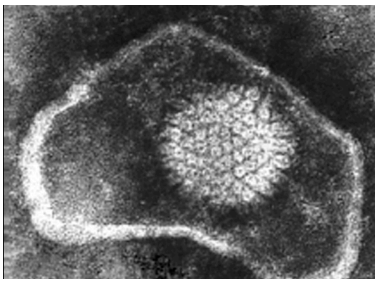
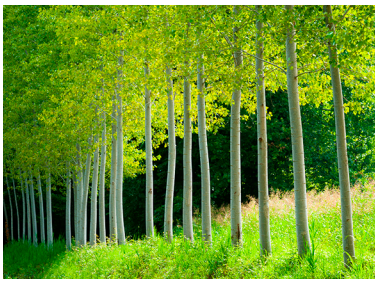
Uncertainty and Knowledge Gaps related to Environmental Risk Assessment of GMOs



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GenØk – Centre for Biosafety (www.genok.no) is an independent research institute founded in 1998 and located in Tromsø, Norway. GenØk is engaged in the field of biosafety and gene ecology research on modern biotechnology, nanotechnology, synthetic biology and other technologies emerging from these. This institution also works on capacity building and advisory activities related to biosafety. GenØk focuses on a precautionary, holistic and interdisciplinary approach to biosafety. In 2007, GenØk was appointed national competence center on biosafety by Norwegian authorities.

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Contents

Executive Summary

This report provides a detailed discussion of uncertainties, knowledge gaps and research needs related to the potential use and introduction of GMOs. It covers GM plants, antibiotic resistance marker genes, GM vaccines, gene therapy and medicinal products containing or consisting of GMOs, as well as GM microorganisms, GM trees and GM salmon.

GMOs are made by inserting a gene (or several genes) encoding a wanted trait into an organism (of the same species or a different species). The environmental risks associated with such alteration of the genome are evaluated on the basis of present scientific knowledge and analysis of available literature. The report identifies many unresolved scientific uncertainties and potential adverse effects related to GMOs and their impacts on ecosystems, as well as on animal and human health. Particular attention has been given to GMOs relevant in a Norwegian context. The report emphasizes today's challenges, but also outlines needs for the future, as new GMOs are becoming increasingly more complex. Examples of such novel GMOs are discussed under the different subchapters.

The most significant identified areas of uncertainty and knowledge gaps in each section are summarized below.

Common themes

This report covers numerous kinds of GMOs in different environmental contexts. However, there are common themes which resonate throughout the report, and which contribute to uncertainty and knowledge gaps related to GMOs in general:

- The novelty of the introduced genes and their products within the context of the recipient organisms, and the potential effects (e.g. pleiotropic effects, interactions with endogenous proteins etc.) that may occur as a result of their introduction.
- Knowledge regarding unintended changes (e.g. recombination, positional effects etc.) introduced into the genomes of recipient organisms due to genetic modification is sparse, and exacerbated by lack of access to test materials and sequence information.
- The imprecision of both the methods used to perform genetic modification, as well the techniques used to characterize the resultant GM organisms.

Uncertainties and knowledge gaps for GM plants

- Most new GMPs are "multistacks", i.e. containing several transgenes for both herbicide tolerance and insect resistance. Data on the adequacy of the use of less complex parental lines for environmental risk assessments (ERA) of multistack plants is lacking.
- Ecosystem complexity and how environmental factors may affect transgene expression is not well understood.

- How co-exposure of one or more herbicides may effect gene expression in the plants is uncertain. Both the expression levels of the transgenic proteins, and other gene products in multistack GMPs is difficult to assess due to lack of data.
- Data on herbicide accumulation in herbicide tolerant GMPs is lacking.
- Unwanted and unintended effects on non–target organisms have been observed and indicate that Cry-toxins are less specific than previously claimed. There is a lack of studies on alternative modes of action for these agricultural toxins.
- Availability of GM plant test material is limited and hampers the possibility for testing by independent scientific institutions. This undermines the quality and credibility of risk assessments.
- There is lack of data for the presence, use and potential transfer of antibiotic resistance (ARM) genes in GMPs. ARM genes may be transferred to other organisms (microorganisms etc.) through horizontal gene transfer.

Uncertainties and knowledge gaps for GM viruses

- Available data on baseline information of naturally occurring viruses is generally lacking in ERA.
- The potential for recombination, complementation and reactivation is difficult to assess due to lack of data.
- Molecular basis for host ranges, molecular characterization and immune status of the host and non-target hosts has little data.
- The potential for GM virus shedding and the potential for infection in non-target species is not well understood.

Uncertainties and knowledge gaps for GM microorganisms (GMMs)

- There is an incomplete understanding of how GMMs become established within an environment.
- Potential impact of resource competition between GMMs and other microbes in the environment.
- Containment strategies used under field conditions for GMMs (GM bacteria, GM algae, GM fungi) are highly challenging, and the potential for escape is not well explored.
- There is little or no data on how to deal with escaped GMMs.
- Knowledge regarding environmental impacts of large populations of GMMs and their effect on the receiving environment is lacking.
- There is a lack of knowledge on the capacity of GMMs to maintain or transfer transgenes within or between populations.
- Potential for, and possible impacts of, horizontal gene transfer (HGT) between GMMs and non-GMM species is not well understood.

Uncertainties and knowledge gaps for GM trees

- The present knowledge on GM trees and the dynamics of gene flow are not fully understood.
- The practical utility of containment strategies for GM trees is not well understood.
- Data is missing on transgene stability of expression over longer time frames. This is highly relevant for GM trees.
- How GM trees will interact with the complex forest, soil and aquatic ecosystems, as a whole, is not investigated.

Uncertainties and knowledge gaps for GM salmon

- Salmon is a highly complex and plastic species, thus there might be many effects of genetic modifications that we will not observe in captivity. The production and the effects of GM salmon on the environment is at present uncertain.
- The knowledge on the impact of escaped GM salmon on wild salmon populations and the ecosystem in general is lacking.
- GM fish health and welfare conditions are unknown.

Recommendations for further research

- There is a need for development of uncertainty analyses that can be used for increasing the quality of ERA of GMOs.
- More open ERA processes are needed, together with scientific institutions' access to GMO test material for independent testing and validation of data.
- The potential for combinatorial effects of transgenes expressed in multistack GMPs are high, and the relevance by the use of parental lines as representative controls of stacked events should be assessed.
- The accumulation of herbicide residues in GMPs must be routinely measured.
- Potential transfer of ARM genes to microorganisms and to other organisms should be investigated.
- Natural environments must be investigated for the presence of reservoirs of ARM genes, in order to elucidate whether the use of ARM-containing GMOs could potentially augment the environmental level of resistance.
- Persistence of GM viruses, and in what form (virion, DNA, RNA or protein) they exist outside their hosts, needs to be elucidated.
- Molecular characterization and the basis for host range of GM viruses, as well as immune status of host and at-risk non-target hosts needs to be investigated.
- Long-term assessment of GM trees is required to determine whether transgene expression is stable over time, through seasonal variations and dormancy cycles.
- Aquatic ecosystems have been neglected in previous ERAs. The effect of GM crops and trees on aquatic organisms must be assessed.

- Effects on welfare and the environment by GM salmon production and use need to be investigated.
- Impacts on wild salmon upon escape by GM salmon must be elaborated.

In a Norwegian context we will in addition recommend:

- Monitoring of baseline data in the environment of ARM, herbicides, natural reservoirs of viruses, target and non-target organisms.
- Evaluation of the relevance of current methods and models in ERA for Norwegian conditions.
- And, if needed, development of new methods and models to be used for ERA and environmental monitoring of GMOs in Norway.

I. Introduction

I.I Background

The production of genetically modified organisms (GMOs) involves recombination, insertion and expression of novel genetic information within a host cell organism. Recombinant DNA techniques enable genetic engineering (GE) of virtually any living cell or virus. The application of GE is now extensively used in basic biological research as well as in applied research to produce GMOs or derived products for commercial use in medicine and agriculture.

The various uncertainties embedded in GE and GMOs relating to health, environment and food security have led to numerous national and international initiatives (OECD, Codex Alimentarius, EFSA) to regulate research-based and commercial use of recombinant DNA technology. The regulatory principles and requirements for the GMOs commercialized so far have been controversial and conflict-laden.

There are many unresolved scientific uncertainties and risks related to GMOs and their impacts on animal and human health, on ecosystems as well as a lack of shared understanding and variable recognition of ethical, social, cultural, legal implications of GMOs.

This report aims to examine the knowledge gaps and research needs related to GMOs, with a focus on environmental issues related to the potential use and introduction of GMOs in general. We intend to map these knowledge gaps and also focus on knowledge gaps and uncertainties related to GMOs in a Norwegian context.

The objectives are to:

- Map and document knowledge gaps and areas of uncertainty related to GMOs
- Identify today's challenges and the future's need
- Identify what is important in a Norwegian context

I.II Organization of the report

The report is divided into the following areas:

1. GM plants
2. GM microorganisms: Viruses and vaccines
3. GM microorganisms
4. GM trees
5. GM salmon

In addition we will provide examples of the various forms of scientific uncertainties present in knowledge production with a specific focus on how it may affect the ERA of GMOs, with examples of uncertainties with relevance for ERA of GMOs.

I.III Sources of information

The main sources of information used in this report are:

- Publicly available relevant literature, mostly scientific peer-reviewed articles, reports and book chapters.
- The technical dossiers of GMO applications assessed by GenØk 2010-2014.
- GenØk policy briefs and reports
- GenØk unpublished research: Field observations gathered / lab experiments carried out by the authors and colleagues.

I.IV Highlighted themes

In this report we have examined available scientific literature (peer-reviewed articles, book chapters and reports) on GM research to highlight potential knowledge gaps of relevance for environmental risk assessments. We have included examples from our own research, as well as experience from doing assessment of technical dossiers in the two first parts, GM plants and GM microorganisms: Viruses and vaccines. The three other parts, GM salmon, GM microorganisms (bacteria, fungi and algae) and GM trees are based on literature studies only.

We have identified some common knowledge gaps that are of high relevance for environmental risk assessments. These touch upon the issue of the change introduced into the genomes of the GMOs, the novelty of the introduced genes and their products, and also that the methods used at present for genetic modification are not entirely predictable. These knowledge gaps raise questions with regard to potentially unforeseen genetic changes that may happen, as well as with regard to the research efforts that need to be initiated and the risk management strategies that need to be performed to detect such unintended changes.

GM plants are important due to their extensive cultivation and use in food and feed. In this context, we have used MON810 as an example due to its long-term use, and since there is available literature on the issues related to the molecular characterization of that event. MON810 is also of high relevance to Norway at present as the Norwegian Environmental Agency has recommended import and processing of this GM plant (http://www.miljodirektoratet.no/no/Publikasjoner/2015/Mars-2015/Genmodifisert-mais-MON810-Helhetlig-vurdering-og-anbefaling-til-vedtak/).

GenØk has many years of experience in research involving vaccine relevant viruses (in particular orthopoxviruses). This has provided competence for environmental risk assessment of applications for marketing authorization of GM vaccines and GM medicinal products, which the institute conducts for the European Medical Agency on behalf of the Norwegian Environmental Agency. The knowledge gaps that are highlighted in this report are based on experiences garnered from assessment of application dossiers and information in the published literature, and includes a wide aspect of GM vaccines and GM medicinal products where viruses or viral vectors are used.

GM microorganisms, fungi and algae have been of interest to scientists for many years due to their great potential in biofuel production. The demand for renewable energy is increasing, and there are many public and private efforts exploring potential sources. Here, GM trees also are exemplified due to the uses as fuel, but also for increased growth under diverse climatic conditions and as fruit (food) producers.

Salmon is an important export trade in Norway. Thousands of tons are produced and exported yearly, a trend which is set to increase. GM salmon have been developed to try to improve the economy of the salmon production by making fish that grow twice as fast on the same amount of food. Other phenotypes have also been developed.

This report is the initiation of a report series that is to be followed up on a regular basis. We have highlighted some of the knowledge gaps we see at present. We aim to follow this up in the future, to look at new GMPs, new GMOs, new emerging techniques and also look back and evaluate whether the gaps in knowledge we have identified have been filled with new knowledge, and if increasing experiences with different GMOs raise new gaps in knowledge and uncertainties.

II. Scientific uncertanties in research and risk assessment of GMOs

II.I Introduction

Uncertainty is a driving force within science and a main driver for new discoveries, creativity and inventions. It plays an inherent part of the dynamics of research, where scientific investigations may contribute to close some knowledge gaps while at the same time new gaps of knowledge are identified, new research questions arise and new uncertainties are revealed (Myhr and Nielsen, 2007). Hence, there will always be a time lag between the science-based regulatory agencies´ immediate need for robust knowledge and the relative, iterative process of knowledge production itself. This time lag is also evident in the production of knowledge related to the use of new technologies such as gene technology. The financial mechanisms driving technological opportunity may be different from those examining the consequences of their uses.

Access to peer-reviewed quality data is essential for a "science-based" risk assessment. However, areas of uncertainty pointing to new knowledge gaps are routinely identified during regulatory risk assessment of GMOs. Uncertainty often arises as a consequence of missing data (lack of relevant studies) that could solve identified potential hazards at the problem formulation stage (i.e. when definition of assessment endpoint to determine what ecological entity is important to protect is decided). GMOs interact with complex ecological and social systems at multiple levels, where lack of full understanding of biological systems leads to uncertainties; including a lack of ability to fully predict unintentional effects of releasing GMOs with new traits into the environment. Hence, these types of uncertainties are not necessarily reduced with more science.

Another source of uncertainty arises from how researchers' and risk assessors' values, beliefs and interests influence knowledge production itself and hence the knowledge base used for biological risk assessment. This influence is caused by bias in e.g. research funding, peer review and selective publication, and in the interpretation and judgments of the validity of study outcomes. Such bias will frequently lead to that published studies, which have too narrow a scope or which have focused on aspects of the biological system with only limited relevance to the biosafety of the GMO itself. The different sources of bias, as well as uneven funding mechanisms of biosafety studies, illustrate how choices made by companies, funding agencies and researchers influence the outcome, and the quality of biosafety studies. In combination, these factors affecting the quality of knowledge production create sources of uncertainty and hence pose challenges for environmental risk assessments (ERA) of GMOs. It is therefore necessary to highlight the need for explicit consideration of uncertainties to improve the quality and robustness of the science underlying the ERA of GMOs.

In this chapter we provide examples of the various forms of scientific uncertainties present in knowledge production with a specific focus on how it may affect the ERA of GMOs, with examples of uncertainties with relevance for ERA of GMOs.

II.II Types of uncertainties

Scientific uncertainties have typically been described as:

(I) 'Knowledge related (epistemological) uncertainties': described as a lack of scientific knowledge or a lack of tools and methodologies resulting in imprecise measurements/observations in experiments and

(ii) 'Variability related (ontological) uncertainties': arising due to the inherent variability and diversity in the population or system under study (Walker et al., 2003).

The characterization of these uncertainties has been attempted through the use of statistics. Quantitative statistical measures have been the principal language to express uncertainties in scientific findings. Importantly, these uncertainties are usually perceived as incomplete knowledge — and reducible through further investigation.

There is however also an increasing awareness of other dimensions of uncertainty that cannot be adequately expressed in quantitative terms (Walker et al., 2003). This has become particularly evident when investigating impacts from applying novel technologies in complex systems. The multidimensional nature of these systems has revealed new dimensions of uncertainty, generally referred to as 'qualitative dimensions'.

Several terminologies (typologies) characterizing these different dimensions of uncertainty have emerged (e.g., Faber et al., 1992; Felt and Wynne, 2007; Funtowicz and Ravetz, 1993; Stirling, 1998, 1999a; Stirling and Gee, 2002; Walker et al., 2003; Wynne, 1992). The different typologies developed usually centre on the concepts of *risk, uncertainty, indeterminacy, ambiguity and ignorance* and is presented in Tables II.I and II.II).

Risk. Each of the typologies takes the concept of risk as a starting point. *Risk* is typically defined as 'magnitude of a possible hazard' multiplied by the 'probability that a hazard will occur' (Stirling and Gee, 2002). This implies that a situation characterized by risk is quite well known — the range of possible hazards is known, and it is possible to numerically calculate the probability that each of these hazards will occur. Still, a situation defined by a quantified risk implies some degree of uncertainty as we cannot know in advance whether an identified hazardous outcome will actually occur or not (we only know the likelihood for it to occur).

Uncertainty describes situations where all hazards associated with an activity are known, but there is a lack of sufficient knowledge to calculate the probabilities that each of the hazards will occur. This is, however, assumed to be solvable with more research. Thus, in this typology both risk and uncertainty are quantitative types of uncertainty which can be adequately expressed in statistical terms, reduced through more research and managed through the conventional approach of risk assessment (e.g., in the sense that scientists are able (or by conducting more research will eventually be able) to identify the range of possible hazards and the respective probabilities for their occurrence).

Table II.I: Typology of quantitative uncertainty in policy-relevant science (adapted from Wickson et al. 2010)

Type of uncertainty	Explanation	Approach/Implications
Risk	We can imagine the range of possible hazards and calculate the probability of those hazards occurring, even though whether any of the hazards will occur or not remains unknown.	Can be dealt with through conventional risk assessment procedures.
Uncertainty	We can imagine the range of possible hazards, but we do not know the probabilities for their occurrence. It is however possible to calculate that probability, but we do not have enough knowledge to do so yet.	Can be dealt with through conventional risk assessments. More research should be initiated to reduce the level of inexactness.

Indeterminacy, *ambiguity* and *ignorance*, in turn, describe qualitative dimensions of uncertainty. *Indeterminacy* is a type of uncertainty that arises due to the complexity of various open-ended social and natural systems. As already described, it is impossible to include all the relevant factors and interactions when investigating complex systems. Hence, knowledge generated about complex systems will always be inherently incomplete. In other words, all scientific studies select their own frames of reference and each of these will necessary be limited in their ability to include all factors of a complex and dynamic reality. Reductionism has been and remains the norm in science.

Ambiguity results from contradictory information and/or the existence of divergent framing, assumptions, interpretation of data and values among different knowledge providers such as scientists, policymakers, impacted parties and the public. Again, this relates to the characteristics of complex systems — they can be described in several equally plausible ways, as there are different ways to understand and approach complex problems and to interpret results. This potential for plural framings exists both in science (especially apparent in the way different disciplines approach the same issue in different ways) and in the socio-political arena (apparent in the way in which diverse interests, perspectives and value frameworks shape understandings of particular issues). Stirling (2007) provides a useful list of places where ambiguity can manifest itself in the framing of scientific risk assessments, starting from how problems are defined and hypotheses formulated, to the choice of tools and methods for the study and its analysis, and how the level of significance for hypothesis testing is defined, and, finally how the results are interpreted and communicated.

Finally, *ignorance* can be described as our inability to conceptualize, articulate and consider the outcomes and causal relationships that lie beyond current frameworks of understanding. It has been described as the things ‘we don’t know that we don’t know’ and represents an inability to ask the right questions, rather than a failure to provide the right answers. The idea here is that there will be potential impacts, which we have not considered, which we have not yet even imagined as possible.

Table II.II: Typology of qualitative uncertainty in policy-relevant science (adapted from Wickson et al. 2010)

Type of uncertainty	Explanation	Approach/ Implications
Indeterminacy	For complex, open, interacting systems, it is impossible to include all the relevant factors and interactions in the calculations	Scientific findings must be treated as partial and conditional explanations, and therefore possibly fallible. Hence, we must expect and be prepared for surprises.
Ambiguity	We can variously frame both the impacts we are interested in and the way we approach, interpret and understand the knowledge and calculations generated about them.	To acknowledge the diversity of possible framings, negotiating across different ones where possible, and at least being transparent about the particular frames that are chosen and the reasons for their selection.
Ignorance	We cannot imagine the possible impact. Not only have we not yet calculated the probability of the event, we are unaware of what we should make calculations for. For instance, the inability to predict unintended effects.	To pursue a diverse range of policy options to maintain flexibility, resilience and reversibility, as well as to consistently and vigilantly monitor for potential surprises. General surveillance as a tool to address ignorance.

II.II.I Values influence hypothesis testing

Choices made by scientists when formulating and testing hypotheses give a good, simple, illustration of how values influence the scientific assessment of risks. Typically, one of two types of hypotheses will be tested in risk research — H_0; 'There is no adverse impact' and H_1; 'There is an adverse impact'. When testing these hypotheses and determining the statistical level of significance, more concern is traditionally given to avoiding Type I errors (false positives — situations where one rejects H_0 and claims there will be an adverse effect, but in fact no adverse consequences manifest themselves) (See table II.III).

Table II.III: Type-I and type-II error in ecological studies. Null hypothesis H_0 = There are no adverse effects.

Reality / Test results	H_0 is true	H_0 is false
The investigation does not show adverse effects	Correct $(1-\alpha)$	Type-II error False negative (β)
The investigation shows adverse effects	Type-I error False positive (α)	Correct Statistical power ($1-\beta$)

What this focus does, however, is to increase the chance of Type II errors (false negatives — situations where one rejects H_1, claims there will be no adverse effect, but in fact adverse consequences do occur). The current scientific focus on avoiding Type I errors (false positives), means that strong evidence is required in order to claim that hazardous consequences may occur. This practice has been accused of favouring the developers of new technologies at the possible expense of human, animal and environmental health. The choice of which type of errors a scientist strives to avoid is in itself a value judgment, and both choices have their respective pitfalls.

In the following we will provide examples of how these various forms of uncertainties are associated with in the science involved in ERA of GMOs.

II.III Uncertainty in the knowledge base of GMOs

II.III.I Knowledge related uncertainties in ERA of GMOs

Knowledge related (epistemic) uncertainty may be due to i) the novelty of the technology, or ii) the complexity involved which causes incomplete description of a mechanism or process. Epistemic uncertainties with GMOs may be related to:

- *Changes of/ in the GMO or its product*. Uncertainty related to the novelty introduced in a GM plant by the genetic material as for instance a) direct effects of the introduced trait such as increased allergenicity or toxicity, b) unintended transfer of genetic material through vertical or horizontal gene transfer, c) unintended non-target effects, and d) unintended environmental consequences.
- *Secondary effects of introduction of the genetic material*. Due to the lack of coherent understanding of how genomes function, it is today not possible to predict precisely how the introduced genes will function in the new host organisms and how the modification will affect the organisms´ own gene functions and regulations. The genetic modification process may cause pleiotropic effects and cause unexpected changes in the host cells protein production and metabolic activities. One example of a pleiotropic effect was published by Prescott et al. (2005), which reported that mice fed with GM peas modified to resist insects elicited an inflammation response in the lungs. The extracted protein did, however, not cause any immune reaction in mouse; hence the adverse effect may have been related to changes in the transgenicplant's protein synthesis or modification.
- *Changes in interactions and responses to environmental conditions.* A GMO released to the environment will interact with the other organisms in this environment. Furthermore, different (not fully predictable) climatic and environmental conditions may affect persistence and dispersal properties of the released GMO (scenario uncertainty).

In the ERA of GMOs sources of epistemic uncertainty typically include:

- Measurement uncertainty (i.e. if the measurement is correct and that the methods for measurement are accurate)
- Parameter uncertainty (i.e. if the relevant parameters have been included for studying cause-effect relationship, this is for example discussed within models for studying non-target effects (see chapter 1.3)
- Sampling uncertainty (if the sample size, places, times are relevant and representative).
- Biological model uncertainty (if the models chosen for laboratory studies represent the causal relationships in nature, this is for example discussed with regard to allergenicity studies and in assessment of ERA of GM vaccines, see chapter 2.1)
- Model system uncertainty (this is for example related to extrapolation form laboratory research and field studies where extrapolation may cause uncertainty, another example is the extrapolation of field studies from one region to another), see chapter 1.3.5 and 3.2.3).
- Scenario uncertainty (into the future there may be release of different GMOs that may interact, influence by climatic changes and/or xenobiotic pollutants, on the GMO/ecosystem interactions, the potential development of resistance in target organisms and weeds))
- Bias in data production (lack of access of test materials etc., see chapter 1.3).

Epistemic uncertainty may be reduced by doing more risk-associated research and by collecting more empirical data. However, if this risk-associated research has too narrow a scope or is biased, or that not adequate methods and or models are employed, or have focused on only parts of the biological system, the outcome may have only limited relevance to the biosafety of the GMO itself. This is also the case if the assessment is based on biased subjective judgements and if the working paradigm is not adequate for achieving scientifically recognised and socially robust understanding.

II.III.II Ambiguity in data production: choice and limitations of methods

In the conduct of research, scientists make assumptions and inferences based on the paradigms within which they are trained, and under the research environment they are socialized into (Kuhn, 1962). The choice of models and methods to test a specific hypothesis is a variable of the research environment, resources, the competencies and instruments at hand, and most importantly, time constraints. Thus, researchers operating in different research environments will invariably choose different models and methods to address the same risk-relevant question (Nielsen and Myhr 2007). An example of this ambiguity is the issue of addressing potential allergenicity of GMO products. This issue is exceedingly complex because the mechanistic aspects of allergy development are not fully understood even within the basic medical sciences. Thus, there is no single biological model or experimental standard available to evaluate the potential allergenicity of new products from GMOs.

Scientists have thus been drawing on the familiarity of the unmodified host organism(s) and have constructed a number of models, assumptions and comparative approaches to determine allergenic

potential and to conclude on the absence of IgE-mediated allergenicity in the GM products[1]. Not surprisingly, the assumptions behind the choice of appropriate models and methodological specifications have been questioned (Spök et al., 2005). Selecting live test organisms, other than humans, inevitably brings up uncertainty due to the need for extrapolation without fully understanding all relevant differences in biology between test species.

Another example is the potential for pollen flow from GM-crops to other crops, weeds and wild-relatives. This is a biosafety-relevant question for regulators and scientists that can be addressed by a range of hypotheses and model choices of a highly complex natural system (Nielsen and Myhr, 2007) Pollen flow raises issues such as:

a) Economic and legal concerns with regard to how GM-crops can be cultivated in co-existence with conventional and organic farming, including issues related to labelling, liability, and socio-economic aspects such as effects on traditional farming practices, product identity, seed quality control and changes in farming infrastructure.
b) Environmental concerns with regard to potential adverse effects from flow of transgenes (introgression) into cultivated species, weeds, and wild species.
c) Health concerns with regard to the potentially changed allergenic properties of pollen caused by the genetic modification, or health impacts caused by pollen flow from GM-plants producing pharmaceutically active compounds into crop plants entering the food chain.

Ambiguity may also arise due to expert subjective judgement and due to linguistic uncertainty (study bias, anchoring, overconfidence, etc.).

These sources of ambiguity affect the risk and problem formulation. The choice of model system and methodological approaches will likely remain a contentious issue in the ERA of GMOs.

These sources of ambiguity affect the risk and problem formulation, and have an impact on the formation of hypotheses on the effect (or lack thereof). The choice of model system and methodological approaches will likely remain a contentious issue in the RA of GMOs.

II.III.III Indeterminacy due to inherent randomness in biological systems

Biological systems are highly complex and may not be easily quantified or explained by quantitative methods. Random variation in baseline data in conjunction with complex, multi-scale network interactions between molecules, cells, organisms, physical environments and environmental

[1] These include (i) computer-assisted bioinformatics-based comparisons of the new proteins (produced by the GMO) to known protein allergens, (ii) examinations of the stability of the protein in experimentally simulated gastrointestinal tract systems and (iii) experimental and theoretical consideration of the overall concentration, composition and stability of the protein (e.g. heat stability). It is clear that these methodologies require numerous subjective decisions regarding the exact experimental conditions applied. Some examples of assumptions that depend on the model choices include assumptions that the allergenic site can be identified in proteins based on 2-D amino acid composition and not 3-D structure, and that the protein digestive capacity of the gastrointestinal tract of humans can be adequately constructed by mixing specific concentrations of enzymes and chemicals in test tubes.

variables (temperature, season, geography, etc.) can lead to meaningless quantification efforts; and hence indeterminacy (Funtowicz and Ravtez, 1990; Wynne, 1992).

Whereas precise numbers (such as the rate of gene flow, or degradation kinetics of a protein) can be obtained within various experimental model systems, their quantitative mean and range as a variable in changing geographical and environmental contexts rarely have the same level of precision. Another source for indeterminacy may be statistical variability.

A closer look at biosafety-relevant studies reveals that indeterminacy is an intrinsic component in many, if not most of these, hence, they may have limited quantitative value.

II.III.IV Ignorance arising from conceptual limitations in the operating paradigms of the biological system

Risk from GMOs arise because there is uncertainty about causal chains in the intervened complex biological system. The ecotoxicological risk perspective (paradigm) has been influential in shaping risk concepts in biosafety. This unwittingly contributes to further ignorance since chemicals follow a different environmental route and degradation pathway than transgenes do (Karlsson, 2006), see chapter 1.3.

II.III Summary

This chapter highlights the importance of recognizing various forms of uncertainties in the science involved in ERA of GMOs.

A main challenge in ERA is how to interpret and weigh conflicting studies, of which some may indicate an undesired effect arising from the activity, whereas others, perhaps the majority, indicate no observable effects or a beneficial effect. Thus, in other words, should ERA be exclusively based on a selected group of scientists' views on what type of studies to pursue and their interpretation of data? And how should contrasting data, and minority views be communicated in the conclusions of a risk assessment? There is no clear policy on how to deal with contrasting studies during ERA, leaving their inclusion or exclusion, and interpretation open to subjective assessments made by the members of the ERA body. Often, the presence of conflicting studies on safety issues in the ERA phase never reach the risk communication phase, due to implicit subjective judgments about their validity and the perceived need of providing the public with an unambiguous risk conclusion.

Numerous methods and tools for uncertainty characterisation and analysis have been developed (see for instance Skinner et el., 2014, and Stirling 2007 for an overview). At GenØk we have used the Walker & Harremöes (W&H) framework (Walker et al, 2003; Gillund et al., 2008a, b). The aim of this framework is to provide a conceptual framework for the systematic treatment of uncertainties in model-based decision support. Without presenting any of these tools in detail we want to emphasise that comprehensive analysis of uncertainties in ERA of GMOs would require the use of such tools.

One approach to acknowledge uncertainties, and conflicting knowledge claims in particular in ERA of GMOs, is to 'open up' risk assessment processes (Stirling, 2009) and allow for deliberation and stakeholder participation in the ERA. The Problem Formulation and Options Assessment Framework is one example of useful tool developed to facilitate stakeholder engagement in ERA of GM plants (Nelson et al., 2009).

References

FABER, M., MANSTETTEN, R. & PROOPS J.L.R. 1992. Humankind and the environment: an anatomy of surprise and ignorance. *Environmental values*, 1, 217-242.

FELT, U. & Wynne, B. 2007. *Taking European knowledge society seriously*. Report on the Expert Group on Science and Governance to the Science, Economy and Society Directorate. Directorate – General for Research. European Commission, Brussels.

FUNTOWICZ, S. O. & RAVETZ, J. R. 1990. *Uncertainty and Quality in Science for Policy*. Dordrecht: Kluwer.

FUNTOWICZ, S.O. & Ravetz, J.R. 1993. Science for the post normal age. *Futures*, 25, 739-755.

GILLUND, F., DALMO, R., TONHEIM, T.C., SETERNES, T. & MYHR, A.I. 2008a. DNA vaccination in aquaculture – expert judgments of impacts on environment and fish health. *Aquaculture*, 284, 25-34.

GILLUND, F., KJØLBERG, K.A., KRAYER VON KRAUSS, M. & MYHR, A.I. 2008b. Do uncertainty analyses reveal uncertainties? Using the introduction of DNA vaccines to aquaculture as a case. *Science of the total environment*, 407, 185-196.

KARLSSON M. 2006. The Precautionary Principle, Swedish chemicals policy and sustainable development. *Journal of Risk Research,* 9, 337-360.

KUHN, T. S. 1962. *The structure of scientific revolutions*. Chicago: University of Chicago press.

NELSON, K. C., ANDOW, D. A. & BANKER, M. J. 2009. Problem Formulation and Option Assessment (PFOA) Linking Governance and Environmental Risk Assessment for Technologies: A Methodology for Problem Analysis of Nanotechnologies and Genetically Engineered Organisms. The *Journal of Law, Medicine & Ethics,* 37, 732-748.

NIELSEN, K. M. & MYHR, A.I. 2007. Understanding the uncertainties arising from technological inventions in complex biological systems: the case of GMOs. In: Traavik, T., Lim, L.C. (eds.) *Biosafety First: Holistic Approaches to Risk and Uncertainty in Genetic Engineering and Genetically Modified Organisms.* Tapir Academic Press, Trondheim. pp. 107-123

PRESCOTT, V. E., CAMPBELL, P. M., MOORE, A., MATTES, J., ROTHENBERG, M. E., FOSTER, P. S., HIGGINS, T. J. & HOGAN, S. P. 2005. Transgenic expression of bean alpha-amylase inhibitor in peas results in altered structure and immunogenicity. *Journal of Agricultural and Food Chemistry*, 53, 9023-30.

SKINNER, D. J. C., ROCKS S. A., POLLARD, S. J. T. & DREW G.H. 2014. Identifying Uncertainty in Environmental Risk Assessments: The Development of a Novel Typology and Its Implications for Risk Characterization*, Human and Ecological Risk Assessment: An International Journal*, 607-640.

SPÖK, A., GAUGITSCH, H., LAFFER, S., PAULI, G., SAITO, H., SAMPSON, H., SIBANDA, E., THOMAS, W., VAN HAGE, M. & VALENTA, R. 2005. Suggestions for the assessment of the allergenic potential of genetically modified organisms, *International Archives of Allergy and Immunology*, 137, 167–180.

STIRLING, A. 1998. Risk at a Turning Point? *Journal of Risk Research*, 1, 97-109.

STIRLING, A. 1999. *On science and precaution in the management of technological risk*. Vol. 1. A synthesis report of case studies. An Esto project prepared for the European Commission – JRC Institute for prospective Technological Studies, Seville.

STIRLING, A. 2007. Risk, precaution and science: towards a more constructive policy debate, *Embo reports*, 8, 309-312.

STIRLING, A. 2009. Participation, precaution and reflexive governance for sustainable development. In: Agder, N. and Jordan, A. (Eds). *Governing Sustainability*, UK: Cambridge university press, 193-225.

STIRLING, A. & GEE, D. 2002. Science, precaution and practice, *Public health reports*, 117, 521-533.

WALKER, W.E., HARREMOÖES, P., ROTMANS, J., VAN DER SLIUIJS, J.P., VAN ASSELT, M.B.A., JANSSEN, P. & KRAYER VON KRAUSS, M.P. 2003. Defining uncertainty a conceptual basis for uncertainty management in model based decision support, *Integrated assessment*, 4, 5–17.

WICKSON, F., GILLUND, F. & MYHR, A.I. (2010). Treating nanoparticles with precaution: recognising qualitative uncertainty in scientific risk assessment. In: Kjølberg, K.L. and Wickson, F. (Eds). *Nano meets macro social perspectives on nanoscale sciences and technologies*, Singapore: Pan Stanford Publishing, 445-473.

WYNNE, B. (1992). Uncertainty and environmental learning: reconceiving science and policy in the preventive paradigm, *Global environmental change*, 2, 111- 127.

1. GM plants

1.1 Introduction

Most of the genetically modified (GM) plants currently commercialized encompass a handful of crop species: soybean, corn, cotton and canola. Other genetically modified plants that have been approved for sale in the U.S. include tomatoes, radicchio, zucchini and yellow squash. The majority of fresh fruits and vegetables on the market are not genetically engineered. In the EU, there are no GM fruits on the market.

The two principal traits introduced into the main GM crops are Bt insect resistance and glyphosate herbicide resistance and are developed by multinational companies. At present there is huge interest and research initiated to achieve disease tolerance, altered compositions, stress tolerance (especially related to drought tolerance) as well GM plants that can be used to produce pharmaceuticals and be used for environmental remediation. The future of GM and innovation in plant improvement technologies (agriculture) will be affected by the future needs of our increasing population.

In the first GM plants, transgenesis was used to transfer one trait in the varieties commercialized, mainly resistance to one pest or tolerance to one herbicide. Today there is a clear trend towards combining two or more transgenic traits present in single events through conventional breeding. The "offspring" plant would then be a stacked event. In addition, plant science has made considerable progress in the recent years towards the development of new plant breeding techniques and new approaches, such as using nanotechnology, RNAi technology and synthetic biology that can be used separately or in combinations. More background information about new emerging techniques can be found in the GenØk report "Current status of unintentional effects and biosafety of emerging technologies for plant breeding: Nuclease-based and oligonucleotide-directed mutagenesis techniques" from 2015.

Genetically modified plants, such as crop plants intended for food and feed or release into the environment, have to undergo risk assessment prior to marked authorization in many countries. Today there is no broad international consensus as to what is at risk from introduction and use of GM foods and crops. There are differences in the regulation of GMOs between countries, with some of the most marked differences between USA and Europe. Regulation varies in a given country depending on the intended use of the products and in general GMOs can be demarcated for import and processing, for food, feed and industrial use and/or cultivation. A

Soy beans
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GMO application can cover one or more of the different categories. In most countries, governmental employees are responsible for the performance of regulatory risk assessments in accordance with international agreements and national laws. In principle, the safety assessment of GM food, feed, releases and marketing of GM plants is assessed on a case-by-case basis.

In this chapter we will focus on the knowledge gaps and uncertainties of some of the main GM crops (maize, soy and rapeseed) and traits (Bt-resistance and herbicide tolerance). In addition, uncertainties and knowledge gaps associated with stacked events and antibiotic resistant marker genes (ARM genes) will be discussed.

The main issues that will be addressed in this chapter are:

- Knowledge gaps and uncertainties connected to the technique of genetic modification
- Knowledge gaps and uncertainties associated with environmental risk assessment

1.2 Molecular characterization

According to EFSA, key steps in risk assessment of food and feed derived from genetically modified plants include several steps concerning the characteristics of the donor and recipient plants, genetic modification and the functional consequences and compositional characteristics of GM plants and derived food and feed (EFSA, 2011). The guidance document calls for characterization of the nucleic acid used for transformation, the complete DNA sequence of the intended inserted DNA in addition to analyses regarding the possible relationship between the inserted gene products and known toxins, anti-nutrients or allergens. In the EFSA guidance document of environmental risk assessment of genetically modified plants, the importance of molecular description and analyses is affirmed by calling for a "detailed molecular description of the DNA sequence inserted into the plant" (EFSA, 2010). Despite the clear statement and intentions in the guidance documents, GenØk's experience has been that claims of low likelihood of molecular interactions and changed characteristics due to modification in the DNA sequence, are often made without providing sufficient scientific evidence – or sometimes without any explanations at all. Reliable claims regarding molecular properties are of course very difficult to make, particularly without proper scientific evidence. Even in the scientific literature there is much we do not know about interactions and protein behavior, one reason is undoubtedly the nature of information in applications of GMOs. Because they are regarded as confidential business information, knowledge and research contribution from non-company researchers is severely hampered on almost all of the traits on the market today. What we do know from independent research has generally been acquired in retrospect of approval. This chapter highlights some, but by no means all, of the molecular aspects of GM crops that we greatly need much more independent research on.

The only events from food and feed crops for which molecular data are available are MON810 and GTS40-3-2. That is why the examples below are mostly drawn from the experiences with these events since their commercial release. As we highlight below, there are still many unanswered questions, even after almost two decades of growing these crops. In this chapter we will focus on the main and long-residing knowledge gaps when it comes to molecular data and characterization of the

transgene inserted into GM crops. Due to the severely limited availability of data, most of what we know is drawn from MON810 and Roundup Ready Soy.

1.2.1 Plant Transformation methods

The first step in creating a GM plant is of course to assemble the DNA construct that is to be inserted into the genome of the plant. This construct normally hails from different kingdoms in the tree of life, such as bacteria, viruses, other plants, or entirely synthetic constructs like Cry1A.105. The latter sometimes mimic the behaviour of bacterial Cry proteins expressed in plants (Hernandez-Rodrigues et al., 2013). After the construction of traits is finished, the plant needs to be exposed to the DNA construct so that it may incorporate it into its genome. Some new technologies are slowly being made available, but the GM crops approved in Europe today are all made by either ballistic bombardment or by *Agrobacterium*-mediated transfer.

1.2.1.1 Transformation by Ballistic Bombardment

Ballistic bombardment involves the use of a particle accelerator (gene gun), which can deliver DNA coated on micro particles into cells. Normally cationic micro particles like gold (i.e. Svarovsky et al., 2008) or tungsten are coated with DNA and driven to ultrasonic speed, and this momentum allows the particles to penetrate the cell membranes and deliver the DNA to the cytoplasm or nuclei of the target cells (Zhao et al., 2012, Zhang et al. 2013). The process puts the DNA under physical stress and the method is known to cause several mutations in the delivered DNA sequence. These mutations are not possible to predict. It is therefore of utmost importance that sequence data of the transgene DNA after integration, as well as sequence data of the DNA construct *a priori* to transfection, are available.

