions, and/or transmit genes to compatible relatives that could overwinter. The risk assessment should consider whether the novel traits and phenotype of the GM plant could cause the plant to become a more

serious weed within production sites. With GM plants with more than a single event (e.g. stacked events), applicants should consider whether the combination of events may lead to enhanced persistence or invasiveness that is more than the simple product of the single traits.

Data on relative persistence and fitness of the GM plant under production conditions may be available in the scientific literature, or new data may be required in the form of:

1) Monitoring of existing GM plants in comparable climatic conditions;
2) Manipulative field experiments comparing GM and conventional plant fitness under a range of environmental conditions representative of Bhutan's receiving environments; and/or
3) Population models parameterised by appropriate field data to explore the long-term persistence of GM traits in relevant crop rotations.

Since relative fitness is dependent upon the environmental context, the most direct way to measure fitness is by conducting experiments at sites in representative regions of Bhutan over a minimum of two years. If this is not feasible then data should be collected from sites in other countries with similar biotic, abiotic and climactic conditions. Glasshouse, growth chamber and microcosm experiments can reveal differences under specific, possibly ideal conditions (e.g. Snow et al., 1999), and such experiments can be more highly replicated and therefore more powerful than field experiments. However, observed differences in controlled conditions do not necessarily translate into field conditions and may require further data or population modelling to allow a complete interpretation (Birch et al., 2007).

Persistence or enhanced fitness of volunteers or hybrids should be considered in the context of typical crop rotations. For example, herbicide tolerant *Brassica napus*, could transmit herbicide tolerance genes to weedy *Brassica rapa*. The presence of herbicide tolerant *B. rapa* may be relatively inconsequential as this weed, and crop volunteers, may be controlled by alternative herbicides. However, persistence of transgenic weedy *B. rapa* crossed with *B. napus* hybrids in *B. napus* crops could have consequences.

Crops vary considerably in their ability to form feral populations and this is extensively recorded in the scientific literature (e.g. Bagavathiannan and Van Acker, 2008). If the conventional crop forms feral populations, then this will allow the GM trait to persist outside production systems, and the consequences of this will need to be assessed (Stage 3). Similarly, there is extensive literature available on the sexual compatibility of crops with their wild relatives, and the assessment should consider whether the GM trait has the potential to move beyond production sites through hybridisation and introgression into wild relatives. If the GM trait is unlikely to move beyond production sites via either of these routes, then the characterisation should stop at Stage 2.

3.1.2.4. Stage 3 information requirements:

Stage 3 information will be required for plants that can form feral populations in semi-natural habitats, or for which there are sexually compatible wild relatives that are likely to be recipients of transgenes.

The risk assessment will need to evaluate whether feral plants, or compatible relatives containing the GM trait, will exhibit changed fitness in semi-natural habitats. If fitness is

enhanced, populations may increase; if fitness is reduced, outbreeding depression may occur.

The potential for changes in fitness may be estimated through:

- Observations from regions growing the GM plant;
- Manipulative field experiments (Crawley et al.,1993,2001);
- Greenhouse, microcosm or growth chamber experiments with additional field data and/or models to aid interpretation; or through
- Knowledge of the ecology of feral crops and wild relatives and the phenotypic consequences of the presence of the GM trait. Fitness will vary depending upon the environmental context (including anthropogenic influences like mowing), particularly upon the presence of inter and intra-specific competitors, the presence of herbivores and pathogens, and the abiotic conditions. The variation in fitness according to biotic and abiotic conditions is often referred to as a genotype-by-environment interaction. It is therefore important that an appropriate range of environmental conditions is considered.

Detailed knowledge of the ecology of feral crops and wild relatives and the phenotypic consequences of carrying the GM trait may lead to the conclusion that the GM trait is extremely unlikely to confer a fitness advantage in semi-natural habitats. This may be supported by information from other events of the same GM trait. For example, it is unlikely that herbicide tolerant genes will influence fitness except in the presence of the herbicide. There is now a body of evidence to support this conclusion (Crawley et al., 1993, Crawley et al., 2001, Warwick et al., 2008).

However, in some cases, the existing evidence may be insufficient to draw firm conclusions, and further experiments may be required. The most direct way to measure relative fitness is via manipulative field trials in a range of suitable habitats and over a minimum of two years. In designing such experiments, field sites should be representative of the receiving environments. The timescale should be sufficient to ensure that a range of abiotic conditions are experienced by the experimental plants.

The number of seasons should also be sufficient to ensure that a range of biotic pressures (pathogen and herbivore pressure for example) are experienced, although this may also be enhanced by experimental treatments. Treatments should always include disturbance, in which perennial vegetation is removed before experimental seed is sown, as many crops are not strong competitors with species in semi-natural habitats, but may be able to exploit disturbed areas in the manner of ruderal species. Other treatments should be guided by the GM trait being considered. For example, enhancing the densities of herbivores within limits not infrequently experienced in the field could simulate years of high herbivore. This would allow the hypothesis to be tested that insect resistant GM crops may have enhanced fitness under these conditions. The experimental design should allow the treatment-by-disturbance interaction to be tested. Fitness advantages in response to certain selection pressures may only be manifested under disturbed or undisturbed conditions. Plot size should be sufficient to allow the subsequent generation to be monitored, following seed dispersal, survival and fecundity of adult plants, to allow the lifetime fitness to be estimated.

Greenhouse, microcosm or growth chamber experiments can be used to manipulate the relevant ecological factors to determine the potential impact on the fitness of feral plants or wild relatives (e.g. Vacher et al., 2004). However the detection of fitness differences from controlled greenhouse experiments requires further information for accurate interpretation. For example, the frequency and intensity of herbivore and pathogen attack under field conditions would be needed to interpret the consequences of the possession of herbivore or pathogen resistance traits in the field. Furthermore, competition is likely to modulate the rate at which individual plants recover from herbivore (Weis et al., 2000) or pathogen attack, and so possession of resistance genes may be more valuable when competition is high. Population models, parameterised by greenhouse and/or field data can be used to explore the conditions under which GM plants may invade and establish (e.g. Damgaard and Kjaer, 2009). This allows worse-case scenarios to be explored and the consequences of any uncertainty in parameter estimates to be explicitly defined.

A form of outbreeding depression may occur if:

1) There are high rates of hybridisation with a wild relative, and if;
2) The GM trait decreases hybrid fitness.

The methods outlined above, specifically manipulative field experiments and/or parameterised population models, could be used to estimate the conditions under which this is likely to occur.

For some GM traits, for example some of the stress tolerance genes (Damude and Kinney, 2008a,b, Newell-Mc Gloughlin, 2008, Roelofs et al., 2008, Ufaz and Galili, 2008, Warwick et al., 2009), it is possible that the GM plant, or any introgressed compatible relative would be able to grow beyond the geographical range of the conventional crop. The methods outlined above, particularly manipulative field experiments, knowledge of the ecology of the feral plant and its compatible relatives, microcosm experiments and modelling approaches, are tools that can address this issue.

For those crops for which no significant changes in fitness can be detected, or are thought likely, for either GM plants or their compatible relatives, then exposure characterisation should stop at Stage 3. However, if fitness differences are detected, then further assessment is required to interpret the potential consequences (Stage 4).

3.1.2.5. Stage 4 information requirements:

Stage 4 information is be required when the presence of the GM trait in either feral crop plants or a compatible relative causes an alteration in fitness, or increases the range of habitats in which the plant may survive and reproduce.

Enhanced fitness may or may not result in population increase of the transgenic plant compared to its appropriate comparator, depending upon the factors limiting or regulating the population. A combination of field experiments, growth chamber data, population models and knowledge of the ecology of the potential recipients of the GM trait would then be required to interpret the potential consequences of enhanced fitness.

Detailed knowledge of the ecology of feral crops and compatible relatives including knowledge of the habitats in which these relatives have established populations, and the factors that limit and regulate populations will facilitate an interpretation of the likely

impact of a GM plant. For example, if specific herbivores are known to have an impact on the fecundity of a particular plant species, and these herbivores are susceptible to insect resistant GM traits, then introgression of those insect resistant GM traits could lead to ecological release – but only when those plant populations are seed limited.

Manipulative field experiments may be required to determine if a plant species is seed or microsite limited. For example, seed addition experiments, in which seeds are added as a supplement to undisturbed habitats, followed by monitoring of subsequent generations (and appropriate controls) can determine the degree to which a species may be seed limited, and may be carried out with conventional counterparts. A reasoned argument may then be presented to assess whether the GM plant would be expected to behave in a similar manner, and whether enhanced fecundity would alter dynamics. Similar experiments may be used to deduce other limiting or regulating factors.

Population models (e.g. stochastic models), parameterised with field data, may be required to interpret the long-term impacts of GM trait presence on field populations. For example, it is likely that more than one biotic or abiotic factor is influential in determining population levels of a plant species over a number of seasons. Parameterised models may allow the impact of the presence of a GM trait to be modelled over several seasons, in which putatively important biotic factors (such as herbivores and pathogens) fluctuate in abundance. The range of conditions under which population increase may occur could then be estimated, in order to determine the occurrence and extent of environmental damage.

Finally, the consequences of an increase in abundance or increased range of the transgenic species or of outbreeding depression could be the decline or even extinction of desirable species, or another form of habitat alteration that is undesirable.

3.1.3. Step 3: Exposure characterisation

An exposure characterisation should be conducted for any hazards identified in the ten questions and four stages of Figure 4. The frequency and extent of intended and unintended releases of GM plants into different receiving environments should be assessed, in order to estimate the occurrence of GM plant populations and whether the GM plant population will establish or persist.

3.1.4. Step 4: Risk characterisation

The answers to the questions posed in Figure 4 lead to the characterisation of possible risks – that of an adverse effect in agricultural areas, in which the GM trait causes the plant and/or its wild relatives to become a more persistent weed in subsequent rotations; and that in the wider environment, where the presence of the GM trait affects plant populations and other species, leading to changes in biodiversity in certain receiving environments. Applicants should characterise these risks e.g. by determination of whether any expected change falls within or outside the range defined as being acceptable during problem formulation.

3.1.5. Step 5: Application of risk management strategies

If the ERA identifies risks related to persistence and invasiveness, strategies to manage these risks shall be required and should be defined by applicants. These strategies might

focus on reducing transgene movement, or be directed at controlling the progeny of GM plants resulting from introductions and dispersal. Applicants should evaluate the efficacy and reliability of any risk mitigation measures and conclude on the final level of risk resulting from their application. Remaining identified risks and risk management measures should be considered when formulating environmental monitoring plans.

3.1.6. Conclusions

The risk assessment should conclude on impacts and consequences of the unintentional release of the GM plant. These include:

1) The impact of the GM plant and/or hybridising relatives in agricultural systems, particularly through increased weediness and more intense weed control;
2) The impact of the GM plant and/or hybridising relatives in semi-natural and natural habitats, through changes in persistence and invasiveness or through changes to biodiversity or ecological functions;
3) The reasons why any anticipated impacts and consequences are considered harmful and unacceptable; and
4) Description of the risk management measures required to mitigate any harm.

3.2. Plant to micro-organisms horizontal gene transfer

Recombinant DNA from the viable GM materials present in the imported GM products may be released into the environment, e.g. into soil, or inside the gut of animals feeding on GM plant material. Therefore it is necessary to consider the likelihood of gene transfer into micro-organisms and its stabilisation e.g. by integration into their genomes. Horizontal gene transfer (HGT) is defined as any process in which an organism incorporates genetic material from another organism without being the offspring of that organism. The evaluation of the impact of this HGT includes analysis of the transfer of recombinant plant DNA to initially receiving micro-organisms and potential transfer to other organisms (microorganisms, plants) and the potential consequences of such a gene transfer for human and animal health and the environment. Although the extent of environmental exposure is likely to differ between applications for import and processing and for cultivation, the issues to be considered in the ERA are expected to be similar.

3.2.1. Step 1: Problem formulation

Micro-organisms, especially bacteria, are capable of exchanging genetic material directly between each other and even across species boundaries using different mechanisms i.e. conjugation, transduction or transformation. HGT can be initiated by uptake of cell free DNA from the environment, which may also include DNA derived from GM plants. The current state of knowledge (EFSA, 2009g) indicates that the HGT from GM plants to micro-organisms with subsequent expression of the transgene and integration of genes from plants into bacteria in the absence of DNA sequence identity, are regarded as rare events under natural conditions.

Integration of DNA fragments in micro-organisms occurs mainly by homologous recombination. For this reason, the presence in the plant DNA of sequences with high similarity to microbial DNA would increase the probability of transfer. Mobile genetic elements present in the vicinity of the insertion site could also enhance the potential for

gene transfer. In addition selection pressure would enhance the likelihood for the dissemination and maintenance of horizontally transferred genes.

For instance, the contribution of antibiotic resistance marker genes to the development and dissemination of antibiotic resistance in pathogenic micro-organisms of clinical relevance should be evaluated (EFSA, 2009g).

After initial HGT from plant to micro-organism, the horizontally transferred genes may be further spread to other micro-organisms. Although HGT from plant to micro-organisms is regarded as a rare event, there may be consequences for human and animal health and the environment and therefore they should be considered in the ERA. This ERA will depend on the potentially acquired character and the prevalence of similar traits in microbial communities (EFSA, 2009g). The problem formulation also needs to consider the routes of exposure in the receiving environment(s) as well as the assessment endpoints being representative of the aspects/parts of the environment(s) that need to be protected from adverse effects.

Therefore the problem formulation should focus on:

- Determining the presence of transgenic DNA in a form that could be horizontally transferred. Products that have been exposed to high temperatures during processing may have only denatured DNA that is not capable of promoting genes.
- Detailed molecular characterisation of the DNA sequences inserted in the plant including information on the potential of the promoter elements that could drive expression in microorganisms;
- Presence of antibiotic resistance marker genes;
- Presence of inserted plant DNA sequences showing similarities with DNA sequences from relevant microbial recipients enhancing the probability of recombination and subsequent stabilisation, or mobile elements;
- Presence of recipient micro-organisms for transgenic DNA in the receiving environment(s);
- Selective conditions (including co-selection) enhancing the probability of dissemination and maintenance of the genetic material from GM plants in natural microbial communities (e.g. the presence of antibiotics in the receiving environment(s);
- Persistence of GM plant material after harvesting, until degradation of the material has occurred;
- Potential for long-term establishment of the genetic material from GM plants in natural microbial communities (Chapter 3.2.4);
- Ecological or human and animal health consequences of a potential HGT from GM plant to micro-organisms.

3.2.2. Step 2: Hazard characterisation

If a hazard has been identified in Step 1 of the ERA (Chapter 3.2.1), the hazard should be further characterised (e.g. the potential spread of antibiotic resistance genes and potentially reduced efficiency of antibiotic treatment). Hazard characterisation should consider information on the prevalence and distribution of genes (similar to the transgene(s) in natural environment(s)) and try to establish potential consequences (e.g. for a gene or trait that is already widespread in the environment).