The most famous crop produced by gene gun methods is undoubtedly the maize event MON810 by Monsanto Company, and it is also an example of why access to the sequence data is so important. In 2003, Hernandez and co-workers published a study characterizing the 3´end of the MON810 transgene inserted into maize, and discovered that the 3´end was truncated and hence had lost the 3´NOS terminator signal originally included in the bacterial plasmid (Hernandez 2003). In addition, the active protein (Cry1Ab) was unexpectedly shortened by 336 base pairs creating a much shorter inserted element and shorter protein than intended. The function of Cry1Ab was retained in the new novel truncated protein, although no studies so far have aimed at comparing the effectiveness of the truncated MON810 compared to a full-length Cry1Ab. It is of major importance that MON810 was approved in Europe in 1998 and the information about the truncation was not available until 4 years later, brought forward by independent research and not declared in the original application of approval. There are some concerns tied to this truncation, particularly to the loss of the terminator, as discussed below in the following sections.

1.2.1.2 Agrobacterium-mediated transfer

The other main method of transferring DNA into a plant cells is by the use of the bacteria *Agrobacterium tumefaciens*. The bacterium is the cause of plant tumours on dicotyledonous plants. In the process of inducing tumours, the bacteria transfers a piece of DNA harboured between two specific genetic elements called T-DNA (transferred DNA) that have certain characteristics – one of them being that anything between two T-DNA sequences is transferred into the plant cell (see Zupan

and Zambryski, 1995 for an historical overview of the discovery of this process). This property is utilized in biotechnology and the creation of GMOs by inserting the desired genes between these T-DNA sequences. One main obstacle and limitation to the method, is that the integration process is poorly understood and appears random in nature with no apparent preference for chromosome loci (Forsbach et al., 2003). In one of the few big studies of this phenomenon, 112 transfected *Arabidopsis thaliana* plants were sequenced around the integration spot and the sequences mapped to the chromosomes. In the majority of cases, rearrangements (deletions, duplications of T-DNA) around the integration site occurred (Forsbach et al., 2003).

1.2.2 Sequence information

Although the applicant is required to submit the integrated DNA sequence as well as flanking regions along with the application for GMO approvals, this information is considered confidential and not released to independent researchers or the public in any form. The inherent problem relying solely on applicant data is clearly illustrated by a publication authored by Windels and co-workers in 2001 (Windels et al., 2001). They discovered that rearrangements in soy event GTS 40-3-2 (Roundup Ready Soy) had occurred at the 3'NOS junction when the T-DNA was delivered and integrated in the soy cell. A 254 bp portion of the EPSPS gene (which confers tolerance to glyphosate) had been duplicated and inserted after the terminator sequence, which was designed to end transcription of the inserted DNA. In addition, 540 bp with no known sequence homology follows immediately after the 254 bp sequence. This is the same line of events that followed the MON810 approval and release, where researchers not involved in the development discovered rearrangement in the final integrated DNA that was not reported by the companies. These two examples are the only events that are fully sequenced in the public literature and thus the only two that independent research can uncover unintentional molecular changes in. For the others released after MON810 and GTS 40-3-2, there are no publicly available data or reports on the DNA sequence, neither before nor after integration, except the minimal information needed for event-specific detection.

The companies are obliged to deposit sequence information and primer sequences to the Joint Reference Center (JRC) for validation of detection methods for the event seeking approval in Europe. One of the reasons is that the validation must be performed by an accredited organization up to the current ISO-standards. The information required is limited to event-specific detection, typically concentrated around the 5' and 3' ends of the transgene insert, which would allow the investigators to distinguish the events from one another. In a screening scheme, one typically starts with broader screening to determine if GMOs are present in the sample or not. Promoters and terminators are typically chosen, as they are common to many GMOs and allow for a cost effective way to rule out GM negative samples for further analysis. Thereafter for the positive samples, the detection process moves to more specific analyses, such as event-specific screening. Sequence information about promoters, terminators etc. are not distributed to detection facilities. Validation of the promoters from JRC relies on public literature, which in a trial and error approach tests different primers and different constructs (Wu et al., 2014). The information on what TYPE of element (CaMV35s promoter etc.) is released, but not what kind of modifications done to the sequence or what part of the full-length P35S promoter is used (Podevin and Jardin, 2012 and references within). This process would

have been a lot easier if promoter sequences were available so that primers could be constructed based on the actual DNA sequence used and not in a blind fashion.

1.2.3 Promoters

The CaMV35S promoter may be of biosafety concern because of the multiple properties inherent in the same sequence. In the cauliflower mosaic virus (CaMV), the 35s sequence encodes a protein called P6 (encoded from gene VI) that harbors many different functions. The naturally condensed state of the chromosome of the virus results in an overlap between gene VI and the part known as P35S used in GMO plants (Podevin and Jardin, 2012). This poses a potential risk, as the degree of overlap between P6 and P35S depends on what variation (i.e. length) of P35S is used in genetic engineering. The concern is that partial expression of P6 may be allergenic (Podevin & Jardin 2012). As shown by Benfey and Chua (1990), Kay et al (1987), Fang et al (1989) and Podevin & Jardin (2012), multiple variants of P35 exists are used today without any knowledge of expressed P6 domains or their potential allergic properties. Podevin and Jardin (2012) suggested a framework to aid risk assessment of living products containing any form of the P35S promoter, but of course this requires detailed knowledge of the inserted DNA sequence and how it overlaps with genomic DNA. Today there is no way for researchers to access this information.

1.2.4 Chromosomal localization of events

Maize is a very complex and highly diverse species. In fact, a detailed comparison with modern molecular biology techniques of two inbred lines, B73 and Mo17, revealed a structural diversity never seen before among eukaryotes (Springer et al., 2009). Although there were large regions of low genetic diversity, a huge number of sequences differed in copy number and also in presence/absence analysis. Nearly 85% of the genome consists of several families of transposable elements (Schnable et al, 2009, Springer et al., 2009), which raises interesting concerns about the chromosomal localization of transgenic events. There has already been a mismatch when analyzing the junction between endogenous DNA and transgene DNA in which chromosome MON810 is located. The 5´end is mapped to chromosome 4, based on a high similarity blast (88%) of 440 bp adjacent upstream of the 5´integration site (Holck et al., 2002). The 3'integration site is followed by a 1222 bp fragment that does not show any identity with any known maize sequence (Hernandez 2003). Extending the downstream sequence to another 345 bp, it has been suggested that the 3´end aligns (99% identity) with sequences on maize chromosome 5 (Rosati et al., 2008). In this case, the target sequence was a BAC clone from maize line B73 (AC185641). Reanalyzing the 5´sequence obtained by Holck (2002) (accession no. AF434709) shows high score hits to chromosome 5, 8 and 10 (see Table 1.1).

Table 1.1: Identity scores from performing a BLAST with AF434709 from Holck et al., 2002 against maize sequences (March 2015)

Target Sequence	Accession No	Identity Score
Genomic DNA, clone Chromosome 8	AC 157487.1	88 %
Genomic DNA, clone Chromosome 10	AC225944.1	86 %
Genomic DNA, clone Chromosome 5	AC205514.6	84%

There are also highly relevant hits to DNA from a 19-KD Zein gene family, confirming the findings from Hernandez (2002). The Zein cluster resides is many loci in the maize genome (e.g. Song and Messing 2002), and as such there is no surprise that blasting sequences from this cluster would result in multiple hits and that they may be on more than one chromosome. It is interesting to note that no hits from the 3'end are suggested to be at chromosome 4 where the 5'end is claimed to be located. Either there are structural differences between the maize sequenced and deposited in the databases, or there are multiple copies of MON810 sequence in the genome of the transgene maize varieties. It is important to understand that this may be possible even if the applicant has submitted correct data to the contrary, because once approved the transgene event can legally be crossed into multiple genomic backgrounds which incorporate the event differently than that of the original event described in the application. This is especially a risk in an event such as MON810 that resides close to genes that may have many loci and hence many possibilities for homology-based crossing.

1.2.4.1 MON810 Variants

The current practice in risk assessment is that once an event is approved, there are no limitations to what genetic background, within the same species, that same event can be crossed into and still fall under the original approval. For instance, over 100 varieties of MON810 exist on the market, yet only a few have been part of the efforts sequencing and characterizing DNA sequences. Given that maize is a species with a very high degree of structural differences, there are uncertainties regarding how crosses between highly divergent variants integrate their genomes.

The genomic divergence also raises some concerns regarding non-GM comparators. The choice of method from the applicants seems to be to choose several maize species, arguing to establish a natural variability baseline, rather than comparing to the most similar non-GM variety (most often the conventional non-GM parental strain of the GM-hybrid). The concern with this approach is that because maize gene expression will vary a lot between varieties due to the high genetic variability in the species, this "natural baseline" will be so variable that it may mask statistically significant differences between the GM and the near-isogenic line.

1.2.5 Unintended insertion effects

Several studies have shown unintended effects on the transgene after insertion in the host genome, either by ballistic bombardment or *Agrobacterium*-mediated transfer. Deletions (Hernandez et al. 2003), recombinations and repeats (Windels et al., 2001), rearrangements (Hernandez et al., 2003) and mutations affecting detection (Morisset et al., 2009) have been reported by independent researchers investigating the molecular features of some events after commercial approval.

1.2.5.1 Unintended transcripts

Transgenes inserted into a host genome will disrupt the locus which it is inserted into. This may give rise to unintended transcripts with unknown fate. In the scientific literature, very little is known about the insertion site (loci) for any event. Genomic localization is important in the context of positional effects, but also the transcripts that may arise due to leakage of the stop signal or the complete lack of it. Rosati and co-workers showed in 2008 that mRNA-species of different length were produced across the junction between the end of Cry1ab and the disrupted HECT gene in the recipient genome of MON810 (Rosati et al 2008). No further attempt published to quantify or deduce the fate of these novel mRNAs has been published. The same discovery was made by Rang and co-workers in 2005 when analyzing roundup Ready soy (Rang et al., 2005). Roundup ready soy has an intact stop signal, but it was still discovered that mRNA species of different lengths were produced by the plant transcription system. This GM plant has undergone a duplication of parts of the CP4-EPSPS gene copied after the stop signal, so that the event would read (from 5'-to 3') [CP4EPSPS-*nos* terminator-duplicated part CP4EPSPS-genomic plant DNA]. It was across this t-*nos* gene that transcription was detected (Rang et al., 2005). There have been no further published studies investigating the fate of these mRNAs.

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1.2.5.2 Unintended proteomic effects

Proteomic analyses are often limited by a number of factors like protein solubility, pH spectrum and abundance. Despite these limitations, a number of proteomic effects have been described for GM maize plants when compared to non-GM maize plants.

The environment has been shown to be a determining factor when it comes to gene expression. The same variety in different environments may have different expression profiles that would mask any variation from GM MON810 maize plants (Barros et al., 2010). Not only non-GM proteins may vary with the environment. In a study from 2013, Agapito-Tenfen and co-workers showed that even within environmental variation, detectable and significant differences between the GM and non-GM plants occurred (Agapito-Tenfen et al., 2013). In fact, 32 proteins assigned to functions like carbohydrate and energy metabolism, genetic information processing and stress response were differentially expressed between GM/non-GM MON810 maize. Several other studies confirm the effects of the environment on gene expression (reviewed in Ricroch et al., 2011); the question is whether GM and non-GM plants with semi-similar genotypes (parental and GM variant) would predictably react in the same manner.

Zolla and co-workers published in 2008 that even though many effects may be due to environmental effects, still many unpredictable changes occurred in the maize MON810 GM genotypes compared to the near-isogenic parental variant. Considering the limitations of proteomic 2D-gel analyses, a

significant number of proteins showed different expression contributed by the genotype (i.e. GM/non-GM). Up and down regulation, truncation and even a potential allergen were detected in GM maize (Zolla et al., 2008). This study confirms an earlier study by Albo et al., showing unpredictable differences in Bt-maize flour (Albo et al., 2007). The importance of Zolla's work is that it is done in maize seeds, whereas the majority of maize proteome studies are performed on maize leaves. While environmentally relevant, from the consumer point of view, seeds are more important. There is a need for much more research on GM crop seeds and the effects on the modification related to different environmental conditions.

1.2.6 Host-specific impacts on GM proteins

One concern that has been raised concerning genetically engineered food or crops are that the toxicological effects of the transgene or other compounds in the plant may change after insertion. Plants have a feature called RNA editing (Takenaka et al., 2013) which induces changes in the mRNA sequence so that it is not complementary to the DNA sequence anymore. This could potentially change the predicted amino acid sequence and thus the function of the protein. More importantly it would not allow the DNA sequence to determine the protein, something that complicates genetic engineering (Knoop, 2011). Mainly located in plastids and mitochondria, this may not be a major issue in genomic inserted DNA, but because no GM proteins have ever been isolated from plants, nobody has confirmed the amino acid sequence in any GM protein.

Due to the random and unpredictable nature of the insertion processes, insertion of foreign DNA into a plant genome could potentially change several aspects of the recipient plant. Such changes may not always be obvious, since the environment plays an important role in determining which genes are switched on or off in the genome. This is of course the reason that the choice of comparator is important and why it is necessary to compare to the closest related strain/variety. It has been found that toxic metabolites have been produced under very specific environmental conditions in GM plants, whereas the levels of these components have been lower in the non-GM counterpart (Mathews et al. 2005). In potato for instance, the levels of toxic secondary metabolites called glycoalkaloids (PGA) are influenced by many biotic and abiotic stresses. The findings of Mathews and co-workers illustrate this point. They found that transforming potato with an inverted gene and a maize promoter can change the levels of PGA significantly in tubers (Mathews 2005), but could not rule out that the changes where due to positional effects rather than processing of the inserted gene. It is clear that this is different from crop to crop, as the PGAs in potato are associated with plant defence systems and thus manipulating pathways of pathogen metabolism often would lead to changes in associated compounds like PGA (Mathews 2005). For other crops, unwanted proteins or metabolites may be associated with other pathways.

1.2.6.1. Importance of studying the correct protein

The principle of genetic engineering is to take a gene from one context and express it in another. The underlying assumption is that the gene product will perform the same tasks and functions in the new host as in the original system. This assumption is apparent in risk assessment of proteins originating from bacteria, like various forms of Bt proteins, where feeding trials and molecular description analyses are performed on the bacterial version of the proteins rather than the proteins produced in

the transgene context. The reason for this, developers claim, is that it is not feasible to produce adequate amount of proteins, thus bacterial versions of proteins must be used. While this may have been more accurate some years ago, the development of protein isolation and purification technologies has dramatically improved the efficiency of these procedures in recent years.

The justification for use of bacterial protein variants is often that they have similar amino acid sequence to the modified plant protein. One important assumption of this approach is that similar (important: not identical) proteins produced in different systems (i.e. bacteria and plants) have the same characteristics and properties. This statement has never been tested in the scientific literature for any Bt proteins. In addition, there are examples from the literature that proteins may change abilities based on what system they are produced in.

Small changes in primary and secondary protein structure can impart important changes to their bioactivity (Haider and Ellar, 1989; Geiser et al., 1996; Walsh and Jefferies, 2007). One well-known example is the study by Prescott et al. in 2005 (Prescott et al., 2005), where a protein from beans was expressed in peas (α-Amylase Inhibitor-1), which gave the seeds protection from plant pests. When expressed in peas, the protein was expressed in an altered structural form that induced inflammatory responses when fed to mice. Moreover, this response was not present in the unmodified form of the protein (i.e. from beans). The authors conclude that transgenic expression of non-native proteins in plants may lead to the synthesis of structural variants possessing altered immunogenicity. Similar analyses have been performed on transgenic soybeans, where a Brazil-nut allergen was detected (Nordlee et al., 1996) and in MON810 maize, where an almond allergen was detected (Zolla et al., 2008). These studies clearly demonstrate the example of the possibility for altered properties for proteins when expressed in different systems, and/or altered allergenic potential of the proteome when the host is genetically modified. These studies also demonstrate the scientific justification for the demand for purified plant produced proteins to be tested.

The phenomenon of altered structural isoforms is also important in light of detection and protein quantification analyses. Protein quantification is based on antibody-reactions between a detection antibody and the protein. Today, antibodies raised against plant-GM produced proteins are exclusively developed by the use of bacterial variants of the GM proteins (often *E. coli*). This may bias all subsequent equivalence testing against the detection of potential novel *in-planta* produced isoforms. It is impossible to say, using evidence often provided in the dossiers, that the polyclonal antibodies would in fact detect all isoforms of the recombinant proteins that might be produced *in-planta*, were they present in the sample.

1.2.6.2 In silico analyses

The applicants often conclude that the protein is not allergenic based on the *in silico* study, yet immunogenic responses do not necessarily follow a dose-response curve. *In silico* analysis is widely accepted, but also requires additional testing (EFSA, 2008a, Gendel 2002). Although a homology to a known toxin or allergen is not found based on sequence analysis, the protein folding and creation of new structures similar to known toxins/allergens should also be considered. The possibility that allergen can be predicted based on non-contiguous stretches of amino acids should also be considered (Gendel 2002, Moreau 2006). The limitation of the *in silico* approach must be considered, as it is limited to identified proteins and epitopes that are not influenced by PTM (Codex 2003).

1.2.7 Lack of molecular data

As emphasized several times above, there is a fundamental lack of molecular information and basic data necessary to perform relevant biosafety research. Because no transgene DNA sequence except from a few older GM crops (see above) is known, it is not possible to detect potential changes and perform stability investigations on GM plants. This is particularly true for newer products and stacks. We, as independent biosafety researchers, have no cost effective way of checking expression variants, amino acid changes and potential allergenic protein variants, DNA mutations or mRNA variants in any GMO except the two first released 20 years ago. As new knowledge of different regulatory systems surfaces, one would like to apply this knowledge also to biosafety research, but without being able to predict interactions between the GM construct and natural pathways and systems (for instance micro RNA target sites), this research will simply not be done. It is also extremely important to understand that such knowledge that would enhance the GM techniques and products would benefit. A good example of this is the resistance in *Busseola fusca* in South Africa to MON810, where molecular knowledge from researchers not connected to the developing companies are promoting research that ultimately would benefit Bt-designs.

1.2.7.1 Quality of applicant Data

Dossiers submitted to regulators and describing the evidence of GMO testing include in most cases PCR and Southern blot data (targeted genome profile) and western blot data (targeted proteome profile). These techniques can be said to be part of routine evaluations.

In many assessments, analyses submitted with technical dossiers for EFSA approval have continually found to be of sub-par quality. The use of oversized probes, too short exposure times, and lack of molecular weight markers have been noted in many assessments, but not necessarily improved upon thus far.

The levels of the expressed GM proteins are most often determined by the use of ELISA and the levels detected are always too low for further use in downstream analysis (toxicology, allergenicity etc). However, these proteins are subjected to western blots for determination and comparison of molecular weight and for analysis of glycosylation patterns. The quality of the western blots in general is good, but molecular weight markers are often lacking. In some cases, there are bands on the gels and membranes that are left out of comments, without explanations. It is not obvious what the reasons for this might be. These bands are thus not further analysed by any method (as for instance MALDI-TOF MS or a similar method) identifying the protein(s) in the band. In some cases, the antibody used for detection of the GM protein lacks sufficient characterization (as in; what antigen was used to make the antibody), something that can be important when one looks at the detection level of the western blot.

1.2.8 Funding effect

In 2011, Domingo and Bordonada surveyed the scientific literature for studies on GM safety assessments (Domingo & Bordonada, 2011). In their conclusion the authors state "…it is worth mentioning that most of the studies demonstrating that GM foods are as nutritional and safe as those obtained by conventional breeding, have been performed by biotechnology companies or

associates, which are also responsible of commercializing these GM plants". The concern is that these studies may be affected by something referred to as the funding effect, demonstrated in 2005 for low-dose effects of Bisphenol A (Saal and Hughes, 2005). During a meta-analysis of the published studies concerning low-dose effects of Bisphenol A, the authors found that studies funded by chemical corporations or performed by researchers with known association to chemical companies always concluded with no-harm of low-dose Bisphenol A (100%). In contrast, studies funded by governmental institutions would find no harm in 10% of the studies and claim harm in 90 % of the studies. There is a history of accepting applicant data regarding GMO safety (feeding trials, genetic description and stability etc.) for risk assessment. When we consider the potential for the funding effect and recall that the original data from MON810 was faulty when originally submitted, one has to conclude that there is a dire need for independent research in all areas of GM crops. It is particularly interesting that MON810 and GTS 40-3-2 are the only events in GM food/feed crops where the DNA sequence is public information.

1.2.9 Conclusion

Access to molecular data for independent research is restricted, if not entirely cut off. This effectively stops any researcher investigating DNA effects over time in any GM crop except MON810 on maize and GTS-40-3-2 in soy, without first undertaking major commitments in time and expense to sequence the artificial DNA. Very few GM companies, if any, are willing to take this upon themselves, also due to the likelihood of being legally challenged by commercial interests for violating trademark laws and revealing confidential business information. This is a serious issue that should be raised by governmental institutions of member countries with EFSA and the European Commission.

1.2.10 Directions for further studies

The main knowledge gap with respect to molecular aspects is by far the availability of molecular data of events. Without this information, it is extremely difficult to investigate fundamental issues like gene stability, mutations and protein structure and function. New GM crops utilizing RNAi uses the sequence to function, thus independent research needs access to these data.

New transformation methods may arise, and new insertion techniques are being developed. There is a need to understand how these new techniques (zinc finger technology, ODM directed mutagenesis etc.) interplay with the natural plant biology to uncover the mechanisms and potential unwanted side effects of these techniques.

Sequence information is crucial, as mentioned several times above, and gene stability and expression rate is completely unknown for almost all events. Research in this field is severely hindered by the lack of sequence data on the genetic construct. As an example, it is impossible to design good probes without knowing the sequence of the target (i.e. transgene).

The common practice of using bacterially produced versions of the genetically modified protein are not optimal given the possibility for host-specific changes and post-translational modifications.

Efforts should be made to isolate and describe the GM proteins actually produced in plants/crops and compared to the bacterial versions used in risk assessment experiments.

The choice of who performs the research is important to consider. As shown above, the funding effect is relevant and history in the field of biosafety research has shown that it is independent research that drives the field forward. In the only two examples in which we know the sequence data in GM food/feed crops, it is independent research that has discovered insertion-effects like truncation and loss of stop signal, transcription of multiple mRNA variants and the potential for fusion-proteins from P35S. In light of this, there is no doubt that independent biosafety research should continue and be strengthened, particularly when we are facing new technologies to be implemented in GM crops (for instance RNAi).

1.3 Environmental Risk Assessment

Most countries agree that biodiversity must be preserved, conserved and used sustainably for the future. This is firmly established through the Convention on Biological Diversity and the Cartagena Protocol, ratified by 194 and 169 countries, respectively.

Further, lack of full scientific certainty of harm should not prevent actions to safeguard biodiversity and natural resources. Thus, there is a strong international standing for the Precautionary Principle, with the goal of preserving genetic, species and ecosystem diversity.

Genetically modified (GM) organisms and the agricultural or forestry practices they belong to may cause negative effects on biodiversity (e.g. non-target organisms) and ecosystems. Therefore, GMOs need to be thoroughly assessed. An environmental risk assessment (ERA) is required for the regulatory evaluation of a GMO within the framework of Regulation (EC) No. 1829/2003 on GM food and feed and under Directive 2001/18/EC on deliberate release into the environment of genetically modified organisms (GMOs) (EFSA Journal 2010).

Depending on the intended uses of a GM plant (import, processing, food, feed and/or cultivation), the pathways and levels of exposure of the GM plant to the environment will vary (EFSA Guidance document on the ERA of GM plants), and as defined in the European Union (EU) legislation, an ERA shall evaluate the "risk to human health and the environment" (EC2001, Annex II). For the Norwegian context, the Gene Technology Act gives additional criteria for evaluation: the use of a GMO should be sustainable, socially acceptable and ethically justifiable. These criteria applied together with the Precautionary Principle can be argued to be particularly important for the environment since long-term and combinatorial effects in complex ecosystems always will be difficult to detect, understand and respond to. Also, we are in a period of mass extinction of biodiversity, caused by various human activities (Woodruff 2001), and new potential stressors to ecosystems that already are under stress (reduced resilience) may trigger synergistic effects and further losses.

Main traits of GM plants grown today

Most GM crop plants grown on a commercial scale have two classes of traits built into their genome: **(1) Insect resistance**, typically from Bt-toxins (Cry-toxins), in theory targeting a narrow range of pest insects; and **(2) Herbicide tolerance**, as of today primarily to Roundup/glyphosate-based herbicides. Both types of traits lead to new exposure routes and other doses of insect toxins and selected herbicides in the environment, and to the organisms that live there.

In this chapter we focus on the two main traits used in GM plants this far: insect resistance and herbicide tolerance and evaluate the following issues (for each of them):

- Effects on non-target biodiversity
- Resistance evolution
- Ecosystem effects
    - Gene flow/spread and persistence of GMOs and transgenes
    - Invasiveness of GMOs
    - Ecosystem genes and phenotypes

In addition we will also discuss knowledge gaps and uncertainties related to

- Stacked traits and combinatorial effects
- Antibiotic resistance marker (ARM) genes

1.3.1 Insect resistant plants / Bt-toxins

The soil bacterium *Bacillus thuringiensis* (Bt) has a long tradition for use in agriculture due to its insecticidal properties. More than 270 different strains of Bt are described and more than 700 *cry* sequences that code for Cry proteins have been identified (Palma et al. 2014). Some of these have been used for controlling insect pests of Lepidoptera, Coleoptera, Diptera and Hymenoptera, as well as nematodes (Bravo, Gill, & Soberon 2007).

Cry genes are inserted into GM crop plants to express Bt-toxins that make them resistant to important insect pest species. Reduced grazing from target herbivore insects leads to improved yield when these insects are abundant (Areal, Riesgo, & Rodriguez-Cerezo 2013). Further, the strategy to make the plants express pesticides internally (as a part of their metabolism) may replace external use, i.e. spraying of (often broad spectrum) pesticides on the plants.

1.3.1.1 Effects on non-target biodiversity

Meta-analyses and reviews on effects

A meta-analysis based on 42 field experiments showed that unsprayed fields of Bt-transgenic maize plants have significantly higher abundance of terrestrial non-target invertebrates than sprayed conventional fields (Marvier et al. 2007). Thus, Bt-plants with a single Bt-gene inserted may represent an improvement for non-target organisms in the environment. However, an indication of some negative effects of the Cry1Ab toxin itself, or the Cry1Ab producing maize plant, on non-target

abundance was shown in the same meta-analysis: when conventional (non-GM) fields were not sprayed, the non-target abundance was significantly higher than in the Bt-fields (Marvier et al. 2007).

Specificity of Bt-toxins (Cry-toxins)

The specificity of Bt-toxins to kill a limited range of target organisms has been considered a major advantage for agricultural application because unwanted negative effects on non-target organisms can be minimized (Avisar et al. 2009;Torres & Ruberson 2008). The toxicity of Cry proteins is studied in some detail, and susceptibility requires ingestion, an alkaline midgut to solubilize and activate the proteins, and presence of Bt protein-binding receptors on the midgut membrane (Aimanova, Zhuang, & Gill 2006;Jurat-Fuentes & Adang 2006).

However, effects on non-target organisms without the relevant receptors point to alternative modes of action (Bøhn et al. 2008;Bøhn, Traavik, & Primicerio 2010;Hilbeck & Schmidt 2006;Then 2009).

The Bt protein in the GM plant is continuously expressed as a water-soluble and active toxin due to its truncated form where the N-terminal part is shortened.

Additionally, the safety testing of Bt-toxins is performed with Bt toxins produced by bacteria, typically in *E. coli*, and not by the protein expressed in the transgenic plants. This gives a mismatch between what we test and what is shed into the environment (see section 1.2.6.1 “importance of studying the correct protein”).

In most of the dossiers for Bt-plants, the applicants fail to address relevant literature on the environmental and health implications of the Cry I class of proteins, and Cry1Ab in particular, in relation to non-target and environmental effects. For example, a meta-analyses of published studies on non-target effects of Bt proteins in insects, (Lövei & Arpaia 2005) documented that 30% of studies on predators and 57% of studies on parasitoids display negative effects to Cry1Ab transgenic insecticidal proteins. However, there has been a bias towards the study of a few predatory species and the collected data is far from adequate to predict general effects of Bt-toxins (Lövei, Andow, & Arpaia 2009). Nevertheless, responses in natural enemies are non-neutral much more often than would be expected by chance, and most of the observed effects are negative (Lövei et al. 2009). A review by (Hilbeck & Schmidt 2006) on all Bt-plants found 50% of studies documenting negative effects on tested invertebrates.

Impacts on soil microflora and fauna, including earthworms (Zwahlen et al. 2003), mycorrhizal fungi (Castaldini et al. 2005) and microarthropods, in response to Cry endotoxins have also been reported (Wandeler et al 2002, Griffiths et al 2006, Cortet et al 2007).

It has been demonstrated that sub-chronic dosages of Cry proteins may affect both foraging behaviour and learning ability in non-target bees (Ramirez-Romero et al. 2008), and may have indirect effects on recipient populations. Given the key-stone role of bees as pollinators, on both primary production and on entire food-webs, more detailed studies are warranted. A meta-analysis based on 25 studies did not detect direct negative effects on honeybee survival (Duan et al. 2008). A more recent feeding study using pollen from a single and a triple Bt-event found no negative effects in honeybees (Hendriksma, Hartel, & Steffan-Dewenter 2011).

The significance of tri-trophic effects of accumulation, particularly of insecticidal Cry toxins (Harwood, Samson, & Obrycki 2006;Obrist et al. 2006) is not firmly established. However, a study by

Zhou et al. showed that two species of spiders showed changes to three metabolic enzymes after feeding on Cry1Ab-containing prey, demonstrating how Cry toxins may impact non-target arthropods in the food-web (Zhou et al. 2014).

Indications of harm to non-target organisms in the environment, and possible impacts to human and animal health prompted the Austrian authorities to invoke a safeguard clause to ban the use of Cry1Ab-containing maize event MON810 (Umweltbundesamt, 2007). We refer to this report as a detailed analysis of potential adverse effects from a Cry1Ab-producing GMO. Similar responses with bans for growing MON810 Bt-maize were performed also in Germany, Hungary, Luxemburg, Greece and France.

Studies of exposure and effects of Bt-toxins in the aquatic environment

Basic aquatic ecology, e.g. through the "river continuum concept", has shown that the energy input to a small stream can be significant and even larger than the local energy production within the stream. The exposure of terrestrial material (energy) into the aquatic system stems from the submerged stratum of the stream and from the cumulative effects of wind, rain and gravity that drive plant material down into the aquatic system (Griffiths et al. 2009). Streams and ponds close to agricultural fields may therefore have a large energy source in agricultural plant parts and byproducts. A field of transgenic Bt-plants will thus represent a significant source not only of energy but also Bt-toxins, particularly after harvest and if crop residues are washed off the field, into a nearby stream or pond by a flood (Figure 1.1).

The exposure of Bt proteins into the aquatic ecosystem may also come from living plants since Bt-toxins are released from the roots of the plants (Saxena & Stotzky 2000) and can leak into the aquatic system in periods of rain or floods. The soil quality may also play a role. Clay particles binds strongly to Cry1Ab-toxin and also reduce the biodegradation of the protein (Palm et al. 1996; Tapp & Stotzky 1995). Soils that contain clay tend to keep Cry1Ab toxin close to the soil surface, therefore a higher rate of bioactive Cry1Ab run-off can be expected in systems with clay-rich soil (Saxena, Flores, & Stotzky 2002).

It has been demonstrated that freshwater mussels near Bt-corn fields contained *Cry1Ab* transgenes in their gills, digestive glands and gonads. The authors claim that the most likely exposure route was mussels filter-feeding on microorganisms which had acquired the transgene via plant to bacteria horizontal gene transfer (Douville et al. 2009). Mussels may however also filter small-sized Bt-maize

Figure 1.1: A Bt-maize crop residue fills up a run-off aquatic ecosystem near a maize field in South Africa 2000. *Photo: Thomas Bøhn*

particles directly. The investigation of Douville and co-workers found a gradient of *Cry1Ab* linked to the exposure from the environment. At the most exposed site, the level of recombinant DNA in organs was at its highest, and this coincided with a significantly reduced condition factor (weight/length) in the mussels (Douville et al. 2009). However, in field studies causal factors are difficult to point out as other factors may be confounding.

Target insect groups of Bt-toxins also inhabit aquatic ecosystems

The insect orders Lepidoptera, Diptera and Coleoptera are all found in aquatic environments. In terrestrial systems species within Lepidoptera, Diptera and Coleoptera are specifically targeted by different Cry-toxins. However, aquatic stages or species in these groups may also be adversely affected, depending on the exposure, feeding strategy and sensitivity of each individual species. Feeding strategies of aquatic insects include filtering of particles like pollen, scraping off the biofilm from surfaces, and direct feeding on organic material like leaf litter. Probably, the timing of feeding in relation to when the plant material enters the system will be important, particularly if Bt-toxins are available in high concentrations but for a limited time, before they are released from the plant material and may be found elsewhere in the system or simply broken down. Also, if the Bt-toxins are quickly released from the plant material, there may be a significant peak in Bt-toxin concentration in the water shortly after new plant material has been washed off from the field and into the aquatic system. If this comes from a flood, the dilution effect due to a large volume of water present must be considered as well.

Effect studies on aquatic organisms

A study conducted by Rosi-Marshall and co-workers investigated the fate of Cry1Ab/Bt transgenic corn byproducts in 12 headwater streams of agricultural production areas in the Midwest of the US. The study revealed that headwater streams receive cobs, litter and pollen from agricultural plants, and that this input decreased with increasing distance from the point of source (Rosi-Marshall et al. 2007). Thus, organisms in headwater streams are exposed to transgenic crop by-products. In addition, they found that 50% of the field-collected caddisflies contained corn pollen in their guts.

Feeding trials from the same study revealed that the caddisfly *Lepidostoma lima* reduced its daily growth rates by more than 50% ($p=0.008$) when fed Bt-corn litter as compared to non-Bt-litter. Another caddisfly, *Helicopsyche borealis*, was shown to have increased mortality, but this response required exposure to a high concentration of pollen, i.e. 2-3 times higher than max pollen density observed in the field (Rosi-Marshall et al. 2007).

Caddisflies are relatively close relatives to the target group of Cry1Ab transgenic corn, the lepidopterans. Both belong to the same superorder *Amphiesmenoptera* and share similarities in traits, life cycle and use of habitat (Grimaldi & Engel 2005). Thus they may also share receptors for active domains in the Cry1Ab toxin and thus be vulnerable to exposure to transgenic proteins. The primary candidates for safety testing and risk assessment of Bt-transgenic plants should therefore be sought among stream insects that feed directly on plant material and that also are closely related to terrestrial target pest species.

1.3.2.2 Resistance evolution to Cry-toxins

The positive effect of replacing the use of broad spectrum pesticides on conventional plants with growing Bt-plants, as referred to above, is clearly reduced or lost if GM Bt-plants must be sprayed with broad spectrum pesticides due to resistance development in the pest insect. Such pest

resistance development has been documented in South Africa. The main target pest for that region, *Busseola fusca,* has become resistant to Bt-maize MON810 (*cry1Ab*) (Kruger, Van Rensburg, & Van den Berg 2009; Kruger, Van Rensburg, & Van den Berg 2012; Van den Berg 2013a; Van den Berg, Hilbeck, & Bohn 2013; Van den Berg 2013b) and has been observed feeding on MON810 plants (Figure 1.2a). The response has been to spray Bt-plants with conventional pesticides (Figure 1.2b). From the 2013 growing season onwards, this issue is dealt with by replacing MON810 with the MON89034 in South Africa, a plant that includes both cry2Ab2 and cry1A.105 genes (Van den Berg et al. 2013). Thus, resistance in pest insects led to stacking of two insect toxins in the plants, with potentially increased effects on non-target organisms.

Figure 1.2: a) Resistance development to Cry1Ab in South Africa. *Busseola fusca*, feeding on a MON810 maize cob (top), and b) insecticide spraying on MON810 Bt-maize in 2012 (bottom). This illustrates that the Cry1Ab maize was not used sustainably in South Africa. *Photos: Thomas Bøhn*

1.3.2.3 Ecosystem effects

Gene flow and unintended spread of GMO: a case study of Bt-maize in South Africa

It is not realistic that GM and non-GM maize can be separated in small-scale farming systems. Iversen and co-workers documented the presence and spread of transgenes in rural agricultural communities in South Africa (Iversen et al. 2014), indicating that important management/permit regulations made for large-scale industrial productions are in practice impossible to follow in small-scale farming, both due to pollen flow (Beckie & Hall 2008) and because farmers share and recycle seeds. The study shows how transgenes are unintentionally mixed into local seed stores. Using these seeds for planting may lead to low-level expression of Cry-toxin genes which may contribute to ecological consequences, such as accelerated resistance development in pest insects. Transgenes mixed into local seed stores in small-scale farming systems may be difficult or impossible to remove, illustrating the problem of co-existence of GM and non-GM farming in small-scale systems (Binimelis 2008; Devos et al. 2009; Iversen et al. 2014; Jacobson & Myhr 2013; Tumusiime et al. 2010).

Do cry-genes have community and ecosystem properties?

When genes have properties that may transform community structure and ecosystem processes, those genes contribute to 'community or ecosystem phenotypes' (Schweitzer et al. 2004; Whitham et al. 2006). For example, the variation in condensed tannins (which are genetically controlled) in poplar trees, give rise to multiple effects on higher level properties of the community and even on ecosystem processes (Schweitzer et al. 2004). This case illustrates how a single gene (or quantitative trait locus) can have significant effects on the entire ecosystem: on the community composition, endophyte community, related aquatic community and also nitrogen mineralization and aquatic decomposition (Whitham et al. 2006). Poplar trees thus serve as a model system to study how a single but well characterized gene/loci/trait may affect not only the individual and population, but also organisms that directly and indirectly interact with that gene (Schweitzer et al. 2004).

Just as tannins reduce herbivory on the foundation species poplar in forests, Bt-toxins may reduce herbivory in foundation species in the agricultural system, e.g. in some of our most important crop species, like maize, cotton and rice grown on millions of hectares of land. A successful Bt-transgenic plant will control pest insects, i.e. strongly reduce the population(s) of key herbivore species, thus significantly altering food-web interactions. In food-webs, species are embedded within matrices of hundreds of species that co-exist in variable environments (Whitham et al. 2006). The most obvious response in such system may be increased populations of some non-target organisms that eventually may become secondary pests (Hagenbucher et al. 2013). A rise in a secondary pest populations may reduce or eliminate the environmental benefits from reduced spraying with pesticides. In China it has even been reported that Bt-cotton are sprayed with *more applications* of pesticides than conventional cotton (Zhao, Ho, & Azadi 2011). A contrasting conclusion, that insecticide applications were strongly reduced by the use of Bt-cotton, and marginally increased by secondary pest development was reached by Wang (Wang, Lin, & Huang 2009). Studies of detailed responses in complex food-webs have indicated indirect interactions through plant metabolites, but not necessarily with negative fitness consequences (Hagenbucher, Waeckers, & Romeis 2014). A review article on spiders indicates different effects for different families of spiders, and in different plant species (corn, cotton, eggplant, rice and potato) tested. Thus new data with higher resolution is needed (Peterson, Lundgren, & Harwood 2011).

1.3.3 Herbicide tolerant (HT) GM crop plants

HT plants dominate the world production of GM food and feed plants with about 80 % of all GM plants grown globally (James 2013). HT plants are modified to tolerate certain herbicides so that these can be sprayed during the growing season of the plant. This has the advantage that emerging weeds are reduced or eliminated during the growing season with little labour, and at relatively low cost. On the other hand, it may lead to increased use of the herbicides which the plants tolerate, and may also lead to accumulation of toxic chemicals in the final plant product (Bøhn et al. 2014; Duke et al. 2003). For all HT GM plants, the herbicide co-technology is an integral part of the production system and will always be used by the farmer. Hence, HT GM plants should be assessed for risk together with the relevant herbicide used on it.

The most commonly used herbicides that GM plants tolerate are: Glyphosate (Roundup), Glufosinate ammonium, 2, 4-D and Dicamba. These can be used as single event, with plants tolerant to only one herbicide, or stacked events where two or more tolerances are combined in a single GM plant. Dominating plant species with HT traits are: soybean, rapeseed (canola), maize, cotton and sugarbeet.

To exemplify HT GM plants: the first-generation glyphosate-tolerant GM-soy plant (event 40-3-2), produced and patented by Monsanto Company, has been genetically modified to tolerate exposure to glyphosate-based herbicides. This is still the predominantly used GM soy on the market. Herbicide tolerance was achieved by insertion into the plant genome of a transgene construct which constitutively expresses the *Agrobacterium* strain CP4 analogue of the plant enzyme EPSPS (5-enolpyruvylshikimate-3-phosphate synthase). The endogenous plant EPSPS is critically important for production of certain essential aromatic amino acids. Glyphosate, the active ingredient of Roundup herbicide formulations, is able to bind to both weed and crop versions of EPSPS. The binding leads to inactivation of the enzyme and consequently death for the plant. Glyphosate binds the CP4 EPSPS expressed in GM-soy cells in a condensed, non-inhibitory conformation. Hence plants engineered to express the CP4 EPSPS enzyme are provided tolerance to glyphosate. Accordingly, the farmer may eradicate all kinds of plant weeds by spraying with glyphosate, and yet not harm his GM crop plants.

1.3.3.1 Effects on non-target biodiversity

Assessing the environmental effects of the use of HT GM plants is not straightforward for a number of reasons.

Firstly, these plants must be evaluated together with their co-technology herbicides. Thus far, the risk assessment of HT plants and their belonging herbicides has been highly disconnected by food safety authorities, also in Europe and Norway, with separated groups working with GM plants and herbicides/pesticides both in EFSA and in the Norwegian VKM. This needs to change.

Secondly, to understand the environmental impact of herbicides, we need to assess acute as well as chronic toxicity of both single and combinatorial exposures to different non-target organisms (with different sensitivities) in different types of ecosystems (terrestrial, soil and aquatic). Further, we need to discuss the issue of (different) *comparators*. Non-GM industrial agriculture typically uses a range of herbicides and pesticides. With this as the comparator, HT GM crops may represent a shift from certain herbicides, potentially from more to less harmful herbicides. A very different comparator

would be agroecological or organic production systems where the use of pesticides and herbicides are reduced to a minimum or eliminated.

Thirdly, a key element of the potentially negative effects of HT plants is accumulation of herbicides in the plants, which has been overlooked in the risk assessment procedures this far. This is the case both for comparative compositional analyses, and for comparative feeding experiments.

Toxicity of glyphosate and Roundup

Glyphosate has been heralded as an ideal herbicide with low toxicity for operators, consumers and the environment surrounding agriculture fields (Duke & Powles 2008), but has received more risk-related attention due to its negative effects on both aquatic and terrestrial ecosystems. For example, studies of effects on non-target organisms indicate that glyphosate herbicides in fresh-water and marine ecosystems can have significant negative effects on aquatic microbial communities (Perez et al. 2007), macrophytes (Lockhart, Billeck, & Baron 1989; Simenstad et al. 1996), cnidaria (Demetrio et al. 2012), sea-urchin embryogenesis (Marc, Mulner-Lorillon, & Belle 2004), fish (Servizi, Gordon, & Martens 1987), amphibians (Mann et al. 2009; Relyea 2005b) and planktonic algae (Perez et al. 2007). However, a majority of relevant publications report low toxicity or no adverse effects from prescribed dosage use. This also corresponds to conclusions in published reviews of glyphosate-based herbicide ecotoxicity potential (Dill et al. 2010;Giesy, Dobson, & Solomon 2000). A recent review of glyphosate herbicide effects in aquatic ecosystems gives a comprehensive overview of individual studies for most investigated taxonomic groups (Perez, Miranda, & Vera 2011).

Fully formulated Roundup herbicide, not glyphosate in isolation, is used in the field. Thus, it is important to consider, not only the active ingredient glyphosate and its breakdown product AMPA, but also the other compounds present in the formulation. Roundup herbicide formulations contain surfactants (adjuvants) to facilitate penetration of glyphosate into the plant tissue. Polyoxyethylene amine (POEA) and polyethoxylated tallowamine (POE-15) are commonly used surfactants in Roundup formulations, and have been shown to contribute significantly to the toxicity of Roundup formulations (Benachour & Seralini 2009;Mesnage, Bernay, & Seralini 2012; Moore et al. 2012). However, glyphosate alone has also been shown to interfere with molecular mechanisms that regulate early development in frogs and chicken, with deformities of embryos as a consequence and the retinoic acid signaling pathway as the affected mediator (Paganelli et al. 2010).

Feeding studies

Nile Tilapia (*Oreochromis niloticus*) fed sub-lethal concentrations of Roundup exhibited a number of histopathological changes in various organs (Jiraungkoorskul et al. 2003). A study of Roundup effects on the first cell divisions of sea urchins (Marc et al. 2004) is of particular interest for ontogenetic and embryological development, relevant in multicellular organisms. The experiments demonstrated cell division dysfunctions at the level of CDK1/Cyclin B activation. Considering the universality among species of the CDK1/Cyclin B cell regulators, these results question the safety of glyphosate and Roundup on higher animal and human health. In another study, a negative effect of glyphosate, as well as a number of other organophosphate pesticides was demonstrated on nerve-cell differentiation (Axelrad, Howard & Mclean 2003). In human placental cells, Roundup is more toxic than its active ingredient. The effects of glyphosate and Roundup were tested at lower non-toxic concentrations on aromatase, the enzyme responsible for estrogen synthesis (Richard et al. 2005). The glyphosate-based herbicide disrupts aromatase activity and mRNA levels and interacts with the

active site of the purified enzyme, but the effects of glyphosate seems to be facilitated by Roundup formulations. This suggests that endocrine and toxic effects of Roundup, not just glyphosate, can be observed in mammals. Thus, the presence of Roundup adjuvants may enhance glyphosate bioavailability and/or bioaccumulation. However, as Roundup is sold as differently formulated products (with secret composition), the effects on wildlife and environmental health of its use are difficult to study and evaluate.

Further follow-up long-term studies on animal and ecosystems health is required to properly assess HT GM plants and herbicides that are applied with them.

Cuhra and co-workers tested chronic effects of glyphosate and Roundup in an invertebrate model and showed that environmental concentrations of 0.05, 0.45 and 1.35 mg/l (active ingredient) cause negative effects on *D. magna* (waterflea) offspring size, reproduction and survival, respectively (Cuhra, Traavik, & Bøhn 2013). Given that: i) such low concentrations (i.e. below the accepted US thresholds for surface waters = 0.7 mg/l) of glyphosate and Roundup cause negative effects in *D. magna* from environmental exposure, and ii) glyphosate has been documented to accumulate at a level of 9.0 mg/kg (on average) in soy on the food and feed market (Bøhn et al. 2014), it is both interesting and important to test if, how, and at what concentration, residues of the same herbicides in plant material may cause negative health effects on organisms in the environment.

1.3.3.2 Resistance evolution and herbicide tolerance traits

As for Bt-toxins, there is a resistance issue for herbicides. For example, the extended use of glyphosate on Roundup Ready glyphosate tolerant GM plants has led to a dramatic increase in the use of glyphosate-based herbicides (Benbrook 2012) which must be seen in relation to the resistance evolution documented for 24 species of weeds globally (Heap 2012). Such development may illustrate a 'transgenic treadmill' (Binimelis, Pengue, & Monterroso 2009) – resistance triggers more applications and higher doses, which leads to stronger selection for resistance, etc. In the end, the use of additional and often more toxic herbicides like atrazine and 2,4D may be the only way out.

For the farmers, such development is not sustainable, for the environment it will contribute to higher exposure rates of toxic agrochemicals, and for non-target consumers it may affect health through plant tissue accumulation of herbicide residues. An important point is that crop and herbicide monoculture makes the agroecosystem more vulnerable to further resistance development (Beckie 2011). Despite resistance challenges, the use of HT plants have increased sharply (James 2013), particularly in the US, Brazil, Argentina that dominate the global production of soy (American Soy Association 2015).

USDA data document dramatic increases in the use of glyphosate-based herbicides and GM soy is a major driver for this development (Benbrook 2012). By 2008, 620 000 tons of glyphosate were produced (Pollak 2011).

1.3.3.3 Ecosystem effects

Gene flow and unintended spread of GMO: a case study of rapeseed in a Norwegian context

Oilseed rape (OSR) species *Brassica napus*, is the most cultivated variety in Norway. Other varieties such as *Brassica rapa* are also cultivated, but less intensively.

The majority of GM OSRs at present are herbicide tolerant and insect resistant. These GM events are not considered interesting in a Norwegian context, as the farmers primarily have other challenges, e.g. due to climate (GenØk Biosafety report 2015/01).

Pollen from OSR may spread several kilometres by wind and insects. However, potential spread of GM OSR through seed spillage is seen as the largest source of potential GM contamination. Seed spillage may result in the establishment of volunteers and feral populations of OSR that further can be spread by pollen.

The following factors for spread have been highlighted (Finne 2006):

- High seed production potential of OSR;
- Great seed losses during harvest (pods that shatter);
- Seeds can survive in the environment for many years;
- Seed size is small which makes them difficult to contain; and
- Seeds are easily lost during handling and transport.

A major issue concerning OSRs is the ability to hybridise with wild relatives and produce fertile offspring. Data from early 1990s show that *B. napus* can produce fertile offspring with many of its wild relatives and especially with *Brassica campestris* (references in Finne, 2006). This species can grow up as far north as Troms county. Any GM OSR in Norway would then potentially spread transgene(s) throughout most of the country. Such unintended spread of transgenes would have an unknown impact on its wild relatives, including hybrid plants and surrounding ecological communities of non-target organisms.

Rapeseed
Photo: Dollarphotoclub_65034309/stefanholm

Other countries, such as Austria, have analyzed the issue of co-existence between GM and non-GM OSR and concluded that it is impossible to maintain co-existence of GM and non-GM OSR (Scientific arguments for an import ban of herbicide resistant rape GT73" by "Bundesministerium für Gesundheit und Frauen (bmgf).

Thus, as a case, GM OSR introduced to Norway would most probably lead to uncontrollable spread of transgenes into the environment, although we lack knowledge regarding the ecological consequences of such spread, or how it would affect other Brassica species hybridizing with the GM OSRs in Norway.

1.3.3.4 Herbicide residues in HT GM plants are overlooked

Generally, the suggested key food and feed nutrients found in the OECD consensus documents, are considered in safety evaluations of new varieties of soybeans and risk assessment of GM plants has

focused on allergenicity and toxicity resulting from the transgenic product itself, or from possible unintended effects of the transformation process (Podevin & du Jardin 2012). However, little attention is given to the residues of herbicides and their metabolites that can potentially accumulate in the final product, and also whether exposure to these herbicides through feeding or exposure to such plant material may affect non-target organisms or the environment.

Therefore, a major knowledge gap comes from the fact that HT GM plants have been tested without its corresponding herbicide co-technology. In early studies of the composition of Roundup-Ready GM soy, the researchers did not spray the tested plants with the recommended herbicide (Millstone, Brunner, & Mayer 1999). This shortcoming was quickly corrected, and sprayed GM soybeans were claimed to be substantially equivalent to non-GM soybeans (Harrigan et al. 2007). Still, and surprisingly, even in these studies, the residues of herbicides were not measured.

The concept of "substantial equivalence" (i.e. close nutritional and elemental similarity between a genetically modified (GM) crop and a non-GM traditional counterpart) has been used to claim that GM crops are substantially equivalent to, and therefore as safe and nutritious as, currently consumed plant-derived foods (Aumaitre 2002). However, we argue that compositional studies that have overlooked (not measured) pesticide residues contain serious shortcomings. Chemical residues, if present, are important because i) they are clearly a part of a plant's composition (including plant residues that are shed into the environment), and ii) they may add toxic properties to the final plant product either by itself or by affecting the plant metabolism. This is particularly relevant for herbicide-tolerant varieties.

A related problem is how research material is produced. In contrast to real-life samples from the market, transgenic crops intended for scientific studies are often produced in well-controlled small experimental plots. In most research studies, application of herbicides has been omitted or application has been done at doses lower than those typically used by farmers, giving test materials that are not representative of actual conditions existing in typical agricultural operation, e.g. with regard to glyphosate residues.

For example, most feeding studies using HT GM crops plants have missed the relevant target and tested unsprayed plants. Viljoen showed that out of 16 feeding studies testing the quality of herbicide tolerant GM crop products, 13 did not spray the GM plants (Viljoen 2013).

Chemical residues of glyphosate and its primary metabolite, AMPA, are documented to be present in herbicide tolerant GM soy (Duke et al. 2003; Bøhn et al. 2014(Box 1.1.)). Data on pesticides including glyphosate residues in glyphosate resistant plants should be regularly monitored. Such data should further be correlated to doses and timing of herbicide applications used in the field. This information is of key importance for regulators/the regulatory approval process and should be part of submitted dossiers for HT GM plants. Up to now, this has not been done.

Box 1.1: Case study of herbicide tolerant GM soy

In research recently published by GenØk's laboratory (Bøhn et al. 2014) soy samples grown under three typical agricultural conditions: organic, GM, and conventional (but non-GM) were collected. The GM soybeans were glyphosate-resistant/Roundup Ready.

In depth compositional data, including residues of pesticides such as organochlorinated pesticides, glyphosate and AMPA and analyses of more than 90 nutritional components, comparing the three different agricultural systems of soy, were provided in this study. Such data from representative field conditions had never before been published, and is therefore a valuable contribution to the literature.

From the study we concluded that Roundup Ready GM-soy had accumulated residues of glyphosate and AMPA (Fig. A), and also differed markedly in nutritional composition compared to soybeans from industrial non-GM and organic production (Fig. B). Organic soybean samples also showed in general a more healthy nutritional profile (e.g. higher in protein and lower in saturated fatty acids) than both industrial conventional and GM soybeans.

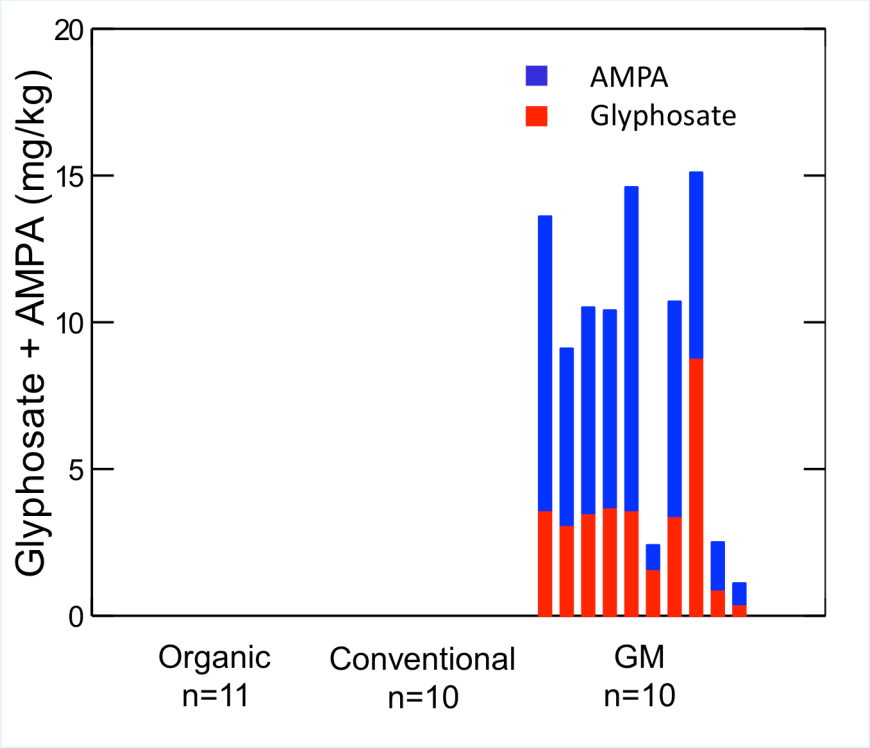

Figure A. Residues of glyphosate and AMPA in individual soybean samples (n=31). For conventional and organic soybeans all measurements were below the detection limit of 0.1 mg/kg. (Reproduced from Bøhn et al. 2014, with permission.)

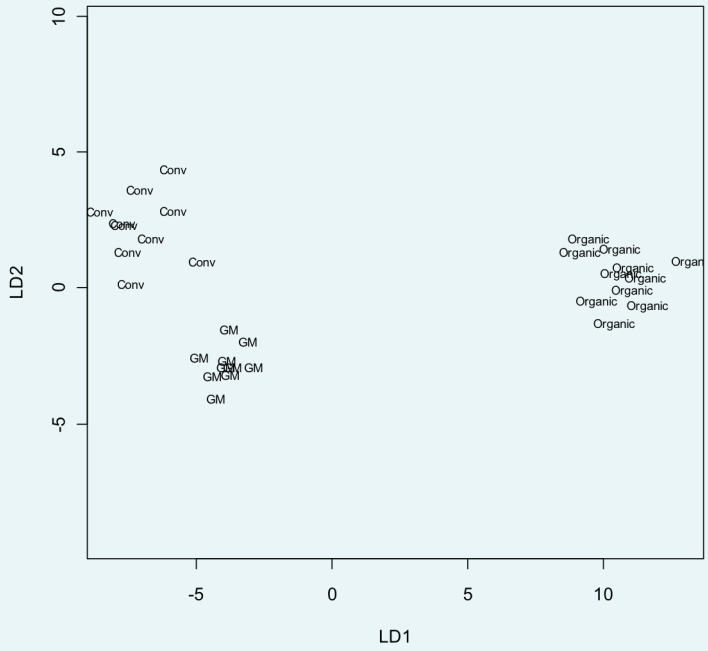

Figure B. Discriminant analysis for GM, conventional and organic soy samples based on 35 variables. Data was standardized (mean = 0 and SD = 1). Glyphosate/AMPA residues are not included (would have separated the GM soy from non-GM soy). (Reproduced from Bøhn et al. 2014, with permission.)

Lack of data on pesticide residues in major crop plants is a serious gap of knowledge with potential consequences for human and animal health. How is the public to trust a risk assessment system that has overlooked the most obvious risk factor for herbicide tolerant GM crops, i.e. high residue levels of herbicides, for nearly 20 years?

Follow-up feeding studies in *D. magna* demonstrated that organic soybeans were better suited as feed compared to non-GM industrial soybeans. HT GM soybeans performed less well than the other two types of soy, particularly for raw soy material (Cuhra, Traavik, & Bøhn 2014).

Further testing also demonstrated that GM soybeans with a high level of glyphosate herbicides resulted in negative effects on the fitness of *D. magna*. Reproduction was delayed and the number of offspring was reduced (Cuhra et al. 2015).

Conclusion

The case study of HT GM soy shows as a proof-of-concept that accumulating herbicides (glyphosate) in the final plant product of a GM plant may cause negative effects on the health of consumer organisms in the environment.

1.3.4 Stacked events – combinatorial effects?

A stacked GM plant is referred to as having the combination of two or more genes of interest in one single plant. In literature, it might also be called "gene pyramiding" or "multigene transfer". There are different gene stacking methods, as described in table 1.2 below.

The combined traits resulting from this are called "stacked traits". An example on this could be a GM plant expressing two different cry proteins or cry proteins and a herbicide tolerance protein. Until recently, the dossiers submitted for market authorization almost only covered single GM events (having one gene of interest inserted). Today there is a clear trend towards combining two or more transgenic traits present in single events through conventional breeding. The "offspring" plant would then be a stacked event.

Table 1.2: Common gene stacking methods used in the production of biotech stacks.

Gene stacking method	Description	Examples of commercial stacks*
Hybrid stacking	A plant harboring one or more transgenes is cross-hybridized with another plant containing other transgenes. Development of a multi-stack hybrid occurs via iterative hybridization.	Maize: Agrisure™ Viptera™ 3220 (Bt11 x MIR162 x TC1507 x GA21) Cotton: Roundup Ready™ Flex Bollgard™ II (MON88913 x MON15985)
Co-transformation	A plant is transformed with two or more independent transgenes. The transgenes of interest are in separate gene constructs and delivered to the plant simultaneously.	Maize: NaturGard™ Knockout™ (Bt176), Bt Xtra™ (DBT418), YieldGard™ (MON810, MON809, MON802)
Linked genes or multigene cassette transformation	A plant is transformed with a single gene construct that harbors two or more linked transgenes.	Maize: Herculex™ I (TC1507), Herculex™ RW (59122), Agrisure™ CB/LL (Bt11) Soybean: Vistive™ Gold (MON87705)
Re-transformation	A plant harboring a transgene is transformed with other transgenes.	Cotton: Bollgard™ II (MON15985)

**The examples of commercial biotech stacks are taken from the online ISAAA GM Approval database[6]. Links to more detailed information on the derivation of the biotech stacks are provided therein (Table from https://isaaa.org/resources/publications/pocketk/42/default.asp).*

Information on how these GM stacked events should be assessed is presented in an EFSA guidance document (EFSA Journal 2011). The assessment data for each single GM events has been taken into account to prove the safety of the whole food/feed (previous use, mode of action, specificity, biological activity, relation to other proteins with a history of safe use, absence of toxicity to mammals, absence of adverse effects on fast growing species, lack of homology to known toxins, lack of resistance to proteolysis, degradation upon heating). Analysis of the proteins in combination would only be performed if there indications of potential harm.

Stacked events are in general more complex than the single events, as there are more genes involved. There has been an increased interest in the possible combinatorial and/or synergistic effects that may lead to unintended and undesirable changes in the plant – like the potential for up- and down regulation of the plants own genes (Halpin 2005, Schrijver et al., 2006, Then 2009). Such changes may critically influence the bioactivity and hence the potential for unintended effects.

The potential combinatorial and synergistic effects must thus be carefully considered in the development and risk assessments of stacked events. Therefore, robust data are necessary to identify whether the combined presence of transgenes influences expression levels of other genes, e.g. by silencing effects.

The analysis of potential change in expression level of the different proteins as compared to the single events requires analysis of expression data as do analyses of protein (amino acid) sequences in order to predict potential protein, protein interaction based on their sequence data. This is done by searching for interacting domains through databases, especially to predict interactions in tertiary structures of proteins.

In the dossiers, the applicants rationalize the exclusion of event specific information based on an assumption of implied non-harm from parent plants already assessed for non-harm in prior submitted dossiers to the EU.

At present, most stacked events combine different classes of Bt toxins and or different classes of herbicide traits. Stacked genes in a single plant enable several traits to be expressed at the same time. However, stacking may also be the result of resistance development, both for insect resistant plants (as documented in South Africa) and for HT traits (both discussed above).

The understanding of resistance evolution, with increased use of agrochemicals, the stacking of HT and insect resistance traits, must be improved upon. Co-exposure of multiple herbicides/pesticides and Bt-toxins may trigger combinatorial effects in non-target organism in the environment. However, this represents a major knowledge gap in the scientific literature.

By 2012, nearly twice as large area of GM plants with both insect protection and herbicide tolerance traits were grown as compared to plants only expressing Bt-toxins (James 2012). In South Africa, approximately 30% of the production of Bt maize is also herbicide tolerant. This means that herbicide co-technology regularly will co-occur with Bt in the environment. Stacked events are relatively easy to produce as conventional breeding is used to combine two or more events that are already approved singularly for the market. For example, the maize hybrid MON 89034 x 1507 x MON 88017 x 59122, from Monsanto and Dow AgroSciences, is broadly resistant to insects with six different *cry* genes (*cry*1A.105, *cry*1F, *cry*2Ab2, *cry*3Bb1-, *cry*34Ab1 and *cry*35Ab1) and herbicide tolerant for both glyphosate and glufosinate ammonium. Stacked events may be expected to have a wider range of effects (Then 2009), both on target pest insects (as intended) and on non-target organisms (unintended and unwanted) from their multiple Bt-toxins.

1.3.4.1 Stacked traits as multiple stressors?

The development towards stacked events as the norm will arguably lead to increased doses/more applications of herbicides per season and a broader range of Cry toxins in insect resistant GM plants. Such development emphasizes the importance of the potential environmental impacts of these

technologies. In addition, since these toxins/chemicals/traits will meet and interact, also each other and other stressors in the environment, the co-exposure and potential combinatorial effects need to be studied (Bjergager et al. 2011;Nørgaard & Cedergreen 2010;Then 2009). Although the potential negative effects of cry toxins and agrochemicals is relevant for a number of non-target species in the agricultural ecosystem (including terrestrial, soil and run-off aquatic systems), lessons can be learned from more precise laboratory studies in established model organisms.

The scientific literature is very limited with regard to studies testing plant material from 'stacked traits'. A couple of studies from industry sources show no effects, respectively in European corn borer/Colorado potato beetle (Raybould et al. 2012), and in rats (Appenzeller et al. 2009). Schuppener et al. showed that feeding activity and survival were negatively affected by a stacked GM-maize trial in a non-target Lepidoptera, *Aglais urticae*, but only at concentrations of pollen that were higher than found in the field (Schuppener et al. 2012).

In addition to the potentially added (or combinatorial) effect of 'multi-Bt-plants', the spraying of agricultural fields throughout the growing season with one or several herbicides on stacked (i.e. Bt + herbicide tolerant) GM plants, may add stress to non-target organisms that live in nearby ecosystems. Bio-active herbicides ultimately get into soil and water systems through processes such as drifting, leaching and surface runoff (Mensah, Muller, & Palmer 2012). Negative effects of herbicides are documented for a number of aquatic species related to the most relevant of the herbicides: glyphosate based formulations (Roundup), i.e. on amphibians (Sih, Bell, & Kerby 2004) (Relyea 2005a;Relyea 2005b;Relyea & Hoverman 2006), shrimps (Mensah, Muller, & Palmer 2011;Mensah et al. 2012), and water fleas (Cuhra et al. 2013).

1.3.5 Antibiotic resistance marker (ARM) genes

In GM plants of the first generation, genes encoding resistance to specific antibiotics have been largely used for selection and rapid identification of transformed cells. The selectable marker gene is usually co-transformed with the gene of interest, and can therefore be transferred to other organisms. Concerns have been raised that the large-scale release of antibiotic resistance marker genes by release of transgenic organisms in fields will increase the rate of antibiotic resistant bacteria leading to reduced therapeutic options for the treatment of infectious diseases (Dona et al 2009).

The most commonly used ARM in the applications for plant cell selection is *nptII* (neomycin phosphotransferase II, also referred to as APH-3`-II or aminoglycoside phosphotransferase 3`-II) encoding resistance to some aminoglycosides, including kanamycin (EFSA 2007). Many GM plants containing *nptII* have been approved for field trails and for marketing in several countries (Carrer et al 1993, EFB 2001, Badosa et al 2004, Bennet et al 2004, Breyer et al 2014). The choice of using this marker gene has been driven by the fact that kanamycin is not important in medical treatments and that kanamycin resistant bacteria are ubiquitous in nature (EFB 2001, EFSA 2009). However, kanamycin has recently been classified as a critically important antibiotic for human and animals (WHO, 2012). Literature surveys indicate that only few data are available on the prevalence of *nptII* gene. The limited data demonstrates that there is a low level presence of *nptII* in naturally occurring bacterial populations from agricultural soils (Smalla et al 1993, Gebhard and Smalla, 1999)

To what extent GMOs containing ARM genes will influence the antibiotic resistance situation is highly debated (EFB 2001, Ramessar et al 2007, Woegerbauer 2007, EFSA 2009, Breyer et al 2014).

In this section we will discuss some of the main knowledge gaps and uncertainties associated with the use of antibiotic resistance marker genes:

- Horizontal gene transfer (HGT) transgenic plants to soil bacteria
- Resistance level occurring in natural environments

Horizontal gene transfer (HGT) from transgenic plants to soil bacteria

Much of the current debate on the relevance and risk of HGT to transgenic plant biosafety is focused on the expected low (if any) probability of HGT from transgenic plants to soil bacteria (Gebard and Smalla, 1999, EFSA 2007, Sung Eun et al 2010,). Plant DNA can be released into the soil from various sources including decaying plant material and root exudates. The degradation kinetics vary considerably depending on cellular and environmental conditions, which means that plant-derived DNA in soil can be rapidly degraded but also persist for a long term in the soil (Lorenz and Wackernagel 1994, Paget et al 1998, Nielsen and Townsend 2004, Nielsen et al 2007, Ma et al 2011). Some of this DNA is sufficiently undamaged that it can transform competent bacteria after re-extraction (Nielsen et al 2007). Horizontal gene transfer from transgenic plants to natural populations of soil bacteria has not been unequivocally demonstrated so far under field conditions (Schlutter and Potrkus, 1995; Nielsen et al 1997, Nielsen et al 2003, Nielsen et al 2004). However, theoretically there is a possibility that HGT from transgenic plants to bacteria may happen but the likelihood of detecting such an event using current methodology is low (ERMA 2006). More details about HGT is discussed in part 3, regarding GM microorganisms.

Resistance level occurring in natural environments

We know that natural environments harbour a diverse reservoir of antibiotic resistance determinants (Berkner et al 2014). However, for an adequate evaluation of the potential effects of the release of GMOs containing *nptII* into the environment, the occurrence of the ARM gene in culturable and nonculturable environmental bacteria should be known.

Selective plating of different environmental samples on kanamycin-containing medium has shown that Kanamycin-resistant bacteria are ubiquitous in nature (Smalla et al 1993, Leff et al 1993, Bennet el at 2004). However, only a fraction of kanamycin resistant bacteria have shown to contain the *nptII* gene, which means that the other resistant bacteria have different genes and/or other mechanisms conferring kanamycin resistance.

The current data on the distribution of ARM genes in microbial populations in soils or other habitats are limited, and when it comes to more specific data about the prevalence of ARM genes today in Norway there are also little data (VKM 2005).

The opinion of the EFSA GMO panel is not necessarily consistent with the precautionary motivated regulations for ARM genes for commercial use in food and feed in Norway (VKM 2005). Since the agricultural usage of antibiotics varies considerable between countries, observations of the prevalence of *nptII* need to be documented in Norwegian environments. Given the low level of phenotypic resistance to aminoglycosides in pathogenic bacteria in Norway, the large scale introduction of the *nptII* gene in food and feed could pose a risk to human and animal health. More

information on *nptII* gene copy number in relevant Norwegian environments may alter this observation.

Despite the controversy, a few GM plants containing ARM genes have still been approved for commercialization in the period 2000-2014. However, over the years we have seen in the dossiers/applications, that the use of antibiotic resistance marker genes for selection of GM plants has been limited to the *nptII* gene. Also, in many cases the *nptII* gene has been excised after selection or other markers such as *epsps* and *pat* genes coding for herbicide tolerance have been used instead.

1.3.4 Directions for further studies

There are significant knowledge gaps as well as uncertainties concerning the fate and potential effects of GM plants in the environment, i.e. in terrestrial, soil and aquatic ecosystems. Further studies should include:

Bt-expression. Substantial variability in Bt-toxin concentrations is observed in GM Bt-corn (Nguyen & Jehle 2007;Saeglitz et al. 2006). Patterns and concentration levels of Bt-toxin in Bt-plants, particularly in stacked events, are far from being understood and merit further investigation. Expression may also be altered by the event, the genotype of the hybrid, the environmental conditions, co-technologies used (e.g. herbicides), climate, stress, etc.

Resistance development. Monitor evolution of resistance and study mechanisms for resistance development in target organisms to Bt-toxin(s)

Potential alternative modes of action of Bt-toxins. Studies indicate that Bt-toxins have alternative mode(s) of action, i.e. in adversely affected non-target organisms that are distantly related to the target organisms (Bøhn et al. 2008;Bøhn et al. 2010;Bravo et al. 2007;Hilbeck & Schmidt 2006);

Dose-response toxicity testing of Bt-toxins on relevant non-target species. Dose-response experiments, ideally including additional control groups fed non-transgenic material with purified Bt-toxin added in order to be able to distinguish between effects of Bt-toxin from other effects of the engineering process;

Combinatorial effects of several Bt-toxins stacked. Emphasis on toxins stacked in plants that are grown commercially.

Combinatorial effects of Bt-toxins and herbicide co-technologies. Emphasis on toxins and herbicides/pesticides that co-occur in the same environment. The combinatorial toxicity of multiple/stacked Bt and HT traits that are in the pipeline may have a broader range of non-target effects. Also, wider interactions and community level effects, including studies of system processes, keystone/threatened (i.e. red list) species and potential effects on biodiversity, need to be addressed.

Accumulation of herbicides in plant products. Data need to be generated on the accumulation of herbicides in HT GM plants. Such data are missing in the scientific literature; these residues have simply fallen outside the remit of regulatory authorities, and have been overlooked both in compositional studies and in feeding studies.

Field studies. Monitor evolution resistance and study mechanisms for development of resistance in weeds to herbicide used in HT plants.

Exposure and break-down rates of Bt-toxins and herbicide/pesticides. Need to improve knowledge on the "cocktail" of potential stressors present in the environment: their presence, interactions and effects. This includes the amount of plant material, toxin concentration of bioactive transgenic components the rates of toxin release and break-down etc. depending on biotic and abiotic factors in the environment

Feeding habits of species at different trophic levels and food-web transport of Bt-toxins.

Sensitivity screening of relevant organisms to Bt-toxins and herbicide co-technology. Testing for acute and chronic effects via long-term feeding/exposure experiments with relevant Bt-transgenic material (kernels, pollen or litter) under controlled conditions in the lab.

Field studies. Testing whether Bt-toxins and co-occurring herbicides/pesticides may affect species or species composition in ecological communities, i.e. by multiple comparisons of diversity and community structure between systems where GM agricultural practices and control systems of non-GM production systems (industrial, agroecological, organic) can be compared.

Field studies. Monitor pollen spread and whether GM plants can hybridise with wild relatives and produce fertile offspring.

Studies on relevant organisms. The exposure and potential effects of GM traits like built-in insecticides and herbicide tolerance must be studied on relevant organisms, either model organisms or test organisms from the relevant environment. Both laboratory studies, in established or new model organisms, and studies in real ecosystems may contribute relevant scientific knowledge.

Access to research material. The restrictions of research material from GM crops strongly limit independent research. This is not acceptable. Relevant research material must be made available for testing by competent and independent research groups.

Antibiotic resistance marker genes. More data about the prevalence of ARM genes in the environment are needed.

References

AGAPITO-TENFEN, S.Z. GUERRA, M.P. WIKMARK, O.G. AND NODARI R.O. (2013) Comparative proteomic analysis of genetically modified maize grown under different agroecosystems conditions in Brazil. Proteome Science. 11(46).

AIMANOVA, K. G., ZHUANG, M. B., & GILL, S. S., 2006. Expression of Cry1Ac cadherin receptors in insect midgut and cell lines. *Journal of Invertebrate Pathology* 92(3), pp. 178-187.

ALBO, A.G. MILA, S. DIGILIO, G. MOTTO, M. AIME, S. AND CORPILLO, D. (2007). Proteomic analysis of a genetically modified maize flour carrying Cry1AB gene and comparison to the corresponding wild-type. Maydica 52. 443-455.

AMERICAN SOY ASSOCIATION. http://www.nass.usda.gov/Statistics_by_Subject/index.php?sector=CROPS. 2015. 7-1-2015.
Ref Type: Online Source

APPENZELLER, L. M., MUNLEY, S. M., HOBAN, D., SYKES, G. P., MALLEY, L. A., & DELANEY, B., 2009. Subchronic feeding study of grain from herbicide-tolerant maize DP-empty set9814empty set-6 in Sprague-Dawley rats. *Food and Chemical Toxicology* 47(9), pp. 2269-2280.

AREAL, F. J., RIESGO, L., & RODRIGUEZ-CEREZO, E., 2013. Economic and agronomic impact of commercialized GM crops: a meta-analysis. *Journal of Agricultural Science* 151(1), pp. 7-33.

AUMAITRE, L. A., 2002. New feeds from genetically modified plants: substantial equivalence, nutritional equivalence and safety for animals and animal products. *Productions Animales* 15(2), pp. 97-108.

AVISAR, D., EILENBERG, H., KELLER, M., REZNIK, N., SEGAL, M., SNEH, B., & ZILBERSTEIN, A., 2009. The Bacillus thuringiensis delta-endotoxin Cry1C as a potential bioinsecticide in plants. *Plant Science* 176(3), pp. 315-324.

AXELRAD, J. C., HOWARD, C. V., & MCLEAN, W. G., 2003. The effects of acute pesticide exposure on neuroblastoma cells chronically exposed to diazinon. *Toxicology* 185(1-2), pp. 67-78.

BADOSA, E., MORENO, C. & MONTESINO, E. (2004) Lack of detection of ampicillin resistance gene trasnfer from Bt176 transgenic corn to culturable bacteria under field conditions. *FEMS Microbiology Ecology*. 48 (x) p.169-178.

BAROS, E. LEZAR, S. ANTTONEN, M.J. VAN DIJK, J.P. ROHLIG, R.M. KOK, E.J. AND ENGEL, K.H. (2010). Comparisons of two GM maize varieties with a near-isogenic non-GM variety using transcriptomics, proteomics and metabolomics. Plant Biotech Journal. 8. p. 436-451

BECKIE, H. J., 2011. Herbicide-resistant weed management: focus on glyphosate. *Pest Management Science* 67(9), pp. 1037-1048.

BECKIE, H. J. & HALL, L. M., 2008. Simple to complex: Modelling crop pollen-mediated gene flow. *Plant Science* 175(5), pp. 615-628.

BENACHOUR, N. & SERALINI, G. E., 2009. Glyphosate Formulations Induce Apoptosis and Necrosis in Human Umbilical, Embryonic, and Placental Cells. *Chemical Research in Toxicology* 22(1), pp. 97-105.

BENBROOK, C. M., 2012. Impacts of genetically engineered crops on pesticide use in the U.S. - the first sixteen years. *Environmental Science Europe* 24:24.

BENFEY, P.N. AND CHUA, N.H. (1990) The Cauliflower Mosaic Virus 35S Promoter: Combinatorial Regulation of Transcription in Plants. Science 250:959-66.

BENNET, P.M., LIVESEY, C.T., NATHWANI, D., REEVES, D.S., SAUNDERS, J.R. & WISE, R. (2004) An assessment of the risks associated with the use of antibiotic resistance genes in genetically modified plants: report of the Working Party of the British Society for Antimicrobial Chemotherapy. *Journal of Antimicrobial Chemotherapy.* 54 (3) p. 418-431.

BERKNER, S., KONRADI, S. & SCHÔNFELD, J. (2014) Antibiotic resistance and the environment – there and back again. *EMBO Report*. 15(7)p. 740-744.

BINIMELIS, R., 2008. Coexistence of Plants and Coexistence of Farmers: Is an Individual Choice Possible? *Journal of Agricultural and Environmental Ethics* 21(5), pp. 437-457.

BINIMELIS, R., PENGUE, W., & MONTERROSO, I., 2009. "Transgenic treadmill": Responses to the emergence and spread of glyphosate-resistant johnsongrass in Argentina. *Geoforum* 40(4), pp. 623-633.

BJERGAGER, M. B. A., HANSON, M. L., LISSEMORE, L., HENRIQUEZ, N., SOLOMON, K. R., & CEDERGREEN, N., 2011. Synergy in microcosms with environmentally realistic concentrations of prochloraz and esfenvalerate. *Aquatic Toxicology* 101(2), pp. 412-422.

BREYER, D., KOPERTEKH, L., & REHEUL, D. (2014) Alternatives to Antibiotic Resistance marker Genes for In Vitro Selection of Genetically Modified Plants – Science Developments, Current Use, Operational Access and Biosafety Considerations. *Critical Reviews in Plant Science*. 33 (x) p.286-330.

BØHN, T., CUHRA, M., TRAAVIK, T., SANDEN, M., FAGAN, J., & PRIMICERIO, R., 2014. Compositional differences in soybeans on the market: Glyphosate accumulates in Roundup Ready GM soybeans. *Food Chemistry* 153, pp. 207-215.

BØHN, T., PRIMICERIO, R., HESSEN, D. O., & TRAAVIK, T., 2008. Reduced fitness of Daphnia magna fed a Bt-transgenic maize variety. *Archives of Environmental Contamination and Toxicology* 55(4), pp. 584-592.

BØHN, T., TRAAVIK, T., & PRIMICERIO, R., 2010. Demographic responses of *Daphnia magna* fed transgenic *Bt*-maize. *Ecotoxicology* DOI 10.1007/s10646-009-0427-x. (Open Access).(19), pp. 419-430.

BRAVO, A., GILL, S. S., & SOBERON, M., 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* 49, pp. 423-435.

BUNDESMINISTERIUM FÜR GESUNDHEIT UND FRAUEN (bmgf) (2006) Scientific arguments for an import ban of herbicide tolerant oilseed rape GT73. *Notification C/NL/98/11*.

CODEX ALIMENTARUS (2003) Principles for the risk analysis of foods derived from modern biotechnology.

CAC/GL 44-2003

CARRER, H., HOCKENBERRY, T.N., SVAB, Z. & MALIGA, P. (1993) Kanamycin resistance as selectable marker for plastid transformation in tobacco. *Mol Gen Genet*. 24(1) p. 49-56.

CUHRA, M., TRAAVIK, T., & BØHN, T., 2013. Clone- and age-dependent toxicity of a glyphosate commercial formulation and its active ingredient in *Daphnia magna*. *Ecotoxicology* 22(2), pp. 251-262 (open access). DOI 10.1007/s10646-012-1021-1.

CUHRA, M., TRAAVIK, T., & BØHN, T., 2014. Life cycle fitness differences in *Daphnia magna* fed Roundup-Ready soybean or conventional soybean or organic soybean. *Aquaculture Nutrition* (in press).

CUHRA, M., TRAAVIK, T., DANDO, M., PRIMICERIO, R., HOLDERBAUM, D., & BØHN, T., 2015. Glyphosate-residues in Roundup-ready soybean impair *D. magna* life-cycle. *Journal of Agricultural Chemistry and Environment* (in press).

DALLEGRAVE, E., MANTESE, F. D., COELHO, R. S., PEREIRA, J. D., DALSENTER, P. R., & LANGELOH, A., 2003. The teratogenic potential of the herbicide glyphosate-Roundup (R) in Wistar rats. *Toxicology Letters* 142(1-2), pp. 45-52.