3.2.3. Step 3: Exposure characterisation

Exposure characterisation should consider the sub-cellular location and copy number of the recombinant DNA, the environmental routes of exposure of the GM plant and the recombinant DNA, and the stability of the DNA in the relevant environment(s). After GM plant degradation, cell free DNA may persist in the environment for up to weeks or even years influenced by a number of biotic and abiotic factors (Nielsen et al., 2007, Pontiroli et al., 2007).

It is recognised that the experimental acquisition of data on DNA exposure levels in complex microbial communities is severely limited by methodological constraints under natural conditions. In most cases, the frequency of HGT will be below the detection threshold of particular experiments. Other limitations are related to sampling, detection, challenges in estimating exposure levels and the inability to assign transferable genes to a defined source (EFSA, 2009g).

In light of such technical limitations, however, applicants are requested to characterise the routes of the hazards characterised under Step 2 considering the various routes of exposure in the receiving environment(s):

- DNA from feral or introduced GM plants might be released into the environment as a result of degradation of plant material and might persist in the field and move to aquatic environment(s));
- GM plants intended for food and feed use is often subject to a variety of processing and storage regimes which will expose them to bacteria;
- DNA of GM plants consumed as food and feed might be in contact with micro-organisms, mainly bacteria present in the gastrointestinal tract, and subsequent routes of environmental exposure. These exposure scenarios should include both vertebrates (including humans) and invertebrates that feed on plants or processed plants and plant ingredients above or below ground. (Gay and Gillespie, 2005, Keese, 2008).

3.2.4. Step 4: Risk characterisation

It is important to focus the risk characterisation on potential impacts on indigenous microbial communities that occur in the various receiving environment(s) (as outlined above in Step 3).

Environmental microbial communities may include certain human or animal pathogens (e.g. *Pseudomonas aeruginosa*, some Enterobacteriaceae), or non-pathogenic bacteria, which could serve as first recipients of genes derived from GM plants (e.g. ARMGs) and the transgenes could be then transferred to other micro-organisms including pathogens (EFSA, 2009g). Any risk identified should be characterised by estimating the probability of occurrence, any positive selection conferred by the horizontally transferred trait and the magnitude of the consequences of the adverse effect(s).

3.2.5. Step 5: Application of risk management strategies

Based on the outcome of the risk characterisation, applicants may need to determine and evaluate targeted risk management strategies. Potential strategies may be related to the avoidance of conditions allowing positive selection.

3.2.6. Conclusions

A conclusion is required of the overall risk i.e. a clear rationale on the potential for plant to microorganism gene transfer and its consequences, taking into account any risk management strategies. The potential impact and consequences of such an event and the long-term persistence of the genetic material from GM plants in natural microbial communities should be assessed.

3.3. Interactions of the GM plant with non-target organisms

If the risk assessment of invasion, establishment and introgression conducted as in Chapter 3.1 indicates that significant populations of GM plants might establish and/or introgress genes into native or crop plant species, then impacts on other biota need to be considered.

Guidance is given here for the risk assessment of impacts on NTOs. If applicants require additional guidance they may also refer to the published scientific opinion of the EFSA GMO Panel on the assessment of potential impacts of GM plants on non-target organisms (EFSA, 2010e) which provides more detailed guidance on assessing the environmental effects of GM plants on NTOs, together with rationales for data requirements in order to complete a more comprehensive ERA for NTOs.

3.3.1. Step 1: Problem formulation

3.3.1.1. Environmental concerns and hazard identification

An important environmental concern is that GM plants may have adverse effects on biodiversity and its functioning at several levels, through interactions with populations of other species associated with or sympatric with the GM plants, which are referred to as non-target organisms (NTOs). In this chapter biodiversity is interpreted broadly and covers both species richness and agro-eco functions providing ecosystem services. Since the environment (including biodiversity) is to be protected from harm according to the protection goals and conservation policies of Bhutan, the protection of species richness and ecological functions should be considered in the ERA.

Applicants shall consider whether a GM plant and its level of introduction are directly and/or indirectly (e.g. through food web interactions, scale of adoption) potentially harmful to species guilds involved in ecosystem functions in managed land and ecosystem services in other areas. Problem formulation starts with the identification of potential hazards through a comparison of the GM plant with its conventional counterpart. The different features of the GM plant are considered the novel stressor since environmental impacts can be a consequence of changes to the GM plant, to its management as well as the effects of the introduced traits. These differences are initially assessed theoretically in the problem formulation process in order to identify the potential environmental consequences of these differences. While some differences may be deemed irrelevant to

the assessment, others will need to be practically evaluated for their potential to cause harm.

In addition, in natural and semi-natural situations where exposure levels might be significant, the problem formulation should consider the exposed ecosystems and their biota. Species potentially at risk through direct or indirect exposure (e.g. food chains) should be identified so that assessments can be made of their sensitivity, the vulnerability of their populations, effects on ecosystem services and food chains.

Details of the problem formulation and testing of NTOs can be found in Appendix 5.

3.3.2. Step 2: Hazard characterisation

Once specific measurement endpoints are chosen, appropriate methods and criteria of measurement should be selected and described. This includes information on studies to be conducted, the appropriate tier for analysis, the design of experimental protocols with the definition of the appropriate statistical power (Marvier, 2002, Lövei and Arpaia, 2005, Perry et al., 2009) (Chapter 2.3.3).

3.3.2.1. Laboratory studies

Two kinds of methodologies are relevant for laboratory studies. First, existing conventional ecotoxicology methodologies (e.g. OECD, ISO, EPPO, IOBC standardized methods) can be used and adapted in order to assess the sensitivity of the NTO to different levels of exposure to the GM plant-produced proteins. The methodologies must be adapted to fulfil the measurement endpoint requirements. Secondly, an *in planta* experimental protocol is required in which the GM plant-NTO interactions are evaluated at exposure levels likely to occur in the field. For *in planta* studies, the testing scheme should ensure that the food used is ecologically relevant for the chosen NTO life stage to be tested (e.g. mimicking the trophic interactions existing in nature), and that specimens are exposed to the expected concentration throughout the study duration.

In addition to the above examples, several first tier studies that have been published in scientific literature can be considered by applicants.
All laboratory tests shall satisfy the following requirements:

- The endpoint and species are unequivocally identified;
- The rationale for the selection of the species and endpoint is given;
- Variability is sufficiently low for precise effect level estimation;
- Optimization of other conditions for survival must be provided by the test substrate and food supply (as demonstrated by low mortality of the comparator/control);
- Exposure to known quantities of testing material is maintained throughout the study;
- The experiment is conducted for a time span adequate to reliably estimate measurement endpoints.

When reproduction is an endpoint, the following requirements shall also be fulfilled:

- The processes of the reproductive biology must be included in the testing phase;
- The life-history must be known: age at maturation, duration of egg development, and instars subjected to exposure;
- Optimization of conditions for growth and reproduction must be provided by the test substrate and food supply.

Applicants can develop their own protocols for particular NTO species that are considered in the ERA.

In this case, it is requested that, among others, the following aspects of the experimental protocols are correctly addressed:

- Organisms used during tests shall be healthy and of similar age;
- The biological performance of organisms used as controls shall be within acceptable limits (control mortality less than e.g. 20% depending on the testing system and organism);
- Environmental conditions in growth chambers, mesocosms and greenhouses shall be described explicitly and justified;
- Plant material shall be checked for transgene expression;
- Direct and indirect exposure pathways shall be clearly identified in the experimental setup.

When designing experiments with natural enemies, the following additional requirements shall be considered:

- The suitability of artificial diet or surrogate host/prey species vs. natural food (e.g. some species do not grow well or do not reproduce when reared on artificial diet);
- Host/prey herbivores have to be properly exposed (possibly from hatching) to the right treatments;
- A uniform supply of prey/host quality, age, etc;
- The availability of additional food sources for species with mixed feeding habits (e.g. availability of pollen, honey or sugar solution, possibility for sucking from plants, etc.);
- The availability of an appropriate oviposition surface for predators;
- The provision of particular microhabitats (e.g. providing additional sources of water-saturated surfaces).

For tier 1a, it is assumed that the test substance can be dosed and conventional testing approaches of chemicals can be followed. The sensitivity of the endpoint must be presented as EC10 and EC50 with confidence intervals. Laboratory practices (e.g. environmental conditions, specimen handling) should be according to standardised and published testing procedures. Limitations of some laboratory protocols should be considered (Lövei and Arpaia, 2005) when designing tests and concluding test results. When novel or non-standardised testing procedures are used, it shall be demonstrated that the method is appropriate, reproducible, reliable and of correct sensitivity.

The *in planta* testing required for tier 1b needs particular consideration as NTOs could be exposed to plant material through whole plants, plant parts (e.g. leaves, pollens) or ground plant material in diets or soil.

For *in planta* tests where feeding is an important route of exposure, it will not normally be possible to produce doses of the GM product that exceed the concentrations in plant tissues. Thus the normal level will act as the maximal exposure concentration in a test. Doses lower than the maximal dose can be made by dilution with a near-isogenic non-GM variety and EC10 and EC50 effect levels may be obtained. Different levels of exposure can also be achieved by mixing levels of GM plant material into the test substrates, e.g. soil, and a true dose-response relationship can be established delivering EC10 and EC50 effect levels. Appropriate controls for the effects of these diet regimes can be made by making similar mixtures with near isogenic non-GM materials.

In order to provide an optimal nutrition in soil ecotoxicological tests, a food source may be added. The amount of additional food source may need to be adjusted in order to ensure worst-case exposure.

When the aim is to demonstrate equivalence of the GM plant to the appropriate comparator, the standard tests should include the appropriate comparator as a negative control at an exposure level identical to the GM plant, as well as a positive chemical control to prove the functionality of the experimental setup, as advised in the relevant pesticide test guideline.

3.3.2.2. Field trials

Experimental complexity and variability increases from tier 1 (e.g. toxicological studies), to bi- and tritrophic studies with plant parts, bi-and tritrophic studies with whole plants, to field assemblage studies. Laboratory testing provides the best way to control and manipulate experimental conditions (environmental factors, set-up) and to limit complexity and variability. In contrast, field tests allow the evaluation of trait and environment interactions, but they exhibit the highest experimental complexity and provide the lowest ability to control experimental conditions due to large natural variability.

The objectives of field trials are:

- To identify and study exposure routes (including trophic relationships) and confirm observed effects in lower tier experiments;
- To discover potential unintended effects not anticipated in lower tier tests;
- To provide feedback for further testing hypotheses;
- To study food chain and indirect effects;
- To determine effects of scale on NTO populations, including effects on generations and other spatio/temporal interactions;
- To study effects of interactions between several NTOs species in natural environment(s).

Field testing for NTOs is of special importance for certain species that cannot be tested in laboratory (e.g. rearing methods and experiences are not available). Field testing provides a very broad range of arthropods in terms of species number, life stages, exposure to

abiotic and biotic stress, complexity of trophic interactions, etc. that cannot be reproduced in the laboratory. Hence, attention should be paid to the trade-off between standardised laboratory tests in lower tiers and the testing of NTO species in field experiments. Moreover, field studies offer the opportunity to estimate the functioning of whole ecological functions in natural conditions (e.g. Naranjo, 2005b, a).

Design and analysis of field trials for NTOs should be performed according to the criteria explained in Appendix 5.

3.3.3. Step 3: Exposure characterisation

A major factor in evaluating the likelihood or probability of adverse effects occurring to the NTO is the characteristics of the environment into which the GM plant is intended to be released, and the manner of release. Several ecological characteristics specific to the crop-trait-receiving environment interactions need to be taken into account to characterise NTO exposure.

The introduction of a GM plant into a production system will indeed introduce two new stressors, the transgene and its products and the GM plant itself. If hazards are identified (Step 1) and hazard characterisation gives sufficient evidence for potential environmental damage (Step 2), an exposure characterisation is conducted (Step 3) to determine whether and to what degree the NTO species comes into contact with the GM plant and the transgene product. This assessment requires information on the phenotypic pattern of transgene expression in the various parts of the plant over the growing season. This exposure can be bitrophic via exposure to the GM plant (or plant parts, e.g. pollen) or can occur in higher trophic level organisms exposed to prey or host feeding on the GM plant (Andow et al., 2006).

Organisms at higher trophic levels can be exposed in different ways to the plant and/or its products, therefore direct, indirect or mixed exposure models needs to be evaluated according to the NTO and the GM plant characteristics. For example, a carnivore in an agro ecosystem including GM plants will be faced with the presence in its diet of the transgene product and/or its metabolites, combined with the constitutive compounds of the prey/host species and the combination of both might interfere with the normal development of the natural enemy.

Based on the specific biological characteristics, the likelihood of exposure needs to be estimated. For this purpose, the highest mean protein expression level in any plant tissue is often taken as the worst-case environmental exposure concentration (EEC) in regulatory risk assessments (e.g. Raybould, 2007).

3.3.4. Step 4: Risk characterisation

Based on the conclusions reached in Steps 3.3.2 and 3.3.3, applicants should estimate each identified risk that a GM plant will cause to NTOs considering the magnitude of the effects detected and the likelihood of their occurrence. Applicants should summarise the outcomes of the ERA considering intended and unintended effects as outlined in Step 3.3.1. Hence, applicants should conclude on risk for intended and unintended effects on NTOs taking into account focal species as well as the overall functionality of the agro-ecosystem. Applicants should provide an assessment of the range of effects likely to occur

in different receiving environments based on the collected data and other relevant information (Chapter 2.3.2).

Considering receiving environment-plant-trait combinations, applicants are also required to characterise the risk:

1) to agricultural and managed ecosystems; and
2) to natural/semi-natural habitats where relevant exposure of sensitive NTO may occur.

Quantification of risks and its relative uncertainties shall be provided in relation to each selected assessment endpoint and upscaling of data from lab, semi-field and field trials to landscapes considering the expected adoption rate of GM plants. The conclusions of risk characterisation should assess the consequences of each identified risk to NTO populations and the ecological significance of these effects.

3.3.5. Step 5: Risk management strategies

In situations where risk to NTO and related ecosystem services has been identified and characterised due to the introduction of viable GM materials through GM products, applicants should provide appropriate risk management strategies to ensure that exposure of viable GM material to the NTOs is adequately reduced to an acceptable level of risk. These strategies should be designed, under assumptions of high exposure scenarios, to reduce the risk to a level considered acceptable (criteria defining this acceptability should be explicitly discussed). The implementation of measures should fit with management practices of the receiving environments concerned.