DE VENDOMOIS, J. S., ROULLIER, F., CELLIER, D., & SERALINI, G. E., 2009. A Comparison of the Effects of Three Gm Corn on Mammalian Health. *International Journal of Biological Sciences* 5(7), pp. 706-721.

DEMETRIO, P. M., ROSSINI, G. D. B., BONETTO, C. A., & RONCO, A. E., 2012. Effects of pesticide formulations and active ingredients on the coelenterate Hydra attenuata (Pallas, 1766). *Bulletin of Environmental Contamination and Toxicology* 88(1), pp. 15-19.

DEVOS, Y., DEMONT, M., DILLEN, K., REHEUL, D., KAISER, M., & SANVIDO, O., 2009. Coexistence of genetically modified (GM) and non-GM crops in the European Union. A review. *Agronomy for Sustainable Development* 29(1), pp. 11-30.

DILL, G. M., SAMMONS, R. D., FENG, P. C., KOHN, F., KRETZMER, K., MEHRSHEIKH, A., BLEEKE, M., HONEGGER, J. L., FARMER, D., & WRIGHT, D., 2010. Glyphosate: discovery, development, applications, and properties. *Glyphosate Resistance in Crops and Weeds: History, Development, and Management, John Wiley and Sons, Inc., Hoboken*, pp. 1-33.

DOMINGO, J.L. AND BORDONABA, J.G. (2011) A literature review on the safety assessment of genetically modified plants. Environment International 37. 734–742

DONA, A. & ARVANITOYANNIS, I.S. (2009) Health Risks of Genetically Modified Foods. *Critical reviews in Food Science and Nutrition.* 49 (X) p. 164-175.

DOUVILLE, M., GAGNE, F., ANDRE, C., & BLAISE, C., 2009. Occurrence of the transgenic corn cry1Ab gene in freshwater mussels (Elliptio complanata) near corn fields: Evidence of exposure by bacterial ingestion. *Ecotoxicology and Environmental Safety* 72(1), pp. 17-25.

DUAN, J. J., MARVIER, M., HUESING, J., DIVELY, G., & HUANG, Z. Y., 2008. A Meta-Analysis of Effects of Bt Crops on Honey Bees (Hymenoptera: Apidae). *Plos One* 3(1).

DUKE, S. O. & POWLES, S. B., 2008. Glyphosate: a once-in-a-century herbicide. *Pest Management Science* 64(4), pp. 319-325.

DUKE, S. O., RIMANDO, A. M., PACE, P. F., REDDY, K. N., & SMEDA, R. J., 2003. Isoflavone, glyphosate, and aminomethylphosphonic acid levels in seeds of glyphosate-treated, glyphosate-resistant soybean. *Journal of Agricultural and Food Chemistry* 51(1), pp. 340-344.

EFB (2001) Antibiotic Resistance Markers in Genetically Modified (GM) Crops.

EFSA (2007) Statement of the Scientific Panel on Genetically Modified Organisms on the safe use of the *nptII* antibiotic resistance marker gene in genetically modified plants.

EFSA (2008) Safety and nutritional assessment of GM plants and derived food and feed: The role of animal feeding trials. Food and Chemical Toxicology. 46. S2-S70.

EFSA (2009) Consolidated presentation of the joint Scientific Opinion of the GMO and BIOHAZ Panles on the "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants" and the Scientific Opinion of the GMO Panel on "Consequences of the Opinion on the Use of Antibiotic Resistance Genes as marker Genes in Genetically Modified Plants on Previous EFSA Assessments of Individual GM plants". *The EFSA Journal*. 1108 p. 1-8.

EFSA. (2010) Guidance on the environmental risk assessment of genetically modified plants. EFSA Journal 8(11). p.1879

EFSA. (2011) Guidance for risk assessment of food and feed from genetically modified plants. EFSA Journal. 9(5). p.2150

FANG, R.X. NAGY, F. SIVASUBRAMANIAM, S. AND CHUA, N.H. (1989) Multiple cis regulatory elements for maximal expres- sion of the cauliflower mosaic virus 35S promot- er in transgenic plants. Plant Cell 1:141-50

Forsbach. A, Schubert, D. Lechtenberg, B. Gils, M. and Schmidt, R (2003) A comprehensive characterization of single-copy T-DNA insertions in the Arabidopsis thaliana genome. Plant Molecular Biology 52: 161–176

FINNE, M. A. (2006) Sameksistens mellom transgene sorter og konvensjonelt og økologisk landbruk i Norge. En litteraturoversikt. *Rapport til Mattilsynet*.

GEISER, M. SCHWEITZER, S. AND GRIMM, C. (1986) The hypervariable region in the genes coding for entomopathogenic crystal proteins of *Bacillus thuringiensis:* nucleotide sequence of the *kurhdl* gene of subsp. *kurstuki* HD1. GENE. 48. 109-118.

GENDEL, S. M. (2002) Sequnce analysis for assessing potential allergenicity. ANN. NY. ACAD. SCI. 964. 87-98.

GIESY, J. P., DOBSON, S., & SOLOMON, K. R., 2000. Ecotoxicological risk assessment for Roundup herbicide. *Rev.Environ.Contam.Toxicol.* 167, pp. 35-120.

GRIFFITHS, N. A., TANK, J. L., ROYER, T. V., ROSI-MARSHALL, E. J., WHILES, M. R., CHAMBERS, C. P., FRAUENDORF, T. C., & EVANS-WHITE, M. A., 2009. Rapid decomposition of maize detritus in agricultural headwater streams. *Ecological Applications* 19(1), pp. 133-142.

GRIMALDI, D. A. & ENGEL, M. S., 2005. Amphiesmenoptera: the caddisflies and Lepidoptera. *Evolution of the insects.New York: Cambridge University Press*, pp. 548-555.

GEBHARD, F. & SMALLA, K. (1999) Monitoring field release of genetically modified sugard beats for persistence of transgenic plant DNA and horizontal gene transfer. *FEMS Microbiology Ecology*. 28 (x) p. 261-272.

HAGENBUCHER, S., WACKERS, F. L., WETTSTEIN, F. E., OLSON, D. M., RUBERSON, J. R., & ROMEIS, J., 2013. Pest trade-offs in technology: reduced damage by caterpillars in Bt cotton benefits aphids. *Proceedings of the Royal Society B-Biological Sciences* 280(1758).

HAGENBUCHER, S., WAECKERS, F. L., & ROMEIS, J., 2014. Indirect multi-trophic interactions mediated by induced plant resistance: impact of caterpillar feeding on aphid parasitoids. *Biology Letters* 10(2).

HAIDER, M.Z AND ELLAR, D.J. (1989) Functional Mapping of an Entomocidal &Endotoxin Single Amino Acid changes Produced by Site-directed Mutagenesis Influence Toxicity and Specificity of the Protein. J.MOL.BIOL. 208. 183-194.

HAMMOND, B., DUDEK, R., LEMEN, J., & NEMETH, M., 2004. Results of a 13 week safety assurance study with rats fed grain from glyphosate tolerant corn. *Food and Chemical Toxicology* 42(6), pp. 1003-1014.

HARRIGAN, G. G., RIDLEY, G., RIORDAN, S. G., NEMETH, M. A., SORBET, R., TRUJILLO, W. A., BREEZE, M. L., & SCHNEIDER, R. W., 2007. Chemical composition of glyphosate-tolerant soybean 40–3-2 grown in Europe remains equivalent with that of conventional soybean (Glycine max L.). *Journal of Agricultural and Food Chemistry* 55, pp. 6160-6168.

HARWOOD, J. D., SAMSON, R. A., & OBRYCKI, J. J., 2006. No evidence for the uptake of Cry1Ab Bt-endotoxins by the generalist predator Scarites subterraneus (Coleoptera : Carabidae) in laboratory and field experiments. *Biocontrol Science and Technology* 16(4), pp. 377-388.

HEAP, I. The International Survey of Herbicide Resistant Weeds. Online. Internet. 2012. www.weedscience.com. 12-9-2012. Ref Type: Online Source

HENDRIKSMA, H. P., HARTEL, S., & STEFFAN-DEWENTER, I., 2011. Testing Pollen of Single and Stacked Insect-Resistant Bt-Maize on In vitro Reared Honey Bee Larvae. *Plos One* 6(12).

HERNANDEZ, M. PLA, M. ESTEVE, T. PRAT, S. PUIGDOMENECH, P. AND FERRANDO, A. (2003) A specific real-time quantitative PCR detection system for event MON810 in maize YieldGard® based on the 3-transgene integration sequence. Transgenic Research 12: 179–189

HERNÁNDEZ-RODRÍGUEZ C.S. HERNÁNDEZ-MARTÍNEZ P, VAN RIE J, ESCRICHE B, FERRÉ J. (2013). Shared midgut binding sites for Cry1A.105, Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa proteins from Bacillus thuringiensis in two important corn pests, Ostrinia nubilalis and Spodoptera frugiperda. PLoS One. Jul 5;8(7). e68164. doi: 10.1371/journal.pone.0068164

HILBECK, A. & SCHMIDT, J. E. U., 2006. Another view on Bt proteins - how specific are they and what else might they do? *Biopestic.Int.* 2(1), pp. 1-50.

HOLCK, A. VA. M. DIDIERJEAN, L. AND RUDI, K. (2002) 5'-Nuclease PCR for quantitative event-specific detection of the genetically modified Mon810 MaisGard maize. Eur Food Res Technol 214:449–453 DOI 10.1007/s00217-001-0473-y

IVERSEN, M., GRØNSBERG, I. M., VAN DEN BERG, J., FISCHER, K., AHETO, D., & BØHN, T., 2014. Detection of transgenes in local maize varieties of small-scale farmers in the Eastern Cape, South Africa. *Plos One* Published online Dec 31, 2014. DOI:10.1371/journal.pone.0116147.

JACOBSON, K. & MYHR, A. I., 2013. GM Crops and Smallholders: Biosafety and Local Practice. *The Journal of Environment and Development* 22(1), pp. 104-124.

James, C. 2013. *Global Status of Commercialized Biotech/GM Crops: 2013.* ISAAA: Ithaca, NY., *ISAAA Brief* No. 46.

JIRAUNGKOORSKUL, W., UPATHAM, E. S., KRUATRACHUE, M., SAHAPHONG, S., VICHASRI-GRAMS, S., & POKETHITIYOOK, P., 2003. Biochemical and histopathological effects of glyphosate herbicide on Nile tilapia (Oreochromis niloticus). *Environmental Toxicology* 18(4), pp. 260-267.

JURAT-FUENTES, J. L. & ADANG, M. J., 2006. Cry toxin mode of action in susceptible and resistant Heliothis virescens larvae. *Journal of Invertebrate Pathology* 92(3), pp. 166-171.

KAY, R. CHAN, A.M.Y. DALY, M. AND MCPHERSON, J. (1987) Duplication of CaMV 35S Promoter Sequences Creates a Strong Enhancer for Plant Genes. Science 1987; 236:1299- 302.

KIM, S.E., MOON, J.S., KIM, J.K., YOO, R.H., CHOI, W.S., LEE, E.N., LEE, S.H. & KIM, S.U. (2010) Monitoring of possible horizontal gene transfer from transgenic potatoes to soil microorganisms in the potato fields and the emergence of variants in Phytophthora infestans. *Journal Microbiology Biotechnology.* 20 (6) p. 1027-1031.

KNOOP, V. (2011) When you can´t trust the DNA: RNA editing changes transcript sequences. *Cell. Mol. Life Sci.* 68. 567-586. DOI: 10.1007/s00018-010-0538-9

KRUGER, M., VAN RENSBURG, J. B. J., & VAN DEN BERG, J., 2009. Perspective on the development of stem borer resistance to Bt maize and refuge compliance at the Vaalharts irrigation scheme in South Africa. *Crop Protection* 28(8), pp. 684-689.

KRUGER, M., VAN RENSBURG, J. B. J., & VAN DEN BERG, J., 2012. Transgenic Bt maize: farmers' perceptions, refuge compliance and reports of stem borer resistance in South Africa. *Journal of Applied Entomology* 136(1-2), pp. 38-50.

LEFF, L.G., DNAN, J.R., McArthur J.V. & SHIMKETS, L.J. (1993) Detection of Tn5-like sequences in kanamycin-resistant stream bacteria and environmental DNA. *Applied Environmental Microbiology.* 59 (2) p. 417-421

LOCKHART, W. L., BILLECK, B. N., & BARON, C. L. 1989. Bioassays with a floating aquatic plant (Lemna minor) for effects of sprayed and dissolved glyphosate. *Environmental Bioassay Techniques and their Application.* Springer, pp. 353-359.

LÖVEI, G. L., ANDOW, D. A., & ARPAIA, S., 2009. Transgenic Insecticidal Crops and Natural Enemies: A Detailed Review of Laboratory Studies. *Environmental Entomology* 38(2), pp. 293-306.

LÖVEI, G. L. & ARPAIA, S., 2005. The impact of transgenic plants on natural enemies: a critical review of laboratory studies. *Entomologia Experimentalis et Applicata* 114, pp. 1-14.

MANN, R. M., HYNE, R. V., CHOUNG, C. B., & WILSON, S. P., 2009. Amphibians and agricultural chemicals: review of the risks in a complex environment. *Environmental Pollution* 157(11), pp. 2903-2927.

MARC, J., MULNER-LORILLON, O., & BELLE, R., 2004. Glyphosate-based pesticides affect cell cycle regulation. *Biology of the Cell* 96(3), pp. 245-249.

MATTHEWS, D. JONES, H. GANS, P. COATES, S. AND SMITH, L.M.J. (2005) Toxic Secondary Metabolite Production in Genetically Modified Potatoes in Response to Stress. J. Agric. Food Chem. 53. 7766−7776

MENSAH, P. K., MULLER, W. J., & PALMER, C. G., 2011. Acute toxicity of Roundup (R) herbicide to three life stages of the freshwater shrimp Caridina nilotica (Decapoda: Atyidae). *Physics and Chemistry of the Earth* 36(14-15), pp. 905-909.

MENSAH, P. K., MULLER, W. J., & PALMER, C. G., 2012. Using growth measures in the freshwater shrimp Caridina nilotica as biomarkers of Roundup (R) pollution of South African freshwater systems. *Physics and Chemistry of the Earth* 50-52, pp. 262-268.

MESNAGE, R., BERNAY, B., & SERALINI, G. E., 2012. Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity . *Toxicology* (in press)(http://dx.doi.org/10.1016/j.tox.2012.09.006).

MILLSTONE, E., BRUNNER, E., & MAYER, S., 1999. Beyond 'substantial equivalence'. *Nature* 401, pp. 525-526.

MOORE, L. J., FUENTES, L., RODGERS, J. H., BOWERMAN, W. W., YARROW, G. K., CHAO, W. Y., & BRIDGES, W. C., 2012. Relative toxicity of the components of the original formulation of Roundup (R) to five North American anurans. *Ecotoxicology and Environmental Safety* 78, pp. 128-133.

MOREAU, V. GRANIER, C. VILLARD, S. LAUNE, D. AND MOLINA, F. (2006) Discontinuous epitope prediction based on mimotope analysis. BIOINFORMATICS. 22. 1088–1095.

MORISSET, D. DEMSAR, T. GRUDEN, K. VOJVODA, J. STEBIH, D. AND ZEL. J. (2009) Detection of genetically modified organisms – closing the gaps. Nature Biotech. 27 (8). p.700-701.

NGUYEN, H. T. & JEHLE, J. A., 2007. Quantitative analysis of the seasonal and tissue-specific expression of Cry1Ab in transgenic maize Mon810. *Journal of Plant Diseases and Protection* 114(2), pp. 82-87.

NIELSEN, K.M., BONES, A.M. & VAN ELSAS, J.D. (1997) Induced Natural Transformation of Acinetobacter calcoaceticus in Soil. *Apllied Envrionmental Mircrobiology*. 63 (10) p. 3972-3977.

NIELSEN, K.M. & TOWNSEND, J.P. (2004) Monitorinf and modeling horizontal gene transfer. *Nature Biotechnology*. 22 (9) p. 1110-1114.

NIELSEN, K.M., JOHNSEN, P.J., BENSASSON, D. & DAFFONCHIO, D. (2007) Release and Persistence of extracellular DNA in the environment. *Environmental Biosafety Research*. 6 (1-2) p. 37-53.

NØRGAARD, K. B. & CEDERGREEN, N., 2010. Pesticide cocktails can interact synergistically on aquatic crustaceans. *Environmental Science and Pollution Research* 17(4), pp. 957-967.

OBRIST, L. B., DUTTON, A., ROMEIS, J., & BIGLER, F., 2006. Biological activity of Cry1Ab toxin expressed by Bt maize following ingestion by herbivorous arthropods and exposure of the predator Chrysoperla carnea. *Biocontrol* 51(1), pp. 31-48.

OVERBALLE-PETERSEN, S. & WILLERSLEV E. (2014) Horizontal transfer of short and degraded DNA has evolutionary implications for microbes and eukaryotic sexual reproduction. *Bioassay*. 36 (10) p. 1005 – 1010.

PAGANELLI, A., GNAZZO, V., ACOSTA, H., LOPEZ, S. L., & CARRASCO, A. E., 2010. Glyphosate-Based Herbicides Produce Teratogenic Effects on Vertebrates by Impairing Retinoic Acid Signaling. *Chemical Research in Toxicology* 23(10), pp. 1586-1595.

PALM, C. J., SCHALLER, D. L., DONEGAN, K. K., & SEIDLER, R. J., 1996. Persistence in soil of transgenic plant produced Bacillus thuringiensis var kurstaki delta-endotoxin. *Canadian Journal of Microbiology* 42(12), pp. 1258-1262.

PALMA, L., MUNOZ, D., BERRY, C., MURILLO, J., & CABALLERO, P., 2014. Bacillus thuringiensis Toxins: An Overview of Their Biocidal Activity. *Toxins* 6(12), pp. 3296-3325.

PEREZ, G. L., TORREMORELL, A., MUGNI, H., RODRIGUEZ, P., VERA, M. S., NASCIMENTO, M. D., ALLENDE, L., BUSTINGORRY, J., ESCARAY, R., & FERRARO, M., 2007. Effects of the herbicide Roundup on freshwater microbial communities: a mesocosm study. *Ecological Applications* 17(8), pp. 2310-2322.

PEREZ, J. E., MIRANDA, L., & VERA, M. A. S. 2011. *Effects of herbicide glyphosate and glyphosate-based formulations on aquatic ecosystems.* INTECH Open Access Publisher.

PETERSON, J. A., LUNDGREN, J. G., & HARWOOD, J. D., 2011. Interactions of transgenic Bacillus thuringiensis insecticidal crops with spiders (Araneae). *Journal of Arachnology* 39(1), pp. 1-21.

PODEVIN, N. & DU JARDIN, P., 2012. Possible consequences of the overlap between the CaMV 35S promoter regions in plant transformation vectors used and the viral gene VI in transgenic plants. *GM Crops and Food* 3(4), pp. 296-300.

POLLAK, P. 2011. *Fine chemicals: the industry and the business.* Hoboken: Wiley.

PRESCOTT, V.E. CAMPELL, P.M. MOORE, A. MATTES, J. ROTHENBERG, M.E. FOSTER, P.S. HIGGINS, T.J.V. AND HOGAN, S.P. (2005) Transgenic Expression of Bean r-Amylase Inhibitor in Peas Results in Altered Structure and Immunogenicity. J. Agric. Food Chem. 53. 9023−9030

RAMESSAR, K., PEREMARTI, A., GÒMEZ-GALERA, S., NAQVI, S., MORALEJO, M., MUÑOZ, P., CAPELL, T. & CHRISTOU, P. Biosafety and risk assessment framework for selectable marker genes in transgenic crop plants: a case of the science not supporting the politics. *Transgenic Research*. 16 (3) p. 261-280.

RAMIREZ-ROMERO, R., DESNEUX, N., DECOURTYE, A., CHAFFIOL, A., & PHAM-DELEGUE, M. H., 2008. Does CrylAb protein affect learning performances of the honey bee Apis mellifera L. (Hymenoptera, Apidae)? *Ecotoxicology and Environmental Safety* 70(2), pp. 327-333.

RANG, A. LINKE, B. AND JANSEN, B. (2005) Detection of RNA variants transcribed from the transgene in Roundup Ready soybean. Eur Food Res Technol. 220:438–443 DOI 10.1007/s00217-004-1064-5

RAYBOULD, A., GRASER, G., HILL, K., & WARD, K., 2012. Ecological risk assessments for transgenic crops with combined insect-resistance traits: the example of Bt11 x MIR604 maize. *Journal of Applied Entomology* 136(1-2), pp. 27-37.

RELYEA, R. A., 2005a. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecological Applications* 15(2), pp. 618-627.

RELYEA, R. A., 2005b. The lethal impact of roundup on aquatic and terrestrial amphibians. *Ecological Applications* 15(4), pp. 1118-1124.

RELYEA, R. A. & HOVERMAN, J., 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. *Ecology Letters* 9, pp. 1157-1171.

RICHARD, S., MOSLEMI, S., SIPAHUTAR, H., BENACHOUR, N., & SERALINI, G. E., 2005. Differential effects of glyphosate and roundup on human placental cells and aromatase. *Environmental Health Perspectives* 113(6), pp. 716-720.

RICROCH, A. BERGE, J.B. AND KUNTZ, M. (2011) Evaluation of genetically engineered crops using transcriptomic, proteomic and metabolomics profiling techniques. Plant Physiology 155. 1752-1761

ROSATI, A. BOGANI, P. SANTARLASCI, A. AND BUIATTI, M (2008) Characterisation of 30 transgene insertion site and derived mRNAs in MON810 YieldGard maize. Plant Mol Biol 67:271–281 DOI 10.1007/s11103-008-9315-7

ROSI-MARSHALL, E. J., TANK, J. L., ROYER, T. V., WHILES, M. R., EVANS-WHITE, M., CHAMBERS, C., GRIFFITHS, N. A., POKELSEK, J., & STEPHEN, M. L., 2007. Toxins in transgenic crop byproducts may affect headwater stream ecosystems. *Proceedings of the National Academy of Sciences of the United States of America* 104(41), pp. 16204-16208.

SAAL, S.F.V. AND HUGHES, C. (2005) An Extensive New Literature Concerning Low-Dose Effects of Bisphenol A Shows the Need for a New Risk Assessment. Environmental Health Perspectives. 113(8).p. 926-933.

SAEGLITZ, C., BARTSCH, D., EBER, S., GATHMANN, A., PRIESNITZ, K. U., & SCHUPHAN, I., 2006. Monitoring the Cry1Ab susceptibility of European corn borer in Germany. *Journal of Economic Entomology* 99(5), pp. 1768-1773.

SAXENA, D. & STOTZKY, G., 2000. Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic *Bt* corn in vitro and in situ. *FEMS Microbiology Ecology* 33, pp. 35-39.

SAXENA, D., FLORES, S., & STOTZKY, G., 2002. Vertical movement in soil of insecticidal Cry1Ab protein from Bacillus thuringiensis. *Soil Biology & Biochemistry* 34(1), pp. 111-120.

SCHLÜTER, K., FÜTTER, J. & POTRYKUS, I. (1995) “Horizontal” gene transfer from a transgenic potato line to a bacterial pathogen (Erwinia chrysanthemi) occurs—if at all-at an extremely low frequency. *Biotechnology (NY)* 13 (10) p. 1094-1098.

SCHNABLE, P.S.,WARE, D. FULTON, R.S. STEIN, J.C. WEI F, ET AL. (2009) The B73 maize genome: complexity, diversity, and dynamics. Science 326:doi:1126/science.1178534.

SCHUPPENER, M., MUEHLHAUSE, J., MUELLER, A. K., & RAUSCHEN, S., 2012. Environmental risk assessment for the small tortoiseshell Aglais urticae and a stacked Bt-maize with combined resistances against Lepidoptera and Chrysomelidae in central European agrarian landscapes. *Molecular Ecology* 21(18), pp. 4646-4662.

SCHWEITZER, J. A., BAILEY, J. K., REHILL, B. J., MARTINSEN, G. D., HART, S. C., LINDROTH, R. L., KEIM, P., & WHITHAM, T. G., 2004. Genetically based trait in a dominant tree affects ecosystem processes. *Ecology Letters* 7(2), pp. 127-134.

SERALINI, G. E., CLAIR, E., MESNAGE, R., GRESS, S., DEFARGE, N., MALATESTA, M., HENNEQUIN, D., & DE VENDOMOIS, J. S., 2014. Republished study: long-term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. *Environmental Sciences Europe* 26(1), p. 14.

SERVIZI, J. A., GORDON, R. W., & MARTENS, D. W., 1987. Acute toxicity of Garlon 4 and Roundup herbicides to salmon, Daphnia, and trout. *Bulletin of Environmental Contamination and Toxicology* 39(1), pp. 15-22.

SIH, A., BELL, A. M., & KERBY, J. L., 2004. Two stressors are far deadlier than one. *Trends in Ecology & Evolution* 19(6), pp. 274-276.

SIMENSTAD, C. A., CORDELL, J. R., TEAR, L., WEITKAMP, L. A., PAVEGLIO, F. L., KILBRIDE, K. M., FRESH, K. L., & GRUE, C. E., 1996. Use of rodeo-« and XGÇÉ77-« spreader to control smooth cordgrass (Spartina alterniflora) in a southwestern washington estuary: 2. Effects on benthic microflora and invertebrates. *Environmental Toxicology and Chemistry* 15(6), pp. 969-978.

SMALLA, K., VAN OVERBEEK, L.S., PUKALL, R. & VAN ELSAS, J.D. (1993) Prevalence of nptII and Tn5 in kanamycin-resistant bacteria from different environments. *FEMS Microbiology Ecology*. 13 (x) p. 47-58.

SONG R, MESSING J. (2002) Contiguous genomic DNA sequence comprising the 19-kD zein gene family from maize. Plant Physiol. 130(4):1626-35

SPRINGER, N.M. YING, K. FU, Y. JI, T. YEH, C.T. ET AL. (2009) Maize Inbreds Exhibit High Levels of Copy Number Variation (CNV) and Presence/Absence Variation (PAV) in Genome Content. *PLOS Genetics*. 5 (11). e1000734

SVAROVSKY, S, BOROVKOV, A. AND SYKES, K. (2008). Cationic gold microparticles for biolistic delivery of nucleic acids. BioTechniques 45:535-540

TAKENAKA M, ZEHRMANN A, VERBITSKIY D, HÄRTEL B, BRENNICKE A. (2013) RNA editing in plants and its evolution. *Annu. Rev. Genet*. 47:335-52. doi: 10.1146/annurev-genet-111212-133519

TAPP, H. & STOTZKY, G., 1995. Insecticidal Activity of the Toxins from Bacillus-Thuringiensis Subspecies Kurstaki and Tenebrionis Adsorbed and Bound on Pure and Soil Clays. *Applied and Environmental Microbiology* 61(5), pp. 1786-1790.

THEN, C., 2009. Risk assessment of toxins derived from Bacillus thuringiensis-synergism, efficacy, and selectivity. *Environmental Science and Pollution Research* 17(3), pp. 791-797.

TORRES, J. B. & RUBERSON, J. R., 2008. Interactions of Bacillus thuringiensis Cry1Ac toxin in genetically engineered cotton with predatory heteropterans. *Transgenic Research* 17(3), pp. 345-354.

TUMUSIIME, E., DE GROOTE, H., VITALE, J., & ADAM, B., 2010. The Cost of Coexistence between Bt Maize and Open-Pollinated Maize Varieties in Lowland Coastal Kenya. *AgBioForum* 13(3), pp. 208-221.

VAN DEN BERG, J., 2013a. Socio-economic factors affecting adoption of improved agricultural practices by small scale farmers in South Africa. *African Journal of Agricultural Research* 8(35), pp. 4490-4500.

VAN DEN BERG, J., HILBECK, A., & BOHN, T., 2013. Pest resistance to Cry1Ab Bt maize: Field resistance, contributing factors and lessons from South Africa. *Crop Protection* 54, pp. 154-160.

VAN DEN BERG, J., 2013b. Evolution in action: field-evolved resistance of African stem borer to Bt maize. *Outlooks on Pest Management* 24(5), pp. 236-239.

VILJOEN, C., 2013. Letter to the editor. *Food and Chemical Toxicology* 59, pp. 809-810.

VKM (2005) An assessment on potential long-term health effects caused by antibiotic resistance marker genes in genetically modified organisms based on antibiotic usage and resistance patterns in Norway.

WALSH, G. AND JEFFRIES, R. (2006) Post-translational modifications in the context of therapeutic proteins. NATURE BIOTECHNOLOGY. 24 (10). 1241-1252

WANG, Z. J., LIN, H., & HUANG, J. K., 2009. Cotton in China: Are secondary insect infestations offsetting the benefits in farmer fields? *Agricultural Sciences in China* 8(1), pp. 83-90.

WHITHAM, T. G., BAILEY, J. K., SCHWEITZER, J. A., SHUSTER, S. M., BANGERT, R. K., LEROY, C. J., LONSDORF, E. V., ALLAN, G. J., DIFAZIO, S. P., POTTS, B. M., FISCHER, D. G., GEHRING, C. A., LINDROTH, R. L., MARKS, J. C., HART, S. C., WIMP, G. M., & WOOLEY, S. C., 2006. A framework for community and ecosystem genetics: from genes to ecosystems. *Nature Reviews Genetics* 7(7), pp. 510-523.

WHO (2012) Critically Important Antimicrobials for Human Medicine

WINDELS, P. TAVERNIERS. I. DEPICKER. A. VAN BOCKSTAELE, E. AND DE LOOSE, M. (2001) Characterisation of the Roundup Ready soybean insert. Eur Food Res Technol 213:107–112. DOI 10.1007/s002170100336

WOEGERBAUER, M., ZEINZINGER, J., SPRINGER, B., HUFNAGL, P., INDRA, A.., KORSCHINECK, I., HOFRICHTER, J., KOPACKA, I., FUCHS, R., STEINWIDER, J., FUCHS, K., NIELSEN, K.M. & ALLERBERGER, F. (2014) Prevalence of the aminoglycoside phosphotransferase genes aph(3')-IIIa and aph(3')-IIa in Escherichia coli, Enterococcus faecalis, Enterococcus faecium, Pseudomonas aeruginosa, Salmonella enterica subsp. enterica and Staphylococcus aureus isolates in Austria. *Journal of Medical Microbiology.* 63 p. 210 – 217.

WOODRUFF, D. S., 2001. Declines of biomes and biotas and the future of evolution. *Proceedings of the National Academy of Sciences of the United States of America* 98(10), pp. 5471-5476.

WU, Y. Wang, Y. Li, J, Li, W. Zhang, L. Li, Y. Li. X, Li, Y. Zhu, L. AND Wu, G. (2014) Development of a general method for detection and quantification of the P35S promoter based on assessment of existing methods. *Scientific Reports.* 4(7358). DOI: 10.138/srep07358

ZHANG, D. DAS, D.B. AND RIELLY, C.D. (2013) Potential of microneedle-assisted micro-particle delivery by gene guns: a review. Drug Deliv. DOI: 10.3109/10717544.2013.864345

ZHAO, J. H., HO, P., & AZADI, H., 2011. Benefits of Bt cotton counterbalanced by secondary pests? Perceptions of ecological change in China. *Environmental Monitoring and Assessment* 173(1-4), pp. 985-994.

ZHAO, H. AVENARIUS, M.R. AND GILLESPIE, P.G. (2012) Improved Biolistic Transfection of Hair Cells. PLOS one. 7 (10). E46765

ZHOU, J., XIAO, K. F., WEI, B. Y., WANG, Z., TIAN, Y., TIAN, Y. X., & SONG, Q. S., 2014. Bioaccumulation of Cry1Ab Protein from an Herbivore Reduces Anti-Oxidant Enzyme Activities in Two Spider Species. *Plos One* 9(1).

ZOLLA, L. RINALDUCCI, S. ANTONIOLLI, AND RIGHETTI, P.G. (2008) Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of gene modification. Journal of Proteome Research. 7. 1850-1861

ZUPAN, J.R. AND ZAMBRYSKI, P. (1995) Transfer of T-DNA from Agrobacterium to the Plant Cell. Plant Physiol. 107: 1041-1 047

ZWAHLEN, C., HILBECK, A., HOWALD, R., & NENTWIG, W., 2003. Effects of transgenic Bt corn litter on the earthworm Lumbricus terrestris. (vol 12, pg 1077, 2003). *Molecular Ecology* 12(8), p. 2279.

2. GM vaccines, Gene therapy and Medicinal products containing or consisting of GMOs

2.1 Introduction

Genetically modified viruses (GMVs) are produced by modifying the genes or genomes of viruses using recombinant DNA technology and/or synthetic biology. These modifications are achieved by targeted deletion of the virus genes/gene fragments and/or insertion of heterologous genes (transgenes) obtained from other organisms. Viruses can infect cells in a target specific manner, transduce them as well as induce immune responses from the infected host. These inherent characteristics recommend viruses as ideal vectors for heterologous antigen expression *in vivo*.

The concept of viral vector was first introduced in 1972 when Simian Virus 40 (SV 40) was engineered to contain lambda phage genes and the galactose operon of *Escherichia coli* (Jackson et al., 1972). Ten years later, it was demonstrated that vaccinia virus (VACV) is an efficient transient gene expression vector (Mackett et al., 1982, Panicali and Paoletti, 1982). Presently, several virus vectors have been developed from many virus families including *Poxviridae*, *Adenoviridae*, *Parvoviridae*, *Retroviridae*, *Flaviviridae*, *Herpesviridae*, *Rhabdoviridae*, *Togoviridae*, *Paramyxoviride*, etc. (Rollier et al., 2011).

Genetically modified viruses (GMVs) have application as vaccines against infectious diseases, immunocontraception for animal pest control and immunotherapy/oncotherapy against cancer as well as in gene therapy for the correction of inherited genetic disorders (Liniger et al., 2007, Jackson et al., 1998, Lichty et al., 2014, Kay, 2011). Several virus-vectored vaccines/gene therapy products are at different stages of clinical trials[2], while some recombinant virus vaccines are already licensed for veterinary applications (Draper and Heeney, 2010). Currently, GLYBERA®, an adeno-associated virus-vectored gene therapy product engineered to express lipoprotein lipase in the muscle for the treatment of lipoprotein lipase deficiency, and IMOJEV®, a yellow fever virus-vectored Japanese encephalitis vaccine for vaccination against Japanese encephalitis are the only products already licensed for human use (Yla-Herttuala, 2012, Appaiahgari and Vrati, 2010).

Virus-vectored products for vaccination and gene therapy have the potential of being the panacea to infectious diseases, cancers and hereditary disorders affecting human, domesticated animals and wildlife. The conundrum is that their usage may result in unintended risk to humans, animals and the environment. In recognition of this, regulatory agencies like the European Medicine Agency (EMA) and the Food and Drug Administration (FDA) have published guidelines on the environmental risk assessment of genetically modified (GM) vaccines, gene therapy and medicinal products containing or consisting of genetically modified organisms (GMOs) (EMA/CHMP/VWP/141697/2009, EMEA/CHMP/ICH/449035/2009).

Advanced therapy medicinal products (ATMP) are very diverse in nature and comprise gene therapy medicinal products (GTMPs), somatic cell therapy medicinal products (sCTMPs) and tissue

[2] https://clinicaltrials.gov/ct2/results?term=Virus+vectors&Search=Search

engineering products (TEPs). Therefore, a flexible approach, known as the 'Risk-based approach', contained in Annex 1 part IV of the directive 2001/83/EC[3] is generally adopted to address and evaluate potential risks to the patient and to third parties. The risk-based approach is aimed at determining the extent of quality, non-clinical and clinical data to be included in the marketing authorization application, in accordance with the scientific guidelines relating to the quality, safety and efficacy of medicinal products, and to justify any deviation from the requirements of this Annex. Detailed information on the risk-based approach and classification of ATMP is contained in the 'Guideline on the risk-based approach according to annex I, part IV of Directive 2001/18/EC applied to advanced therapy medicinal products'[4].

In the EU, Directive 2001/18/EC regulates the placement on the market of any GMO or a product containing GMOs. It requires the Applicant to submit a Part C notification that includes relevant administrative and scientific information, an ERA, a summary, and information on proposed monitoring and risk management strategies, to the designated competent authority (CA) of the member state in the territory where the GMO is intended to be placed on the market for the first time. For human biotechnological medicinal products that contain or consist of GMOs, their regulation is governed by reciprocal provisions in Directive 2001/18/EC and the European Commission Regulation (EC) 726/2004 (Articles 6.2 and 6.3)[5]. The Directive defines 'effect on the environment' to mean effects exerted on any living or non-living human or non-human inhabitant, component or compartment of the global ecosystem, with the exception of those effects exerted on the intended patients as a direct result of the administration of the product.

Examination of peer reviewed literature available in public databases, as well as the environmental risk assessment dossiers submitted for marketing application authorization (MAA) to EMA, indicates that there are areas of biological characterization of the virus-vectored products that need further research in order have reasonable data to evaluate the potential risk of these virus-vectors to human health and the environment. Thus, there are knowledge gaps, uncertainty and omitted research in the biological characterization of potential hazards associated with the use of virus vectors for vaccination and gene therapy.

Insufficient data or gaps in knowledge exist in the areas of (i) choice of an appropriate comparator to the GMV, (ii) baseline information on the nature and distribution of naturally circulating viruses, (iii) interactions between the GMV and naturally circulating virus relatives including recombination and complementation, (iv) cell tropism of the GMVs including the basis for host restriction, (v) genome stability of the GMVs following multiple passages, (vi) authentic *in vivo* models to study the GMVs cell tropism, distribution and shedding, (vii) unintended transgene effects on the virus and host metabolomes, (viii) epigenetic and pleiotropic effects, (ix) GMVs tissue tropism, distribution and shedding in immune-compromised individuals, (x) transgene instability including host and viral factors that modulate the stability of the transgene, etc. In this chapter, we will discuss the aforementioned gaps in knowledge and suggest ways to address them.

3 http://ec.europa.eu/health/files/eudralex/vol-1/dir_2001_83_consol_2012/dir_2001_83_cons_2012_en.pdf
4 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500139748.pdf
5 http://apps.who.int/medicinedocs/en/d/Js17139e/

2.1.1 Viruses, vaccination and gene therapy – definition

Viruses are obligate intracellular microbes that replicate only within living cells. Viruses are metabolically inactive outside the host cell. A virus particle (virion) is made up of nucleic acid and a protein coat, although some viruses can have an extra coat called an envelope. The nucleic acid of viruses can either be DNA or RNA or both (at different stages of the life cycle). The shape of the nucleic acid can be linear, circular or segmented, while the strands may be single or double. The polarity of the viruses with RNA genomes can be described as positive sense, negative sense or ambisense. Generally, viruses have high replication throughput resulting in the production of billions of virus particles within a short time. While this may be an advantage for a GMV with respect to the quantity of expressed antigen, the high rate of multiplication will result in generation of mutant viruses and quasi species. Viruses infect all life forms (Koonin et al., 2006), thus in principle virus vectors can be engineered to express transgenes from any life form.

A vaccine is a biologically preparation that stimulates the host immune system to develop immunity against a pathogen. Traditional vaccine types may be classified as (i) inactivated, (ii) attenuated, (iii) virus-like particles and (iv) subunit vaccines. These traditional vaccine types have proved ineffective for major human diseases like malaria, tuberculosis, human immunodeficiency virus (HIV), Ebola, cancers, etc. The failure of traditional vaccine forms, especially against diseases caused by intracellular pathogens, necessitates the search for alternative but robust vaccine platforms (Plotkin, 2009). Virus-vectored vaccines seem promising as an effective platform to prevent and treat diseases where traditional methods of vaccine development have failed (Small and Ertl, 2011, Parks et al., 2013, Stanley et al., 2014). Gene therapy can be defined as the introduction of nucleic acids coupled to a vector into cells with the intention of altering gene expression in order to prevent, halt, reverse or reduce the severity of a pathological process (Kay, 2011). Gene therapy can alter the host gene expression by gene addition, gene correction/alteration or by gene knockdown. Gene therapy products can be administered in vitro where cells are modified outside the patient's body and then transplanted back into the patient (also called ex vivo), or in vivo where the genetic modification is achieved within the patient. The natural ability of viruses to infect cells, deliver their nucleic acid to the nucleus where it either integrates into the host chromosome (as in retroviruses) or exists as episome (as in some herpes viruses), has been exploited in developing virus-vectored gene therapy products.

2.1.2 Overview of major virus vectors for vaccination and gene therapy

(a) Poxvirus vectors

Poxvirus vectors are derived from viruses belonging to family *Poxviridae*. Poxviruses have linear, double stranded genomes and their entire morphogenesis is in the cytoplasm of infected cells. Since 1982, poxvirus-vectored vaccines have emerged as promising candidates for the prevention of infectious diseases and cancers in humans, domestic animals and wildlife (Moss, 1996, Panicali and Paoletti, 1982, Altenburg et al., 2014, Izzi et al., 2014). Poxviruses present several advantages as live vaccine vectors. These include a wide host range, multiplication only in the cytoplasm of infected cells, resistance to heat and other environmental stress, ease of large scale production, etc. In addition, poxvirus vectors are able to carry up to 25 kbp of transgenic cDNAs. Several multiplication

competent poxvirus-vectored vaccines have been evaluated, but only vaccinia virus (VACV) – vectored rabies vaccine (RGV) has been licensed and marketed so far. RGV was successfully used in the field to vaccinate against rabies in feral animals in Europe and North America (Rupprecht et al., 1986).

At present, the use of multiplication competent poxvirus vectors for vaccines (except in virus-vectored oncotherapy), especially in humans, is disfavoured due to biosafety concerns (Casey et al., 2005, Mempel et al., 2003). These biosafety concerns were in part addressed by the development of multiplication incompetent poxviruses like Modified vaccinia virus Ankara (MVA), NYVAC, ALVAC and TROVAC. These multiplication deficient poxvirus vectors form the backbone of most poxvirus-vectored vaccines that are in clinical trials or already licensed (only for veterinary applications) (Draper and Heeney, 2010). Although no vaccines based on these replication deficient poxviruses have been approved for human use, the MVA vector itself, Imavax® (MVA-BN), an MVA variant developed by Bavarian Nordic, has been approved by EMA for vaccination against smallpox virus infection[6].

The characteristics of the poxvirus vector and the poxvirus-vectored vaccine relevant to environmental risk assessment include (i) productive infection in human and mammalian cells, (ii) clonal purity of the vector, (iii) phenotypic and genetic stability of the transgene, (iv) recombination between the poxvirus-vectored vaccine and naturally circulating poxviruses, (v) biological activity of the transgenes and its effect on the virus and host metabolome, virus shedding and spread to the environment, (vi) baseline information on the nature and occurrence of naturally circulating poxviruses, etc. (Goossens et al., 2013, Okeke et al., 2006, Hansen et al., 2004, Okeke et al., 2012, Okeke et al., 2014).

(b) Adenovirus vectors

Adenovirus (Ad) vectors are derived from viruses with Family *Adenoviridae*. Adenoviruses have linear, double stranded genomes and they multiply in the nucleus of infected cells. There about 57 human Ad serotypes and the human Ad serotype 5 (Ad5) is widely used as a vector for vaccination and gene therapy. Adenovirus genes are divided into early (E) and late (L) genes. Early genes are responsible for virus DNA replication and modulation of the host metabolism while the late genes code for virus structural proteins (Thomas and Mathews, 1980). Although Ad replicates its DNA in nucleus of infected cells there is no evidence of the integration of the virus DNA into the host genome.

Recombinant Ad vectors are widely used for gene therapy and to a lesser extent for vaccination because of their high transduction efficiency, broad cell tropism, good ability to infect dividing and non-dividing cells, high level of transgene expression, accommodation of large transgene expression cassette as well as producing high virus titres under GMP conditions (Capasso et al., 2014). Ad vectors are the most used vector in gene therapy trials (Ginn et al., 2013). However, in spite of their wide usage, Ad vectors have shown some severe adverse effects in clinical trials, including the death of a patient following intravenous infusion of Ad vector designed to cure ornithine transcarbamylase

[6] (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-Summary_for_the_public/human/002596/WC500147899.pdf).

deficiency (Raper et al., 2003). In addition, a phase 2b clinical trial designed to test the efficacy of Ad-vectored HIV vaccine was halted following concerns that the vaccine may enhance susceptibility to HIV infection (Gray et al., 2010).

The biological characteristics of this vector that may modulate cell tropism, shedding and transmission (and hence of relevance to ERA) include (i) contamination of vaccine stock by helper viruses especially in third generation Ad vectors, (ii) inflammatory responses and cytokine storm, (iii) antibody, cell or innate immunity mediated enhancement of infection, (iv) anti-vector immunity, (v) oncogenicity of human Ad strains in animal, and (vi) high level of *in vivo* toxicity (Liu and Muruve, 2003, Capasso et al., 2014).

(c) Adeno-associated virus vectors.

Adeno-associated virus (AAV) is a small, non-enveloped virus with a single-stranded DNA genome measuring about 4.7 kbp in size. They belong to family *Parvoviridae* and genus *Dependovirus*. There are about twelve AAV serotypes in humans and more than 100 serotypes in different animal species. AAVs are non-pathogenic in humans and require a helper virus for productive virus infection. They require complementation by adenovirus VA, E1, E2 and E4 proteins. These helper functions can either be provided by plasmid co-transfection or by co-infection with a helper virus (Schaffer et al., 2008, Kotin, 2011).

Several AAV serotypes are being evaluated as vectors but the human AAV2 is commonly used in most pre-clinical and clinical trials (Nieto and Salvetti, 2014). GLYBERA®, the first gene therapy product approved by EMA is vectored by an AAV. Recombinant AAV vectors are generated by deletion of the *Rep* and *Cap* coding regions that are located between the inverted terminal repeats (ITRs), and inserting a transgene expression cassette between the ITRs. AAV vectors can transduce both dividing and non-diving cells and have broad cell tropism. The characteristics of AAV vectors that are relevant to ERA include (i) insertional mutagenesis due to integration into the recipient genome, (ii) contamination of vector stock by helper viruses during high scale production, (iii) pre-existing immunity, (iv) high level of vector DNA persistence either as episomes or integrated into the recipient genome, (v) tumorigenesis in animals and (vi) infection of off-target cells and hosts (Donsante et al., 2001).

(d) Retrovirus vectors

Retrovirus vectors are derived from viruses belonging to family *Retroviridae*. Retroviruses are enveloped viruses with a diploid 7 – 12 kbp single-stranded, positive sense RNA genome. Retroviral life cycle is characterized by the reverse transcription of their RNA genome to a double-stranded DNA. The double stranded virus DNA is integrated into the host genome as a provirus, and virus gene expression is from the provirus. Long-term stable expression of the transgene is achieved with retro vectors because of their integration to the host chromosome. The gamma (γ) retrovirus, like the Moloney Murine Leukemia Virus (MoMuLV), and lentivirus like the Feline immunodeficiency virus (FIV), are commonly used as vectors for gene therapy and vaccination (Maetzig et al., 2011, Segura et al., 2013). Unlike the γ-retrovirus vectors, lentivirus vectors can transduce both dividing and non-dividing cells.

Potential adverse effects attributable to retroviral vectors used for gene therapy and vaccination include (i) generation and propagation of replication competent retroviruses (RCR) due to mobilization of the vector during manufacture or because of recombination/complementation

between pro-viral and host sequences, (ii) oncogenesis due to insertional mutagenesis, (iii) uncontrolled growth of transduced cells and RCR breakouts (iv) immunogenicity of the transgene inserted into the vector, (v) vertical transmission, (vi) shedding of free virus and transduction of non-target cells, (vii) perturbation of the host gene expression including cytokine expression profile, and (viii) reactivation of endogenous viruses like human endogenous retroviruses (HERVs), cytomegalovirus (CMV) or Epstein Bar Virus (EBV) (Pauwels et al., 2009, Deichmann and Schmidt, 2013).

(e) Flavivirus and pestivirus vectors

The genera *Flavivirus* and *Pestivirus* belong to family *Flaviviridae*. Viruses in both genera are enveloped and contain approximately 10 – 13 kbp of non-segmented, single-stranded, positive sense RNA genome. Their genome organization is characterized by 5^1 and 3^1 non-coding regions and a single polyprotein open reading frame. Members of the genus *Flavivirus* whose vectors have been developed and extensively evaluated include yellow fever virus (YFV), Japanese encephalitis virus (JEV) and dengue virus (DV). IMOJEV ®, a recently licensed vaccine against Japanese Encephalitis in human is vectored with YF-17D backbone (Appaiahgari and Vrati, 2010). Most pestivirus vectors for vaccination especially against animal diseases are derived from bovine viral diarrhoea virus (BVDV) and classical swine fever virus (CSFV) (Reimann et al., 2010, Zemke et al., 2010, van Gennip et al., 2000).

The biological characteristics of the vector (flavirus and pestivirus) and its interaction with the host or other viruses that are relevant to ERA include (i) the emergence of quasi species, (ii) recombination between the fittest strain and the variants, (iii) phenotypic and genetic stability of the chimeric vaccine/transgene, (iv) reversion to wild type by the chimeric vaccine following adaptation in the host, (v) transplacentral transmission in immune-compromised host, (vi) spread to immune-compromised non-target hosts, (vii) modulation of chimeric virus vaccine biology in the presence of persistent infection by non- cytopathic (ncp) BVDVs, etc.

(f) Herpesvirus vectors

The family *Herpesviridae* is a large group of DNA viruses that cause infections in humans and animals. The alphaherpesviruses, especially those that belong to genera *simplexvirus* (Herpes simplex virus 1, HSV-1 and Herpes simplex virus 2, HSV-2), and *mardivirus* (Gallid herpesvirus 3, Herpes virus of Turkey, HVT) have been used as vectors for vaccination against infectious diseases of humans and animals as well as in cancer immunotherapy (Lichty et al., 2014, Esaki et al., 2013, Gimeno et al., 2011).

The biology of the alphaherpesvirus vector relevant to characterization of potential hazard during ERA include (i) intraspecific recombination between vaccine virus and naturally circulating strains, (ii) restoration of virulence following passage *in vitro* and *in vivo*, (iii) integration of viral DNA into the host genome and the risk of insertional mutagenesis (mardiviruses only), (iv) establishment of latent infection in cells and hosts, (v) reactivation of cells latently infected with virulent field isolates, (v) infection and dissemination in non-target species, (vi) dissemination in target but immune-compromised hosts, (vii) adverse effect on reproductive performance, (viii)transgene instability, and (ix) genome/proteome stability.

Apart from the major virus vectors described above, viruses from other families like *Togaviridae, Rhabdoviridae*, *Paramyxoviriade* are also been developed and evaluated as vaccine vectors.

2.2 Knowledge gaps and omitted research in risk assessment of GM vaccines and gene therapies

2.2.1 Appropriate comparators

We suggest that revertant viruses should be included as a second comparator in all the studies aimed at biological characterization of the GMV in vitro and in vivo. The use of the correct comparator is essential for a comparative evaluation of the characteristics of the GMV compared to the unmodified virus from which it was derived. Ideally, there should be two comparators to the GMV namely (i) an unmodified parental strain from which the GMV was *directly* derived and (ii) a revertant virus in which all the genetic modification introduced have been removed. The unmodified parental strain (comparator 1) should be identically to the revertant virus. At present, ERA of GMVs only use the unmodified virus as the comparator. The inclusion of revertant viruses as comparator 2 to the GMV is more robust than comparison to comparator 1 alone as it can reveal changes in characteristics of the GMV that are not attributable to the expressed transgene. In cases in which the characteristics of comparator 1 differ significantly from the revertant virus; it might suggest that changes in the region outside the loci of modification may have taken place. Revertant viruses are commonly used as controls in functional analysis of virus genes (McKelvey et al., 2002).

Biological characterization of the GMV should be informed by this observation and comparator 1 to the GMV should be the wild type parental virus from which the GMV was directly derived from. Another area of omitted research or gap in knowledge is the inconsistent use of the comparator 1. The comparator should be used in all studies involving the GMV but it is common to find in public databases as well as ERA dossiers that comparator 1 is only used in some of the studies. For example, comparator I will be used in virus replication kinetics studies but will not be used in reversion to virulence tests or in transmission to non-target effect studies. The implication of this is that no data is provided to enable risk assessors to ascertain if any changes observed in the GMV treatment group is solely due to the genetic modification. Often, GMV producers in the ERA of their product try to rationalize this flaw by referring to studies done with viruses belonging to the same species as their comparator 1. Within the same species, there are many strains with significant differences in terms of virulence, attenuation, virus shedding and spread. Human herpes simplex virus 1 (HSV-1) (for example) has more than 6410 strains and the characteristics of these strains are not similar or identical[7]. Thus, a GMV based on HSV-1 strain H129 should be compared to wild type HSV-1 strain H129 and not to other HSV-1 strains like JSI. The genetic and phenotypic diversity of strains within a species exclude any of the strains from being the representative of the species in terms of biological characteristics.

[7] http://www.viprbrc.org/brc/home.spg?decorator=herpes

2.2.2 GMV multiplication *in vitro* and *in vivo*

The host range and distribution of wild type viruses that form the vector backbone of GMVs are important in the ERA of GMVs. Such information provides the baseline data to which the host range, spread and transmission of the GMV to both target and off target animals can be compared. Often, these data are not provided in the ERA dossier of GMVs. These omissions are rationalized with sweeping generalizations like ***" canarypox does not infect other birds except canaries and possibly passerine birds"*** (Taylor et al., 1995), "***MVA does not undergo productive infection in human cells***" (Cottingham and Carroll, 2013), "***knowledge of pestivirus tropism in vitro and in vivo is only fragmentary***" (Reimann et al., 2004). These catch phrases are meant to convey the impression that the scientific evidence in support of the assertions is overwhelming and indisputable, thus there is no iota of uncertainty. This implies that there is no need to provide data on these issues since the scientific debate is already closed. But is the debate closed? Is there contradictory scientific evidence, omitted research and gaps in knowledge? There are over 10000 species of birds in the ecosystem and the host range of canarypox virus has only being studied in few bird species. Canarypox virus or canarypox-like virus has been found to be endemic in passerine birds (Thiel et al., 2005). The susceptibility of many wild bird species to canarypox virus infection is unknown and there is also paucity of data on the susceptibility of several native bird species from different continents to canarypox virus infection. Wild (especially migrant birds) and native bird species may play a role in the transmission of canarypox virus (ALVAC) vectored vaccine from vaccinated birds to non-vaccinated birds. Although ALVAC and ALVAC derived recombinants have been shown not to multiply in mammalian cells, only very few cells have been investigated. Besides, some host restricted avipoxviruses have been shown to multiply efficiently in mammalian cell lines (Sainova et al., 2005, Weli et al., 2005). Taken together, the host range and cell tropism of Canarypox virus in most bird species is unknown.

MVA is regarded as the vector of choice due to its extreme attenuation *in vivo*, inability to infect human and most mammalian cells, as well as its un-impaired transgene expression even in non-permissive cells. Although the basis of the host range restriction of MVA is unknown (Wyatt et al., 1998, Meisinger-Henschel et al., 2010), it is strongly asserted that MVA cannot undergo productive infection in human cells. Like the canarypox described above, studies on cell tropism of MVA and its host range are not provided in the ERA dossier of MVA vectored vaccines because it is claimed that there is overwhelming scientific evidence to show that MVA cannot multiply in human cells. EMA seem to agree to this because in granting license to Imanvex ® (MVA-BN) to be used within the European Union (EU), EMA Committee for Medicinal Products for Human Use (CHMP) stated "***With regard to safety, the vaccinia virus in Imvanex cannot replicate in human cells and hence is less likely to cause side effects than previous smallpox vaccines. Imvanex would therefore be beneficial for people who cannot be given vaccines containing replicating viruses, such as patients with a weakened immune system***"[8].

However, it is highly unlikely that a virus will be non-productive in all human cells, and yet the basis for such host restriction remains elusive. Since MVA host restriction in human and most mammalian

[8] (http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002596/human_med_001666.jsp&mid=WC0b01ac058001d124)

cells was achieved by multiple serial passage of parental chorioallontois vaccinia virus Ankara (CVA) in chicken embryo fibroblast (CEF), we hypothesized that multiple serial passage of MVA in human cell may yield virus variants that can productively infect human cells. We purified MVA (ATCC VR-1508) and used it to infect human caco-2 cells at a multiplicity of infection of 0.01 and blindly passaged for 40 times. We were able to isolate MVA variants that efficiently infect human Caco-2 cells (Fig 2.1). Transmission electron microscopy also shows the production of mature virions (Figure 2.2). Our results demonstrate for the first time that MVA can productively infect a human cell.

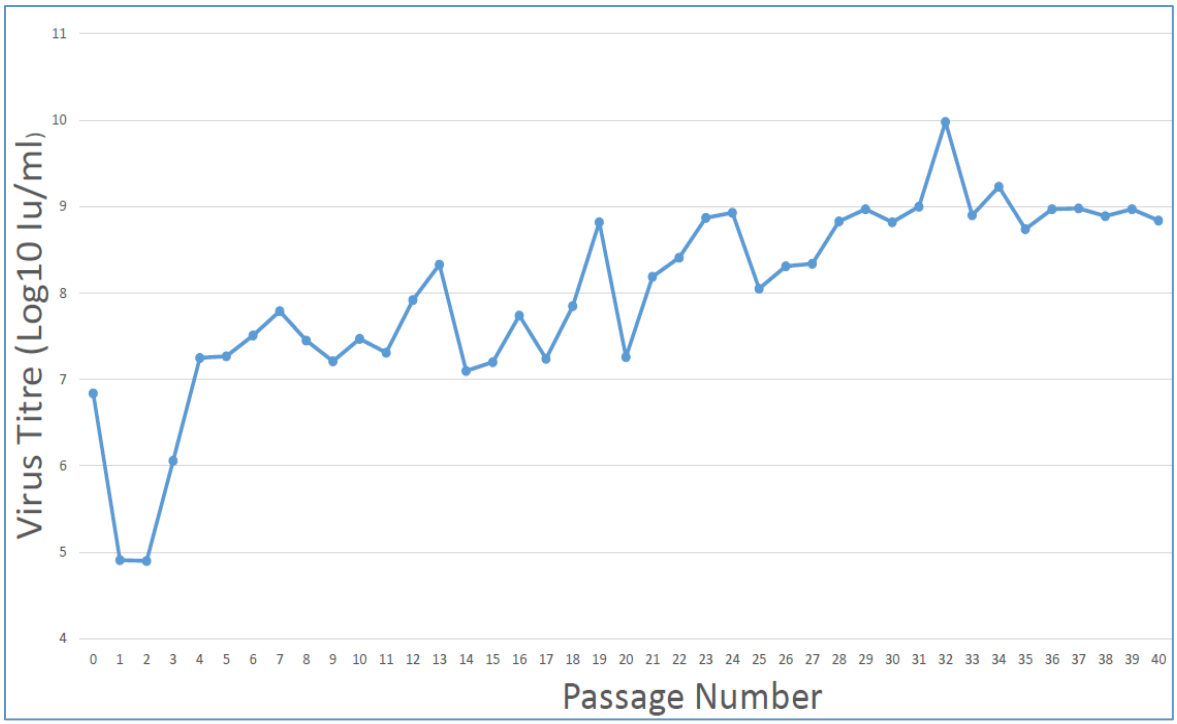

Fig 2.1. Serial passage of MVA (ATCC VR-1506) in human cell. Purified MVA virions were blindly passaged in Caco-2 cells for 40 times. At each passage, infected cells were harvested, freeze-thawed three times and virus titre was determined as we previously described (Okeke et al., 2006). Ofor et al. 2015 (*Unpublished)*.

Further work will examine if these MVA viruses can productively infect other human cell lines (apart from Caco-2), primary human cells, and intact animals. If the productive infection observed with human cell line is replicated in primary cells and in intact animals, it will have a significant implication for the environmental risk assessment of MVA vectored vaccines. At the very least, these MVA variants capable of productive infection will serve as molecular tools to unravel the molecular basis of MVA host restriction in mammalian cells. It will also throw light on whether these viruses were already present as sub-species in ATCC VR-1508 and were selected via serial passaging or that they adapted to multiply efficiently in human cells.

Pestiviruses like BVDV have broad host range and can infect a wide range of even-toed ungulates. They also have broad tissue tropism. Non-bovine host like rabbit may play a role as reservoir especially since they are abundant and live near livestock pastures. BVDV-vectored vaccines have been reported to be unable to infect rabbit during experimental infection (Konig et al., 2011). However, a recent report has shown that BVDV was propagated in rabbit during experimental

infection (Bachofen et al., 2014). The role of rabbits as a reservoir for transmission of BVDV or BVDV vectored-vaccine need to be clarified as it is relevant to assessment of non-target infections of pestivirus vectored GMVs. The *in vitro* multiplication studies aimed at characterizing BVDV and recombinant BVDVs are often carried out in MDBK-2 cells but this cell line is not the most permissive to BVDV infections. It is known that higher virus titres can be obtained using bovine testicular (BT) cells (Dubovi, 2013). Using a less permissive cell line can mask the differences in virus multiplication between the comparator and the GMV.

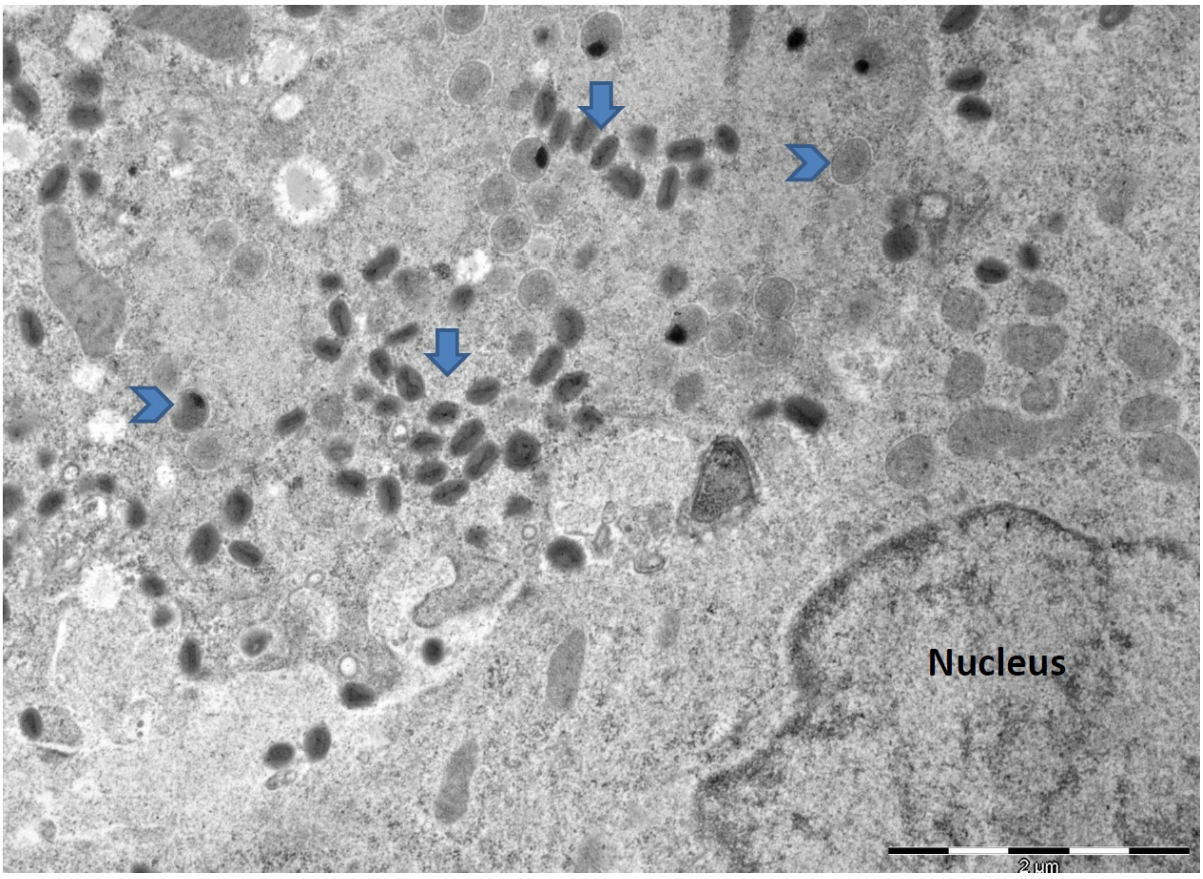

Figure 2.2 Electron micrograph of cell section of Caco-2 cells infected with MVA obtained from passage 32 (P32). Confluent caco-2 cells were infected with MVA obtained from passage 32 for 24 hours post infection. The infected cells were fixed and processed for electron microscopy. Arrows indicates mature virions while arrow heads point to immature viruses. Similar electron micrographs were obtained for viruses harvested from P28 to P40 (data not shown). No mature virions were observed from cells infected with un-passaged MVA (data not shown). Bar: 200 nm. Ofor et al. 2015 (*Unpublished*).

In addition, *the three examples with canarypox, MVA and pestivirus demonstrate that there is uncertainty, omitted research, gaps in knowledge, and new knowledge concerning the host range and cell tropism of these virus vectors. Thus GMV producers should provide data on these issues to enable a more authentic evaluation of the ERA dossier. The argument that the debate is closed because there is scientific consensus is invalid. In addition, regulatory agencies like the EMA should re-assess application status of approved GMVs in the light of new information.*

2.2.3 Genetic and phenotypic stability

Relevant EU directives on recombinant live virus vaccines require that the GMV vaccine is genetically and phenotypical stable at a passage level that yield virus titres equivalent to what is present in the production batch. This includes stability of the transgene following multiple serial passages, the stability of the entire genome, and the clonal purity of the vaccine taking into account the emergence of variants and quasi species. Often the stability of the GMV from master seed virus (MSV) +1 to MSV+5 is deemed acceptable. Usually, data on the stability of the transgene cassette is provided. However such data only takes into account one cell type/line or host. However, information on the stability of the transgene in different cells and hosts is missing. A transgene that is stable in one infection model can become unstable in another infection model (Okeke et al., 2009). The number of studies on viral and host factors that modulate transgene stability is miniscule. The effect of insertion sites, promoters, expression levels of the transgene, sequence and structure of the transgene/flanking regions are poorly understood except for poxvirus vectors where some mechanisms have been elucidated (Wyatt et al., 2009).

In view of this obvious gap in knowledge, it is not uncommon that some GMVs have the transgene expression cassette inserted into regions of the vector that are non-essential for replication. The implication is that instability or truncation of such non-essential regions will result in mutant viruses that are viable, as opposed to transgene insertion between essential genes. In the later scenario, truncation of the flanking sequences will lead to non-viable mutant viruses. *Since the transgene is the only logical tag for monitoring non-target effects/spread of the GMV as well as Differentiating Infected from Vaccinated Animals (DIVA), it is essential that causes of transgene stability are studied so that pre-emptive actions can be taken in the vector design (prior to ERA) to eliminate transgene instability.*

At present, data on the genome stability of the GMV across MSV+1 to MSV+5 in both *in vitro* and *in vivo* infection models are not provided for ERA because relevant EU directive (Directive 2001/I8/EC) has not made the provision of such data obligatory. Thus, mutations outside the region of transgene insertion as well as the emergence of variants or quasi species go unreported. The role of quasi species and virus sub-populations on the attenuation, infectivity, virulence, persistence and host range of GMVs has not been reported, nor the host and virus factors that modulates the selection of the dominant haplotype. The lack of proof-reading function of the RNA dependent RNA polymerase suggest that quasi species will be more common in GMVs whose backbone is from an RNA virus, for example pestivirus (Becher and Tautz, 2011). However, its presence in GMVs whose backbone is a DNA virus cannot be excluded. Indeed both the MVA vector and the smallpox vaccine Dryvax has been shown to be polyclonal (Suter et al., 2009, Qin et al., 2011). *The availability of high through-put next generation sequencing technologies* (Grada and Weinbrecht, 2013, Kircher and Kelso, 2010) *capable of genome-wide mapping of GMV sub-populations and clones at relatively low costs leaves no reason for not investigating the genome stability of GMVs. Genome-wide data can shed light to issues relevant to risk characterization of GMVs like vector persistence, residual virulence, altered tropism and host range and allow for a more robust biological characterization of GMVs. We recommend that regulatory agencies like EMA should revise the relevant directives so that the stability of GMVs is extended to the entire genome and not just to the locus of transgene insertion.*

Stable expression of the transgene at MSV+1 to MSV+5 in relevant *in vitro* and *in vivo* infection models is regarded as satisfactory for the ERA of GMVs. Phenotypic stability of the transgene should be considered both in terms of transgene expression (qualitative) as well as the amount of protein (qualitative) that was produced. The data on the latter (quantitative expression) is often not provided. However the quantity of expressed protein is relevant to ERA since over or under expression can influence toxicity of the product, transgene stability, interaction of the expressed protein with endogenous proteins, etc. The detection of phenotypic instability is only as good as the detection test that is used. Assays used to detect if the transgene is stably expressed across MSV+1 to MSV+5 rely on the transgene expression by whole virus population and not on individual clones. The implication of this is that contamination of GMV stock by unmodified parental virus or virus clones that have lost the ability to express the transgene may go undetected. *To exclude this possibility, assays used to examine stable expression of the transgene should be able to detect in the same petri-dish GMV clones expressing the transgene and virus clones in which the transgene is not expressed.*

2.2.4 Recombination

The potential for recombination between GMV vaccine strains, and between vaccine strains and field isolates is significant in terms of ERA since recombination has the potential of producing hybrid viruses with altered host range, tissue tropism, virulence and transmission. It can also result in the rescue of an attenuated GMV vaccine by a wild type virus and subsequent reversion to virulence. Homologous recombination is a common occurrence among many virus families from which virus vectors are derived, and their evolution is in part driven by recombination (Kameyama et al., 2006, Weber et al., 2015, Gubser et al., 2004). Although relevant directives from regulatory agencies advise that the potential for recombination of the GMV vaccines with field isolates be explored, no such data is provided because the risk for recombination is considered low or negligible for the virus vectors currently in use. Thus, instead of demonstrating experimentally that recombination is unlikely, effort is spent on marshalling out possible reasons to show that the theoretical risk of recombination between GMV vaccines and naturally occurring relatives or between two GM vaccines is low or negligible. *But is there a scientific justification for considering the risk of recombination low or negligible?*

The potential for recombination between GMV vaccines derived from avian alphaherpesviruses like HVT and naturally occurring isolates has not been investigated because the risk is considered low or negligible. Proponents of this view argue that recombination between HVT-vectored vaccines and field or vaccine strains of avian alphaherpesviruses have not been reported in spite of the fact that (i) in Europe and America, HVT (serotype 3) is used as a polyvalent vaccine containing serotypes 1 (Rispens) and 2 (SBI) strains, and (ii) vaccination with HVT has been in use for more than 35 years. Furthermore, it is stated that super-infection exclusion will significantly limit co-infection and subsequent recombination. *However, there is no evidence from scientific literature to show that post release monitoring of recombination has been conducted in those regions in which HVT vaccine have been in use. If a potential risk factor is not investigated, there is likely to be no report about it except if the recombinant is very virulent and possess a fitness advantage. The absence of report on recombination is not evidence for its absence.*

Recombination is a significant hazard that can occur with the use of alphaherpesviruses (herpesvirus in general) as vaccines or vaccine vectors and need to be experimentally excluded in studies conducted for marketing application authorization of GMVs based on HVT or other avian alphaherpesviruses. Intraspecific recombination between alphaherpesviruses has been shown to occur frequently *in vivo* (Thiry et al., 2005). Recombination between two Mareks Disease Virus serotype 1 (MDV-1) (*gallid herpes virus 2*) have been demonstrated in chicken (Jarosinski, 2012). Attenuated infectious laryngotracheitis virus (ILTV) vaccines have recombined to form virulent field isolates (Lee et al., 2012). There is some suggestion that vaccination against MD with homologous alphaherpesviruses like attenuated MDV-1 (serotype 1), non-oncogenic MDV-2 (serotype 2) and HVT (serotype 3) have driven field MDV-1 strains to increased virulence, probably as a result of intraspecific homologous recombination between vaccine strain and field isolates (Baigent et al., 2006).

Taken in tandem, the above reports show that recombination between vaccine and field isolates among avian alphaherpesviruses is not uncommon, thus there seems to be no scientific reasons why data on recombination is not provided in ERA dossier submitted for MAA. *In evaluating the risk of recombination, emphasis should not only be placed on the likelihood or frequency of occurrence but also on the impact or consequences of such occurrence. The likelihood of recombination and the emergence of a virulent strain may be rare but such a rare event can bring a fitness advantage that allows the recombinant virulent strain to outcompete the parental strains. This has been demonstrated in a devastating way with the emergence of two highly virulent field isolates of ILTV following a rare recombination event between ILTV vaccine strains* (Lee et al., 2012).

Except for some *in vitro* experiments (Hansen et al., 2004), not much is known about the potential of MVA-vectored vaccines to recombine with naturally occurring *Orthopoxviruses* (OPVs) during co-infection and superinfection. The potential of recombination between MVA-vectored vaccines and naturally occurring OPVs is considered negligible at the moment. Presently, the ERA of MVA-vectored vaccines does not evaluate the potential for recombination (Goossens et al., 2013). The non-multiplication of MVA in human and most mammalian cells (Wyatt et al., 1998, Okeke et al., 2006), as well as superinfection exclusion (Lin and Evans, 2010) are adduced as reasons why recombination between MVA-vectored vaccines and naturally occurring OPVs is highly unlikely. However recombination does not require productive virus infection, only the presence of homologous DNA templates. Since MVA DNA synthesis is not impaired, even in semi or non-permissive cells, then recombination with a naturally occurring OPV during co-infection and superinfection cannot be excluded.

To examine the potential of recombination in cells in which MVA poorly multiplies, as well as the stringency of superinfection exclusion, we (i) co-infected Vero cells with MVA-vectored influenza vaccine and a naturally isolated feline cowpox virus CPXV, (ii) infected Vero with MVA vectored influenza vaccine or CPXV and then superinfected with CPXV or MVA. We were able to isolate recombinant viruses from both co-infected and super-infected cells. *"Our results show that (i) MVA vectored vaccine can undergo recombination with naturally isolated CPXV in cells in which MVA multiplies poorly, (ii) superinfection exclusion is insufficient in preventing co-infection and subsequent recombination between MVA-vector vaccines and naturally occurring OPVs, and (iii) hybrid viruses with presumably complex genomes are produced as a result of recombination between poxvirus-vector vaccines and wild-type OPVs"* (Oludotun, 2014). These results are relevant to the ERA of MVA

vectored vaccine. If these results are reproduced in intact animal models, then the potential for recombination should no longer be considered negligible and must be evaluated experimentally.

The examples shown with poxvirus and avian alphaherpesvirus vectors can also be found in GMVs derived from other virus families. They demonstrate that contrary to the arbitrary assignment of negligible risk to the potential for recombination between GMV vaccines and field isolates, there is enough scientific evidence to suggest that the risk of recombination is not negligible or low. *In order to fill gaps in knowledge concerning recombination between GMV vaccines and field isolates or naturally occurring virus relatives, we recommend as follows: (i) mapping of naturally occurring viruses or field isolates in areas in which the GMV will be released in future (ii) co-infection and superinfection experiments in relevant cells as well as in immunocompetent and immune-compromised intact hosts, and (iii) post release genome-wide mapping of circulating virus strains in regions in which the GMVs have previously been released.*

2.2.5 Biodistribution, shedding and persistence of vaccine strain virus/vector

Vaccine strain virus/vector could spread at the site of administration; disperse from the site of administration to other cells, tissues and organs within the patient's body (biodistribution); or disseminate into the environment via urine, faeces, sweat, saliva, nasopharyngeal fluids, blood, breast milk, skin lesions, and semen of treated patient (shedding). Following shedding, and depending on its biological properties and the nature of the environment, the virus/vector can survive for some time outside the host (persistence). The evaluation of exposure pathways through which virus/vectors and/or their inserted gene products may interact with humans (other than patients receiving treatment with the virus/vector) or the environment is critical in ERA. In this section, the relevance of biodistribution, shedding and persistence in the ERA of GM vaccine strain viruses/vectors is briefly discussed. Areas in which ERA-relevant knowledge is lacking are highlighted together with common omissions usually encountered in the risk assessment of application dossiers.

2.2.5.1 Biodistribution

Any kind of administration other than through the alimentary canal can potentially result in a dispersion of the virus/vector/recombinant product through the entire body with a possible risk of germline transmission (and the attendant risk of transmission to offspring). Some viruses used in GM vaccine/vector engineering, e.g. MVA, are known to reach target tissues other than the site of administration (Ramirez et al., 2000). MVA is replication incompetent and is rapidly cleared from the tissues (Stittelaar et al., 2001), but the same cannot be said for replication (albeit conditional) competent viruses/vectors derived, for example, from Herpes simplex virus, Adenovirus, Lentivirus or Retrovirus.

In both replication competent virus/vectors (RCVV) and replication defective virus/vectors (RDVV), it is important to ascertain whether the genetic modification has altered cellular/tissue tropism compared to the wild-type virus as this will affect biodistribution. Altered cellular/tissue tropism will result in infection of cells which naturally are not permissible to infection by the original parent virus. *Data in the published literature on biodistribution of vaccine strain viruses/vectors in humans and animals are lacking. Thus, the practice, as observed in application dossiers, where only few targeted body fluids are examined to evaluate biodistribution is inadequate. It is recommended that a mapping*

of the dissemination of the recombinant vector in different body fluids of the treated patients during early phases of clinical studies and post marketing monitoring is conducted for each virus/vector construction. In addition, basic science studies should be designed to ascertain whether, and the degree to which, a GM modification has altered cellular and tissue receptors and other determinants of tropism in cells and tissues. This should be done on a case-by-case basis using the biological properties of the wild-type strain as a guide, and in consideration of the target population, such as their immune status.

Biodistribution may also influence the period/routes of shedding from the subject, and the likelihood of transmission to third parties (including vertical transmission and transmission to animals/cross species transfer) of the virus/vector and its recombinants. Further, biodistribution of the virus/vector in immune competent individuals is expected to be different from that of the immune-compromised. *Therefore, mapping the biodistribution in immune-compromised individual in each case of virus/vector will provide a broader worst-case scenario picture*.

2.2.5.2 Shedding

Different from dispersion of virus/vector to other parts of the body from the administered sites of treated patient, is the spread, externally, of the virus/vector via body fluids of the patients. This is one of the scenarios whereby personnel, non-patients and/or the environment may be exposed to the virus/vector. Factors that are usually considered when evaluating shedding of virus/vectors include biological properties of the virus/vector, animals host, immunocompetence of the host, dose and route of administration, sampling frequency and duration of sampling, sample collection, methods for sample analysis and data interpretation within the context of clinical and non-clinical (i.e. animal models) settings.

Information on the known biological properties of the wild-type strain from which the virus/vector under consideration was derived is used to guide design of shedding evaluations. For example, some viruses are spread through aerosols, and if the virus/vector product is shed through saliva or found in nasopharyngeal swabs, this could pose a higher likelihood for transmission as compared to a different route of shedding such as through urine.

Replication competence is an important property because RCVVs might persist in the patient for extended periods and can increase in amount leading to higher shedding of infectious particles with the attendant greater likelihood of transmission. For such RCVVs it is important to analyze molecular variants, which can also impact virus/vector shedding. Based on the available data on the current generation of clinical RCVVs, the basic assumptions in their risk assessments are that there is a likelihood of the spread of the viral vector from the treated patient into the environment (van den Akker et al., 2013). As is the case in biodistribution, immune status of the recipient can affect shedding; this hazard becomes higher when an RCVV is the case under evaluation. For example, in the case of Dryvax, which is a RCVV vaccine strain derived from vaccinia, it will be useful to derive information from use of vaccinia in vaccination of smallpox in the 80s. In the course of the vaccination, vaccinia related side effects were reported. However, vaccinia-related side effects in third parties are rare, but can result in serious consequences if they occur. Contact with vaccinia may result in serious complications in third parties that include progressive vaccinia, eczema vaccinatum or accidental infection of the eye (Neff et al., 2008).

Interpretation of shedding information derived from animal models (non-clinical) data may be useful in guiding design of clinical studies such as sample types, sampling frequency, and duration. However, they should not be substitutes for clinical studies. Even when shedding is absent during the non-clinical studies, it should not preclude assessing virus/vector shedding during clinical and post-market monitoring. *A general setback often experienced by evaluators of risk assessment dossiers is that information on shedding studies in clinical trials is rarely reported in the published literature or often have been limited to the first two weeks (Schenk-Braat et al., 2007).*

There is also an ongoing omission in the ERA of gene therapy medicinal products in lieu of shedding. For GTMP where the virus/vector is stably integrated into the host genome, it is often considered unlikely that the vector/drug product (DP) will be shed into the environment. However, there are scenarios in which shedding can occur. For example, in the case of the use of retroviral vectors for stable corrections of genetic defects (Barquinero et al., 2004), production of the DP is by ex vivo transduction of donor T-cells with the genetically modified retroviral vector supernatant. It is highly likely that some free viral particles are present in the DP at the end of the formulation process. Thus, free GM retroviral vector can potentially be co-administered, leading to horizontal spread and integration of viral particles in non-target tissues, and increasing shedding in body fluids. It cannot also be ruled out that replication competent retroviral (RCR) particles can be generated when integrating into the host non-target cell. Generation of RCR can also occur due to complementation between pro-viral and host sequences. Productive transmission of the virus to human non-target population may occur systemically, through mucosae contact, or exposure of broken skin to infectious aerosol. This will be more likely if the GTMP expresses epitopes that have receptors on a broad range of cells.