These mitigation measures may include measures to reduce exposure by limiting introduction, dissemination, dispersal and establishment of GM plant material, and by removing feral plants. Also, the establishment and maintenance of buffer or quarantine zones which protect habitats from the spread of GM plants can be established. These mitigation measures and strategies should be devised in the light of long-term management and maintenance of NTOs and ecosystem services and functions in rural landscapes.

3.3.6. Conclusions

Applicants should conclude on the risk of intended and unintended effects on NTOs taking into account the sensitivity of focal species and considering all relevant ecosystem services/functions. Applicants should provide an assessment of the range of effects likely to occur in relevant receiving environments based on the collected data and other relevant information. Applicants are also required to characterise the risks both in and outside managed landscapes in different regions considering relevant exposure routes. Quantification of risks and its relative uncertainties shall be provided in relation to each selected assessment endpoint in comparison to relevant baselines. The consequences of these risks for all relevant protection goals, including the overall functionality of the ecosystems, should be considered.

The conclusions of risk characterisation should assess the consequences of each identified risk to NTO populations and their survival and applicants should propose appropriate risk management measures where levels of risk exceed acceptable threshold levels.

3.4. Effects on human and animal health

An assessment is required of whether the GM plant products present new hazards for human and animal health. In particular, if a potential hazard has been identified, the risk to persons working with the GM plant product, coming into contact with it or exposed to products such as pollen or dust from processed plants should be assessed. This assessment is meant for GM plant products imported for processing purposes (for non-food and non-feed uses), such as for the production of industrial or medicinal/cosmetic products, biofuel, etc.

For GM plant product applications for food and feed purposes, the applicant is requested to refer to the requirements detailed in the "Guideline for risk assessment of food and feed products derived from GM plant products " and where relevant, the EFSA opinion on "assessing the allergenicity of GM plants and microorganisms and derived food and feed" (EFSA, 2010d).

3.4.1. Step 1. Problem Formulation and Hazard Identification

The strategy for human and animal safety focuses on:

(i) The characteristics of the newly expressed protein(s);
(ii) The characteristics of new constituent(s) other than protein(s) and/or possible changes in the level of constituents occurring naturally in the respective unmodified plant species;
(iii) The characteristics of the whole GM plant.

The availability of appropriate non-GM plant comparators is important when performing comparisons of newly expressed products and phenotype.

3.4.2 Step 2: Hazard Characterisation.

Compositional analyses have to be carried out to determine the expression level of:

(i) The newly expressed protein(s);
(ii) The new constituent(s) other than protein(s) and/or possible changes in the level of constituents occurring naturally in the respective unmodified plant species; as well as
(iii) To identify and quantify possible unintended changes in the composition of the whole GM plant. This type of information is necessary in order to evaluate potential risks of exposure of humans, animals and organisms in the biotic environment to the GM plant or plant parts.

For identification of intended and unintended alterations in the GM plant product, the strategies should be followed as recommended in the Guideline for Risk Assessment of Food and Feed Products derived from GM plant products. Analyses should be carried out using established and validated analytical methods according to appropriate quality standards.

The extent of the compositional and agronomic analyses for GM plant products used for non-food or non-feed purposes (i.e. the type and number of components and agronomic and phenotypic parameters to be compared) may vary, taking the nature of the plant, the

possible non-food or non-feed use and the nature of the genetic modification of the plant into account. The selection of compounds must follow an interdisciplinary approach and should be based on expert knowledge.

Toxicology

The requirements of toxicological testing of GM plants used for non-food or non-feed purposes are to be considered on a case-by-case basis and will be determined by the outcome of the assessment of the differences identified between the GM plant and derived food/feed products and their conventional counterparts in the comparative analysis of composition, agronomic and phenotypic traits.

The risk assessment must consider the presence of new proteins expressed as a result of the genetic modification, the presence of other new constituents and/or possible changes in the level of constituents occurring naturally in the respective unmodified plant species (according to the Guideline for Risk Assessment of Food and Feed Products derived from GM plant products). For example, in the case of GM plants containing a substance(s) with pronounced biological activity, the risk assessment should be tailored accordingly. Moreover data on potential genotoxicity, metabolism and toxico kinetics should be generated when required on a case-by-case basis.

To establish the safety of new constituents having no history of safe use, information analogous to that described in the "Guidance on submissions for food additive evaluations by the Scientific Committee on Foods" (SC, 2001). This implies the submission of information on a core set of studies and the consideration of whether or not any other type of study might also be appropriate. This core set will consist of the results from molecular characterization, compositional and agronomical analysis, together with information on the toxicological profile and allergenic potential of newly expressed proteins and other plant constituents, and information on potential exposure routes and patterns, should be evaluated.

Normally, the core set includes information on metabolism/toxicokinetics, sub-chronic toxicity, genotoxicity, chronic toxicity, carcinogenicity and reproduction and developmental toxicity. Genotoxicity has to be considered on a case-by-case basis and justification for carrying out genotoxicity testing would be necessary. As an example, the expression of particular antigens in plants could require genotoxicity testing. Genotoxicity would be tested with plant extract.

Allergenicity

It is considered that the case-by-case risk assessment for allergenicity of GM plants for non-food or non-feed purposes should cover at least one of the two possible hypotheses, namely:

1. That the plant and/or one of its products is already known as allergenic; or alternatively
2. That the plant and/or one of its products is not known to be allergenic.

In the first case, when the GM plant product is used for non-food or non-feed purposes, and one of the products is known to be allergenic, the risk of an allergic reaction should be managed by reducing exposure of humans and animals.

In the second case the Guideline for Risk Assessment of Food and Feed derived from GM Plant is applicable and to be followed. Respiratory exposure (e.g. via dust or pollen) may need particular attention. Regarding the assessment of allergenicity of the newly expressed proteins, a strategy is applied which is in accordance with the recommendations of the Codex ad hoc Intergovernmental Task Force on Foods Derived from Biotechnology (Codex Alimentarius, 2003).

A comprehensive set of in vitro and in vivo tests is available to study potential toxicity resulting from skin, eye and inhalatory exposure. The studies required should be determined on a case-by-case basis depending on the expected route of exposure, the characteristics of the plant and the changes resulting from the genetic modification. For example, the Local Lymph Node Assay (LLNA) using mice or the Guinea Pig Maximisation Test (OECD Guideline 429 (OECD, 2002) and OECD Guideline 406 (OECD, 1992)) may be required to test extracts from GM plants for their potential to induce skin sensitization. Internationally agreed protocols to test for skin and eye irritation/corrosion as well as for acute and repeated dose dermal and inhalation toxicity are also available (OECD Guidelines for the Testing of Chemicals).

3.4.3. Step 3. Exposure assessment

The exposure assessment should consider sources, routes, levels, frequency and duration of exposure in order to determine anticipated intake and external exposure. It is recommended that the exposure assessment is carried out in parallel to the hazard identification because the information on exposure may be needed in order to determine the requirements of the safety testing.

Exposure can result directly from the GM plant products and all potential exposure routes (see below) should be considered. For GM plant products used for non-food or non-feed purposes accidental intake by humans, livestock and wildlife animals, the exposure of farmers and workers handling the GM plant products should be taken into account.

Oral exposure and general considerations on the use of GM plants

For GM plant products used for non-food or non-feed purposes, there is not likely to be chronic oral exposure. However, accidental intake via inadvertent entry of the GM plant product into the regular food and feed chain (e.g. through admixture) cannot be ruled out. In addition, accidental intake through unintentional consumption of the GM plant products by humans, livestock or wildlife animals may occur.

Applicants should determine the likely levels of unintended oral exposure in relation to the intended uses of the GM plant products and also in relation to unintended release, admixture or escape of GM plant products.

Dermal, ocular and inhalatory exposure to GM plants

The applicant has to assess potential dermal, ocular and inhalatory exposure routes in relation to the intended uses of the GM plant products. For instance, in the case of GM plant products which produce pollen or dust, an assessment of the inhalatory exposure to dust and, where applicable, any new constituents expressed therein will be required.

For farmers and workers handling or working in the environment of the GM plant products, the exposure assessment should take into account working and handling practices for workers who produce or process products. It is expected that the procedures applied during the import, transport, storage and processing of GM plant products, differ widely between different production systems. Therefore, a detailed description of the systems applied is required. These descriptions should focus on the identification of critical points where dermal (skin) and eye contact and/or inhalation of GM plant products could occur as well as the level, frequency and duration of exposure at these points. The measures intended to minimise the exposure of farmers and workers handling the GM plant products should be described and the expected impact of these measures should be assessed.

3.4.4 Steps 4 and 5: Risk assessment and risk management for human and animal health

Applicants shall identify risks to humans and, where risks to health are indicated, determine appropriate management measures. These usually involve reducing exposure by protecting workers individually (e.g. with protective clothes, masks, respirators, eye protectors, etc) or by improving the working environment to reduce exposure levels (e.g. with extractor fans, dust screens, isolation cabinets, air filters, etc.).

Where GM non-food plant products are likely to enter the food chain, then measures should be taken to prevent or minimise introductions by careful screening and segregation of supply chains.

Similarly applicants should identify risks to animals and, where health risks are indicated, develop appropriate risk management measures to reduce exposure. For farm animals this generally relates to the modifying rearing and housing conditions so as to reduce exposure. For wild animals, measures need to be taken to reduce exposure to GM plant products considering all routes of dispersal and dissemination as described in Section 3.1. Where GM non-food/feed plant products are likely to enter the animal feed chain, then measures should be taken to prevent and/or minimise introductions by careful screening and segregation of supply chains.

3.5. Overall risk evaluation and conclusions

On the basis of the ERA performed under Chapter 3.1 to 3.4, the weight-of-evidence and the conclusions reached under each chapter, the applicant is requested to perform an overall evaluation of the risk(s) of the introduction of the GM plant product in the receiving environment(s). The overall evaluation of the risk(s) of the GM plant product should take into account the risk characterisation (Step 1 to Step 4) and any risk management strategies proposed (Step 5).

The overall risk evaluation should be expressed in a form of a summary, in a concise way, of the overall risk(s) from the introduction and deliberate release of the GM plant products, including the overall uncertainties. The quality of existing data and information should be discussed, an explanation on how the body of information has been taken into account and the potential uncertainties. The overall risk evaluation should result in informed qualitative, and if possible quantitative guidance to risk managers. The applicant

should explain clearly what assumptions have been made during the ERA, and what is the nature and magnitude of uncertainties associated with establishing the (se) risk(s).

The applicant should provide a summary of the overall risk evaluation in a way that conclusions can be drawn up for the environmental management and Monitoring (Chapter 4).

4. ENVIRONMENTAL MANAGEMENT AND MONITORING

4.1 Risk Management

In Bhutan, the environmental hazards and identified risks will be associated with GM plant products imported for food, feed, processing and industrial purposes and their products. The main hazards will be associated with unintended presence of viable GM plant materials as these may have the ability to grow, survive, establish, spread, disperse and introgress into established plant populations.

Non-viable GM plant products will only have an environmental impact if they are released into the environment in such quantities that they disturb exposed ecosystems. This could happen with GM plant products containing viable GM material are released in large amounts in effluents and waste products from farms or processing plants. The results of the risk assessment in Section 3.1 will determine whether this is likely and where environmental harm is most likely to occur. Management measures should be put in place to reduce or remove environmental exposure to the waste products.

Viable GM Plants

Unintended and unlicensed introduction of GM plant material can be introduced through the importation of GM plant products and this can be prevented effectively by plant health and quarantine regulations and requirements which are enforced at the borders.

In order to avoid environmental hazards and potential risks, a number of different approaches can be taken and some examples are listed:

1. ***Reducing the hazard***: this can be achieved by restricting imports to non-viable material of the approved GM plant or by reducing or destroying the viability of the GM plant.

    a. For example: grain (e.g. rice) imports could be restricted to milled grain which does not have a viable embryo;
    b. If GM plant product containing viable GM material is imported, it could be taken directly to a centralised processing plant where it is processed to a non-viable form before distribution to other areas.

2. ***Reducing the exposure***: a primary source of unintended release of a GM plant will be due to handling, transport and usage practices. Spillage and dispersal of the GM plant products containing viable materials during transportation may occur. In such cases, measures can be put in place to reduce transport and distribution by limiting the number of end users of the GM plant products.. Transporters and handling systems can be improved to prevent spillage.

3. ***Monitoring of adventitious presence of GM plants:*** monitoring should be conducted to discover the establishment of feral populations of GM plants (see below) and these populations should be destroyed to prevent secondary spread or dispersal.

Another possible route for the introduction of viable GM plant is in the adventitious presence of seeds of GM plants in imported non-GM seeds. This seed could then be used on farms and hence GM plants grow and establish in farm crops. The management of this is through requirements that all imported seeds are certified or that they are tested for adventitious presence of GM.

Applicants should describe the management measures that will be put in place to prevent or restrict unintended release of GM plants into the environment and this should be linked to the plans for monitoring unintended releases of GM plants (see 4.2).

4.2 Monitoring

Monitoring can be defined as the systematic measurement of variables and processes over time and assumes that there are specific reasons to collect such data, for example, to ensure that certain standards or conditions are being met or to examine potential changes with respect to certain baselines. Thus, monitoring should be targeted rather than considering every possible environmental aspect. Applications concerning only food/feed or ingredients (i.e. imported but not cultivated) will thus not normally be required to describe a detailed environmental monitoring plan if the applicant has clearly shown that environmental exposure to viable GM plants is absent and that the GM plant products do not present a risk to the environment.

The results of the environmental risk assessment determine whether monitoring is required and its objectives. The environmental monitoring of the unintended presence of GM plants will have two aims:

1) To study any possible adverse effects of the GM plant products identified in the risk assessment; and
2) To identify the occurrence of adverse unforeseen effects of the GM plant product or its use which were not anticipated in the ERA.

Where there is scientific evidence of a potential adverse effect linked to the genetic modification, then case-specific monitoring should be carried out after placing on the market, in order to confirm the assumptions of the ERA. Consequently, case-specific monitoring is not obligatory and is only required to verify the risk assessment, whereas a general surveillance plan must be part of the application. Applicants who consider there is no requirement for case-specific monitoring must provide arguments in support of this position. These arguments should relate to the assumptions applicants have made in the ERA as well as to the lack of any identified adverse effects.

These general principles for monitoring are also appropriate for stacked events. Requirements for case-specific monitoring should take into account the results of the ERA, plus any monitoring already proposed or established for the single events previously

assessed. Consideration should be given to any additional environmental exposure or other effect due to the combination of events identified in the ERA.

A general monitoring or surveillance is recommended for the occurrence of adverse unforeseen effects of the GM plant products or its use which were not anticipated in the ERA, and to allow for unexpected effects that may occur after longer periods of environmental exposure. The focus of this general surveillance should be to detect and observe the establishment of any feral populations of GM plants so that they can be destroyed or managed, so as to minimise environmental effects.

Monitoring methods will depend on the particular GM plant product, trait, environment and usage combination. Therefore, it is advisable to develop an environmental monitoring plan for each GM plant product which describes in detail the monitoring strategy, methodology, analysis, reporting and review, and any follow up management measures and their efficacy.

4.3. Case-specific GM plant product monitoring

The main objective of case-specific monitoring is to determine the significance of any adverse effects identified in the risk assessment (Chapter 3). Case-specific monitoring should be targeted at those environmental factors most likely to be adversely affected by the GM plant and its products which were identified in the ERA. The scientific approach should be designed in order to test the specific hypothesis of expected adverse effects derived from the environmental risk assessment. The monitoring programme design should also reflect levels of exposure in different geographical regions and other specific site influences. Such monitoring may be carried out at a limited number of sites ('local monitoring'), where exposure is greatest and intensive recording and data collection can take place.

The methods selected, the duration of the monitoring, the extent or number of areas and the parameters to be monitored will be determined on a case-by-case basis. Whilst the planning and execution of case-specific monitoring is under the applicant's responsibility, it may be appropriate for the applicant to involve public institutions to contribute to the agreed work.

4.4. General surveillance for unanticipated adverse effects

The objective of general surveillance is to identify the occurrence of unanticipated adverse effects of the GM plant products on human health or the environment that were not anticipated in the environmental risk assessment. General surveillance applies where no adverse effect has been identified in the ERA, but is always required in order to detect unanticipated adverse effects (EC, 2002).

An effect can be defined as an alteration that results in values that fall outside the normal range, given the variation due to the constant changes in the agricultural practices, rural environment and associated biota in the Bhutan.

A major challenge of general surveillance is determining whether:

- An unusual effect has been observed;
- The effect is adverse; and

- The adverse effect is associated with the GM plant products.

The use of a range of monitoring systems to supply data and the ability to compare data from these different sources will help to indicate whether an effect is unusual and adverse. The identification of an adverse effect which is potentially linked to specific GM plants would trigger the need for a specific study to evaluate harm and determine cause.

An important task within general surveillance is to link monitoring to the environmental protection goals of Bhutan. Environmental damage was defined as a measurable adverse change in a natural resource or measurable impairment of a natural resource service which may occur directly or indirectly (EC, 2004).

Within a broader concept of environmental issues, unanticipated adverse effects on human health have also to be addressed in the monitoring plan presented by the applicant. The scope of monitoring for unanticipated adverse effects on human health is defined as monitoring for unanticipated adverse effects that may result from handling of the GM plant products.

It might prove very difficult to design monitoring (including general surveillance) for unanticipated adverse effects on human health. However, knowing that the release of GM plant products needs to be considered in context of their interaction with other environmental components, monitoring for health effects could be considered in conjunction with human population screening methods currently used by public health organisations (for assessing such elements as incidences of allergic reactions) and as part of the monitoring of GM Food and Feed.

4.4.1. Approach and principles of general surveillance

In the case of non-viable GM material (e.g. derived products not containing any living GMOs) the applicant does not have to provide any environmental monitoring plan (including general surveillance).

General surveillance plans as part of applications for import and processing of GM plant products which contain viable materials will need to take account of the modified characteristics specific to the GM plants in question, their intended use and the receiving environment(s). The extent of the general surveillance plan will depend on the level of environmental exposure, the establishment, persistence and spread of the GM plant. The applicant has to establish monitoring which indicates what level of environmental exposure is occurring.

The establishment, persistence and spread of a GM plant is not necessarily an environmental hazard in itself. Similarly, dispersal of pollen and seeds and gene flow *per se* are not environmental hazards and thus, the focus of general surveillance should be on recording establishment of GM plants and any unanticipated consequences, such as unforeseen increases in weediness, invasiveness or changes in plant population dynamics or populations of biota associated with the GM plants. Unanticipated adverse effects are most likely to occur where the level of environmental exposure is highest. Thus, an evaluation of how and where the GM plant is likely to occur and the associated environmental exposure is considered a good starting point in any general surveillance plan.

If unusual effects on human health or the environment are reported, more focussed in-depth studies should be carried out in order to determine cause and relationship with GM plants. Such additional studies would be case-specific monitoring studies as they would require an experimental approach to confirm the specific hypothesis that an observed effect is associated with the GM plant.

General Surveillance should complement available general environmental monitoring. The higher the ecological integration and scale (from the individual to a population, from single farm to regions) the more difficult it is to distinguish potential effects of the GM plants from other factors.

Initially, general surveillance should focus on each event individually. Additionally, when several GM plants have been commercialised, any interactions between these GM plants and their management may need to be considered where appropriate.

4.5. Reporting the results of monitoring

Following the placing on the market of a GM plant product not intended for non-food and non-feed, the applicant has a legal obligation to ensure that monitoring and reporting are carried out according to the conditions specified in the consent. The applicant is responsible for submitting the monitoring reports to Bhutan Agriculture and Food Regulatory Authority. Applicants should describe the methods, frequency and timing of reporting in their monitoring plan.

Although no timeframe for reporting is specified, reports allowing for case-specific adaptations, preferably should be submitted:

- Annually confirming that monitoring has been carried out according to the given consent together with a summary of major preliminary results that are important for a short-term feedback on the ERA ('annual reports'); and
- Periodically (e.g. every third year) covering longer periods in which observations and data collected are reported and analysed in detail and which therefore, provide more comprehensive reports that are important for a longer term feedback on the ERA ('comprehensive report').

The comprehensive monitoring report should include in more detail the results of any relevant monitoring by third parties, including the independent surveyors, local, regional and national environmental surveyors. In addition, the applicant should evaluate these results and incorporate full analysis and conclusions in the submitted monitoring report. If appropriate, the applicant should provide access to raw data for stimulating scientific exchange and co-operation.

The applicant should advise third parties to immediately inform them of any unusual occurrence including establishment of GM plants.

If GM plants become established:

- The applicant should immediately take the measures necessary to protect the environment, and inform Bhutan Agriculture and Food Regulatory Authority;

- The applicant should carry out a preliminary examination in order to verify whether a GM plant-related effect has really occurred and provide BAFRA with a report on the result of its preliminary investigations, including an assessment of potential harm;
- If information becomes available to BAFRA which could have consequences for the risks of the GM plant(s) to human health or the environment it should immediately consider appropriate action to be taken in conjunction with the applicant.

4.6. Monitoring Review and adaptation

4.6.1 Monitoring and review of ongoing research & development and scientific literature

There is considerable research and development work around the world on the management, cultivation and impacts of GM Plants. These studies include experimental research, developmental and advisory studies on crop cultivation, variety registration and variety performance trials. The results of these studies should be reviewed and the implications of the results considered for the risk assessment and risk management of the GM plant products.

Monitoring plans should not be viewed as static. It is fundamental that the monitoring plan and associated methodology are reviewed at appropriate intervals and may need to be modified and adapted depending on the results of the monitoring information collected. The monitoring plan might also be adapted based on an assessment of the appropriateness and cost effectiveness of the monitoring plan. Implementation of the revised monitoring plan remains the responsibility of the applicant unless otherwise determined by BAFRA.

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APPENDICES

APPENDIX 1: CHOICE OF COMPARATORS

Single events

Where feasible and appropriate, similarities and differences in the interactions between the GM plant and the environment due to genetic modification and induced changes in management should be estimated in relation to a conventional counterpart (also see Chapter 2.3.3).

In the case of vegetatively propagated crops, the conventional counterpart shall, in principle, be the near-isogenic variety used to generate the transgenic lines (EFSA, 2009e).

In the case of crops that reproduce sexually, the conventional counterpart shall have a genetic background comparable to the GM plant (EFSA, 2009e). Since many crops used to produce food and feed are developed using back-crossing, a conventional counterpart with a genetic background that is as close as possible to the GM plant shall be selected. On a case-by-case basis, and if there is explicit justification, applicants may instead consider the use of a non-GM variety with as similar agronomic properties to the GM plant as possible, as the appropriate comparator for ERA. In all cases, information on the breeding scheme (pedigree) in relation to both the GM plant and all chosen comparator(s) and justification for the use of the selected use of all chosen comparator(s) shall be provided.

In some circumstances, it may be advantageous for the ERA to include an additional comparator with a closer genetic background to the GM plant than the conventional counterpart (such as a negative segregant). In all cases where an additional comparator is used, the motivation and choice shall be justified explicitly.

It is recognised that appropriate management is site- and year-specific; management of field studies should therefore follow standard farming practices and clearly document deviations. Applicants must provide detailed management records to give sufficient confidence that management practices are appropriate. Any additional treatments and/or comparators should be fully integrated within the experimental design, randomised and replicated in the same way as the GM plant and its conventional counterpart.

The ERA of effects of persistence and invasiveness requires a wide variety of information from specific experiments. The effects studied include: reproduction, germination, seed persistence, invasiveness, and hybridisation. Selection of the comparator should therefore be done on a case-by-case basis.

If no extra comparator is employed, it may still be necessary to consider the use of some form of positive control (Perry et al., 2009) in order to demonstrate post-hoc that the study was capable of detecting the desired effects (for example that there was a sufficient population density of organisms available in the experimental area to be sampled). If the positive control is external to the experiment, for example on a single unrandomised plot, then data from the control may not enter the statistical analysis in any form.

In this ERA guideline, the term 'GM plant' refers to the specific GM event for which approval is requested. However, in practice, commercially available GM varieties are often

produced from crosses of this event with other varieties. Applicants should discuss potential risks arising from the genetic background of varieties which might subsequently include the GM event and how these might alter the conclusions of the risk assessment. On a case-by-case basis, depending on the nature of the event and according to the scope of the application, data may be required on the safety of the event when present in different genetic backgrounds.

Stacked events

Single events that have been combined (stacked) by conventional crossing should first have been full characterised and risk assessed as GM plants with single events. Then the characteristics and risks of the single event GM plants can be compared with those of the GM plant with stacked events in order to establish whether the combination of events raises safety concerns due to any interactions between the events and their expression and any instability. In addition for stacked events, a conventional counterpart consisting of a very similar genetic crossed combination but lacking the GM events, if available, should be used as the comparator.

Note that in the harvested seed of a multiple event stack, there will be segregants consisting of sub-combinations of events and the single events. For ERA, field trials for comparative analysis will normally comprise the stacked event under assessment and its conventional counterpart. However comparisons with single events or lesser stacks may also be required in order to determine whether interactions are occurring which are not anticipated from knowledge of the single events.
If differences in stability and/or interactions are indicated by experiments then further, more detailed, studies may be required in order to determine the nature of the instability or interaction.

To assess whether interactions between events affect protein expression levels, events which have been risk assessed, and which contain between them all the events present in the stacked events, should be included as comparators.

In case a conventional counterpart is not available, different comparator(s) may be appropriate depending upon the issue(s) under consideration. In particular, where studies utilise data arising from field trials for food and feed risk assessment (often used to assess agronomic and phenotypic characteristics), the comparators will be identical to those referred to in these studies.

To evaluate the impact on non-target organisms and the effects on persistence and invasiveness, the conventional counterpart can be substituted, on a case by case basis, by either a non-GM line derived from the breeding scheme used to develop the GM plant, or by a non-GM line with agronomic properties as similar as possible to thestackedevents. Applicants must justify the choice explicitly in such cases.

Since the assessment of the effects on persistence and invasiveness of the GM plant requires information from specific experiments which tend to be of a case-specific, research-driven nature, the selection of the appropriate comparator should be done on a case-by-case basis according to the effect studied. Applicants must justify the choice explicitly in such cases.

Applicants should consider whether the use of extra comparators, such as negative segregants or the parental lines, may be appropriate.

For herbicide-tolerant GM plants that are stacked events, GM plants treated with conventional herbicides are not required for field trials for ERA, because the primary concern of these trials is to provide data to establish that the combination of events does not raise any additional safety concerns over protein and trait expression compared with the single events. However, if these initial trials identify unintended effects that raise safety concerns then further, more detailed experimentation is required which includes additional comparator(s). However, on a case-by-case basis, it may be necessary to include GM plants treated with conventional herbicides as an additional comparator.

APPENDIX 2: GENERAL STATISTICAL PRINCIPLES

2.1 Statistical Principles

This chapter applies to data collected from experiments in which specific hypotheses are tested. When such experiments are conducted in the field they are termed 'trials' throughout this chapter. This chapter does not apply to data obtained from surveys or observational data.

For ERA, applicants shall list explicitly in words all the questions that each study, be it a field trial, a trial in semi-field conditions or a laboratory study, was designed to address. In addition, each of these questions shall be re-stated in formal terms, in the form of the precise null hypothesis that was tested to answer the question. This shall apply equally to those studies that seek confirmatory data on unintended effects when some evidence already exists, as to those that take an ecotoxicological approach with a specific null hypothesis (Chapter 3.4).

For field trials, applicants shall provide a clear and explicit statement concerning the minimum levels of abundance acceptable for each taxa sampled, below which results would lack credibility (for an example, see Heard et al., 2003 section 2F). Applicants shall supply justification for the values chosen. In mathematical modelling for the assessment of long-term or large-scale effects, applicants shall state explicitly all assumptions made and provide justifications for each. The principles underlying the statistical tests of difference and equivalence (EFSA, 2009e) described below are to provide information with quantified uncertainty that may be used by biologists in risk characterisation of those endpoints for which differences or lack of equivalence are found. In order to place differences or lack of equivalence into context, allowance must be made for the distinction between statistical and biological significance. The two approaches are complementary: statistically significant differences may point to biological changes caused by the genetic modification, but these may or may not be relevant on safety grounds (see limits of concern, below). For risk assessment it is not the function of statistical analysis to provide results that lead automatically to a particular decision; instead, the case-by-case approach shall remain paramount.

The ERA is often hampered by the difficulty of conducting experiments with sufficient statistical power (see below). The use of meta-analysis (Marvier et al., 2007) is an option for applicants to consider, but is not mandatory. It may be useful to quantify studies that may not all have the power to be individually significant, in the statistical sense, and also to provide an overview of broad patterns when individual studies appear to contradict each other.

The comparative analysis referred to above shall involve two approaches: (1) a proof of difference, to verify whether the GM plant is different from its conventional counterpart(s) and might therefore be considered a potential risk depending on the type of the identified difference, extent and pattern of exposure; and (2) a proof of equivalence to verify whether the GM plant is equivalent or not to its conventional counterpart(s) (Perry et al., 2009) within bounds defined by so-called 'limits of concern' (see below). For each measurement endpoint, the level of environmental protection to be preserved is expressed, directly or indirectly, through the setting of 'limits of concern' which may take one of two forms. For lower-tier studies (Chapter 3.4) the limits of concern will usually be

trigger values which, if exceeded, will usually lead to further studies at higher tiers. Then the relationship of the limits of concern to environmental protection goals is indirect. For higher tier studies, especially field studies, the limits of concern shall reflect more directly the minimum ecological effects (in positive and negative directions) that are deemed biologically relevant. For field studies, at least one of the limits of concern shall represent the minimum effect that is considered by applicants potentially to lead to environmental harm (also see Chapter 2.3.3.2). If this limit is exceeded then detailed quantitative modelling of exposure may be required to scale up adverse effects at the field level both temporally (to seasons, generations, rotations) and spatially (to farms, landscapes, regions and ecosystems) (EFSA, 2008).