2.2.5.3 Persistence of virus/vector in the environment

Survivability (and duration thereof) of infectious particles outside the host after they have been shed is an important aspect of ERA. It gives an indication of the risk of transmission to susceptible non-target hosts in the environment –persistence of the viral vector could increase the likelihood of exposure. Some viruses are relatively stable and persist in the environment, whereas others are highly unstable. Persistence of the most commonly used viral vectors in GM vaccines and gene therapy has been discussed in (Baldo et al., 2013). Evaluation of modified virus/vector persistence should be done on case-by-case basis taking into consideration that the specific genetic modification may have caused a difference in survivability between the GM virus/vector and the wild type. Inserted sequences can influence the genome stability of the viral vector. This can result in an improperly packaged, enlarged and unstable genome which is prone to rearrangement. This can influence the persistence of the GM virus/vector such that its survivability outside the host differs from the wild-type parent. *Additionally, the duration of the insert genetic material in the environment before it is degraded should also be determined because such genetic materials could be available for transfer to related species in the wild*.

There are numerous factors that can affect persistence of viral particles and DNA in an environment (reviewed in England, (1998)). Persistence of viral particles may be affected by the presence or absence of proteolytic microorganisms, pH, temperature (freezing/thawing cycles), lipid and protein materials serving as protective shields to the particles. Persistence of nucleic acids may be affected by presence or absence of DNases, pH and DNA polymer length. *While the persistence in different materials outside the host of wild-type viruses currently used as vectors in vaccines and GTMP is*

available, there is paucity of information on persistence of GM viruses/vectors. This often results that applicants assume that persistence of the GM viruses/vectors is the same or similar to those of the wild-type strains, ignoring the fact that genetic manipulations may have affected some biological properties of the GM virus/vector that could result in differences in persistence outside the host. A ready example is as stated above where sequence insertion can influence genome stability and may lead to unexpected changes in the biological properties of the virus/vector.

2.2.5.4 Analytical assays for detection of virus/vector

Suitable and robust analytic methods are important in the evaluation of shedding and biodistribution. Assays should be sensitive, specific and reproducible. The current methods used for shedding evaluation are based on polymerase chain reaction (PCR) and cell-based infectivity assays. While the PCR-based methods are sensitive, reproducible and rapid, they are unable to differentiate between intact virus/vector and non-infectious or degraded virus/vector. The infectivity assays can detect infectious particles in shed samples, but they are inherently less sensitive. Thus, a combination of the two is often recommended depending on the case under evaluation.

A major hurdle in evaluating shed data is that available published literature does not provide information on the sensitivity of assay used, especially where non-quantitative assay approach is employed. This becomes more difficult when application dossiers lack full description of tests (cell-based infectivity assay, end point PCR, (q)RT-PCR) and validation assays used. Further, sensitivity is expressed in various ways affecting conclusions on level of shed particles (Schenk-Braat et al., 2007). Another general omission in the use of PCR-based assay is non-use of robust primer sets. Applicants generally design primers that are limited to the detection of only GM products but not potential recombinants and variants resulting from other types of mutations. Sometimes the detection assay is unable to discriminate between the GM virus/vector and wild-type. In such instances it becomes difficult to evaluate productive infection in target tissues, and spread in non-target tissues. Also, the shedding data becomes difficult to interpret because the culprit in a positive sample is unknown -it can either be the GM virus/vector or the wild-type virus or both. Samples that are positive for DNA should further be tested for infectivity. In some application dossiers, applicants limit their biodistribution and shedding evaluation to detection of nucleic acids. This does not provide useful information in respect of ERA; shed infectious particles are what are relevant because they can further be transmitted to susceptible hosts in the environment.

2.2.6 Transmission of shed virus/vector/gene therapy medicinal product (GTMP)

When shedding of infectious particles of virus/vector is observed, the potential for transmission to non-target host is usually investigated. In addition, transfer of inserts (in the case of transgenic modification using foreign genes) to wild-type strains circulating in the environment is evaluated. As stated in earlier sections, the environment in this case includes persons other than the individual receiving treatment. Therefore, wild-type virus strains resident in the individual should be considered important in ERA.

2.2.6.1 Transmission of GM vaccine strain virus/vector and Gene therapy virus-derived vector (GTV)

In transmission to non-target hosts, i.e. to a third party, persons considered at risk are those that come in close contact with the virus/vector recipient. These are usually family members, health care personnel, pets and domesticated animals. *In the case of clinical trial recipients, a wider scope of potential close contacts can be envisaged. This is because clinical trial recipients may not be sick and thus not stay long in the hospital. In addition, they may not be restricted as much as sick patients and may mingle with a larger part of the society. In this case co-workers and friends constitute potential close contacts. Applicants rarely consider this sub-population as those also potentially at risk.* The immunological status of the third party should be considered. A good proportion of the population might already have pre-existing immunity to the virus/vector; in this case clearance should be effective in those individuals. However, where the close contacts have compromised immunity, e.g. the elderly, very young, sick, and medicating individuals, clearance may be inefficient. Thus, the consequences of infection might be more significant in these individuals.

A significant knowledge gap in the ERA of GM vaccines and GTMP is the lack of baseline information on naturally occurring viruses. These include viruses naturally occurring in the non-target host and the environment external to the host. These viruses serve as reservoirs for gene pooling through recombination, which can affect the efficacy of the vaccine or DP. Through recombination and/or complementation, a hitherto attenuated virus/vector can revert to a wild-type virulent strain or to a recombinant with phenotype different from the vector/vaccine strain or the wild-type. GM vaccine strain/vector can, through recombination and/or complementation, provide genes/factors required for reactivation of inactive/dormant strains. This can lead to a situation of activation of latent infection or aggravation of an existing infection.

Interactions (exchange of genetic materials, helper functions of gene products/factors) between GM viruses/vectors and resident natural viruses could also lead to generation of species that are unstable and constantly mutate (quasi species) leading to, e.g., species with altered epitopes and thus less infectivity. This is often seen as an attenuation and hence less virulent –*this is true only ex-vivo. In-vivo however, altered epitope can lead to weak binding to receptors on the immune cells, resulting in less efficient immune clearance and establishment of chronic, latent or persistent infections. Additionally, this could lead to a situation where non-neutralizing antibodies facilitate an infection of other cells (antibody-dependent enhancement of infection is discussed in later section). The quasi species may develop the ability to integrate genetic materials into the host chromosome, or pass transgenic materials to circulating resident strains that have the ability to integrate into host genome. This increases the possibility of transmission of GM vaccine/vector into the germ cells and hence transmission to offspring (vertical transmission). Added to this is the fact that virus/vector can be transmitted to other people by organ or blood donation, due to virus/vector persistence as a result of poor clearance from the system, with adverse consequences for the recipient depending on the characteristics of the vector and its transgene. The probability of occurrence increases in the case of clinical trials, especially when a clinical trial includes healthy volunteers in clinical trial situations*.

2.2.6.2 Transmission of gene therapy virus-derived vectors (GTVs)

As in the GTMP product (Barquinero et al., 2004), stated in Section 2.5.2, horizontal transmission of the GTVs from patient to non-target individuals may occur through accidental contact, especially if this involves the mucosae, known to be the primary and privileged tissue to be infected. In this light, patient administration is the most critical phase and hospital personnel involved are the most

exposed subjects. In a retroviral-derived GTV for example, the transgenic product or viral vector is integrated as a provirus in the genome of the human host cells; release into the environment of retroviral particles via patient urine or feces requires either the generation of new retroviral particles by recombination or complementation.

Mobilization of retroviral particles and associated unintended transfer to non-target tissues of individuals may occur in the case of generation of replication competent retroviruses (RCRs) or through shedding of residual retroviral vector particles associated with the drug product at the end of manufacturing. Genetic transfer from the integrated provirus may also occur through sequence complementarity between the GTV integrated sequence and human endogenous retroviruses (HERVs). Genetic material may also be exchanged because of interaction of viral sequences present in the construct e.g. retroviral sequences and promoter sequences, with other wild-type viruses present in the T-cells and the patient, which may go undetected in shed samples and can easily be transmitted to individuals other than the treated patient.

2.2.7 Immune Response

In Section 2, the importance of the immune status of at-risk non-target hosts was discussed in relation to biodistribution, shedding and transmission of virus/vectors. In this section, other aspects of the non-target host's immunity relevant to ERA and in which there are ongoing omissions/knowledge gaps are discussed. The ERA is not concerned with the immune response of target/treated patient or healthy volunteer (as in the case of clinical trial), but with the immune response of the non-target individuals in the vicinity of the patient.

Non-target individuals exposed to a viral vector in which immune evasion genes have been deleted or modified might result in more effective clearance of the viral vector during infection; however, it can also lead to an increased acute response. Examples of this phenomenon, in animal models, are available with the E3-deleted adenovirus (Sparer et al., 1996) or the interleukin (Il) 18-deleted poxviruses (Born et al., 2000). On the other hand, pathogenesis in the host may be affected in the case of a transgenic vector encoding novel immune modulatory functions, e.g. where GM viruses expressing Il-4 were more pathogenic in an animal experiment than the wild-type virus. In this case, the modification was found to inhibit appropriate immune response for the effective clearance of viral infection (Sparer et al., 1996, Born et al., 2000). *The potential effects of increased proliferation, in the case of an RCVV, of an immune evading vector in a non-target individual who may be (or become in the future) immune compromised is of importance in ERA.*

Further, the immune system of a good proportion of the population is primed against the current major vectors used in GM vaccines and gene therapy (for a review of the major gene therapy viral vectors see (Thomas et al., 2003)). For example, antibodies against adenovirus and vaccinia virus are prevalent in the population. For the patient, this can result in reduced effectiveness of the GM vaccine/GTMP; the ERA is however concerned with the fate/effect in non-target host whose immune system may have been primed against the vector. In such an individual, aggravated immune response, such as acute inflammation, can occur. *Therefore, as part of determining the immune status of target hosts/at-risk non-target individuals, mapping of prevailing immunity in the population which might react against the GM virus/vector should be considered*.

Similarly, and as pointed out in Section 2.6.1, antibody-dependent enhancement (ADE) of infection is an important immune response to be considered in non-target hosts in lieu of GM vaccines/GTMP. Altered antigenic epitopes as a result of genetic modification can lead to generation of non-neutralizing antibody against GM virus/vector in people to which the virus/vector may inadvertently be transmitted. In ADE immunopathology, the antibody is not only unable to neutralize the virus/vector which is bound at its antigen binding site, it helps to spread the virus/vector to cells which normally do not express receptors for the virus/vector but express receptors for the antibody (Fc receptors expressed on cell plasma membranes). *Although this phenomenon is rare in vivo except for dengue virus, it cannot be ruled out completely in GM vaccines/GTMP given that modification can alter antigenic epitopes.*

Immune modulation, comprising of immunostimulation (e.g. in the use of adjuvants) or immunosuppression are two manipulations of the immune system often applied in GM therapeutic vaccines to either suppress the acquired immune response or reduce the immunogenicity of vectors. Both strategies are two sides of the same coin, though immunosuppression may gain the upper hand after multiple vaccinations and thus lead to a detrimental outcome. Studies have shown that the utility of immunostimulants can lead to unexpected results (Faries et al., 2009, Slingluff et al., 2009). For example, multiple vaccinations have been shown to induce as much immunosuppression by regulatory T-cells as immunostimulation (LaCelle et al., 2009). In the case of immune-adjuvants, the use of granulocyte macrophage colony-stimulating factor (GM-CSF) as an immune-adjuvant in an allogeneic cell-based vaccine led to over stimulation of patients' immune systems (Faries et al., 2009). Genetic changes in the immune modulatory properties of a virus may influence environmental risks - for example by directly influencing viral replication, pathogenesis, persistence and shedding as discussed in Section 2.5. *Therefore, immune monitoring endpoints in the assessment of GM therapeutic and cancer vaccines must be clarified to reflect the true nature of the immune response of the host and potential non-target hosts. This is a major challenge because in vivo effects are hard to predict (Aerts, 2010), and surrogate endpoints used in preclinical analyses often do not reflect true in vivo immune response in clinical and post clinical evaluations.*

2.2.8 Unintended effects of GM modification/transgene on virus/vector and/or host

The first step in ERA is hazard characterization of the GM vaccine or vector. This implies characterization of the effects (hazards) of the transgene product, hazards due to the virus/vector, hazards due to changes (both quantitative and qualitative) in the virus/vector that are induced by the genetic modifications, and the overall effect (both of the insert and vector) on individuals and the ecosystem in the vicinity of the target recipient. *To date, the approach to hazard characterization related to transgene in transgenic GM vaccines and vectors has been reductionist in that potential hazards are narrowed only to the inserted gene sequences and transgene product.* For a review on potential hazardous substances used as transgenes in viral vector-based gene therapy see (Bergmans et al., 2008). *The changes that the inserts can induce in virus/vectors, which can themselves be hazardous, are often ignored. While the expressed gene product may have intrinsic hazardous properties, (e.g. toxic or allergenic properties), its actual hazard in gene therapy trials depends very much on the genetic and physiological context of the parental virus in which it is introduced and of the conditions of use.*

If therapeutic transgenes are present in the vector, the effects of these insertions on the host should be evaluated in the context of the viral vector. First of all, the level of transgene expression and therefore the magnitude of the effect will depend on the endogenous viral or ectopic promoter preceding the transgenic insert in the vector. Second, in the case of effects of vector on immune modulation, an attempt should be made to describe the overall effects of the entire vector (not just the transgene) on the immune system taking into account both the effects of the transgene and the viral genes involved in immune modulation. The hazards associated with the transgene will be different in the context of non-replicating virus vectors versus replication competent vectors. For a review on effect of inserted sequences in the context of replication competent viral vectors see (van den Akker et al., 2013).

Risk assessors must thus rely only on previously known characteristics of transgene materials to characterize the potential hazards of the inserts. However, and as stated above, in order to fully characterize these hazards, the effect of the transgenes must be evaluated within its new genetic and physiological context in the vector or virus. A way to achieve this is to determine the effect on the physiology of the host virus as well as host response to the transgenic viral vector using global approaches such as proteomics and metabolomics. This approach has the advantage of identifying unintended hazardous effects of the transgene. This approach has been used to successfully characterize complex events, for example, cancer and related research (Shajahan-Haq et al., 2015), (Uhlen et al., 2015), (Zamanian-Azodi et al., 2015). *Unfortunately, such global information on viral transgenic effect related to the use of virus/vector in gene therapy and GM vaccines is lacking in the published literature. The result is a less than comprehensive characterization of transgenic virus vector during ERA of GM vaccines and gene therapy in which viruses and virus-derived vectors are used.*

2.3 Conclusion

In this chapter, we have highlighted knowledge gaps, omitted research and areas of uncertainty with respect to the ERA of GMVs. We have also made some recommendations. The ERA of GMVs is only as good as the regulatory framework governing it. Scientifically valid questions will not be addressed in so far as the provisions in the regulation do not explicitly demand it. One way to influence regulation is to provide scientific data on some of the risk issues discussed in this chapter.

2.4 Directions for further studies

- Changes that the inserts induce in virus/vectors during genetic modifications
- Changes in host(s) response(s) to genetic modifications in viruses
- Mapping of the dissemination of the recombinant vector in different body fluids of the treated patients during early phases of clinical studies and post marketing monitoring for each virus/vector constructs.

- Basic science studies to ascertain whether, and the degree to which, a GM modification has altered cellular and tissue receptors (and other determinants of tropism in cells and tissues) to GM viruses and constructs.
- Mapping the biodistribution in immune-compromised individual in each case of virus/vector.
- Duration in the environment of common and frequently used insert genetic materials.

References

AERTS, K. F., KAPTEIN, L., WAGEMAKER, G. 2010. Immune modulation in gene therapy studies -points to consider for Environmental Risk Assessment. Erasmus MC. *Available at: http://re-pub.eur.nl/res/pub/21967/. Access date 15.03.2015*.

ALTENBURG, A. F., KREIJTZ, J. H., DE VRIES, R. D., SONG, F., FUX, R., RIMMELZWAAN, G. F., SUTTER, G. & VOLZ, A. 2014. Modified vaccinia virus ankara (MVA) as production platform for vaccines against influenza and other viral respiratory diseases. *Viruses, 6, 2735-61.*

APPAIAHGARI, M. B. & VRATI, S. 2010. IMOJEV((R)): a Yellow fever virus-based novel Japanese encephalitis vaccine. *Expert Rev Vaccines, 9, 1371-84.*

BACHOFEN, C., GRANT, D. M., WILLOUGHBY, K., ZADOKS, R. N., DAGLEISH, M. P. & RUSSELL, G. C. 2014. Experimental infection of rabbits with bovine viral diarrhoea virus by a natural route of exposure. *Vet Res, 45, 34.*

BAIGENT, S. J., SMITH, L. P., NAIR, V. K. & CURRIE, R. J. 2006. Vaccinal control of Marek's disease: current challenges, and future strategies to maximize protection. *Vet Immunol Immunopathol, 112, 78-86.*

BALDO, A., VAN DEN AKKER, E., BERGMANS, H. E., LIM, F. & PAUWELS, K. 2013. General considerations on the biosafety of virus-derived vectors used in gene therapy and vaccination. *Curr Gene Ther, 13, 385-94.*

BARQUINERO, J., EIXARCH, H. & PEREZ-MELGOSA, M. 2004. Retroviral vectors: new applications for an old tool. *Gene Ther, 11 Suppl 1, S3-9.*

BECHER, P. & TAUTZ, N. 2011. RNA recombination in pestiviruses: cellular RNA sequences in viral genomes highlight the role of host factors for viral persistence and lethal disease. *RNA Biol, 8, 216-24.*

BERGMANS, H., LOGIE, C., VAN MAANEN, K., HERMSEN, H., MEREDYTH, M. & VAN DER VLUGT, C. 2008. Identification of potentially hazardous human gene products in GMO risk assessment. *Environ Biosafety Res, 7, 1-9.*

BORN, T. L., MORRISON, L. A., ESTEBAN, D. J., VANDENBOS, T., THEBEAU, L. G., CHEN, N., SPRIGGS, M. K., SIMS, J. E. & BULLER, R. M. 2000. A poxvirus protein that binds to and inactivates IL-18, and inhibits NK cell response. *J Immunol, 164, 3246-54.*

CAPASSO, C., GAROFALO, M., HIRVINEN, M. & CERULLO, V. 2014. The evolution of adenoviral vectors through genetic and chemical surface modifications. *Viruses, 6, 832-55.*

CASEY, C. G., ISKANDER, J. K., ROPER, M. H., MAST, E. E., WEN, X. J., TOROK, T. J., CHAPMAN, L. E., SWERDLOW, D. L., MORGAN, J., HEFFELFINGER, J. D., VITEK, C., REEF, S. E., HASBROUCK, L. M., DAMON, I., NEFF, L., VELLOZZI, C., MCCAULEY, M., STRIKAS, R. A. & MOOTREY, G. 2005. *Adverse events associated with smallpox vaccination in the United States, January-October 2003. JAMA, 294, 2734-43.*

COTTINGHAM, M. G. & CARROLL, M. W. 2013. Recombinant MVA vaccines: dispelling the myths. *Vaccine, 31, 4247-51.*

DEICHMANN, A. & SCHMIDT, M. 2013. Biosafety considerations using gamma-retroviral vectors in gene therapy. *Curr Gene Ther, 13, 469-77.*

DONSANTE, A., VOGLER, C., MUZYCZKA, N., CRAWFORD, J. M., BARKER, J., FLOTTE, T., CAMPBELL-THOMPSON, M., DALY, T. & SANDS, M. S. 2001. Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. *Gene Ther, 8, 1343-6.*

DRAPER, S. J. & HEENEY, J. L. 2010. Viruses as vaccine vectors for infectious diseases and cancer. *Nat Rev Microbiol, 8, 62-73.*

DUBOVI, E. J. 2013. Laboratory diagnosis of bovine viral diarrhea virus. *Biologicals, 41, 8-13.*

ENGLAND, L. S., HOLMES, S.B., TREVORS, J.T. 1998. Review: Persistence of viruses and DNA in soil. *World J Microbiol & Biotech, 14, 163-169.*

ESAKI, M., NOLAND, L., EDDINS, T., GODOY, A., SAEKI, S., SAITOH, S., YASUDA, A. & DORSEY, K. M. 2013. Safety and

efficacy of a turkey herpesvirus vector laryngotracheitis vaccine for chickens. *Avian Dis, 57, 192-8.*

FARIES, M. B., HSUEH, E. C., YE, X., HOBAN, M. & MORTON, D. L. 2009. Effect of granulocyte/macrophage colony-stimulating factor on vaccination with an allogeneic whole-cell melanoma vaccine. *Clin Cancer Res, 15, 7029-35.*

GIMENO, I. M., CORTES, A. L., GUY, J. S., TURPIN, E. & WILLIAMS, C. 2011. Replication of recombinant herpesvirus of turkey expressing genes of infectious laryngotracheitis virus in specific pathogen free and broiler chickens following in ovo and subcutaneous vaccination. *Avian Pathol, 40, 395-403.*

GINN, S. L., ALEXANDER, I. E., EDELSTEIN, M. L., ABEDI, M. R. & WIXON, J. 2013. Gene therapy clinical trials worldwide to 2012 - an update. *J Gene Med, 15, 65-77.*

GOOSSENS, M., PAUWELS, K., WILLEMARCK, N. & BREYER, D. 2013. Environmental risk assessment of clinical trials involving modified vaccinia virus Ankara (MVA)-based vectors. *Curr Gene Ther, 13, 413-20.*

GRADA, A. & WEINBRECHT, K. 2013. Next-generation sequencing: methodology and application. *J Invest Dermatol, 133, e11.*

GRAY, G., BUCHBINDER, S. & DUERR, A. 2010. Overview of STEP and Phambili trial results: two phase IIb test-of-concept studies investigating the efficacy of MRK adenovirus type 5 gag/pol/nef subtype B HIV vaccine. *Curr Opin HIV AIDS, 5, 357-61.*

GUBSER, C., HUE, S., KELLAM, P. & SMITH, G. L. 2004. Poxvirus genomes: a phylogenetic analysis. *J Gen Virol, 85, 105-17.*

HANSEN, H., OKEKE, M. I., NILSSEN, O. & TRAAVIK, T. 2004. Recombinant viruses obtained from co-infection in vitro with a live vaccinia-vectored influenza vaccine and a naturally occurring cowpox virus display different plaque phenotypes and loss of the transgene. *Vaccine, 23, 499-506*

IZZI, V., BULER, M., MASUELLI, L., GIGANTI, M. G., MODESTI, A. & BEI, R. 2014. Poxvirus-based vaccines for cancer immunotherapy: new insights from combined cytokines/co-stimulatory molecules delivery and "uncommon" strains. *Anticancer Agents Med Chem, 14, 183-9.*

JACKSON, D. A., SYMONS, R. H. & BERG, P. 1972. Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of Escherichia coli. *Proc Natl Acad Sci U S A, 69, 2904-9.*

JACKSON, R. J., MAGUIRE, D. J., HINDS, L. A. & RAMSHAW, I. A. 1998. Infertility in mice induced by a recombinant ectromelia virus expressing mouse zona pellucida glycoprotein 3. *Biol Reprod, 58, 152-9.*

JAROSINSKI, K. W. 2012. Dual infection and superinfection inhibition of epithelial skin cells by two alphaherpesviruses co-occur in the natural host. *PLoS One, 7, e37428.*

KAMEYAMA, K., SAKODA, Y., TAMAI, K., NAGAI, M., AKASHI, H. & KIDA, H. 2006. Genetic recombination at different points in the Npro-coding region of bovine viral diarrhea viruses and the potentials to change their antigenicities and pathogenicities. *Virus Res, 116, 78-84.*

KAY, M. A. 2011. State-of-the-art gene-based therapies: the road ahead. *Nat Rev Genet, 12, 316-28.*

KIRCHER, M. & KELSO, J. 2010. High-throughput DNA sequencing--concepts and limitations. *Bioessays, 32, 524-36.*

KONIG, P., BLOME, S., GABRIEL, C., REIMANN, I. & BEER, M. 2011. Innocuousness and safety of classical swine fever marker vaccine candidate CP7_E2alf in non-target and target species. *Vaccine, 30, 5-8.*

KOONIN, E. V., SENKEVICH, T. G. & DOLJA, V. V. 2006. The ancient Virus World and evolution of cells. *Biol Direct, 1, 29.*

KOTIN, R. M. 2011. Large-scale recombinant adeno-associated virus production. *Hum Mol Genet, 20, R2-6.*

LACELLE, M. G., JENSEN, S. M. & FOX, B. A. 2009. Partial CD4 depletion reduces regulatory T cells induced by multiple vaccinations and restores therapeutic efficacy. *Clin Cancer Res, 15, 6881-90.*

LEE, S. W., MARKHAM, P. F., COPPO, M. J., LEGIONE, A. R., MARKHAM, J. F., NOORMOHAMMADI, A. H., BROWNING,

G. F., FICORILLI, N., HARTLEY, C. A. & DEVLIN, J. M. 2012. Attenuated vaccines can recombine to form virulent field viruses. *Science, 337, 188.*

LICHTY, B. D., BREITBACH, C. J., STOJDL, D. F. & BELL, J. C. 2014. Going viral with cancer immunotherapy. *Nat Rev Cancer, 14, 559-67.*

LIN, Y. C. & EVANS, D. H. 2010. Vaccinia virus particles mix inefficiently, and in a way that would restrict viral recombination, in coinfected cells. *J Virol, 84, 2432-43.*

LINIGER, M., ZUNIGA, A. & NAIM, H. Y. 2007. Use of viral vectors for the development of vaccines. *Expert Rev Vaccines, 6, 255-66.*

LIU, Q. & MURUVE, D. A. 2003. Molecular basis of the inflammatory response to adenovirus vectors. *Gene Ther, 10, 935-40.*

MACKETT, M., SMITH, G. L. & MOSS, B. 1982. Vaccinia virus: a selectable eukaryotic cloning and expression vector. *Proc Natl Acad Sci U S A, 79, 7415-9.*

MAETZIG, T., GALLA, M., BAUM, C. & SCHAMBACH, A. 2011. Gammaretroviral vectors: biology, technology and application. *Viruses, 3, 677-713.*

MCKELVEY, T. A., ANDREWS, S. C., MILLER, S. E., RAY, C. A. & PICKUP, D. J. 2002. Identification of the orthopoxvirus p4c gene, which encodes a structural protein that directs intracellular mature virus particles into A-type inclusions. *J Virol, 76, 11216-25.*

MEISINGER-HENSCHEL, C., SPATH, M., LUKASSEN, S., WOLFERSTATTER, M., KACHELRIESS, H., BAUR, K., DIRMEIER, U., WAGNER, M., CHAPLIN, P., SUTER, M. & HAUSMANN, J. 2010. Introduction of the six major genomic deletions of modified vaccinia virus Ankara (MVA) into the parental vaccinia virus is not sufficient to reproduce an MVA-like phenotype in cell culture and in mice. *J Virol, 84, 9907-19.*

MEMPEL, M., ISA, G., KLUGBAUER, N., MEYER, H., WILDI, G., RING, J., HOFMANN, F. & HOFMANN, H. 2003. Laboratory acquired infection with recombinant vaccinia virus containing an immunomodulating construct. *J Invest Dermatol, 120, 356-8.*

MOSS, B. 1996. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proc Natl Acad Sci U S A, 93, 11341-8.*

NEFF, J., MODLIN, J., BIRKHEAD, G. S., POLAND, G., ROBERTSON, R. M., SEPKOWITZ, K., YANCY, C., GARDNER, P., GRAY, G. C., MAURER, T., SIEGEL, J., GUERRA, F. A., BERGER, T., FLANDERS, W. D., SHOPE, R., ADVISORY COMMITTEE ON IMMUNIZATION, P. & ARMED FORCES EPIDEMIOLOGICAL, B. 2008. Monitoring the safety of a smallpox vaccination program in the United States: report of the joint Smallpox Vaccine Safety Working Group of the advisory committee on immunization practices and the Armed Forces Epidemiological Board. *Clin Infect Dis, 46 Suppl 3, S258-70.*

NIETO, K. & SALVETTI, A. 2014. AAV Vectors Vaccines Against Infectious Diseases. *Front Immunol, 5, 5.*

OKEKE, M. I., HANSEN, H. & TRAAVIK, T. 2012. A naturally occurring cowpox virus with an ectromelia virus A-type inclusion protein gene displays atypical A-type inclusions. *Infect Genet Evol, 12, 160-8.*

OKEKE, M. I., NILSSEN, O., MOENS, U., TRYLAND, M. & TRAAVIK, T. 2009. In vitro host range, multiplication and virion forms of recombinant viruses obtained from co-infection in vitro with a vaccinia-vectored influenza vaccine and a naturally occurring cowpox virus isolate. *Virol J, 6, 55.*

OKEKE, M. I., NILSSEN, O. & TRAAVIK, T. 2006. Modified vaccinia virus Ankara multiplies in rat IEC-6 cells and limited production of mature virions occurs in other mammalian cell lines. *J Gen Virol, 87, 21-7.*

OKEKE, M. I., OKOLI, A. S., NILSSEN, O., MOENS, U., TRYLAND, M., BOHN, T. & TRAAVIK, T. 2014. Molecular characterization and phylogenetics of Fennoscandian cowpox virus isolates based on the p4c and atip genes. *Virol J, 11, 119.*

OLUDOTUN, T. G. 2014. Isolation and characterization of recombinant viruses obtained from co-infection and super-

infection in vitro with a vaccinia virus vectored influenza vaccine and a naturally occuring feline cowpox virus isolate. *Masters, IMC University of Applied Sciences Krems, Austria.*

PANICALI, D. & PAOLETTI, E. 1982. Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. *Proc Natl Acad Sci U S A, 79, 4927-31.*

PARKS, C. L., PICKER, L. J. & KING, C. R. 2013. Development of replication-competent viral vectors for HIV vaccine delivery. *Curr Opin HIV AIDS, 8, 402-11.*

PAUWELS, K., GIJSBERS, R., TOELEN, J., SCHAMBACH, A., WILLARD-GALLO, K., VERHEUST, C., DEBYSER, Z. & HERMAN, P. 2009. State-of-the-art lentiviral vectors for research use: risk assessment and biosafety recommendations. *Curr Gene Ther, 9, 459-74.*

PLOTKIN, S. A. 2009. Vaccines: the fourth century. *Clin Vaccine Immunol, 16, 1709-19.*

QIN, L., UPTON, C., HAZES, B. & EVANS, D. H. 2011. Genomic analysis of the vaccinia virus strain variants found in Dryvax vaccine. *J Virol, 85, 13049-60.*

RAMIREZ, J. C., GHERARDI, M. M. & ESTEBAN, M. 2000. Biology of attenuated modified vaccinia virus Ankara recombinant vector in mice: virus fate and activation of B- and T-cell immune responses in comparison with the Western Reserve strain and advantages as a vaccine. *J Virol, 74, 923-33.*

RAPER, S. E., CHIRMULE, N., LEE, F. S., WIVEL, N. A., BAGG, A., GAO, G. P., WILSON, J. M. & BATSHAW, M. L. 2003. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab, 80, 148-58.*

REIMANN, I., DEPNER, K., TRAPP, S. & BEER, M. 2004. An avirulent chimeric Pestivirus with altered cell tropism protects pigs against lethal infection with classical swine fever virus. *Virology, 322, 143-57.*

REIMANN, I., DEPNER, K., UTKE, K., LEIFER, I., LANGE, E. & BEER, M. 2010. Characterization of a new chimeric marker vaccine candidate with a mutated antigenic E2-epitope. *Vet Microbiol, 142, 45-50.*

ROLLIER, C. S., REYES-SANDOVAL, A., COTTINGHAM, M. G., EWER, K. & HILL, A. V. 2011. Viral vectors as vaccine platforms: deployment in sight. *Curr Opin Immunol, 23, 377-82.*

RUPPRECHT, C. E., WIKTOR, T. J., JOHNSTON, D. H., HAMIR, A. N., DIETZSCHOLD, B., WUNNER, W. H., GLICKMAN, L. T. & KOPROWSKI, H. 1986. Oral immunization and protection of raccoons (Procyon lotor) with a vaccinia-rabies glycoprotein recombinant virus vaccine. *Proc Natl Acad Sci U S A, 83, 7947-50.*

SAINOVA, I. V., KRIL, A. I., SIMEONOV, K. B., POPOVA, T. P. & IVANOV, I. G. 2005. Investigation of the morphology of cell clones, derived from the mammalian EBTr cell line and their susceptibility to vaccine avian poxvirus strains FK and Dessau. *J Virol Methods, 124, 37-40.*

SCHAFFER, D. V., KOERBER, J. T. & LIM, K. I. 2008. Molecular engineering of viral gene delivery vehicles. *Annu Rev Biomed Eng, 10, 169-94.*

SCHENK-BRAAT, E. A., VAN MIERLO, M. M., WAGEMAKER, G., BANGMA, C. H. & KAPTEIN, L. C. 2007. An inventory of shedding data from clinical gene therapy trials. *J Gene Med, 9, 910-21.*

SEGURA, M. M., MANGION, M., GAILLET, B. & GARNIER, A. 2013. New developments in lentiviral vector design, production and purification. *Expert Opin Biol Ther, 13, 987-1011.*

SHAJAHAN-HAQ, A. N., CHEEMA, M. S. & CLARKE, R. 2015. Application of metabolomics in drug resistant breast cancer research. *Metabolites, 5, 100-18.*

SLINGLUFF, C. L., JR., PETRONI, G. R., OLSON, W. C., SMOLKIN, M. E., ROSS, M. I., HAAS, N. B., GROSH, W. W., BOISVERT, M. E., KIRKWOOD, J. M. & CHIANESE-BULLOCK, K. A. 2009. Effect of granulocyte/macrophage colony-stimulating factor on circulating CD8+ and CD4+ T-cell responses to a multipeptide melanoma vaccine: outcome of a multicenter randomized trial. *Clin Cancer Res, 15, 7036-44.*

SMALL, J. C. & ERTL, H. C. 2011. Viruses - from pathogens to vaccine carriers. Curr Opin Virol, 1, 241-5.

SPARER, T. E., TRIPP, R. A., DILLEHAY, D. L., HERMISTON, T. W., WOLD, W. S. & GOODING, L. R. 1996. The role of

human adenovirus early region 3 proteins (gp19K, 10.4K, 14.5K, and 14.7K) in a murine pneumonia model. *J Virol, 70, 2431-9.*

STANLEY, D. A., HONKO, A. N., ASIEDU, C., TREFRY, J. C., LAU-KILBY, A. W., JOHNSON, J. C., HENSLEY, L., AMMENDOLA, V., ABBATE, A., GRAZIOLI, F., FOULDS, K. E., CHENG, C., WANG, L., DONALDSON, M. M., COLLOCA, S., FOLGORI, A., ROEDERER, M., NABEL, G. J., MASCOLA, J., NICOSIA, A., CORTESE, R., KOUP, R. A. & SULLIVAN, N. J. 2014. Chimpanzee adenovirus vaccine generates acute and durable protective immunity against ebolavirus challenge. *Nat Med, 20, 1126-9.*

STITTELAAR, K. J., KUIKEN, T., DE SWART, R. L., VAN AMERONGEN, G., VOS, H. W., NIESTERS, H. G., VAN SCHALKWIJK, P., VAN DER KWAST, T., WYATT, L. S., MOSS, B. & OSTERHAUS, A. D. 2001. Safety of modified vaccinia virus Ankara (MVA) in immune-suppressed macaques. *Vaccine, 19, 3700-9.*

SUTER, M., MEISINGER-HENSCHEL, C., TZATZARIS, M., HULSEMANN, V., LUKASSEN, S., WULFF, N. H., HAUSMANN, J., HOWLEY, P. & CHAPLIN, P. 2009. Modified vaccinia Ankara strains with identical coding sequences actually represent complex mixtures of viruses that determine the biological properties of each strain. *Vaccine, 27, 7442-50.*

TAYLOR, J., MEIGNIER, B., TARTAGLIA, J., LANGUET, B., VANDERHOEVEN, J., FRANCHINI, G., TRIMARCHI, C. & PAOLETTI, E. 1995. Biological and immunogenic properties of a canarypox-rabies recombinant, ALVAC-RG (vCP65) in non-avian species. *Vaccine, 13, 539-49.*

THIEL, T., WHITEMAN, N. K., TIRAPE, A., BAQUERO, M. I., CEDENO, V., WALSH, T., UZCATEGUI, G. J. & PARKER, P. G. 2005. Characterization of canarypox-like viruses infecting endemic birds in the Galapagos Islands. *J Wildl Dis, 41, 342-53.*

THIRY, E., MEURENS, F., MUYLKENS, B., MCVOY, M., GOGEV, S., THIRY, J., VANDERPLASSCHEN, A., EPSTEIN, A., KEIL, G. & SCHYNTS, F. 2005. Recombination in alphaherpesviruses. *Rev Med Virol, 15, 89-103.*

THOMAS, C. E., EHRHARDT, A. & KAY, M. A. 2003. Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet, 4, 346-58.*

THOMAS, G. P. & MATHEWS, M. B. 1980. DNA replication and the early to late transition in adenovirus infection. *Cell, 22, 523-33.*

UHLEN, M., FAGERBERG, L., HALLSTROM, B. M., LINDSKOG, C., OKSVOLD, P., MARDINOGLU, A., SIVERTSSON, A., KAMPF, C., SJOSTEDT, E., ASPLUND, A., OLSSON, I., EDLUND, K., LUNDBERG, E., NAVANI, S., SZIGYARTO, C. A., ODEBERG, J., DJUREINOVIC, D., TAKANEN, J. O., HOBER, S., ALM, T., EDQVIST, P. H., BERLING, H., TEGEL, H., MULDER, J., ROCKBERG, J., NILSSON, P., SCHWENK, J. M., HAMSTEN, M., VON FEILITZEN, K., FORSBERG, M., PERSSON, L., JOHANSSON, F., ZWAHLEN, M., VON HEIJNE, G., NIELSEN, J. & PONTEN, F. 2015. Proteomics. Tissue-based map of the human proteome. *Science, 347, 1260419.*

VAN DEN AKKER, E., VAN DER VLUGT, C. J., BLEIJS, D. A. & BERGMANS, H. E. 2013. Environmental risk assessment of replication competent viral vectors applied in clinical trials: potential effects of inserted sequences. *Curr Gene Ther, 13, 395-412.*

VAN GENNIP, H. G., VAN RIJN, P. A., WIDJOJOATMODJO, M. N., DE SMIT, A. J. & MOORMANN, R. J. 2000. Chimeric classical swine fever viruses containing envelope protein E(RNS) or E2 of bovine viral diarrhoea virus protect pigs against challenge with CSFV and induce a distinguishable antibody response. *Vaccine, 19, 447-59.*

WEBER, M. N., STRECK, A. F., SILVEIRA, S., MOSENA, A. C., SILVA, M. S. & CANAL, C. W. 2015. Homologous recombination in pestiviruses: identification of three putative novel events between different subtypes/genogroups. *Infect Genet Evol, 30, 219-24.*

WELI, S. C., NILSSEN, O. & TRAAVIK, T. 2005. Avipoxvirus multiplication in a mammalian cell line. *Virus Res, 109, 39-49.*

WYATT, L. S., CARROLL, M. W., CZERNY, C. P., MERCHLINSKY, M., SISLER, J. R. & MOSS, B. 1998. Marker rescue of the host range restriction defects of modified vaccinia virus Ankara. *Virology, 251, 334-42.*

WYATT, L. S., EARL, P. L., XIAO, W., AMERICO, J. L., COTTER, C. A., VOGT, J. & MOSS, B. 2009. Elucidating and

minimizing the loss by recombinant vaccinia virus of human immunodeficiency virus gene expression resulting from spontaneous mutations and positive selection. *J Virol, 83, 7176-84.*

YLA-HERTTUALA, S. 2012. Endgame: glybera finally recommended for approval as the first gene therapy drug in the European union. *Mol Ther, 20, 1831-2.*

ZAMANIAN-AZODI, M., REZAEI-TAVIRANI, M., HASANZADEH, H., RAHMATI RAD, S. & DALILAN, S. 2015. Introducing biomarker panel in esophageal, gastric, and colon cancers; a proteomic approach. *Gastroenterol Hepatol Bed Bench, 8, 6-18.*

ZEMKE, J., KONIG, P., MISCHKALE, K., REIMANN, I. & BEER, M. 2010. Novel BVDV-2 mutants as new candidates for modified-live vaccines. *Vet Microbiol, 142, 69-80.*

3. GM microorganisms

3.1 Introduction

Directive 2009/41/EC of the European Parliament defines genetically modified microorganisms as: *a micro-organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination* by means of the following techniques:

- *Recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation.*
- *Techniques involving the direct introduction into a microorganism of heritable material prepared outside the microorganism, including micro-injection, macro-injection and micro-encapsulation.*
- *Cell fusion or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally (THE COUNCIL OF THE EUROPEAN UNION 2009).*

For the purposes of this section of the report, the focus will be on genetically modified bacteria, archaea, fungi (filamentous fungi and yeasts), microalgae (hereafter algae), as well as synthetic biology products, which are considered as GMMs by the European Commission (see box below).

The microorganisms being considered under this section of the report have been grouped together under the umbrella of "GMMs"; however, small size is one of the few common factors of these extremely diverse organisms. While general concerns regarding knowledge gaps relating to the effects of GMMs on the environment will be discussed, there may be additional concerns for individual transgenic microbes.