Baseline data can be used to define the limits of concern. Purely as a guide, for laboratory studies, a multiplicative effect size of 20% is often taken as the trigger value for further, higher-tier studies. Similarly, for semi-field testing, a trigger value of 30% has been used previously. For field studies, several studies, both in the USA and in the EU (Heard et al., 2003), have adopted 50% as a limit of concern, which is a reasonable level. By contrast, the effect size threshold for classification set by IUCN for butterflies is a reduction in population size of at least 30% over three generations (but here 'population' is defined at a larger than field scale).

Note that, unless there is explicit justification, limits of concern for lower-tier studies shall usually be less than those for higher-tier studies, since it makes no sense for the results from laboratory studies to exclude from further study effects that might be manifest in the field. Whatever are the limits of concern adopted, applicants shall state their value and justify the choice explicitly, for each measurement endpoint. For field studies, it will usually be the lower limit, which might correspond for example to a decrease in the abundance of a particular species in the presence of the GM plant relative to that for the conventional counterpart, that will be defined as the threshold effect deemed to be of just sufficient magnitude to cause environmental harm. Notwithstanding this general approach, it is acknowledged that the multiplicity and diversity of questions that might be posed in an ERA may demand alternative statistical approaches, on a case-by-case basis.

All test materials, the GM plant and conventional counterpart(s), whether in the field, in semi-field conditions or in the laboratory, shall be fully randomised to the experimental units. Other aspects of experimental design are addressed below.

Whether analysis is of field, semi-field or laboratory data, results shall be presented in a clear format, using standardised scientific units. Applicants shall provide the raw data and the programming code used for the statistical analysis in an editable form. Other aspects of reporting and analysis are addressed below.

2.2. Testing for difference and equivalence

In testing for a difference the null hypothesis is that there is no difference between the GM plant and its conventional counterpart, against the alternative hypothesis that a difference exists. In testing for equivalence the null hypothesis is that there is lack of equivalence, in the sense that the difference between the GM plant and its conventional counterpart is at least as great as a specified minimum size, against the alternative hypothesis that there is no difference or a smaller difference than the specified minimum between the GM plant and its conventional counterpart. Rejection of the null hypothesis (i.e. a finding that the difference is no greater than this minimum size) is required in order to conclude that the

GM plant and the conventional counterpart are unambiguously equivalent for the measurement endpoint considered. The two approaches are complementary: statistically significant differences may point to biological changes caused by the genetic modification, but these may or may not be relevant from the viewpoint of environmental harm.

For studies that use extra comparators, the analysis shall encompass separate difference tests (between the GM plant and each of its different comparators) and separate equivalence tests (between the GM plant and each of its different comparators), and these shall be reported similarly. Further discussion of the principles of equivalence testing, with practical examples, is given in EFSA (EFSA, 2009e).

2.3 Specification of the effect size and the limits of concern

Major parts of the risk assessment dossier are problem formulation and risk characterisation. Notwithstanding the well-known distinction between biological relevance and statistical significance (Perry, 1986) risk characterisation cannot be done without relating effects to potential harm. Therefore, it is essential to specify for each variable studied a minimum effect size which is considered to potentially have a relevant impact on the receiving environment(s). Based on such effect sizes, power analyses aid transparency and may engender public confidence that risk to the consumer is well-defined and low (Marvier, 2002); these require specification of the magnitude of the effect size that the study is designed to detect.

Good scientific studies are planned carefully enough for the experimenters to have a reasonable idea of the size of effect that the study is capable of detecting. For all these reasons, for each study, whether in the field, in semi-field conditions or in the laboratory, applicants shall state explicitly the size of the effect that it is desired to detect in the study, for each measured endpoint. The effect size may be asymmetric, and in particular may be set as zero in one direction to yield a non-inferiority form of the equivalence test (Laster and Johnson, 2003).

The magnitude of the effect size that the study is designed to detect will generally be greater for trials designed to provide confirmatory field data for the assessment of unintended effects on non-target organisms than for specific hypotheses (Chapter 3.4). The effect size will often be placed on the multiplicative scale; however, the natural scale or some other scales are admissible alternatives, on a case-by-case basis. In principle, where more than one comparator is used different effect sizes may be specified for the different comparators; however, this is unlikely to be necessary in practice. Applicants shall provide a full justification for all effect sizes chosen.

The applicant shall state explicitly how the chosen effect size(s) relates to the limits of concern through the minimum relevant ecological effect that is deemed biologically relevant. Usually, these quantities will be identical; applicants shall justify cases where this is not so. Applicants shall state explicitly the limits of concern that were used for each equivalence test. If justified appropriately, more than one pair of limits of concern may be set for each measurement endpoint; an equivalence test shall then be performed for each pair of limits.

2.4. Power analysis

For each study, applicants shall ensure that the design is such that the difference test has sufficient statistical power to provide reasonable evidence (Perry et al., 2009). Statistical power is the probability of detecting an effect of a given size, when such a real effect exists.

It is recognised that for ecological GM field trials the restriction on the land available for experimentation combined with unavoidable environmental heterogeneity usually necessitates some compromise between the replication required for high power and the experimental resources available (Perry et al., 2003). Notwithstanding, optimal experimental design shall be directed to attain power as high as possible.

For each study, applicants shall provide an analysis that estimates the power for each difference test on each measurement endpoint, based on the stated effect size and assuming a 5% type I error rate. The analysis shall be done at the planning stage of the study. The power analysis shall use only information verifiable as available prior to the study; under no circumstances shall data from the study itself be used. For field trials, since each field trial at a site on a particular occasion shall have sufficient replication to be able to yield a stand-alone analysis if required (see below), this power analysis shall relate to a single site, not to the entire set of trials. For situations where many species are sampled such as in field trials, the power analysis is required only for those species of prime importance and those expected to be the most abundant.

2.5 Experimental environment

The first decision in conducting a study is whether the questions asked are best answered by data produced in the laboratory, mesocosm, semi-field, field or region. As is clear from Chapters 2.2.1 and 3.4.1.4, the effect of plant-environment interactions can be studied starting from studies that encompass a range of environmental scales. For this, hazards are evaluated within environments that progress from worst-case scenario conditions with laboratory experiments, up to ecological field trials with relatively large plots.

The laboratory environment is favoured for studies where it is important to control and define closely the conditions for tested organisms. Since environmental variability and interfering factors which can mask potential effects are minimised, laboratory studies yield results of relatively high precision. The laboratory environment is used particularly for the identification of acute and direct impacts of GM products and metabolites on individuals. In particular dose-response relationships may be well described. It also provides the possibility to study indirect and multi-trophic effects at small scales.

Trait-environment interactions may be studied in the laboratory, but only to a limited extent. The laboratory is often used as an initial environment in the tiered approach, particularly for tier 1 studies (Chapter 3.4.1.4). In a laboratory study, decisions must be made whether test materials should be of synthetic or *in planta* form (Chapter 3.4.1.4).

Semi-field trials are manipulative test systems that are designed to control the inherent variability of the environment. They usually incorporate some form of protected environment or containment, such as field cages or screen houses, designed both to isolate the organisms under test and exclude unwanted biotic (e.g. predators) or non-biotic (e.g. rainfall) factors. Semi-field trials allow exposure to ambient weather and light

conditions. The larger cages may result in more natural behavioural interactions between the organisms and plants tested. The semi-field environment is not subject to large variations in the ecology of habitats, and any variability due to different receiving environments is suppressed. Semi-field trials may have greater sensitivity than less-controlled open field trials and it may be that lower levels of statistically significant differences may therefore be detected. Examples include studies on possible indirect effects on non-target pollinators using bees in screen house trials (Chapter 3.4.). Mesocosms are experimental ecosystems that can be used to perform tests under realistic semi-field conditions.

Field trials allow the study of indirect and multi-trophic effects at larger scales, including at some cases the population level. Trait-environment interactions may be tested validly. Although they must, by definition, suffer from less ability to control environmental conditions and therefore produce results subject to greater environmental variability, they provide the only way in which relevant lower-tiered results may be validated under natural conditions. They allow experimental tests of parameters of importance in ecosystem functioning (such as the predation and/or parasitism rate of a species, the decomposition rate of plant residues, etc.) and the estimation of overall ecosystem functions (such as pollination, natural pest control, etc.). Another advantage of field trials is that genotype x environment interactions may be studied in the receiving environment(s).

Field surveys are scientifically designed studies without a hypothesis and where there is no experimental imposition of treatments. However, data are collected in the receiving environment(s) and these may provide appropriate data relevant to the identification of unintended effects on non-target organisms (Chapter 3.4.1) and to changes in plant fitness (Chapter 3.1.1).

The importance of field trials in the ERA of GM plants is widely accepted (EFSA, 2008). One crucial aspect is the increase in ecological realism that can be achieved as the scale of tests move up from laboratory through mesocosm to semi-field, field and region. For example, when any organism is in contact with a GM plant within a multi-trophic context, identification of the impacts on ecological functioning is facilitated by an increase of scale of the experimental arena.

Field studies (semi-field, field trials and field surveys) for environmental effects of GM plants is of special importance because there are organisms for which particular ecological or behavioural tests in the laboratory fail to encompass realistic conditions (for example in some studies of species that are highly mobile, such as adult butterflies or bees; or species for which rearing methods are inadequate (Chapter 3.4). Field testing allows a wide range of arthropod characteristics to be assessed (such as species number, life stages, exposure to abiotic and biotic stress, complexity of trophic interactions) that cannot easily be reproduced in laboratory settings.

Conversely, laboratory studies may incorporate controlled conditions that are impossible to reproduce in the field, which may prevent the identification of causal relationships. Attention shall therefore be paid to the differences in inferences that may be drawn between standardised tests and field testing.

2.6 Experimental design

Experimental designs for laboratory experiments shall conform to accepted international standards and protocols such as those published, for example, by OECD or similar organisations specialising in ecotoxicology.

For field trials, the principle shall be followed that each field trial at a site on a particular occasion shall have sufficient replication to be able to yield a stand-alone analysis if required, although the main analysis shall derive inferences from averages over the complete set of field trials at all sites and years.

The level of within-site replication shall be informed by the power analysis referred to above. Notwithstanding this, it is most unlikely that less than three replicates per site would provide an adequate design. A completely randomized or randomized block experimental design is usually appropriate; appropriate extensions to these designs are discussed by (Perry et al., 2009). Applicants shall justify explicitly why the different sites selected for the trials are considered to be representative of the range of receiving environments where the crop will be grown, reflecting relevant meteorological, ecological, soil and agronomic conditions.

The choice of plant varieties shall be appropriate for the chosen sites and shall also be justified explicitly (Chapter 2.3.2). Within each site the GM plant and its conventional counterpart(s) and any additional test material, where appropriate, shall be identical for all replicates. Environmental variation is manifest at two scales: site-to-site and year-to-year. The primary concern is not environmental variation per e.g., but whether potential differences between the test materials vary across environmental conditions (i.e. statistical interactions between test material and environmental factors, often referred to as genotype by environment (GxE) interactions).

Hence, in addition to within-field replication there is a need to replicate over sites and years to achieve representativeness across geography and climate. Unless explicit appropriate justification is given by applicants, each field trial shall be replicated over at least two years, within each of which there shall be replication over at least three sites. In the case that sites cover a very restricted geographic range, further replication of trials, over more than two years, may be required. The use of data from different continents may be informative, but applicants must justify explicitly why the sites within these continents are representative of the range of receiving environments where the GM plant will be grown, reflecting relevant meteorological, ecological, soil and agronomic conditions.

However, these explicit requirements above for replication to achieve representativeness do not apply to confirmatory field data for the assessment of unintended effects, for example, on non-target organisms when some evidence already exists (see below and Chapter 3.4), or to the great variety of field trials designed to provide data for a wide range of purposes, to assess aspects of potential persistence and invasiveness (Chapter 3.1). Many experimental designs used for research purposes are available in the literature as a guide for the very specific requirements for such trials. Data concerning phenotypic and agronomic characteristics of plants is often derived from the same trials designed to supply data for compositional analysis; statistical guidance (EFSA, 2009e,a) has already been prepared for compositional trials and the requirements above do not apply to them. However, for some non-food, non-feed applications for cultivation, such as potatoes modified to enhance the content of the amylopectin component of starch, compositional

trials may not be conducted. Then, the experimental design of phenotypic and agronomic trials shall follow the guidance in this chapter.

For non-target organisms, plant performance and data on environmental measurement endpoints (e.g. agronomic characteristics, including herbivore interactions with the plant, responses to specific environmental exposure) may provide indications concerning the likelihood or otherwise of unintended effects (Chapter 3.4). This may, for example, include evidence for unchanged ecosystem functions. Under the weight of evidence approach (Chapter 2.2), data from field trials may be used to provide such confirmatory data to underpin conclusions that unintended effects are unlikely. While the requirement for statistical power for these field trials shall be carried out as outlined in Chapter 2.3.3.3, the requirements for representativeness may be relaxed. Hence, as long as there is explicit justification, under these circumstances, there is no requirement for a minimum number of sites and/or years.

Experimental units (field plots) that are of the spatial scale of a whole or half-field are probably of most use for post-commercialisation studies, for monitoring or mitigation. For pre-commercialisation experimentation, smaller plots, where variation may be controlled and defined treatments imposed more easily, are more appropriate for experimental units (Perry et al., 2009). It is recommended to separate plots within sites, often by strips of bare soil of specified width, and to sample towards the centre of plots to avoid edge-effects. Unless the experiment is set up specifically to study residual effects from one season to the next or to study long-term effects, it is recommended not to utilise exactly the same plots over more than one year at a particular site (Perry et al., 2009).

When it is desirable to assess several different GM plants for one crop species (e.g. Zea mays) the generation of data for the comparative assessment of these different GM varieties may be produced simultaneously, at the same site and within the same field trial, by the placing of the different GM plants and their appropriate conventional counterparts in the same randomized block. This is subject to two conditions which shall be strictly met: (1) each of the appropriate counterpart(s) shall always occur together with its particular GM plant in the same block; (2) all the different GM plants and their counterpart(s) shall be fully randomized within each block. For further details, and for the use of partially balanced incomplete block designs see EFSA, 2009e.

In general, it is easier to impose controlled conditions in semi-field trials, and these are not subject to environmental variability to the same extent as are field trials. However, if semi-field trials do not control conditions then the need to test in different environments (at different sites and/or in different years) shall be considered.

For some GM perennial plants (e.g. trees), the plants themselves may be more appropriate experimental units than are field plots (Petersen, 1994). Care should be taken to choose an experimental design that does not suffer unduly from loss of plants during the trial. Whilst it is largely unnecessary to control for positional variation, plant-to-plant variability should be minimised when selecting experimental material.

2.7 Analysis and reporting

It is recommended that applicants prepare an experimental design protocol and a statistical analysis protocol for each study (Perry et al., 2009 for a suggested checklist). It

is recommended that the experimental design protocol comprises full information on the study, which includes;

- A list of the measurement endpoints, and why they were included;
- A description of and justification for of the experimental design;
- A description of the experimental units including dimensions;
- The blocking structure of the experimental units, in terms of the factors that represent it, their levels and whether the factors are nested or crossed;
- The sampling regime, within and between experimental units, and through time;
- Any repeated measurements made in the study;
- The test materials and the justification for their inclusion;
- The treatment structure of the study, in terms of the factors that represent it and their levels;
- A list of the interactions, if any, that are of interest, and why they are; and
- A description of how the treatment factors listed will be randomized to the experimental units specified in the blocking structure above.

It is recommended that the statistical analysis protocol comprises full information on the analysis, and includes but is not restricted to;

- A description of the generic form of the analysis and why it was chosen;
- The criteria for identifying outliers;
- A description of the likely transformations planned, with reasons;
- Justification for any distributional assumptions;
- The scale on which the effects in the experiment are assumed to be additive; and
- Justification for any other assumptions made in the analysis.

For field trials, the protocols shall also include:

- Details of the management of the fields before sowing including the cropping system and rotation;
- The dates of sowing;
- The soil types;
- Insecticide and herbicide use and use of any other plant protection products or techniques;
- climatic and other cultivation/environmental conditions during growth, and where appropriate during harvest;
- Relevant details of the field margins and neighbouring fields;
- Brief descriptions of pest and disease infestations.

When many measurement endpoints have been included in a study (e.g. where the endpoints represent several NTO species), the results of all endpoints for which sufficient records have been obtained shall be reported, not just those deemed to be of particular biological or statistical interest. Data transformation may be necessary to ensure normality and to provide an appropriate scale on which statistical effects are additive. As is routine in ecological applications, for many measurement endpoint response variables, a logarithmic transformation (or a generalized linear model with a logarithmic link function) may be appropriate. In such cases, any difference between the GM plant and any other test material is interpreted as a ratio on the natural scale. However, for other

measurement endpoints the logarithmic transformation may not be optimal and the natural scale or another scale may be more suitable.

Allowance must be made for possible correlations between repeated measurements from the same experimental units. This is especially important where:

- Sampling is repeated over several occasions during a season; and
- The GM plant is a perennial.

Analyses will involve a test for difference and a test for equivalence. Specifically, for a particular measurement endpoint, the mean difference(s) between the GM plant and its conventional counterpart(s) is computed and a 90% confidence interval constructed around it, as in Perry et al., (2009). This mean, the confidence limits and all equivalence limits shall be displayed on a graph(s) similar to Figure 1 of (EFSA, 2009e), but where values are plotted relative to a zero baseline defined by the mean of the GM plant test materials (Figure 3 of Perry et al., 2009) and example therein). The line of zero difference on the logarithmic scale corresponds to a multiplicative factor of unity on the natural scale. The horizontal axis shall be labelled with values that specify the change on the natural scale. In the case of logarithmic transformation, changes of 2x and ½x will appear equally spaced on either side of the line of zero difference.

Both the difference test and the equivalence test may be implemented using the well-known correspondence between hypothesis testing and the construction of confidence intervals. In the case of equivalence testing the approach used shall follow the two one-sided tests (TOST) methodology (e.g. Schuirmann, 1987) by rejecting the null hypothesis when the entire confidence interval falls between the equivalence limits. The choice of the 90% confidence interval corresponds to the customary 95% level for statistical testing of equivalence. Since the confidence interval graph is used also for the test of difference, each difference test will have a 90% confidence level. Although 1 in 10 of these tests is expected to yield a significant result by chance alone, applicants shall report and discuss all significant differences observed between the GM plant, its conventional counterpart and, where applicable, any other test material, focussing on their biological relevance within the context of risk characterisation.

Regarding the simultaneous tests of difference and equivalence, each outcome from the graph shall be categorised and the respective appropriate conclusion shall be drawn, exactly as described in EFSA (2009e).

2.8 Statistical analysis of field trials

The main analysis shall address all field trials simultaneously and shall be based on the full dataset from all sites. Accordingly, the form of the equivalence test shall be that termed 'average equivalence' in the drug testing literature (Wellek, 2002). The use of a statistical mixed model is an important feature of analysis for food-feed assessments because of the need to estimate the natural variation of the commercial varieties. However, as stated in Chapter 2.3.3.2 above, for ERA it is recommended that equivalence limits are set explicitly. Therefore, the use of commercial varieties for this purpose is not necessary, although it might be appropriate for other biological reasons. Hence it is not recommended that statistical mixed models be required forms of analysis, as they are for food-feed assessments (Perry et al., 2009). Indeed, it is recommended to use simple

statistical models; effects due to environmental factors such as seasons and sites may be represented by fixed factors if desired.

Applicants shall ensure that each analysis has the potential to identify any interactions between sites and years and the test materials. For each measurement endpoint studied, applicants shall make an explicit statement concerning the presence or absence of any such interactions. If interactions are found, the possible reasons for their existence and the implications for the inferences drawn from the trials shall be discussed. Applicants shall also provide a table or graph giving, for each site and year and for each (transformed) measurement endpoint, the means and standard errors of means of the GM plant and its conventional counterpart(s), and any other test material, where applicable.

Diversity indices are not recommended for general risk assessment in pre-commercialisation studies, because it is most unlikely that studies will yield sufficient samples of individuals to characterise indices adequately or that a sufficient degree of ecological background information will exist to give confidence that biodiversity can be represented adequately as a single number. By contrast, multivariate approaches may be useful, especially for summarising data and for analysing principal response curves (Perry et al., 2009).

Particular recommendations apply for the very wide range of possible studies of persistence and invasiveness, and the related estimation of selective advantage and disadvantage (Chapter 3.1). Further discussions and motivations underpinning the above statistical guidance (Chapters 2.3.3) may be found in Perry et al. (2009).

2.9 Uncertainties

The ERA has to take into account uncertainty at various levels as the effect sizes, the likelihood of occurrence and the environmental impacts are each associated with uncertainty.

Uncertainties may arise due to problem formulation, limitations in the data (e.g. limited exposure data), gaps in the effect database, model choice, the limitation of the test systems and measurement endpoints selected, inadequacy of study designs and the uncertainties in extrapolating between species (EFSA, 2009b. Scientific uncertainty may also arise from differing interpretations of existing data, publication bias or lack of some relevant data. Uncertainty may relate to qualitative or quantitative elements of the analysis. The level of knowledge or data for a baseline is reflected by the level of uncertainty, which shall be discussed by applicants. Applicants shall in addition assess the degree of uncertainty within the ERA in comparison with the current uncertainties displayed in the scientific literature.

Although it may be impossible to identify all the uncertainties, the assessment shall include a description of the types of uncertainties encountered and considered during the different risk assessment steps. Their relative importance and their influence on the assessment outcome shall be described (EFSA, 2009b). Any uncertainties inherent in the different steps of the ERA (Steps 1 to 5) shall be highlighted and quantified as far as possible; this might be done by adapting the methodology outlined by (Risbey and Kandlikar, 2007). Distinction shall be made between uncertainties that reflect natural variations in ecological and biological parameters (including variations in susceptibility in populations or varieties), and possible differences in responses between species.

Estimation of uncertainties in experimental data shall be handled by proper statistical analysis, while quantification of uncertainties in assumptions (e.g. extrapolation from environmental laboratory studies to complex ecosystems) may be more difficult, but shall be discussed fully. The absence of data essential for the environmental risk assessment shall be indicated and the quality of existing data shall be discussed.

It should be clear from the discussion how this body of information has been taken into account when the final risk characterisation is determined. Risk characterisation may be qualitative and, if possible, quantitative depending on the issue to be addressed and the available data. The terms for the expression of risks and associated uncertainties shall be as precise as possible. For instance, expressions like 'no/negligible/acceptable/significant risk' need, where possible, further numerical quantification in terms of probability of exposure and/or occurrence of adverse effects (also see Chapter 2.2.1).

It is recognised that an ERA is only as good as our state of scientific knowledge at the time it was conducted. Thus, under current EU legislation, ERAs are required to identify areas of uncertainty or risk which relate to areas outside current knowledge and the limited scope of the ERA. These include such factors as the impact of the large-scale exposure of different environments when GM plants are commercialised, the impact of exposure over long periods of time and cumulative long-term effects. When uncertainty factors (EFSA, 2009b) are used, an explanation of their basis and a justification of their appropriateness need to be provided, or a reference to documents where that information may be found shall be included. When point estimates are used for uncertain quantities, justification for the values chosen and assessment of their influence on the assessment shall be included (EFSA, 2009b).

Predicting impacts of GM plants on complex ecosystems which are continually in flux is difficult and largely based on experiences with other introductions and an understanding of the robustness of ecosystems. It is recognised that an environmental risk assessment is limited by the nature, scale and location of experimental releases, which biospheres have been studied and the length of time the studies were conducted. Probabilistic methods could be used to determine ranges of plausible values rather than single values or point estimates, which are subsequently combined in order to quantify the uncertainty in the end result. These methods could provide a powerful tool to quantify uncertainties associated with any steps in the environmental risk assessment. When such probabilistic approaches are used, the outcome of the environmental risk assessment should be characterised by reporting a distribution of the risk estimates. However, the use of quantitative methods does not remove the need for a qualitative evaluation of the remaining uncertainties (EFSA, 2009b).

Scientific knowledge from the literature and experience gained from growing GM plants encompassed in PMEM following past applications and approvals may also inform the risk assessment process.

APPENDIX 3: LONG-TERM EFFECTS
(Including techniques for their assessment)

Predicting and assessing (adverse) long-term effects requires information about the GM plant and the receiving environment(s) (also see Chapter 2.3.2), both in terms of the baseline conditions in the receiving environment(s) and temporal changes in these conditions independently of the GM plant and following GM plant introduction. The rate and degree to which the baseline is likely to change independently of the GM plant (e.g. as a result of new crops and agronomy) will vary among production systems.

The consideration of long-term effects in the ERA should address effects that might arise up to a minimum of 10 years after the introduction of the GM plant and should in all cases cover the time period over which progeny of the GM plant might persist and appear as volunteers or ferals. Thus, the analysis should be conducted case-by-case and applicants should fully justify their approach.

3.1. Categories of long-term effects

Long-term effects might result from a diversity of primary causes and secondary interactions, which make it difficult to generalise on methods of investigation. Long term effects can be considered in two broad categories:

I. Long-term or chronic exposure to a particular GM plant or practice results in a delayed response by organisms or their progeny (Category I). It may be in some instances that a response occurs immediately, but is not detected by the measuring tools or the particular indicators employed. For example, exposure over time may affect a specie or community by suppressing certain functional forms in relation to others, or acting on natural mutations that exist at very low frequency such as occurs when pests develop resistance to a pesticide.

II. Effects which occur as the result of an inevitable increase in spatial and temporal complexity, determined by the number of possible interactions that a GM plant would have with biota and the physical and chemical environment as it is grown more widely throughout the landscape and in more extended sequences of cropping. There may not necessarily be a chronic or delayed effect as in the first category; rather, the effect occurs in certain contexts that are outside those experienced in the initial testing, or that have arisen as entirely new contexts due to global environmental change or the adoption of new forms of management. The latter may indeed arise as a downstream effect of the introduction of the GM plant cultivation itself, if this causes a change in the sequence or range of plants grown in the production system.

An estimate of whether long-term effects of both Categories are expected to occur and how PMEM should be followed after commercialisation should be given in any application. Based on the characteristics of the GM plant, the ERA should consider these long-term effects by reference to existing examples, long-term datasets, and in some instances modelling, as indicated below. The analysis and conclusions should be presented in the form of a desk study based on the interpretation of existing information.

2.3.4.2. Techniques and information required to assess long-term effects

Some effects of Category I might already have been investigated within constrained experimental systems maintained over several generations of the GM plant/trait combination under study. While some potential long-terms effects might be revealed by such studies, questions will still remain, as to how much the constrained system restricts the range of possible reactions or encourages untypical reactions, such as caused by a reduced choice in the foraging range and food available to invertebrates that are kept for months or years in controlled environment chambers or restricted to intensely managed field plots. Information from such studies might be useful for defining the primary mechanisms by which the GM plant might interact with other organisms and their abiotic environment, but would not be sufficient alone as a basis for assessment of long term effects in an agricultural or ecological context.

Category II, by definition, cannot be investigated through an initial experimental phase of testing, even at the scale of the field plot, half-field or paired field, none of which can provide the range of complexity experienced after full commercial release into a range of environments under different managements and over a period of time. Category II effects can only be investigated by reference to existing examples and case histories that provide evidence of rates and magnitudes of environmental impact due to changes in land management, agricultural practices (e.g. pesticides, crop type) or external (e.g. weather and climate change) factors, including GM cultivation in neighbouring and other countries.

Despite these uncertainties, there is now a great deal of information in the published literature, and in accessible reports and databases, on long term ecological and environmental effects due to changes in land usage and management. Applicants should conduct appropriate desk-based studies to assess long-term environmental effects of the GM plant in relation to both categories of long-term effects. It is not the intention here to give precise instruction to applicants on which data, processes and indicators should be considered, since they will vary case-by-case. However, examples of the type of information that could be used in assessment are:

- Experience of cultivating the GM plant or long-term environmental exposure to GM cultivation in other regions;
- Experience from cultivation of similar plants (GM and non-GM);
- Long-term ecological or environmental datasets applicable to the receiving environment(s); e.g. information on current and future planned land management in Bhutan;
- The results of studies on introduced species and GM plants that have examined effects or plants similar to those of the GM plant under assessment; e.g. studies of the unintended introduction and spread of GM oilseed rape in several countries (Devos et al, 2011);
- Results of studies of gene flow and introgression in other countries (Ellstrand et al 2013) ;
- The results of meta-analyses drawing together data from different sources (e.g. Marvier et al.,2007, Duan et al., 2009, EC, 2009b);
- The use of models of ecological processes to explore or test scenarios: mathematical models of ecological processes are unlikely to be considered justification on their own, but may be used to argument or interpret data or to

demonstrate that possibilities have been explored; descriptions would be necessary of the model, its verification using existing data, the input variables, etc;

- Foreknowledge of relevant change in the production system and wider environment that can be expected in the years following release; an example is the commercial cultivation of GM crops in countries adjacent to Bhutan.