3.1.1 Uses of GM microbes

Genetically modified (also known as recombinant) microorganisms have been utilised as production systems for proteins, vaccines and enzymes for a number of years. Selection criteria for microbial expression systems are based on the size of the protein to be produced, as well as requirement for glycolysation and post-translational modifications. Bacterial (especially *E.coli*) systems tend to be used for proteins smaller than 30 kD which do not require additional modification, while yeasts or other eukaryotic systems are selected for larger, more complex proteins (Demain, Vaishnav 2009, Çelik, Çalık 2012). Recently, algal expression systems have also been developed for such pharmaceutical applications (Gressel, van der Vlugt, Cécile JB & Bergmans 2013).

The success of contained use of GMMs in pharmaceutical production has not extended to environmental and agricultural applications to the same degree. While GM bacteria have been developed as inoculants (Amarger 2002) and pest control agents (Knudsen 2012) for use in agriculture, as well as bioremediation agents (Singh et al. 2011), their use has not become a widespread practice. It has been suggested that failure on the part of scientists to familiarise themselves with risk assessment policies, and adapt their work accordingly, is at least partly to blame for the lack of commercial success of potentially beneficial GMMs (Ezezika, Singer 2010).

Box 3.1: Synthetic Biology

Synthetic biology has recently attracted a lot of attention due a number of developments, and for its burgeoning potential which has seen a large amount of growth over the last few years. A unified definition of what is encompassed by the term "synthetic biology" has yet to be reached (Schmidt 2015), but a few key ideas and goals can be discerned (Boyle, Silver 2012, Wang, Wei et al. 2013, Baldwin, Bayer et al. 2012, Kogge, Richter 2013, Schmidt 2009):

- An engineering approach to biology, in terms of rational design and production using standard parts (currently synthetically designed DNA)
- Creation of biologically-based systems and devices, with properties which do not exist in nature, which are programmed to carry out specific functions
- Make such systems more systematic, predictable and efficient by reducing complexity and/or using orthogonal approaches to genetically and biochemically isolate organisms

A broader definition was recently offered by the committees who were involved in the formulation of the European Commission's Opinion on Synthetic Biology

- SynBio is the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in living organisms.

While many synthetic biology products (SBPs) may be considered GMMs/GMOs, there is also a large number which would not fall under this definition. Certain SBPs such as protocells and bioparts and are not recognised as living organisms, genetically modified or otherwise (Winter 2015) (also EC opinion).

The need for renewable, sustainable energy sources has generated a great deal of interest in biofuels – fuels produced from biomass which has not been fossilised. Initially, the focus was on crop plants such as maize, sugar cane and rapeseed (Brennan, Owende 2013). However, interest is shifting towards microalgae as a source of biofuel production (both carbon and hydrogen-based) for a number of reasons. Most importantly, algal generation times are much shorter, and the rate of biomass accumulation much higher with very little input, compared to plants. This is mainly due to their higher photosynthetic efficiency and the fact that all cells are autotrophic - no resources are "wasted" maintaining non-photosynthetic structures such as roots (Henley, Litaker et al. 2013). Production of compounds important for biofuel production is high among algae – some species' lipid production may reach 75% of their dry weight, and triglycerol production is also higher in algae than in plants (Brennan, Owende 2013).

Despite their efficiency compared to traditional cropping systems, many feel that there is scope for improvement in algal biomass production which can be achieved through genetic modification (Beer, Boyd et al. 2009, Henley, Litaker et al. 2013, Gressel, van der Vlugt, Cécile et al. 2013) and even

synthetic biology (Hlavová, Turóczy et al. 2015). Genetic manipulation allows metabolic engineering, offering control over biochemical pathways and potentially a more reproducible and predictable systems, while increasing the diversity of available phenotypes (Beer, Boyd et al. 2009).

Synthetic biology (see box 3.1) is a diverse field which has a number of applications, some of which pertain to GMMs, while others (e.g. protocells and bioparts) do not. Some SynBio products are GMMs themselves, for example: an *E. coli* expression system was engineered to produce artemisinic acid, the precursor of the antimalarial drug artemisinin (Keasling 2012), while reprogramming of *Penicilluim Chrysogenum* allowed it to produce the anti-cholesterol drug Pravastatin (McLean et al. 2015). Other applications have used SynBio to develop biocontainment strategies for GMMs – and have coined the term GRO (Genetically Recoded Organism) to describe the results. By recoding microorganisms to be dependent on synthetic metabolites (non-canonical amino acids), their ability to survive outside of controlled conditions is compromised, as is their ability to undergo horizontal gene transfer or escape via mutagenesis (Mandell et al. 2015, Rovner et al. 2015).

3.2 Uncertainties regarding environment risks associated with genetically modified microorganisms (GMMs)

Genetically modified microorganisms may be put to a variety of uses, as noted in the section above. This also means that a wide variety of environments (receiving environments) will be exposed to GMMs, either deliberately or accidentally. GMMs will, for the most part, be entering into ecosystems which already contain functional communities of organisms, such as the animal gastrointestinal tract, rhizosphere of plants, aquatic or marine environments, wastewater treatment facilities and bulk soil (see figure 3.1 below). In some cases, the possible influence of GMMs on such environments may include impacts on human and animal health, due to disruption of the microbiome, or due to the spread of antibiotic resistance genes. As crucial components of food webs, genetic modifications or microorganisms (especially algae) may also affect non-target consumers and bring about changes in food webs within their environments (Henley et al. 2013). Ecosystem services of receiving environments may also be disrupted, if the GMM causes an alteration in community structure (Allison, Martiny 2008), or produces toxic metabolites. Such effects may not manifest immediately. Changes in environmental conditions and selective pressure may give GMMs, or organisms which have received their transgenic traits through horizontal gene transfer, an advantage over other indigenous organisms under specific conditions.

In other cases, it is possible that no impact will be observed at all. Microbial communities in particular can be very difficult for a new organism to infiltrate, even when that organism is beneficial. Establishing a functioning population of a GMM in certain environments may be a challenge, especially if the transgenic trait actually puts the GMM at a competitive disadvantage (Shukla, Singh & Sharma 2010, Amarger 2002). Microbial communities may also display a degree of resilience and ability to recover after disturbances, although there may be a considerable time lapse before they are able to return to their pre-disturbance state, if this occurs (Allison, Martiny 2008).

Predicting whether the outcome of release of GMMs into an environment will be beneficial, harmful, or neutral (see figure 3.1 below), is complicated by two main factors: the inherent adaptability of

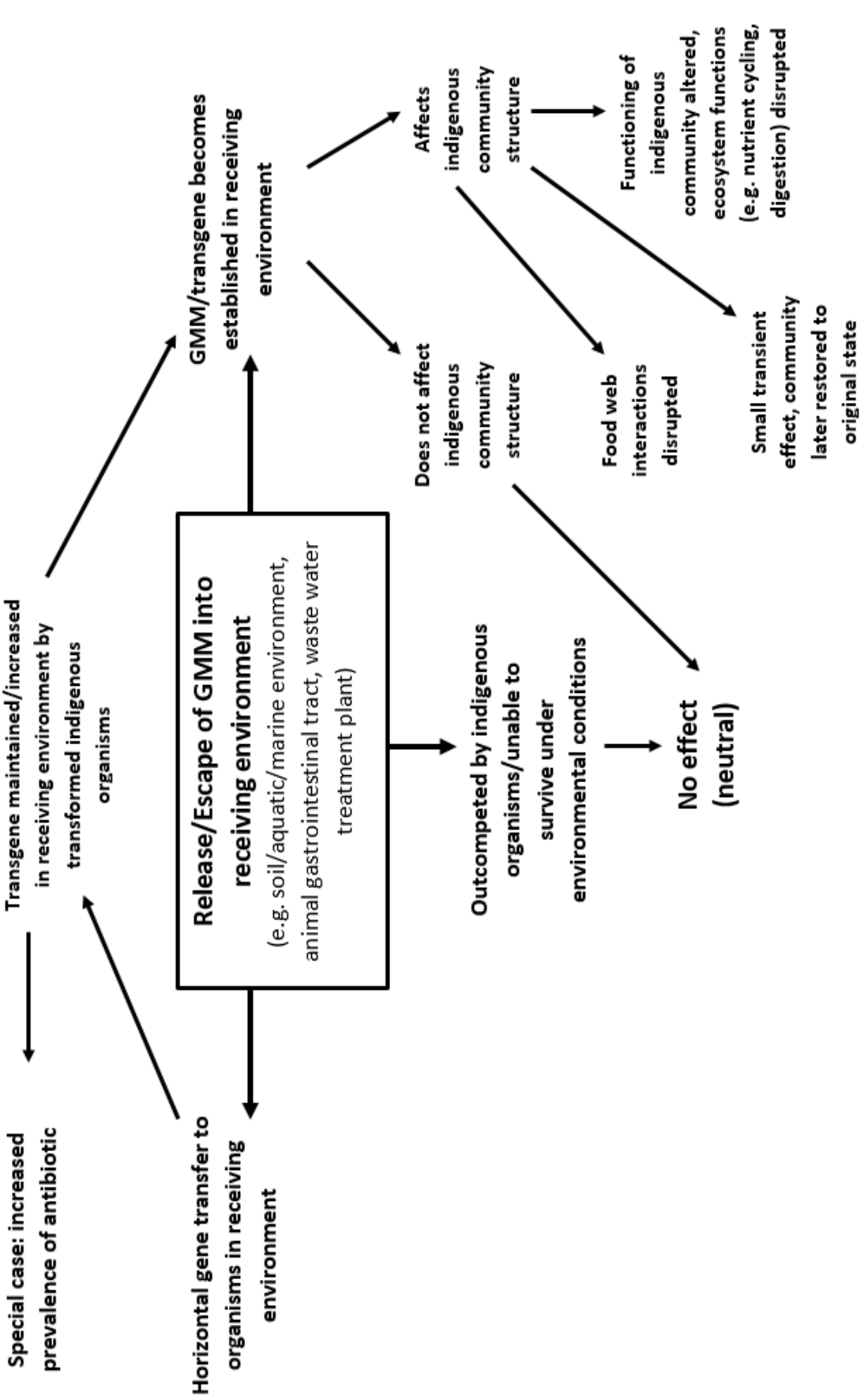

Figure 3.1: Conceptual diagram of the perceived risk scenarios associated with the use, release or escape of GMMs in the environment. Adapted from Henley et al. (2013).

microorganisms themselves; and the complexity and variability of various environments. The interactions between microorganisms and their environments (including other organisms) are dynamic, and seldom completely understood. Knowledge gaps exist concerning how individual GMM events may affect their environment, as well regarding the impact of GMMs in general. Knowledge gaps also exist regarding the efficacy of containment strategies and the possible effects if they should fail.

3.2.1 Release, escape and containment

GMMs will be deliberately established in specific environments to perform specific functions, such as bioremediation of a contaminated site, production of biofuels etc. This constitutes contained use, if measures are in place to prevent negative impacts on the environment and human health, as well as to avoid accidents (EC directive 2009/41). When no specific containment measures are taken to prevent their interaction with the general population and the environment, this constitutes deliberate release (EC directive 2001/18). Escape refers to the accidental release of a GMM into an environment. Within this context of contained use and deliberate release, there may be effects on the indigenous population which are intended (as in the case of population shifts brought about by probiotics), or unintended (food web effects due to GM algae, for example). When accidental release occurs, any effects will be unintended, though not necessarily detrimental.

In the case of accidental release, competition with indigenous populations is viewed as a form of natural containment strategy. In many cases it is assumed that the fitness cost which many transgenes exert on GMMs, will make them uncompetitive in environments in which they do not receive assistance from humans (Flynn et al. 2010, Gressel, van der Vlugt, Cécile JB & Bergmans 2013). The established community, unburdened by such fitness costs and adapted to their environmental niches, provide a line of defense against incoming GMMs and may thus prevent negative impacts on their environment. However, under circumstances where selective pressure is applied (presence of an antibiotic, for example), the transgene may confer a selective advantage to the GMM, potentially allowing it to thrive while other organisms are inhibited or killed (Heinemann, Traavik 2004).

3.2.2 Species composition

In all of the environments in which GMMs will be employed, microbial community composition is critical for the functioning of the ecosystem and the provision of ecosystem services (Allison, Martiny 2008, Neish 2009, Heisler et al. 2008). While some ecosystems may be resistant to the establishment and influence of an incoming microbe (GMM or not), others may be more sensitive. Structural and functional shifts in the resident microbial community may occur as a result of the introduction of a GMM (De Carcer et al. 2007).

A microcosm study evaluated the rhizosphere and bulk soil microbial communities surrounding willow trees after the addition of genetically modified strains *Pseudomonas fluorescens*. The GM bacteria contained transgenes for improved rhizoremediation of soil contaminated with PCBs (polychlorinated biphenyls). While the bulk soil communities remained essentially unchanged

following the addition of the GMM, the rhizosphere communities exhibited significant changes in composition, relative to the control. The observed changes in composition were found to be due to the activity of the transgene and its associated reduction of PCB concentration. Graduating such experiments from microcosms to field trials will not only lead to better understanding of the functioning and long-term efficacy of bioremediation GMMs (De Carcer et al. 2007), they would also help elucidate the potential effects and interactions between GMMs, plants and microbial communities.

An extreme example of how microorganism community structure can affect ecosystems can be seen in the case of harmful algal blooms (HABs), where one algae species overwhelms the environment. During an algal bloom, a species of algae undergoes dramatic growth, but is only considered an HAB when accompanied by adverse consequences for the surrounding ecosystem. Possible consequences of HABs include: physical damage to other organisms (e.g. fish) due to the high cell density; depletion of resources by the large population; development of anoxic conditions associated with decomposition of biomass after an HAB; and the production of substances such as phycotoxins. Phycotoxins are of particular concern because of their capacity for bioaccumulation and magnification within food webs (McLean, Sinclair 2013).

The capacity for GM algae to form HABs may be affected by their genetic modifications. On one hand, depending on the trait, transgenes may be accompanied by a fitness cost which makes development of HABs under natural conditions unlikely. Some GM traits also aim for algal "domestication", which would prevent such organisms from functioning in natural conditions (Gressel, van der Vlugt, Cécile JB & Bergmans 2013). On the other hand, other GM traits may not mitigate, or may even enhance, the transformed algae's natural capacity for causing HABs. Flynn et al. (2013) conducted simulations to investigate the potential of GM algae, optimized for biofuel production, to form HABs. The results of the simulations indicated that such GM algae would likely have greater potential to form HABs than naturally occurring species, due to traits conferring improved nutrient utilisation efficiency, changes in photosynthetic configurations and poor palatability for grazers, compared to naturally occurring species (Flynn et al. 2013).

3.2.3 Horizontal gene transfer

Horizontal gene transfer (HGT) has been defined as "...all forms of gene transfer that do not involve parent-to-offspring transfer" (Keese 2008). There are numerous mechanisms by which HGT can take place (Table 3.1). Among prokaryotes, the three main mechanisms are transformation, conjugation and transduction. Eukaryotes are also subject to HGT, although not to the same extent as prokaryotes. Clear understanding of the mechanisms by which genetic material is transferred into eukaryotic genomes has yet to be reached in many cases (Andersson 2005).

Potential horizontal gene transfer from transgenic microorganisms to other organisms in the environment is considered one of the main risks pertaining to the release or escape of GMMs. Though beneficial HGT events are thought to be quite rare, (Nielsen, Townsend 2004), HGT represents a potential loss of control over the transgene into a possibly unfamiliar host, with possibly detrimental effects on the environment and human health. Most attention in this regard has been

focussed on HGT of antibiotic resistance marker genes from GMMs into bacteria in the surrounding environment – which includes the human gastrointestinal tract, soil and aquatic ecosystems (EFSA Panel on Genetically Modified Organisms (GMO). 2011). Considering the impact of the spread of antibiotic resistance genes, this is not unreasonable (Midtvedt 2014). However, other transgenes which confer a possible selective advantage to environmental organisms should not be overlooked.

Type of HGT	Definition
Transformation	During transformation, exogenous (free) DNA is taken up from the bacteria cell's immediate surroundings (Chen, Dubnau 2004).
Conjugation	Conjugation is the term for the specialised processes which result in the direct transfer of DNA between donor and recipient cells by means of a conjugation apparatus. Although conjugation occurs primarily between bacteria, transfer from bacteria to yeast, plants, and filamentous fungi is not unheard of (Grohmann, Muth & Espinosa 2003, Heinemann, Sprague 1989, Zupan, Zambryski 1995, De Groot et al. 1998).
Virus mediated transfer and transduction	Viruses may act as shuttles for genetic material between different host cells, and have been observed to do so in bacteria, plants, algae and other eukaryotes (Bock 2010, Paul 1999, Monier et al. 2009). The term transduction describes the process by which DNA is transferred between prokaryotes due to the action of viruses known as bacteriophages.
Anastomosis	During anastomosis, the hyphae of different fungi become fused, causing the cytoplasm of the hyphae involved to become interconnected, which may allow HGT to occur (Richards et al. 2011, Xie et al. 2008).
Phagotrophy	During the process of taking in other cells (prokaryotic or eukaryotic) as a food source, phagotrophic organisms import the DNA of these food-cells into their own cells. This DNA may be destroyed, used as a nutrient, or in some cases incorporated into the phagotroph's genome (Andersson 2005).
Endosymbiotic transfer	The transfer of DNA fragments from organelles (plastids) of endosymbiotic origin, to the nuclear genomes of eukaryotic organisms. This process is thought to occur widely and is likely to be ongoing (Nikoh et al. 2008).

Table 3.1: Examples and definitions of known mechanisms of horizontal gene transfer among both prokaryotes and eukaryotes

Transfer of transgenic traits which confer a growth advantage from GM algae into environmental algal populations, for example, could impact negatively on aquatic ecosystems (Henley et al. 2013), though some authors find this scenario unlikely (Gressel, van der Vlugt, Cécile JB & Bergmans 2013). Occurrences of HGT from GMMs *in situ* were demonstrated by Williams et al. (1996). During these experiments, *Acinetobacter calcoaceticus* recipient cells, both as single culture and as part of an epilithon (a biofilm community which develops on the surface of stones in aquatic environments) were shown to have undergone transformation with DNA sourced either from a crude lysate or plasmid-bearing donor cells (GMMs), while being incubated in a river. This study was the first to demonstrate *in situ* transformation in bacteria incorporated into an indigenous community (Williams et al. 1996). This study also found that although there was a general trend of transformation frequency increasing with increases in temperature, no optimal temperature for transformation

could be determined (Williams, Day et al. 1996). Soil microcosm experiments also utilising *A. calcoaceticus* investigated the influence of abiotic factors on natural transformation of this microbe with chromosomal DNA, and found that factors such as soil moisture and phosphate concentration affected transformation success (Nielsen, Bones & Van Elsas 1997).

In both the experiments discussed above, the recipient organism and various other parameters were under the control of the investigators. Detecting HGT events in complex natural environments, to uncharacterised recipients, has proven to be an extremely onerous task, however. There are several factors which contribute to the challenging nature of such an undertaking, which will be discussed briefly below (but for a comprehensive review, see (Nielsen, Bøhn & Townsend 2013)).

Rate of HGT: A selective advantage is needed for long term establishment of transferred genetic material in the recipient organism's genome. Such occurrences are rare in prokaryotes, and even more so in eukaryotes (Nielsen, Bøhn & Townsend 2013, Andersson 2005). Though deleterious or neutral HGT event may occur more frequently, they are not likely to be maintained. Which events constitute selective advantages is dependent on interactions with the environment.

Population size: Populations of microorganisms in receiving environments are likely to be extremely large. For example, in 1 g of soil, there are estimated to be 10^7-10^9 bacteria. Despite the high numbers of microbes present, only a small percentage of these are amenable to being cultured on laboratory media, which severely limits the ability of culture-based methods to detect putative HGT events (Rizzo et al. 2013). Such methods result in huge sampling efforts: Gebhard and Smalla screened 4000 potential isolates for HGT events, after an initial screening of 10 000 isolates on selective media, for example. Despite the effort, such methods are usually not sensitive enough to detect most HGT events.

Strength of selective advantage: Many studies investigating HGT events employ a strong selective agent, such as an antibiotic, in order to screen cells for presence of transgenes (Gebhard, Smalla 1998). However, not all traits offer a very strong selective advantage, and these may be missed by such screening procedures. Also, HGT of a transgene is not necessarily the final modification which it undergoes: recombination, deletion or incomplete transfer are all factors which could affect an organism's response to a specific selective pressure, yet still be valuable to the organism over time (Nielsen, Bøhn & Townsend 2013).

Next generation sequencing (metagenomic) techniques have been applied in order to develop techniques which are not culture-dependent. The prevalence of tetracycline resistance genes in the human mouth microbiome was assayed using such techniques (Seville et al. 2009). For other (non-clinical) environments, however, there is currently a shortage of reference genomes and genetic markers, which reduces the usefulness of metagenomic and transcriptomic approaches for monitoring the prevalence and persistence of certain genes within such environments (Rizzo et al. 2013).

It is reasonable to question whether HGT events which occur so infrequently as to escape detection, may have a measureable effect on the receiving environments. There is some debate regarding this issue. Some authors (Gressel, van der Vlugt, Cécile JB & Bergmans 2013) believe that low selective

pressure combined with a high metabolic cost for maintaining transgenic DNA are likely to mean that HGT will not have a significant effect, especially when antibiotic resistance markers are not part of the GMM in question. Others (Heinemann, Traavik 2004, Nielsen, Townsend 2004, Henley et al. 2013) point out that in such large populations, even rare HGT events occur frequently enough to be non-trivial. For example, Heinemann and Traavik (2004) calculated that the development of the present levels of penicillin resistance in *Streptococcus pnuemoniae* may have resulted from the occurrence of HGT events at frequencies several orders of magnitude lower than those estimated to occur in soil.

The perceived risk of HGT between GMMs and other organisms has piqued the interest of those working in synthetic biology, and a possible biocontainment solution has been developed in the form of GROs (as mentioned in a previous section). The developers report metabolic dependence and genetic isolation as a result of these reprogramming developments, and foresee that their products could add an additional level of safety to GMMs released into the environment (Mandell et al. 2015, Rovner et al. 2015). However, these SynBio containment strategies have yet to be applied to a GMM which has a specific purpose (e.g. bioremediation), and it is not yet clear how compatible such strategies will be with the complex metabolic processes required in some GMMs or variable environmental conditions. SynBio GMMs and GROs often have reduced genomes due to the way in which they are constructed, which can limit their ability to respond to changes in environmental conditions (Güttinger 2013).

Knowledge gaps and uncertainties relating to HGT between GMMs and other organisms arise both from an incomplete understanding of how the process works (especially for algae), as well as methodological limitations. Thus the scope of HGT, as well as the environmental interactions which lead to the maintenance or loss of transferred transgenic traits also remain to be fully understood. The effects of HGT may not manifest until very long after the event has taken place, or may not manifest as a realised environmental effect at all.

3.3 Directions for further studies

Knowledge gaps and uncertainty concerning GMMs largely arise from a shortage of data relating to the interactions of GMMs with complex natural environments. While some studies on the effects of GMMs on soil microbiota in agricultural and bioremediation applications have been done, data relating to aquatic ecosystems has yet to be generated. Unsurprisingly, considering the novelty of such innovations, the efficacy of SynBio GMMs in complex (natural) environments has also yet to be determined, as have any effects on resident communities in such environments. The logistics of how such organisms are to be sustained and managed in natural environments, considering the artificial nutrient requirements of the SynBio GMMs/GROs discussed in this chapter, are also issues in need of resolution.

Horizontal gene transfer of transgenic genetic material from GMMs in various environmental contexts still requires investigation. Methodological limitations may be at the heart of the present lack of data, but adapting recent technological advances (e.g. next generation sequencing and

metagenomics) has shown potential to aid detection and understanding of HGT events (Seville et al. 2009).

In terms of GM algae specifically, significant levels of uncertainty are present concerning large scale cultivation of algae, even before genetic modification. Algal population dynamics, responses to changes in the environment, sexual reproduction and potential for horizontal gene transfer are all poorly understood at this point (Henley et al. 2013). Though the potential for harnessing these organisms as cellular factories producing biofuels, pharmaceuticals and food and feed may yield considerable advantages, the risks inherent in cultivating huge populations of a single species of algae, especially one carrying a transgenic trait, represent a large degree of uncertainty (Flynn et al. 2013). Research into algal population dynamics and interaction with the environment, and how these may be affected by the insertion of transgenes is still needed.

Considering the level of uncertainty relating to GMM-environmental interactions, especially relating to GM algae, strategies monitor these interactions and detect deleterious effects early on should be considered. The potential for negative environmental impacts reinforces the need for research into possible non-target and unintended effects (still lacking at this point), as well for strategies to keep informed on potential effects on the surrounding ecosystems and the efficacy of containment strategies. An opportunity exists at this stage of GMM utilisation to ensure the consideration and inclusion of ecological principles, in order to minimise potential environmental effects (Olivares, Wijffels 2013, Henley et al. 2013, Flynn et al. 2013, Midtvedt 2014, Singh et al. 2011).

References

ALLISON, S.D. and MARTINY, J.B., 2008. Colloquium paper: resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences of the United States of America,* **105 Suppl 1**, pp. 11512-11519.

AMARGER, N., 2002. Genetically modified bacteria in agriculture. *Biochimie,* **84**(11), pp. 1061-1072.

ANDERSSON, J.O., 2005. Lateral gene transfer in eukaryotes. *Cellular and Molecular Life Sciences CMLS,* **62**(11), pp. 1182-1197.

BOCK, R., 2010. The give-and-take of DNA: horizontal gene transfer in plants. *Trends in plant science,* **15**(1), pp. 11-22.

ÇELIK, E. and ÇALIK, P., 2012. Production of recombinant proteins by yeast cells. *Biotechnology Advances,* **30**(5), pp. 1108-1118.

CHEN, I. and DUBNAU, D., 2004. DNA uptake during bacterial transformation. *Nature Reviews Microbiology,* **2**(3), pp. 241-249.

DE CARCER, D.A., MARTÍN, M., MACKOVA, M., MACEK, T., KARLSON, U. and RIVILLA, R., 2007. The introduction of genetically modified microorganisms designed for rhizoremediation induces changes on native bacteria in the rhizosphere but not in the surrounding soil. *The ISME journal,* **1**(3), pp. 215-223.

DE GROOT, M.J., BUNDOCK, P., HOOYKAAS, P.J. and BEIJERSBERGEN, A., 1998. Agrobacterium tumefaciens-mediated transformation of filamentous fungi. *Nature biotechnology,* **16**.

DEMAIN, A.L. and VAISHNAV, P., 2009. Production of recombinant proteins by microbes and higher organisms. *Biotechnology Advances,* **27**(3), pp. 297-306.

EFSA PANEL ON GENETICALLY MODIFIED ORGANISMS (GMO)., 2011. Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. . *EFSA Journal,* **9**(6), pp. 2193.

EZEZIKA, O.C. and SINGER, P.A., 2010. Genetically engineered oil-eating microbes for bioremediation: prospects and regulatory challenges. *Technology in Society,* **32**(4), pp. 331-335.

FLYNN, K.J., GREENWELL, H.C., LOVITT, R.W. and SHIELDS, R.J., 2010. Selection for fitness at the individual or population levels: Modelling effects of genetic modifications in microalgae on productivity and environmental safety. *Journal of theoretical biology,* **263**(3), pp. 269-280.

FLYNN, K.J., MITRA, A., GREENWELL, H.C. and SUI, J., 2013. Monster potential meets potential monster: pros and cons of deploying genetically modified microalgae for biofuels production. *Interface focus,* **3**(1), pp. 20120037.

GEBHARD, F. and SMALLA, K., 1998. Transformation of Acinetobacter sp. strain BD413 by transgenic sugar beet DNA. *Applied and Environmental Microbiology,* **64**(4), pp. 1550-1554.

GRESSEL, J., VAN DER VLUGT, CÉCILE JB and BERGMANS, H.E., 2013. Environmental risks of large scale cultivation of microalgae: Mitigation of spills. *Algal Research,* **2**(3), pp. 286-298.

GROHMANN, E., MUTH, G. and ESPINOSA, M., 2003. Conjugative plasmid transfer in gram-positive bacteria. *Microbiology and molecular biology reviews : MMBR,* **67**(2), pp. 277-301.

GÜTTINGER, S., 2013. Creating parts that allow for rational design: Synthetic biology and the problem of context-sensitivity. *Studies in History and Philosophy of Science Part C: Studies in History and Philosophy of Biological and Biomedical Sciences,* **44**(2), pp. 199-207.

HEINEMANN, J.A. and TRAAVIK, T., 2004. Problems in monitoring horizontal gene transfer in field trials of transgenic plants. *Nature biotechnology,* **22**(9), pp. 1105-1109.

HEINEMANN, J.A. and SPRAGUE, G.F.,Jr, 1989. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. *Nature,* **340**(6230), pp. 205-209.

HEISLER, J., GLIBERT, P.M., BURKHOLDER, J.M., ANDERSON, D.M., COCHLAN, W., DENNISON, W.C., DORTCH, Q., GOBLER, C.J., HEIL, C.A. and HUMPHRIES, E., 2008. Eutrophication and harmful algal blooms: a scientific consensus. *Harmful algae,* **8**(1), pp. 3-13.

HENLEY, W.J., LITAKER, R.W., NOVOVESKÁ, L., DUKE, C.S., QUEMADA, H.D. and SAYRE, R.T., 2013. Initial risk assessment of genetically modified (GM) microalgae for commodity-scale biofuel cultivation. *Algal Research,* **2**(1), pp. 66-77.

ISAAA, 2015-last update, ISAAA's GM Approval Database. Available: http://www.isaaa.org/gmapprovaldatabase/ [September 29, 2015].

KEASLING, J.D., 2012. Synthetic biology and the development of tools for metabolic engineering. *Metabolic engineering,* **14**(3), pp. 189-195.

KEESE, P., 2008. Risks from GMOs due to horizontal gene transfer. *Environmental Biosafety Research,* **7**(03), pp. 123-149.

KNUDSEN, G.R., 2012. International Deployment of Microbial Pest Control Agents: Falling between the Cracks of the Convention on Biological Diversity and the Cartagena Biosafety Protocol. *Pace Envtl.L.Rev.,* **30**, pp. i.

MANDELL, D.J., LAJOIE, M.J., MEE, M.T., TAKEUCHI, R., KUZNETSOV, G., NORVILLE, J.E., GREGG, C.J., STODDARD, B.L. and CHURCH, G.M., 2015. Biocontainment of genetically modified organisms by synthetic protein design. *Nature,* .

MCLEAN, T.I. and SINCLAIR, G.A., 2013. Harmful Algal Blooms. *Environmental Toxicology.* Springer, pp. 319-360.

MCLEAN, K.J., HANS, M., MEIJRINK, B., VAN SCHEPPINGEN, W.B., VOLLEBREGT, A., TEE, K.L., VAN DER LAAN, J.M., LEYS, D., MUNRO, A.W. and VAN DEN BERG, M.A., 2015. Single-step fermentative production of the cholesterol-lowering drug pravastatin via reprogramming of Penicillium chrysogenum. *Proceedings of the National Academy of Sciences of the United States of America,* **112**(9), pp. 2847-2852.

MIDTVEDT, T., 2014. Antibiotic resistance and genetically modified plants. *Microbial Ecology in Health and Disease,* **25**.

MONIER, A., PAGARETE, A., DE VARGAS, C., ALLEN, M.J., READ, B., CLAVERIE, J.M. and OGATA, H., 2009. Horizontal gene transfer of an entire metabolic pathway between a eukaryotic alga and its DNA virus. *Genome research,* **19**(8), pp. 1441-1449.

NEISH, A.S., 2009. Microbes in gastrointestinal health and disease. *Gastroenterology,* **136**(1), pp. 65-80.

NIELSEN, K.M., BØHN, T. and TOWNSEND, J.P., 2013. Detecting rare gene transfer events in bacterial populations. *Frontiers in microbiology,* **4**.

NIELSEN, K.M. and TOWNSEND, J.P., 2004. Monitoring and modeling horizontal gene transfer. *Nature biotechnology,* **22**(9), pp. 1110-1114.

NIELSEN, K.M., BONES, A.M. and VAN ELSAS, J.D., 1997. Induced Natural Transformation of Acinetobacter calcoaceticus in Soil Microcosms. *Applied and Environmental Microbiology,* **63**(10), pp. 3972-3977.

NIKOH, N., TANAKA, K., SHIBATA, F., KONDO, N., HIZUME, M., SHIMADA, M. and FUKATSU, T., 2008. Wolbachia genome integrated in an insect chromosome: evolution and fate of laterally transferred endosymbiont genes. *Genome research,* **18**(2), pp. 272-280.

OLIVARES, J. and WIJFFELS, R., 2013. Responsible Approaches to Genetically Modified Microalgae Production: editorial. *Algal Research,* **2**(1), pp. 1-1.

PAUL, J.H., 1999. Microbial gene transfer: an ecological perspective. *Journal of Molecular Microbiology and Biotechnology,* **1**(1), pp. 45-50.

RICHARDS, T.A., LEONARD, G., SOANES, D.M. and TALBOT, N.J., 2011. Gene transfer into the fungi. *Fungal Biology Reviews,* **25**(2), pp. 98-110.

RIZZO, L., MANAIA, C., MERLIN, C., SCHWARTZ, T., DAGOT, C., PLOY, M., MICHAEL, I. and FATTA-KASSINOS, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Science of the Total Environment,* **447**, pp. 345-360.

ROVNER, A.J., HAIMOVICH, A.D., KATZ, S.R., LI, Z., GROME, M.W., GASSAWAY, B.M., AMIRAM, M., PATEL, J.R., GALLAGHER, R.R. and RINEHART, J., 2015. Recoded organisms engineered to depend on synthetic amino acids. *Nature,* **518**(7537), pp. 89-93.

SCENIHR (SCIENTIFIC COMMITTEE ON EMERGING AND NEWLY IDENTIFIED HEALTH RISKS), SCCS (SCIENTIFIC COMMITTEE ON CONSUMER SAFETY), SCHER (SCIENTIFIC COMMITTEE ON HEALTH AND ENVIRONMENTAL RISKS), 25 September 2014, 2014-last update, Synthetic Biology I Definition. Available: http://ec.europa.eu/health/scientific_committees/consultations/public_consultations/scenihr_consultation_21_en.htm.

SCHER (SCIENTIFIC COMMITTEE ON HEALTH AND ENVIRONMENTAL 15 RISKS), SCENIHR (SCIENTIFIC COMMITTEE ON EMERGING AND NEWLY IDENTIFIED HEALTH RISKS), 16 SCCS (SCIENTIFIC COMMITTEE ON CONSUMER SAFETY), 2014-last update, Biology II - Risk assessment methodologies and safety aspects. Available: http://ec.europa.eu/health/scientific_committees/consultations/public_consultations/scenihr_consultation_26_en.htm [June 6 2015, 2015].

SEVILLE, L.A., PATTERSON, A.J., SCOTT, K.P., MULLANY, P., QUAIL, M.A., PARKHILL, J., READY, D., WILSON, M., SPRATT, D. and ROBERTS, A.P., 2009. Distribution of tetracycline and erythromycin resistance genes among human oral and fecal metagenomic DNA. *Microbial Drug Resistance,* **15**(3), pp. 159-166.

SHUKLA, K.P., SINGH, N.K. and SHARMA, S., 2010. Bioremediation: developments, current practices and perspectives. *Genetic Engineering and Biotechnology Journal,* **3**, pp. 1-20.

SINGH, J.S., ABHILASH, P., SINGH, H., SINGH, R.P. and SINGH, D., 2011. Genetically engineered bacteria: An emerging tool for environmental remediation and future research perspectives. *Gene,* **480**(1), pp. 1-9.

WILLIAMS, H.G., DAY, M.J., FRY, J.C. and STEWART, G.J., 1996. Natural transformation in river epilithon. *Applied and Environmental Microbiology,* **62**(8), pp. 2994-2998.

XIE, J., FU, Y., JIANG, D., LI, G., HUANG, J., LI, B., HSIANG, T. and PENG, Y., 2008. Intergeneric transfer of ribosomal genes between two fungi. *BMC evolutionary biology,* **8**, pp. 87-2148-8-87.

ZUPAN, J.R. and ZAMBRYSKI, P., 1995. Transfer of T-DNA from Agrobacterium to the plant cell. *Plant Physiology,* **107**(4), pp. 1041-1047.

4. GM trees

4.1 Introduction

The need to produce more [wood, food, goods, etc.] by using less [land, time, chemicals, etc.] is an oft-cited formula justifying the development and use of genetically modified organisms, GM trees being no exception (Häggman et al. 2014, Van Acker et al. 2014, Sedjo 2011). In Norway, especially with regard to wood production, this drive towards increased production may not seem pertinent, as the country has been harvesting forest trees at a rate below the annual growth for years, leading to an increase in forest biomass (Trømborg, Bolkesjø & Solberg 2008). However, the growing global population is expected to create increased demands for both food and bioenergy (Häggman et al. 2014). The demand for sustainable forms of biofuel in particular is expected to rise as countries move away from fossil fuels towards more renewable sources. For example, in order to move towards its goal of carbon neutrality by the year 2030, the Norwegian government plans to offer incentives to encourage the utilization of energy derived from wood and forest residues, as alternatives to fossil fuels (Khanam et al. 2014, Norwegian Ministry of the Environment 2012).

Genetically modified plants have been discussed in chapter one in this report, but genetically modified trees deserve special attention for several reasons. Among these, the most obvious are the longevity of trees compared to crop plants; and the fundamental role they fill within ecosystem structures. These considerations mean that, for GM trees, the risk of transgene escape and the capacity to do harm within an ecosystem may be increased, compared to annual crop plants and conventional (non-transgenic) varieties of trees. Furthermore, unlike crop plants and fruit trees, forest trees are largely undomesticated and thus unpredictable (Hjältén et al. 2007).

4.2 Types of transgenic trees

It is the longevity of trees that makes the idea of genetically modifying trees so enticing. Traditional breeding approaches may take many years to develop desirable traits, or may not be able to select for certain traits at all (Séguin et al. 2014, Harfouche, Meilan & Altman 2011). Genetic modification may offer a faster route of improvement. GM trees include varieties of fruit trees, forest trees and other woody perennials such as tea (Bhattacharya, Saini & Ahuja 2006). Although many types of GM trees have entered field trials, more than 700 field trials have been reported (Walter, Fladung & Boerjan 2010), the only types of GM trees listed on the ISAAA GM Approval Database (ISAAA 2015) are papaya (4 events), plum (1 event), poplar (2 events) and most recently, apple (2 events). Commercially grown GM trees are limited to Bt poplar in China (Ewald, Hu & Yang 2006), and papaya resistant to Papaya ringspot virus (PRSV) in Hawaii (Fuchs, Gonsalves 2007).

The shift in focus from fossil-based energy sources to biofuels has especially encouraged the development of transgenic trees better able to meet this increased demand (Sedjo 2011). This includes trees with altered lignin production (reducing the chemical processing needed to strip the lignin away), increased biomass production, and faster growth. Despite the enthusiasm for trees with reduced lignin production, the only transgenic plant with this trait on the GM approval database, is alfalfa (ISAAA, 2015).

Poplar trees
Photo: Dollarphotoclub_67497084/peggy

Reduced loss due to insect pests, weeds and disease is also a strong motivator for the development of transgenic crops which are less susceptible to these issues. For insect resistance, trees containing genes for Bt toxins (Ewald, Hu & Yang 2006, Hu et al. 2001) as well as proteinase inhibitors (Tian et al. 1999) have been developed, with varying degrees of success. Currently being employed commercially in China, field trials of Bt transgenic trees have also taken place in Sweden (Axelsson 2011) as well as other countries. To combat weeds, especially during the early stages of tree growth before a canopy is formed, herbicide tolerance genes have been inserted into some trees, for example glyphosate tolerant poplar (Meilan et al. 2002). Diseases, such as Dutch elm disease in American elm, blight in chestnut (Newhouse et al. 2007, Merkle et al. 2007) and ringspot virus in Papaya (Fuchs, Gonsalves 2007), have decimated certain tree species. In order to restore such species, and to prevent these diseases from causing large scale losses again, transgenic traits which confer a degree of resistance to their infectious agents have been investigated.

Traits which would allow trees to grow under stressed conditions, such as drought or high salinity soil (Oguchi et al. 2014), as well as areas contaminated by heavy metals (Hur et al. 2011) or organic pollutants (Doty et al. 2007), are also being developed. These kinds of traits might allow land otherwise considered unusable to be utilised by transgenic trees, and undergo phytoremediation in the process (Behera 2014). Utilising marginal land would also help reduce competition for arable land between forest trees and crops, pastureland and protected areas (Häggman et al. 2014).

4.3 Uncertainties regarding ecological risks associated with transgenic trees

The surging demands on the forestry industry, coupled with increased environmental awareness and decreasing available land, mean that planted forests (both transgenic and conventional) will likely have to fulfil more than just production functions in future (Sedjo 2011). Planted forests may relieve some of the pressure on natural forests in terms of sources of wood, and may also act as surrogate

havens of biodiversity in landscapes where natural forests have been reduced or destroyed entirely (Brockerhoff et al. 2013). As foundation organisms in forest ecosystems (Whitham et al. 2006), trees influence the composition and structure of the community of organisms. Ecological risks associated with transgenic forest trees include both extensions of those associated with planted (monoculture) forests, as well as risks due to effects which are specifically related to the genetic modifications of the trees involved.