3.2 Other guidance for applicants

Applicants should conclude for each of the Chapters 3.1 to 3.7 – where appropriate - on the outcomes of the risk assessment for long-term effects by summarising:

- The methods and approaches used to reach the conclusions, including the published long-term or large-scale experiments, reference datasets, analysis and models used directly in the assessment;
- The basis of and justification for a conclusion specific to the GM plant or its management (whether a conclusion is for or against the likelihood of a long-term effect);
- Identification of parts of the management and monitoring that are designed to manage and detect possible long-term effects (Chapter 4).

APPENDIX 4: RISK ASSESSMENT OF GM PLANTS CONTAINING STACKED TRANSFORMATION EVENTS

In the context of this guideline, the term "stacked transformation event" or "stacked events" will refer to a GM plant derived from conventional crossing of GM plants consisting of one or more events. It does not include retransformation of GM plants to introduce additional GM events. The ERA of stacked events should follow the general strategies described in Chapter 2.1 and 2.2 of this guideline, i.e. it should include a comparative safety assessment and follow the 6 Steps of the ERA (Figure 2).

The ERA of the single events is a pre-requisite for the risk assessment of stacked events. The ERA of stacked events shall describe the finalised risk assessment of each single event and compare the stacked event with the single events particularly in relation to gene and trait expression, as well as with non-GM comparators. If some single events in a stacked GM plant do not exist separately, then the stacked event GM plant should be assessed de novo as a new event with an appropriate comparator and also compared with single events which do exist.

Since single events have been risk assessed, there is a general assumption that hybridisation of the events and their combination will have the risks which are predicted from the sum of the single events. However the combination of events might result in interactions (e.g. synergistic or multiplicational effects) that would raise new safety concerns compared with the single events.

Similarly stacked events containing three or more events combined by conventional crossing (defined as higher stacked events), should be compared to already risk assessed sub-combinations (defined as lower stacked events)as well as with single events. The appropriate comparator for stacked events should be selected in accordance with the requirements defined in Chapter 2.3.1. Applicants should justify the choice of all comparators.

The ERA of a higher stacked events shall cover all sub-combinations of these events that can occur by natural segregation, since it is likely that imported grain and seeds produced from GM plants with stacked events will contain segregants as well as the stacked event.

The ERA of stacked events shall mainly focus on the characterisation and potential consequences of issues related to:

- stability of the inserts;
- expression of the events;
- potential synergistic, additive or antagonistic effects resulting from the combination of the events;

The impacts of these characterisations on the phenotype, the adaptive and fitness traits and the interactions of the stacked event GM plant with other biota need to be considered in the areas of risk described in Chapter 3 on a case-by-case basis.
Applicants shall provide a scientific rationale justifying the range and extent of information used to support the risk assessment of sub-combinations.

3.3.1.2. Definition of assessment endpoints

Because protection goals are general concepts, they need to be translated into measurable assessment endpoints. Thus the assessment endpoint is an explicit expression of the environmental value that is to be protected. This necessitates defining (a) species and (b) ecosystem functions or services that could be adversely affected by the GM plant, and that require protection from harm.

In any ecosystem, there is usually a high number of NTO species that may be exposed to GM plants. Considering that not each of these species can be tested, a representative subset of NTO species (referred to as 'focal species') shall be selected, on a case-by-case basis, for consideration in the risk assessment of each GM plant. To lead applicants to a decision on which focal NTO species are to be used as assessment endpoints, species selection shall be performed according to the following four steps outlined also in Figure 5.

Step 1 - *Identification of functional groups*

As a first step in species selection, it is necessary to identify the ecosystem functions and services (including maintenance of herbivores as part of food web, pollination, regulation of arthropod pest populations by natural enemies and decomposition of plant material) provided to the receiving environment (e.g. agro-ecosystem, natural forest) and the functional groups of species involved, in the environment(s) where the GM plant is likely to establish.

Step 2 - *Categorisation of NTO species from identified functional groups*

In the second step, the main species linked to the functional groups identified in the previous step should be listed, considering the GM plant and the organisms associated to in its receiving environment(s) (Birch et al., 2004, Hilbeck et al., 2006). An indicative list detailing the ecological role for common invertebrates is provided in Table 3. Some taxonomically related species and/or life stages of the same species may have different ecological roles (e.g. different feeding habits) and this aspect should be considered.

In the categorisation of relevant NTO species, additional species of economic or aesthetic or cultural value, or species of conservational importance considered as threatened or endangered will also need to be included.

Table 3: Examples of functional groups (exposure through trophic interactions)

Functional group		Examples of taxonomic groups
Herbivores		Phloem-feeders: aphids (*Hemiptera: Aphididae*), leafhoppers (e.g. *Hemiptera: Cicadellidae*), certain *Heteroptera* Cell-content feeders: thrips (*Thysanoptera: Thripidae*), spider mites (*Acarina*) and *Nematoda* (*Tylenchida: Meloidogynidae*) Chewing: leaf beetles (*Coleoptera: Chrysomelidae*), *Lepidoptera* larvae, *Diptera* larvae, grasshoppers (*Orthoptera Ensifera*), gastropods (*Mollusca, Gastropoda*
Natural enemies	Predators	Beetles: *Coleoptera* (e.g. *Coccinellidae, Carabidae, Staphilinidae*) Predatory bugs: *Heteroptera* (e.g. *Nabidae, Anthocoridae*) Predatory flies: *Diptera* (e.g. *Syrphidae*) Lacewings: *Neuroptera* (e.g. *Chrysopidae, Hemerobidae*) Thrips: *Thysanoptera* (e.g. *Aeolothrips*) Spiders & harvestmen: *Araneae* and *Opiliones* Mites: Acarina (e.g. *Phytoseiidae*) Nematoda (e.g. *Mononchus* sp)
	Parasitoids	Hymenoptera (e.g. *Ichneumonidae, Braconidae, Aphelinidae*)
	Parasites & Pathogens	Bacteria, fungi, viruses
	Entomopathogenic organisms	*Nematoda* (e.g. *Heterorhabditidae, Steinernematidae*), pathogenic microorganisms
Pollinators		Solitary and social bees (*Hymenoptera: Apidae*), hover flies (*Diptera: Syrphidae*); Coleoptera (e.g. *Melyridae, Curculionidae, Scarabaeidae*)
Decomposers		Diptera larvae (e.g. *Phoridae, Sciaridae*), Nematoda (e.g. *Rhabditidae, Dorylaimidae*), springtails (*Collembola*), mites (*Acarina*), earthworms (*Haplotaxida: Lumbricidae), Isopoda*, microorganisms
Plant symbionts		rhizobacteria, mycorrhiza

Step 3 - Ranking species based on the ecological criteria:

From the list built in Step 2 of species selection, applicants shall prioritise NTO species from each relevant functional group (Birch et al., 2004, Hilbeck et al., 2006).

The main criteria to be considered in this prioritisation process are:

- **Species exposure to the GM plant in different receiving environments, specifically considering life stages present during the period of exposure;**
- **Known sensitivity of the species to the product(s) expressed in the GM plant;**
- **Linkages to the agricultural and natural habitats, and presence of alternative food sources;**
- **Abundance;**

- Species vulnerability (i.e. are certain populations already threatened and thus more vulnerable to additional pressures?).

Step 4 - Final selection of focal species:

Based on the considerations addressed in the previous steps of species selection, a restricted number of focal species needs to be selected from each functional group. A theoretical framework for focal species selection is presented in Figure 3. At this stage, some practical criteria may be considered in the final selection of focal species. It may be that, among the prioritised species, some can be tested more effectively under laboratory conditions, or are more likely to be available in sufficient numbers in the field to give statistically meaningful results (Gathmann et al., 2006a, Gathmann et al., 2006b, Todd et al., 2008). Legal constraints may limit testing of certain NTOs (e.g. protected species), so this aspect may also influence the final choice of focal species.

It is expected that, at the end of the selection process, applicants have selected at least one focal species from each relevant functional group identified in the problem formulation for further consideration in the ERA. Different possible sources of exposure for each focal species (in the most relevant developmental stages) to be tested should be considered in the focal species selection process.

For field trials, estimation of ecosystem functions and services could complement or replace data on focal species. Ecological functions (such as pollination, biological control, soil functions) depend on the number of species, their abundances and different types of assemblages. In a particular assemblage, the abundance of any species naturally fluctuates and the decline of a certain population might be compensated by another species within the same guild without adversely affecting functionality (Naranjo, 2005b,a). For example, the overall predation rate of a guild of predatory species could be selected as an assessment endpoint in field trials (Arpaia et al., 2009). Likewise, evaluating the earthworm community as a whole might provide data that are more ecologically relevant than measuring the effects on a single (focal) earthworm species.

A theoretical framework for focal species selection is presented in Figure 5.

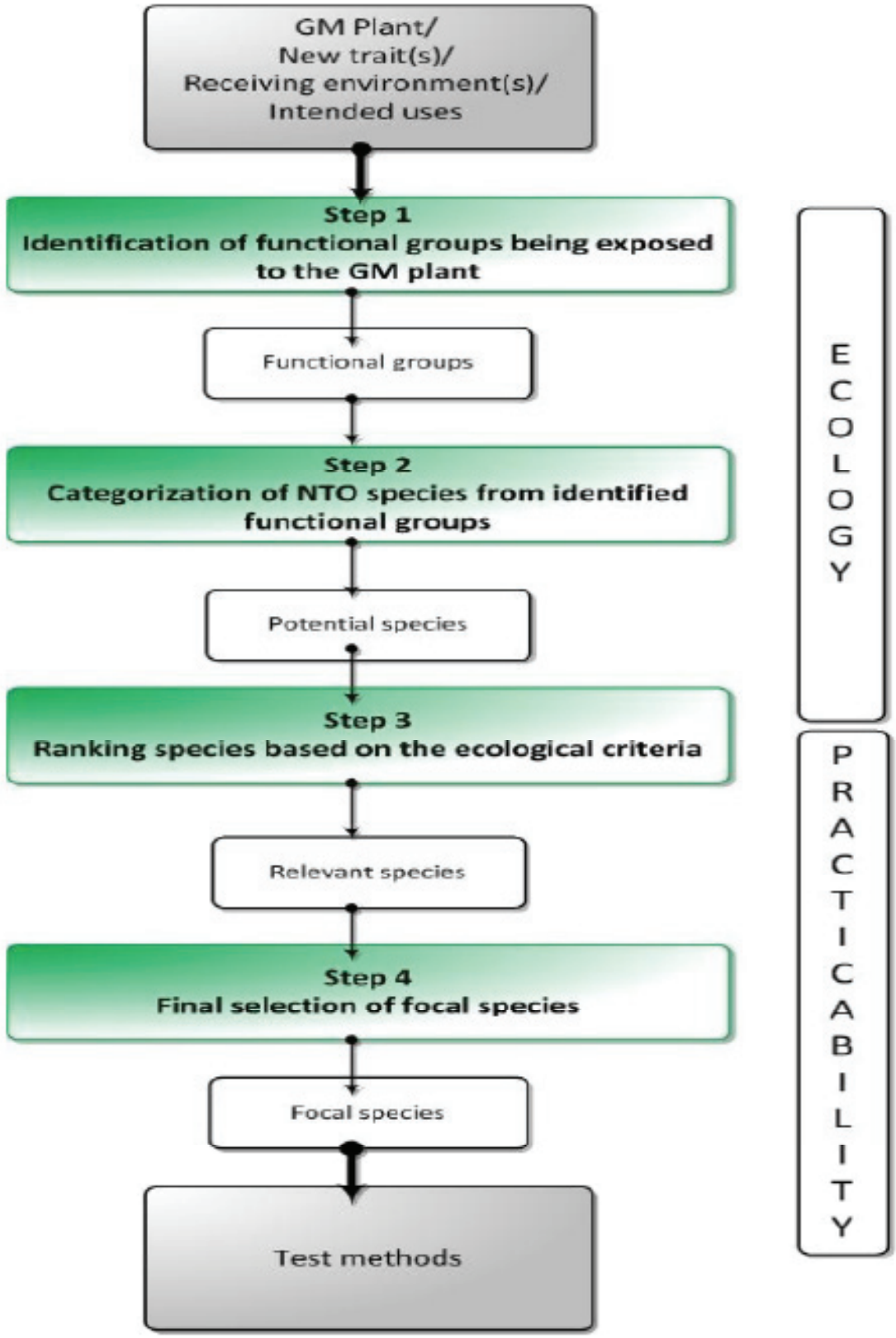

Figure 5: Four steps for selecting focal NTO species to be tested (modified after Birch et al., 2004, Hilbeck et al., 2006).

3.3.1.3. Considering the exposure patterns to NTOs

The overlap of the life cycle and developmental stages of the focal species and the phenology of the GM plants needs to be evaluated. Exposure may also happen after the transgene has moved via dispersal of pollen and grain/seed in and away from the growing site of the GM plant (e.g. pollen deposited on leaves of host plants for non-target Lepidoptera and Coleoptera). Moreover, gene flow via out crossing may result in gene expression in related species and result in additional levels of exposure to other NTO species.

Where the application does not include cultivation, direct environmental exposure of NTOs to the GM plant is via the accidental release into the environment of seeds or propagules of the GM plant during transportation and processing.

The level of exposure of NTOs to the GM plant will depend on the numbers of GM plants that establish and the areas in which they establish:

- If there is sporadic occurrence of feral GM plants then exposure of NTO populations is likely to be negligible;
- Exposure to GM plant products used as food and/or feed can also be through manure and faeces from the animals fed the GM plant;
- Exposure may also occur via by-products of GM plants during industrial processes (e.g. processing of imported GM timber);
- The level of environmental exposure is estimated on a case-by-case basis depending upon several factors. These include the biological and ecological characteristics of the GM plant and its transgene(s), the range of expected scales and frequencies of GM plant dissemination, the receiving environment(s) where the GM plant is likely to establish, and the interactions among these factors.

If gene flow to cross-compatible wild/weedy relatives and feral plants is likely to occur then exposure of NTOs to these GM plants and their products over life cycles and seasons should be assessed.

3.3.1.4. Definition of measurement endpoints

Through the formulated hypotheses, assessment endpoints are made operational into quantitatively measurable endpoints, termed measurement endpoints. Indicators of change, that will be recorded as part of the comparative risk assessment, need to be defined and established by applicants through measurement endpoints. These measurement endpoints should constitute measures to characterise both exposure and/or hazard, and shall be selected when there is an interpretation of the biological data, i.e. how to relate the results to the assessment endpoint.

An alteration in plant metabolism could substantially affect components of the life history of organisms associated with these plants and consequently alter the growth of NTO populations (Charleston and Dicke, 2008). Both lethal and sub-lethal effects are relevant in the assessment of a possible hazard for a given NTO species.

Testing for sub-lethal effects is important since it can also give indications of possible long-term effects. An appropriate measurement endpoint for NTO testing is relative

fitness (or some component of relative fitness), which is the relative lifetime survival and reproduction of the exposed versus unexposed non-target species (Birch et al., 2004). It is therefore required that NTO tests consider both toxic effects (short-term mortality, longevity) and sub-lethal effects. The latest can be assessed through growth pattern, development rate, reproduction parameters (e.g. number and size of offspring, percentage of eggs hatching, sex ratio of progeny, age of sexual maturity), and, when appropriate, behavioural characteristics (e.g. searching efficiency, predation rates, food choice).

In field conditions, the abundance and species diversity of certain groups of NTOs at a relevant life-stage are typical measurement endpoints. The choice of specific measurement endpoints shall be done according to the problem formulation on a case-by-case basis.

Long-term effects on NTO populations or functional guilds are a substantial element of the ERA, meaning that, in the context of NTO testing, reproduction parameters and testing over multiple generations are considered as appropriate endpoints. In addition modelling and/or post-market environmental monitoring can also be suitable methods for addressing potential long-term effects.

Measures of hazard

Measures of hazard represent the measurable change of the measurement endpoint(s) in response to the GM plant and/or its products to which it is exposed (Sturkey et al., 2008). Measures of hazard may be an acute lethal concentration resulting in the death of, e.g. 50% of the organisms tested or the effective response concentration for chronic effects measured or altered reproduction (e.g. fecundity), growth, development and behaviour in a receptor population (Wolt et al., 2010). These measurements can be expressed as effective concentration affecting a x percentage of individuals (ECx).

In addition, it is necessary to consider reproduction parameters (e.g. number and size of offspring, percentage of eggs hatching, age of sexual maturity), growth pattern, development rate and behavioural characteristics (e.g. searching efficiency, predation rates, food choice) may also be appropriate measures of hazard for long-term effects. At population level, an important predictor is the intrinsic rate of increase (rm) that integrates measures of survivorship and fecundity (Romanow et al., 1991, Stark and Wennergren, 1995). Moreover, the calculation of the instantaneous rate of increase (ri) allows a good estimate of rm for the study of insect populations at lower tiers (Walthall and Stark, 1997a,b).

Measures of exposure

Measures of exposure shall describe the contact or co-occurrence of the GM plant with the valued entity, and can be expressed as predicted (or estimated) environmental concentrations (PEC or EEC). The description of the novel attribute of the GM plant (e.g. transgenic protein) in terms of the route, frequency, duration, and intensity of exposure for the change relative to the valued entity is considered relevant information (Wolt et al., 2010). Both plant and NTO features assume an important role here, for instance overlapping of the NTO biology (e.g. life cycle stages) with the spatio-temporal concentration of the transgenic product(s) are to be considered to quantify exposure. If a non-target species is not directly exposed to the transgene and/or its product(s) from the plant but indirectly via other target or non-target species, these pathways of exposure need to be evaluated.

3.3.1.5. *Hypotheses testing & tiered approach*

A case study approach describing how the GM plant may adversely affect NTOs or their ecological functions is proposed as outlined in Table 4. Based on plant-trait-NTO interactions, five possible cases can be foreseen. On one hand, GM plants may express new proteins/metabolites that have (Ia) toxic properties; (Ib) non-toxic properties; or (Ic) unknown toxicity. On the other hand, GM plants may have an altered composition, in which metabolic pathways known to affect NTO-plant relationships (e.g. glucosinolates in Brassicaceae, alkaloids in Solanaceae, lignin in trees) are altered (IIa), or not altered (IIb).

In all of those five cases, the metabolism and/or the composition of the GM plants may in addition be unintentionally altered as a consequence of the genetic modification in a way that could affect NTO-plant relationships ('unintended effects'). The presence of unintended effects in GM plants can be due to different reasons (e.g. pleiotropic effects) and it is well documented in the scientific literature (BEETLE_report, 2009).

Only in some of the five identified cases (i.e. Ia, Ic and IIa), can a specific hypothesis be formulated to assess plausible intended effects (e.g. a GM plant intentionally altered to produce biologically active compounds may produce the same effects on non-target species).

To test these hypotheses and thus assess possible adverse effects on NTOs, relevant data need to be supplied and considered by applicants. For the two remaining classes of GM Plants, only the absence of possible unintended effects on NTOs needs to be demonstrated according to the principle described below.

Table 4: Identified cases and hypotheses testing

	GM plants expressing new proteins/metabolites with:			GM plants with intentionally altered composition	
	Toxic properties	Non-toxic properties	Unknown toxicity	Alteration of metabolic pathways known to affect NTO plant relationships	No Alteration of metabolic pathways known to affect NTO plant relationships
	I a	**I b**	**I c**	**II a**	**II b**
Possible effects of the transformation process	Intended and unintended	Unintended	Intended and unintended	Intended and unintended	Unintended
Could specific hypotheses be defined?	Yes	No, but see Chapter 3.4.1.6	Yes	Yes	No, but see Chapter 3.4.1.6

Source: EFSA 2010

3.3.1.6. *Specific hypothesis-driven investigation*

For the case studies Ia, Ic, and IIa, specific hypotheses can be formulated and assessed (e.g. the new metabolite can be toxic to some non-target species, or the change in the metabolic pathway will possibly influence the plant's interactions with other organisms on various trophic levels) according to the flow chart illustrated in Figure 6.

Stacked events expressing biocidial compounds, may have different adverse effects on NTOs than the single events due to synergistic, additive or antagonistic effects.
Applicants shall perform studies (or provide existing data) with combined administration of proteins when the genetic modification results in the expression of two or more proteins in the GM plant. *In planta* tests with the stacked event shall be included in tier 1 studies. Testing should follow the same approach as described for single events.

Based on specific hypotheses, NTO risk assessment can be performed in a tiered manner; whereby, hazards are evaluated within different tiers that progress from worst-case scenario conditions framed in highly controlled laboratory environments to more realistic conditions in the field. Three main tiers can be used, which comprise experimental tests under controlled conditions (e.g. laboratory tests under tier 1a and 1b and semi-field tests under tier 2), and field tests (tier 3). Tier 1a refers to in vitro tests carried out with purified metabolites, whereas Tier 1b refers to *in planta* testing using bi- or multi-trophic experiments according to the focal species selected.

Semi-field tests: outdoors tests carried out with some containment that controls for variability, with manipulation treatments on relatively small experimental units (e.g. caged plants, screen houses).

Within a tier, all relevant data shall be gathered to assess whether there is sufficient information to conclude on the risk at that tier. In case no reliable risk conclusions can be drawn, further data might be needed. Decision of moving between tiers needs to be driven by trigger values. These values shall be set for the species under consideration taking into account the intrinsic toxicity (e.g. estimated by effective concentration (ECx) of the newly expressed products and the expected concentration in the plant), and the sensitivity of the NTO developmental stages (examples of trigger values for NTOs are provided in EPPO36 guidelines).

Based on the experience with Cry toxins, tier 1 tests generally seem to represent useful predictors for results at higher tier tests (Duan et al., 2009) provided that designs include all ecologically relevant ways of exposure. When laboratory studies are performed, both in vitro and *in planta* tests (tiers 1a and 1b) should be done to reach a reliable risk conclusion after tier 1. Tier 1a testing is of crucial importance for the ERA if no or little data on the metabolites expressed by similar GM traits are available (e.g. Table 4: case Ic). Tier 1a tests require purified metabolites in the same form as expressed in the GM plant. Tier 1b complements the results as they give indications on possible interactions between plant compounds and reflect realistic exposure conditions through bioavailability.

In fact, Duan et al. (2008) demonstrated that laboratory studies incorporating tri-trophic interactions of Cry1-expressing plants, herbivores and parasitoids were better correlated with the decreased field abundance of parasitoids than were direct exposure assays. Where purified metabolites are not available, only tier 1b studies shall be conducted using GM plant material that guarantees exposure to both transgene products and the plant.

Likewise, it is possible that for some NTO focal species no reliable protocols for performing such experiments exist, in this case applicants may perform this type of test on some focal species only. In all justified cases where testing on a lower tier is not appropriate (e.g. test organisms cannot be reared in the laboratory), applicants can perform tests at the next tier.

The diet regime for each focal species (in the most relevant developmental stages) to be tested must reflect the different possible sources of exposure in nature. Some impacts on multi-trophic interactions and ecosystem functions may not be observed in tier 1 tests. Higher tier testing may therefore be needed on a case-by-case basis before decisions on the level of risks can be made. In particular, field testing is essential to investigate trait versus environment interactions when laboratory tests give reason to assume a possible adverse effect.

The NTO testing phase can be finalized when sufficient information is compiled to reject the tested hypotheses. Applicants, who conclude that further tests are not required, based on available information, are required to explain the rationale for this conclusion. If at any tier adverse effects are detected, a hazard characterisation is required to determine the biological relevance of these effects.

Also, the use of more NTO species in the same functional group might help to clarify how common these adverse effects might be for the specific ecosystem. In some cases it might necessary to go back to the problem formulation phase, to redefine a hypothesis and to design additional experiments to generate the data needed.

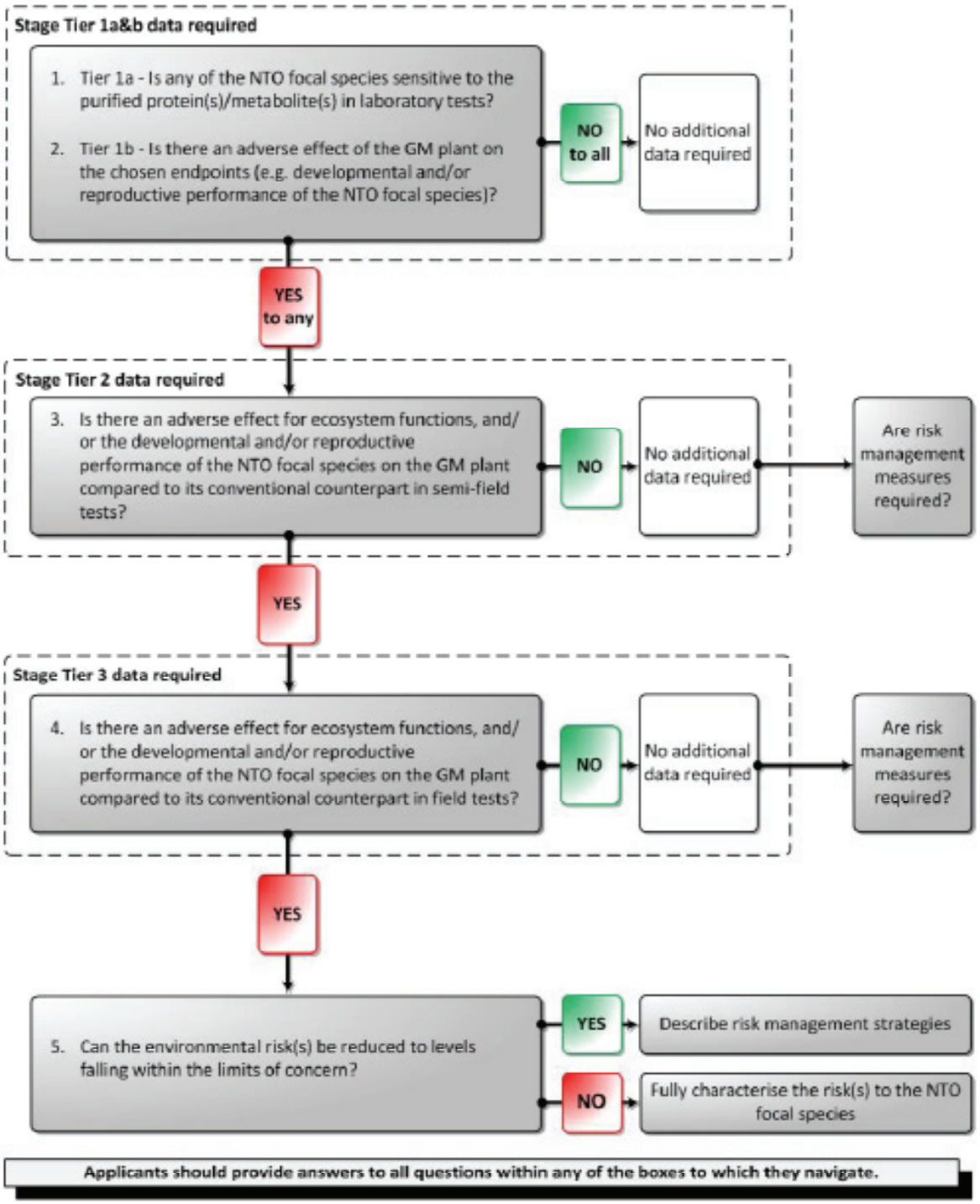

Figure 6: ***Decision tree for carrying out a specific-hypothesis driven investigation. Applicants shall provide answers to all questions within any of the boxes to which they navigate. The questions are divided into three stages (tiers 1.2.3). Only if all the questions of a stage are answered negatively (answer: NO), are no additional data required. If at least one question of a stage is answered positively (answer: YES), applicants shall move to the next stage and address all the questions of that stage.***

3.3.1.7. *Data requirement for the evaluation of possible unintended effects*

GM plants may have unintended adverse effects on biodiversity through interactions with populations of other species associated or sympatric with the GM plant. It is important that species richness and ecological functions, especially considering guilds that provide ecosystem services, are not disrupted to the extent that populations decline and/or vital functions are impaired. Unintended impacts of GM plants on species richness and ecological functions shall be considered in the ERA.

Problem formulation thus seeks to collect all available information to decrease uncertainty of unintended effects to an acceptable level. The evidence to exclude the likelihood of unintended effects on NTOs can come from numerous sources including data already collected for other parts of the risk assessment, collating all the appropriate information from these data sources to provide a weight-of-evidence approach. Data sources relative to plant-environment interactions are always necessary to support the possible exclusion of unintended effects.

The sources of data, which should be properly justified, are described under Chapter 2.1.

Applicants are requested to consider all the information available from these different data sources and to ensure that some field generated data are included. The use of field-generated data may be informative in this context, but applicants must justify why these data are relevant to the ecological functionality of receiving environments in Bhutan where the GM plant might establish.
Since unintended effects are to a large extent event specific, data from other events or from similar events in other plant species will carry little weight in supporting an application.

For stacked events not expressing biocidal compounds, if scientific knowledge does not indicate possibility of synergistic, additive or antagonistic interactions between these compounds that may affect NTOs, then no specific testing is necessary.

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