Uncertainty relating to how transgenic trees affect the environments they enter into includes both epistemological and ontological uncertainties. As stated in a previous chapter, epistemic uncertainties arise from (i) changes in the GM plant and its product, (ii) secondary effects due to the introduction of genetic material (e.g. pleiotropic effects), and (iii) changes in interactions and responses to environmental conditions. It is also possible for more than one type of uncertainty to manifest at once and for various factors to influence each other. For example, uncertainties regarding unintended non-target effects, and uncertainties regarding ecological interactions and responses, may manifest concurrently, and be related to each other. Ontological uncertainties arise due to the complexity and variability which are inherent in the natural systems into which transgenes are inserted. In this section, areas of uncertainty related to how transgenic crops interact with and affect the environment will be considered.

4.3.1 Gene Flow

Gene flow, simply put, refers to the transmission of genes between compatible populations (Farnum, Lucier & Meilan 2007). When transgenic organisms are involved, the more emotive term *genetic pollution* is often used to refer to the entry of transgenes into the non-transgenic population, especially when considering contamination of a plant's centre of origin (Linacre, Ades 2004, Barbour et al. 2010). Gene flow is one of the main environmental safety concerns regarding GM plants, and may have very serious ecological consequences - ranging from development of invasiveness of the transgenic plants (Hoenicka, Fladung 2006), to disruption of food webs (Farnum, Lucier & Meilan 2007) and extinction of local populations (Linacre, Ades 2004) if not given proper consideration. These concerns apply equally to exotic genera and non-transgenic hybrids introduced into ecosystems where crossing with local species may occur. [Trans]Gene escape via gene flow is something which may be impossible to reverse once it has occurred (Brunner et al. 2007).

The longevity of trees singles them out from other transgenic plants when considering the opportunity for gene flow to occur. With annual plants, the type of crop or type of transgene could, in theory, be changed every year. With transgenic trees, the crop and the transgenes being produced are fixed for several years (potentially decades, depending on the type of tree and planned use of the wood), until the trees are considered mature enough for harvest (Farnum, Lucier & Meilan 2007). Although the trees may take several years before reaching maturity, the potential for numerous years of pollen, seed and root sucker production exists.

Practical reasons, such as the time needed to reach maturity (Ahuja 2009), as well as regulations and restrictions (Strauss et al. 2010) regarding the cultivation of GM trees, often prevent field trials for the study of gene flow due to pollen from transgenic to non-transgenic plantations and wild relatives, although some studies using the *GUS* marker gene have been done (Tyson, Wilson & Lane 2011).

Studies of gene flow between non-transgenic populations of a number of different tree species have been performed and modelled in order to better understand the dynamics of the process (Bialozyt 2012, Slavov et al. 2009). While these models likely represent the phenomenon of pollen migration very well under specific environmental conditions, they may not represent the situation in terms of establishment of hybrid transgenic offspring which are the result of such pollination.

Two other routes of gene flow exist: seed dispersal, and vegetative propagation (Farnum, Lucier & Meilan 2007). Vegetative reproduction sidesteps the sexual reproduction process, and is not in itself a means of gene flow unless the offspring become established in an area where hybridization with members of another population could take place (Häggman et al. 2014). Root suckers are clonal shoots which develop from adventitious buds found on lateral roots (Häggman et al. 2014). The fact that in species such as poplar root suckers may reach lengths of up to 10m, complicates efforts to control their spread into other fields. In a field trial in Germany between 1996 and 2001, Fladung et al (2003) detected over 200 root suckers, more than half of which were of transgenic origin, and several of which had escaped the confines of the experimental stand (Fladung et al. 2003).

Successful establishment of such progeny relative to those of non-transgenic origin is dependent on a number of factors, not least of which is the potential competitive advantage (or disadvantage) which could be afforded to offspring carrying the transgene. Difazio et al (2012) created a model to predict gene flow, particularly transgene flow between tree populations. They found that ecological context (in terms of selective pressure) was likely to affect whether the progeny of a transgenic tree were able to become established and spread, but that this was difficult to predict. In order to improve understanding of the dynamics of gene flow and selective pressure in the environmental context, they recommended an integrated approach involving both laboratory studies and field trials (DiFazio et al. 2012).

4.3.2 Pleiotropic effects, epigenetics and transgene stability over time

Pleiotropy refers to the capacity which certain genes have, whereby they are able to influence multiple phenotypic traits (Hodgkin 1998). The relevance of this for transgenic research is that, when attempting to alter a specific trait by introducing changes to the gene coding for it, one may unintentionally influence other traits as well. Effects such as stunted growth or stem deformities are fairly easy to notice (Grünwald et al. 2001), and may lead to affected plants being eliminated during screening procedures. However, more subtle effects require more sophisticated analyses to weed out, and may not be immediately obvious (see also discussion below regarding phenotypic plasticity and gene silencing). The concern with pleiotropic effects in transgenic plants, is whether they will lead to unintended, potentially harmful effects on the plants or the organisms they interact with, which could not have been anticipated during development.

Several lines of transgenic aspen have displayed unexpected changes after transformation. Hjältén et al. (2007) demonstrated that a genetic modification causing hybrid aspen (*Populus tremula* x *Populus tremuloides*) to over-express the sucrose phosphate synthase (SPS) gene, had unintentional consequences for traits involved in plant-herbivore interactions. The transgenic lines experienced increased biomass accumulation (which was the function of the transgene), but one line also experienced altered levels of phenolic metabolites such as condensed tannins and salicyclates as well

as nitrogen. During a feeding trial involving material from the same trees, a typical aspen herbivore (*Phratora vitellinae*) was found to have a decreased preference for the transgenic leaves of the line mentioned above, compared to the control line. This is thought to be due to the noted changes in phenolic metabolite composition (Hjältén et al. 2007). Brodeur-Campbell et al (2006) studied aspens (*Populus tremuloides*) modified to contain the antisense *Pt4CL* gene, which was used to bring about reduced lignin production. They noted reduced survival of gypsy moth larvae feeding on one of the modified lines. Since only one of the modified lines was found to have this effect, the authors reasoned that it was not the low-lignin phenotype, but positional effects due to the location of the transgene insertion, which were responsible for the reduced larval survival (Brodeur-Campbell et al. 2006).

Studies such as these indicate that pleiotropic effects due to transgene insertion can alter the way in which trees interact with other organisms, especially herbivores. They also indicate that these effects may be event or context specific. The mechanisms which underlie the observed effects are incompletely understood, as are the implications in a broader ecological context.

Transgene stability has been a concern for genetically modified organisms overall (Kohli, Miro & Twyman 2010), and is of special interest in the case of transgenic trees because of their long lifespans (Ahuja 2009, Fladung, Hoenicka & Ahuja 2013). Reliable transgene expression is required in order for the benefit of the transgenic trait to be realised, and gene silencing and reversion to an unmodified state may have negative consequences for the trees as well as the environment (Fladung, Kumar 2002). For example, silencing of an insect resistance gene would leave trees vulnerable to insect attack (especially if grown in an area of high pest pressure), potentially for several years; while silencing of sterility genes may lead to gene flow via production of pollen.

Instability of transgene expression may be brought about due to several factors. A number of these have to do with positional effects (such as chromatin architecture, proximity of regulatory sequences, sequence of surrounding DNA) and locus structure features (like copy number, intactness, orientation) of the transgene itself (Kohli, Miro & Twyman 2010). Factors such as these are often the reason behind the variability observed in transgene expression in different transgenic events, despite the use of clonal individuals and the same transgene. Fladung et al (2002) noted several different types of reversions among the transgenic aspen clones they transformed. Multiple copies of transgenes inserted into one locus, or 2 loci with one or more transgenes in varying states of completeness, were noted among the individuals with unstable transgene expression (Fladung, Kumar 2002). This long-term experiment furthermore revealed the importance of genetic background and environmental influence in transgene stability. It was noted that some lines were more prone to transgene instability and reversion to wild type than others, while changes in environmental conditions (such as shifting the plants to the greenhouse or field) were found to bring on reversions in some plants (Kumar, Fladung 2001). Stable expression of transgenes under greenhouse conditions for more than a decade, as well as *in vitro* for 19 years were also noted (Fladung, Hoenicka & Ahuja 2013).

Epigenetic processes, which play a role both in the regulation of gene expression and defence against foreign DNA (including viruses and transposons), may also affect transgene stability (Dietz-Pfeilstetter 2010, Bhaskar, Jiang 2010). Epigenetic effects are frequently divided into two main groups: transcriptional gene silencing (TGS) and post-transcriptional genome silencing (PTGS) (Ahuja

2009, Fladung, Kumar 2002). PTGS is also known as RNA silencing or RNA interference (RNAi) and has been harnessed as a tool for altering gene expression in crop plants (Bhaskar, Jiang 2010). Epigenetic processes are known to play a role in adaptive plant responses, but methylation and other epigenetic tools related to gene expression are not yet fully understood, particularly not in the context of the timescales associated with growing trees (Fladung, Hoenicka & Ahuja 2013).

This is further complicated by the fact that trees often have a degree of phenotypic and genomic plasticity which allows them to adapt to an environment which is changing around them – abilities which are especially important for these long-lived organisms unable to move to a place where conditions are more favourable (Bräutigam et al. 2013). Phenotypic plasticity allows trees to display a range of phenotypes as a function of a single genotype; while genomic plasticity refers to changes in genome structure leading to the formation of new phenotypes, and may be due to factors such as movement of transposable elements, mutational hotspots, somatic recombination and so on. Both forms of plasticity occur in response to environmental triggers (Nicotra et al. 2010).

Gene instability is thought to be common during the *in vitro* culturing stage of transgenic plant development, and generally less common in later stages such as field trials (Harfouche, Meilan & Altman 2011). However, interactions with the environment may bring about responses in GM trees which alter transgene expression. Stout et al (2014) investigated the growth of 12 transgenic lines of poplar containing 3 different lignin reduction transgene constructs, but all derived from the same clone. Different levels of lignin reduction were seen between the different lines. Although reduced lignin concentration was seen initially, this trend was later reversed. After 3 years in the field, an increase in lignin concentration of between 31.7 - 37.8% compared to greenhouse levels was seen in all lines. The authors postulated that the ability of these trees to upregulate lignin production, despite a genetic modification working against this, allowed the trees to survive periods of environmental stress (Stout et al. 2014).

A degree of uncertainty exists surrounding the interaction between epigenetics, pleiotropy and environmental context, and how these factors may influence transgene maintenance and expression, particularly under variable environmental conditions. How such effects on transgene expression may extend to other organisms in the trees' ecosystems are also unknown. Inclusion of multiple (stacked transgenes) may add an additional dimension to this issue.

4.3.3 Effects on non-target organisms

Considering the role of trees as foundation organisms in forest ecosystems, their capacity to influence other organisms is extensive (Whitham et al. 2006). Such effects are often associated with insect resistance traits which may affect non-target insects. However, other genetic modifications (such as traits for lignin reduction, sterility, and antibiotic resistance markers) may affect plant material quality which in turn may affect organisms which interact with transgenic trees (Hjältén et al. 2008). Aside from affecting herbivores which feed directly on transgenic tree tissues, the predators which prey on them may also be affected. Such disruptions of community structure may lead to secondary pest development and loss of ecosystem functions (Axelsson et al. 2011b).

Effects on non-target organisms: Insect resistance traits

In order to curb losses caused by pest insects, insect resistance traits from the suite of Bt toxins have been inserted into some transgenic trees. They have been employed against insects of the orders Lepidoptera (Cry1Ac (Hu et al. 2001), Cry1Aa (Kleiner et al. 2003)) and Coleoptera (Cry3Aa (Zhang et al. 2011, Axelsson et al. 2011b, Axelsson, Hjältén & LeRoy 2012, Hjältén et al. 2012, Génissel et al. 2003)). These studies comprise both field studies and laboratory feeding trials.

Presence-absence tests were performed on Cry3Aa expressing aspen trees under semi-natural field conditions in Sweden. Although the Bt trees experienced a significant reduction in damage, it was noted that *Bycticus populi* individuals of the order Coleoptera (which is the target order for Cry3Aa) were present in similar numbers on both Bt and control lines, and utilized all lines equally for feeding and oviposition. This suggests potential for secondary pest development for this insect (Axelsson, Hjältén & LeRoy 2012). Chinese field tests of a different line, but also expressing a version Cry3A, found that arthropod communities were similar between transgenic and control stands. The authors did, however, indicate the need to perform such studies over a longer time period and a larger plantation area, in order for these results to be more representative of actual field conditions (Zhang et al. 2011).

Axelsson et al showed that the non-target generalist slugs (*Deroceras reticulatum* and *D. agreste*) actually preferred mature Cry3Aa-expressing aspen leaves over those of the control. This was not the case for younger leaves (Axelsson et al. 2011b). During feeding trials of Cry1Aa-expressing poplar, leaf consumption by gypsy moth (*Lymantria dispar*) increased as phenolic glycoside content of the leaves decreased as the leaves matured. In this study, the effect of the Cry1Aa protein was, at best, additive to the trees' natural defences (Kleiner et al. 2003). Both studies found that there was an interaction between leaf ontogeny and Bt proteins, which affected the phytophagous organisms. Phytochemistry between leaves at various stages of maturity has been found to differ, to the point that some herbivores tend to be associated with specific ontogenic stages (Axelsson et al. 2011b). The studies discussed above indicate that the effectiveness of the transgenes may be affected by such changes in phytochemistry, and suggest that transgenes enter into a complex system of pre-existing interactions between trees and the environment and various organisms. How transgenes may affect (or be affected by) changes in phytochemistry and other traits over longer periods of time, or under conditions of stress, may require additional investigation.

Effects on non-target organisms: Non-resistance transgenes

Traits other than those for insect resistance may also have non-target effects on organisms. These may be directly due to changes in tissue quality brought about by modifications such as reduced lignin traits, or due to pleiotropic effects which cause alteration in the expression of other genes (Hjältén et al. 2007) (some of which are discussed in a previous section).

Deroceras reticulatum
Photo: Dollarphotoclub 15596007/Zbyszek Nowak

Altered lignin biosynthesis has received a great deal of attention as a transgenic trait, mainly because of the improvements to the cellulose production process which it offers the pulp and paper and biofuels industries, specifically reduced

use of toxic chemicals to separate lignin from cellulose fibres (Pilate et al. 2002, Li, Weng & Chapple 2008). There are several enzymes whose genes are targeted in order to reduce or alter lignin biosynthesis. These include 4CL (4(hydroxyl)cinnamoyl CoA ligase), CCoAOMT (caffeoyl CoA O-methyl transferase), COMT (caffeate/5-hydroxyferulate O-methyl transferase) and CAD (cinnamyl alcohol dehydrogenase) (Li, Weng & Chapple 2008). Lignin reduction is brought about by inserting antisense transgenes to reduce expression of these genes (Brodeur-Campbell et al. 2006, Pilate et al. 2002). In addition to its role as a structural component of cell walls, lignin is related to trees' defence mechanisms against pathogens (Bagniewska-Zadworna et al. 2014) and some insects (Wainhouse, Cross & Howell 1990). Thus, by altering the lignin character of the tree, its interactions with herbivores, pests and pathogens may be altered. This does not preclude the influence of pleiotropic effects caused by the insertion of the transgene, which may affect these interactions in ways not directly related to lignin content and composition (Brodeur-Campbell et al. 2006).

Silver birch is an economically important tree species in the Nordic region. Leaves of silver birch plants transformed with antisense COMT constructs, as well as those of an untransformed control, were fed to boreal herbivores of the orders Lepidoptera and Coleoptera. Transgenic leaves were preferred over those of the control by *Aethalura punctulata* and *Cleora cintaria* larvae, while the other insects did not favour any particular leaves. *A. punctulata* grew best on leaves of the transgenic line with the lowest lignin content, though the other insects' relative growth rates did not differ between the lines. The feeding preferences were not directly linked to lignin content, since although the ratio of lignin monomers was altered in two of the transgenic lines, no significant reduction in lignin content (compared to the control) was induced in any of the four transgenic lines. The authors ascribed the differences in feeding preference to either pleiotropy or natural variation (Tiimonen et al. 2005).

A four-year multi-site field trial of poplars carrying antisense transgenes which down-regulated CAD and COMT expression was performed by Pilate et al. At neither the French, nor the British locations, did they detect significant differences in insect interactions, soil microbial community composition or differences in incidence of rust between transgenic lines and control lines (Pilate et al. 2002). However, in a later study involving these same events, as well as two other altered lignin constructs (CCoAOMT and CCR), Blomberg found that susceptibility to the rust fungus *Melampsora populnea* differed significantly between all of the transgenic events and their controls, as well as between different lines containing the same construct. In all cases, the transgenic lines were less susceptible to the fungus than the relevant control trees (Blomberg 2007). An explanation for the differences in rust susceptibility at the two different time periods was not offered.

Transgenes which deliberately confer reduced susceptibility to fungal pathogens have been investigated. There has been particular interest in developing chestnut (*Castanea dentate*) resistant to blight fungus (*Cryptophonectria parasitica*), and elm (*Ulmus americana*) resistant to Dutch elm disease fungus (*Ophiostoma novo-ulmi*) as a possible means of restoring populations which have been decimated by these pathogens (Newhouse et al. 2007, Merkle et al. 2007). Transgenic chestnuts expressing a gene coding for oxalate oxidase, which reduces susceptibility to blight, were investigated for potential effects on three generalist foliovores. Of the three, only one was affected by the modification: gypsy moth larval growth was boosted on transgenic chestnuts compared to the control. The lack of more than one transgenic line in this study means that at present, it is not

possible to determine whether this result was due to expression of the oxalate oxidase gene, or was due to unintended effects caused by transgene insertion (Post, Parry 2011).

Soil microbes

Soil microorganisms perform invaluable ecosystem services, such as nitrogen fixation, decomposition and phosphorous acquisition. Soil microbiota often exist in close associations with plants, which may be beneficial, such as symbiosis between plants ectomycorrhizal fungi; or harmful, as in the case of pathogens (Van Der Heijden, Marcel GA, Bardgett & Van Straalen 2008). Plants – as well as any agricultural practices which are associated with crops – may also have the capacity to influence the microbial community. There is concern that transgenic plants, especially transgenic trees, may negatively influence the structure and functioning of the rhizosphere community (Lottmann et al. 2010). In addition to concerns regarding the disruption of ecosystem functions, there is concern that the transgenes of genetically modified plants may be taken up by the soil microbial community via horizontal gene transfer (HGT) (Zhang, Hampp & Nehls 2005).

A number of investigations into the possible effects of transgenic trees on soil microbial community structure have been done. In most cases, microbial community composition remained relatively unaffected by the growth of transgenic trees in their midst (Kaldorf et al. 2002, Stefani et al. 2009, Stefani et al. 2010, Lamarche, Hamelin 2007, D'Amico et al. 2015). When significant differences were noted, they tended to be slight, inconsistent and of temporary duration. For example: two lines of poplar, genetically modified to have altered cytokine levels, displayed microbial community structures which differed from the control, but during two months of the experiment only (Nam et al. 2014). A Canadian study detected significant differences in microbial communities associated with white spruce trees expressing the Bt transgene *Cry1Ab* in conjunction with *uidA* and *nptII*, as well as only *uidA* and *nptII*. However, this study was limited to one sampling date, so could not determine whether these changes had become established, or were of transient duration (LeBlanc, Hamelin & Filion 2007). Denaturing gradient gel electrophoresis (DGGE) revealed significant differences among the Alphaproteobacteria and Actinobacteria groups in soil associated with transgenic pines expressing *leafy* and *nptII* transgenes. These differences were not considered large enough to have a significant impact on microbial functions, however (Lottmann et al. 2010).

Often, the larger impact of factors such as soil type, season and tree genotype dwarf the variations in soil microbiota seen between transgenic trees and control trees. Differences in tree genotype (regardless of genetic modification or lack thereof) were associated with variances in ectomycorrhizal colonization of silver birch (Pasonen et al. 2009) and poplar (Danielsen et al. 2013), for instance. However, it is also possible that parameters such as location/soil type can affect the degree of influence of transgenic trees on soil communities. Bradley et al. (2007) noted that the microbial communities of three different soils responded differently to the same lines of transgenic (reduced lignin) aspen. Nam et al. (2014) also observed that differences in rhizosphere community structure between transgenic poplar lines and the control were most prominent at one of their three sites. Such studies indicate the importance of considering multiple environmental contexts when evaluating the potential effects of transgenic trees on the soil microbiome and associated ecosystem functions (Bradley et al. 2007, Nam et al. 2014).

Horizontal gene transfer (HGT) of transgenes carrying antibiotic or herbicide resistance traits could have potential negative impacts for human (and animal) health and the environment, respectively

(Zhang, Hampp & Nehls 2005, Keese 2008). Due to their close association with roots and root exudates, ectomycorrhizal fungi have been considered as potential recipients of transgenes via HGT. An investigation of 35 000 ectomycorrhizal fungi isolates, grown in association with transgenic poplar carrying the *bar* herbicide resistance gene, was conducted to determine whether HGT to the fungi had taken place. Although 102 of these passed the initial selection procedure by growing on selective agar plates, none were able to grow after transfer to fresh selective media. PCR of DNA isolated from these fungi confirmed that none contained the *bar* transgene (Zhang, Hampp & Nehls 2005). After a similar screening procedure using kanamycin enriched media, Lu et al (2014) were likewise unable to detect transgenes in the genomic DNA of bacteria which had been growing in close contact with poplar carrying the *DREB* (saline tolerance) and *nptII* transgene for 7 years (Lu et al. 2014).

While these negative results are encouraging, it is possible that the chosen methodology is causing possible microbial transformants to be overlooked. Screening procedures which rely on culturing - such as that employed in the studies mentioned above, for example - restrict detection to those organisms which are amenable to being cultured (Nielsen, Townsend 2004). This approach, while valuable, excludes a large portion of microorganisms – the majority, in fact (Stewart 2012). This should be taken into account when interpreting the results. Culture-independent metagenomic antibiotic screening techniques (Seville et al. 2009), may be adjustable for use in screening soil microbes.

Aquatic organisms

The focus of investigations on non-target effects due to transgenic plants tends to be on terrestrial ecosystems and organisms, particularly those in direct contact with the plants. Less attention has been given to aquatic ecosystems, which represents a gap in environmental risk assessment (Carstens et al. 2012). Plant detritus from riparian vegetation is an important energy and nutrient input in many aquatic ecosystems – particularly in low order streams where primary production may be limited due to shading (Vannote et al. 1980). Quality of plant detritus therefore has the potential to affect aquatic organisms which consume it directly, as well as others in the aquatic food web which prey on these consumers. Differences in quality of leaf litter entering streams has been shown to cause measurable effects on the aquatic community. Such differences have been noted even for genetic variation among trees of the same species (LeRoy et al. 2007, LeRoy et al. 2006). Entry of material from transgenic trees (particularly those expressing insecticidal toxins) into adjacent streams, may thus affect aquatic ecosystems.

Since commercialisation of most transgenic tree varieties has yet to take place, an opportunity exists to perform pre-release evaluations of transgenic events on aquatic ecosystems – in contrast to the post-release investigations conducted several years after transgenic maize was commercialised, for example (Axelsson et al. 2011a). Studies were conducted on transgenic poplar carrying the Bt transgene (Axelsson et al. 2011a) as well as lignin-modification transgenes (Axelsson et al. 2010) - *Populus tremula* x *Populus tremuloides* and *Populus tremula* x *Populus alba*, respectively. In both investigations, evidence was seen which suggested that genetic modifications in trees could affect aquatic ecosystems. These examples are discussed in more detail below.

Two lines transformed with a *Cry3Aa* transgene were investigated. Leaves from both transgenic lines, as well as from an untransformed isogenic control were placed in litter bags and placed in the

Tavelån stream in Sweden. Analysis of invertebrate community structure in the litter bags after periods of exposure showed an increase in abundance of aquatic invertebrates in the litter bags containing the Bt leaves. This increase was 33% and 25% for the two Bt lines. However, this difference was ascribed to the number of species encountered, and not to a major shift in species composition. The analyses also revealed that different species respond differently to the different events. The community assemblages of the Bt events were similar to each other, while both differed from the control. Twenty six phytochemical variables were analysed, which showed no significant correlation to the differences in insect community. Given the varying response of different species, the ecological implications of this study could not be determined by the authors (Axelsson et al. 2011a).

Two reduced lignin lines were also tested along with an isogenic control. One of the transgenic lines suppressed the CAD enzyme, while the other suppressed the COMT enzyme. After exposure to different streams, litter bags containing leaves from the three hybrids were assessed. In terms of invertebrate assembly and abundance, no significant differences could be detected between the litter types. However, it was noted that the litter of the CAD event decomposed at a rate significantly slower than either the other transgenic event or the control. The mechanism underlying this result is not known at present, although several factors may have played a role. For example, differences in secondary compound composition were suggested as an explanation – however, in this case the control and CAD event were more similar in composition than the control and the COMT event were. Another possible explanation is that the differences in decomposition rate were not due to insect action, but to interactions with the microbial population which are incompletely understood at this point (Axelsson et al. 2010).

The Axelsson studies were among the only examples which analysed the possible effects of transgenic trees on aquatic ecosystems. This dearth of information was noted by Close in an investigational report to the Minnesota Department of Natural Resources in 2005. Clearly there is a need for further investigation, involving other tree species, transgenic events and aquatic environments. Additionally, possible effects on organisms at higher trophic levels (e.g. fish) should be investigated.

4.3.4 Combinatorial effects with pesticides

Combinatorial effects between transgenic crops and herbicide co-technologies is discussed in chapter one of this report. How transgenic trees might interact with herbicides and insecticides, and how such interactions may affect the ecological interactions between the trees and organisms in their environment is a subject for which there is currently a paucity of information.

4.4 Directions for further studies

A summary of the uncertainties pertaining to the environmental effects of transgenic trees is given below.

Gene flow: Not enough is known about the reproductive behaviour of GM trees – most gene flow studies have been done on unmodified trees, and it is difficult for models to account for the possible advantages conferred by transgenic traits (especially when there is selective pressure). There is a need to better understand how environmental context influences the establishment and spread of transgenic progeny. Furthermore, few data are available regarding the efficacy of containment strategies under varying environmental conditions. An integrated approach, including laboratory-based studies, field work and modelling, may lead to improved knowledge in this area.

Pleiotropic and Epigenetic effects: the ability of these effects to influence both transgene stability and unintentionally affect the interaction between transgenic trees and other organisms has been noted in several studies, but is as yet incompletely understood. While a broader understanding of how pleiotropy and epigenetics can influence transgene expression is needed, specific knowledge regarding different events and tree lines should be obtained. Generalisations are inadvisable since effects are often event-specific. There is also a lack of knowledge regarding how these effects manifest under varying environmental conditions and triggers. Holistic investigations which take both the molecular and environmental context into account may help to close this knowledge gap. Next generation sequencing and proteomics, coupled with field trials and feeding studies, may better illuminate these issues.

Non-target effects: There is a lack of long-term studies documenting the interaction between transgenic trees and other organisms in their environment, as well as potential development of pest resistance and secondary pest development. Furthermore, the possibility that changes in environmental conditions may influence transgene stability and expression over time, which may in turn affect the trees' ecological interactions, also creates a need for longer term investigations. Field trials of longer duration, and possibly also long term monitoring of commercially grown transgenic trees, should be done.

Very few data are available regarding possible effects of transgenic trees on aquatic ecosystems. Considering the influence that riparian vegetation has on streams, especially low order streams, this ecological interaction should not continue to be overlooked. There is also a general lack of data regarding sub-lethal effects (fecundity, larval duration etc.) of transgenic trees on non-target organisms, as well as multi-trophic level effects in both aquatic and terrestrial ecosystems. In addition, possible combinatorial effects of transgenic trees when used in conjunction with herbicides and pesticides, as well as trees containing stacked events where multiple insect resistance and/or herbicide tolerance genes are inserted, have yet to be thoroughly investigated. As mentioned in the previous paragraph, field trials representative of actual cultivation conditions should be done. These should be supplemented by laboratory investigations, particularly where sub-lethal effects are concerned.

Lastly, potential incidences of HGT may possibly be overlooked by culture-dependent techniques currently in use. Next generation sequencing and metagenomics-based approaches to screening for potential incidences of HGT could be useful in determining whether this is the case.

A common theme which can be noted throughout this chapter, is the need for more field trials, of longer duration and which more closely mimic the conditions under which transgenic tree plantations will grow (Zhang et al. 2011, LeBlanc, Hamelin & Filion 2007). Farnum et al summarised this very neatly: "Regardless of what trait is being assessed, trees must be monitored for transgene expression and phenotypic stability under field conditions, in a variety of environments, using several transgenic lines (independent events), and over many years (i.e., after several dormancy cycles). In addition to baseline and year-to-year measurements, the potential for seasonal changes in expression also should be examined" (Farnum, Lucier & Meilan 2007). In most cases, commercialisation of transgenic trees has yet to take place. Thus there is a window for more research to be done, and an opportunity for many of the epistemic uncertainties noted above to become better understood, before large scale cultivation takes place.

In the event of commercialisation/large scale cultivation of transgenic trees, long term monitoring of transgene stability, insect pressure and environmental effects may be advisable. As mentioned in a previous chapter of this report, it is not possible to account for all variables when conducting investigations involving complex, changing biological and ecological systems such as trees and ecosystems. It is possible that unforeseen effects may manifest themselves several years into a tree's life cycle, in response to factors such as climate change, water shortage, pollution etc. Long term monitoring should be considered in order to better understand the interaction of transgenic trees and their environments.

References

AHUJA, M.R., 2009. Transgene stability and dispersal in forest trees. *Trees,* 23(6), pp. 1125-1135.

AXELSSON, E.P., HJÄLTÉN, J. and LEROY, C.J., 2012. Performance of insect-resistant Bacillus thuringiensis (Bt)-expressing aspens under semi-natural field conditions including natural herbivory in Sweden. *Forest Ecology and Management,* 264, pp. 167-171.

AXELSSON, E.P., HJÄLTÉN, J., LEROY, C.J., JULKUNEN-TIITTO, R., WENNSTRÖM, A. and PILATE, G., 2010. Can leaf litter from genetically modified trees affect aquatic ecosystems? *Ecosystems,* 13(7), pp. 1049-1059.

AXELSSON, E.P., HJÄLTÉN, J., LEROY, C.J., WHITHAM, T.G., JULKUNEN-TIITTO, R. and WENNSTRÖM, A., 2011a. Leaf litter from insect-resistant transgenic trees causes changes in aquatic insect community composition. *Journal of Applied Ecology,* 48(6), pp. 1472-1479.

AXELSSON, E.P., HJÄLTÉN, J., WHITHAM, T.G., JULKUNEN-TIITTO, R., PILATE, G. and WENNSTRÖM, A., 2011b. Leaf ontogeny interacts with Bt modification to affect innate resistance in GM aspens. *Chemoecology,* 21(3), pp. 161-169.

AXELSSON, P., 2011. Target and non-target effects of genetically modified trees, Swedish University of Agricultural Sciences.

BAGNIEWSKA-ZADWORNA, A., BARAKAT, A., ŁAKOMY, P., SMOLIŃSKI, D.J. and ZADWORNY, M., 2014. Lignin and lignans in plant defence: Insight from expression profiling of cinnamyl alcohol dehydrogenase genes during development and following fungal infection in Populus. *Plant Science,* 229, pp. 111-121.

BARBOUR, R.C., WISE, S.L., MCKINNON, G.E., VAILLANCOURT, R.E., WILLIAMSON, G.J. and POTTS, B.M., 2010. The potential for gene flow from exotic eucalypt plantations into Australia's rare native eucalypts. *Forest Ecology and Management,* 260(12), pp. 2079-2087.

BEHERA, K.K., 2014. Phytoremediation, transgenic plants and microbes. *Sustainable Agriculture Reviews.* Springer, pp. 65-85.

BHASKAR, P.B. and JIANG, J., 2010. Silencing as a Tool for Transgenic Crop Improvement. *Transgenic Crop Plants.* Springer, pp. 187-199.

BHATTACHARYA, A., SAINI, U. and AHUJA, P.S., 2006. Transgenic tea. *Inter J Tea Sci,* 5, pp. 39-52.

BIALOZYT, R., 2012. Gene flow in poplar-experiments, analysis and modeling to prevent transgene outcrossing. *iForest-Biogeosciences and Forestry,* 5(3), pp. 147.

BLOMBERG, P., 2007. Non-target Effects of Genetically Modified Trees.

BRADLEY, K.L., HANCOCK, J.E., GIARDINA, C.P. and PREGITZER, K.S., 2007. Soil microbial community responses to altered lignin biosynthesis in Populus tremuloides vary among three distinct soils. *Plant and Soil,* 294(1-2), pp. 185-201.

BRÄUTIGAM, K., VINING, K.J., LAFON-PLACETTE, C., FOSSDAL, C.G., MIROUZE, M., MARCOS, J.G., FLUCH, S., FRAGA, M.F., GUEVARA, M. and ABARCA, D., 2013. Epigenetic regulation of adaptive responses of forest tree species to the environment. *Ecology and evolution,* 3(2), pp. 399-415.

BROCKERHOFF, E.G., JACTEL, H., PARROTTA, J.A. and FERRAZ, S.F., 2013. Role of eucalypt and other planted forests in biodiversity conservation and the provision of biodiversity-related ecosystem services. *Forest Ecology and Management,* 301, pp. 43-50.

BRODEUR-CAMPBELL, S.E., VUCETICH, J.A., RICHTER, D.L., WAITE, T.A., ROSEMIER, J.N. and TSAI, C., 2006. Insect herbivory on low-lignin transgenic aspen. *Environmental Entomology,* 35(6), pp. 1696-1701.

BRUNNER, A.M., LI, J., DIFAZIO, S.P., SHEVCHENKO, O., MONTGOMERY, B.E., MOHAMED, R., WEI, H., MA, C., ELIAS, A.A. and VANWORMER, K., 2007. Genetic containment of forest plantations. *Tree Genetics & Genomes,* 3(2), pp. 75-100.

CARSTENS, K., ANDERSON, J., BACHMAN, P., DE SCHRIJVER, A., DIVELY, G., FEDERICI, B., HAMER, M., GIELKENS, M., JENSEN, P. and LAMP, W., 2012. Genetically modified crops and aquatic ecosystems: considerations for environmental risk assessment and non-target organism testing. *Transgenic research,* 21(4), pp. 813-842.

D'AMICO, K.M., HORTON, T.R., MAYNARD, C.A., STEHMAN, S.V., OAKES, A.D. and POWELL, W.A., 2015. Comparisons of ectomycorrhizal colonization of transgenic american chestnut with those of the wild type, a conventionally bred hybrid, and related fagaceae species. *Applied and Environmental Microbiology,* 81(1), pp. 100-108.

DANIELSEN, L., LOHAUS, G., SIRRENBERG, A., KARLOVSKY, P., BASTIEN, C., PILATE, G. and POLLE, A., 2013. Ectomycorrhizal colonization and diversity in relation to tree biomass and nutrition in a plantation of transgenic poplars with modified lignin biosynthesis. *PloS one,* 8(3), pp. e59207.

DIETZ-PFEILSTETTER, A., 2010. Stability of transgene expression as a challenge for genetic engineering. *Plant science,* 179(3), pp. 164-167.

DIFAZIO, S.P., LEONARDI, S., SLAVOV, G.T., GARMAN, S.L., ADAMS, W.T. and STRAUSS, S.H., 2012. Gene flow and simulation of transgene dispersal from hybrid poplar plantations. *New Phytologist,* 193(4), pp. 903-915.

DOTY, S.L., JAMES, C.A., MOORE, A.L., VAJZOVIC, A., SINGLETON, G.L., MA, C., KHAN, Z., XIN, G., KANG, J.W., PARK, J.Y., MEILAN, R., STRAUSS, S.H., WILKERSON, J., FARIN, F. and STRAND, S.E., 2007. Enhanced phytoremediation of volatile environmental pollutants with transgenic trees. *Proceedings of the National Academy of Sciences of the United States of America,* 104(43), pp. 16816-16821.

EWALD, D., HU, J. and YANG, M., 2006. Transgenic forest trees in China. *Tree Transgenesis.* Springer, pp. 25-45.

FARNUM, P., LUCIER, A. and MEILAN, R., 2007. Ecological and population genetics research imperatives for transgenic trees. *Tree Genetics & Genomes,* 3(2), pp. 119-133.

FLADUNG, M. and KUMAR, S., 2002. Gene Stability in Transgenic Aspen-Populus-III. T-DNA Repeats Influence Transgene Expression Differentially among Different Transgenic Lines. *Plant biology,* 4(3), pp. 329-338.

FLADUNG, M., HOENICKA, H. and AHUJA, M.R., 2013. Genomic stability and long-term transgene expression in poplar. *Transgenic research,* 22(6), pp. 1167-1178.

FLADUNG, M., NOWITZKI, O., ZIEGENHAGEN, B. and KUMAR, S., 2003. Vegetative and generative dispersal capacity of field released transgenic aspen trees. *Trees,* 17(5), pp. 412-416.

FUCHS, M. and GONSALVES, D., 2007. Safety of Virus-Resistant Transgenic Plants Two Decades After Their Introduction: Lessons from Realistic Field Risk Assessment Studies*. *Annu.Rev.Phytopathol.,* 45, pp. 173-202.

GÉNISSEL, A., LEPLÉ, J., MILLET, N., AUGUSTIN, S., JOUANIN, L. and PILATE, G., 2003. High tolerance against Chrysomela tremulae of transgenic poplar plants expressing a synthetic cry3Aa gene from Bacillus thuringiensis ssp tenebrionis. *Molecular Breeding,* 11(2), pp. 103-110.

GRÜNWALD, C., RUEL, K., JOSELEAU, J. and FLADUNG, M., 2001. Morphology, wood structure and cell wall composition of rolC transgenic and non-transformed aspen trees. *Trees,* 15(8), pp. 503-517.

HÄGGMAN, H., SUTELA, S., WALTER, C. and FLADUNG, M., 2014. Biosafety Considerations in the Context of Deployment of GE Trees. *Challenges and Opportunities for the World's Forests in the 21st Century.* Springer, pp. 491-524.

HARFOUCHE, A., MEILAN, R. and ALTMAN, A., 2011. Tree genetic engineering and applications to sustainable forestry and biomass production. *Trends in biotechnology,* 29(1), pp. 9-17.

HJÄLTÉN, J., AXELSSON, E.P., WHITHAM, T.G., LEROY, C.J., JULKUNEN-TIITTO, R., WENNSTRÖM, A. and PILATE, G., 2012. Increased resistance of Bt aspens to Phratora vitellinae (Coleoptera) leads to increased plant growth under experimental conditions. *PloS one,* 7(1), pp. e30640.

HJÄLTÉN, J., LINDAU, A., WENNSTRÖM, A., BLOMBERG, P., WITZELL, J., HURRY, V. and ERICSON, L., 2007. Unintentional changes of defence traits in GM trees can influence plant–herbivore interactions. *Basic and Applied Ecology,* 8(5), pp. 434-443.

HJÄLTÉN, J., LINDAU, A., WENNSTRÖM, A., BLOMBERG, P., WITZELL, J., HURRY, V., ERICSON, L., MORITZ, T. and KARLSSON, J., 2008. Vole response to unintentional changes in the chemistry of GM poplars. *Chemoecology,* 18(4), pp. 227-231.

HODGKIN, J., 1998. Seven types of pleiotropy. *International Journal of Developmental Biology,* 42, pp. 501-505.

HOENICKA, H. and FLADUNG, M., 2006. Biosafety in Populus spp. and other forest trees: from non-native species to taxa derived from traditional breeding and genetic engineering. *Trees,* 20(2), pp. 131-144.

HU, J., TIAN, Y., HAN, Y., LI, L. and ZHANG, B., 2001. Field evaluation of insect-resistant transgenic Populus nigra trees. *Euphytica,* 121(2), pp. 123-127.

HUR, M., KIM, Y., SONG, H.R., KIM, J.M., CHOI, Y.I. and YI, H., 2011. Effect of genetically modified poplars on soil microbial communities during the phytoremediation of waste mine tailings. *Applied and Environmental Microbiology,* 77(21), pp. 7611-7619.

ISAAA, 2015-last update, ISAAA's GM Approval Database. . Available: http://www.isaaa.org/gmapprovaldatabase/. [23/03/2015, 2015].

KALDORF, M., FLADUNG, M., MUHS, H. and BUSCOT, F., 2002. Mycorrhizal colonization of transgenic aspen in a field trial. *Planta,* 214(4), pp. 653-660.

KEESE, P., 2008. Risks from GMOs due to horizontal gene transfer. *Environmental Biosafety Research,* 7(03), pp. 123-149.

KHANAM, T., MATERO, J., MOLA-YUDEGO, B., SIKANEN, L. and RAHMAN, A., 2014. Assessing external factors on substitution of fossil fuel by biofuels: model perspective from the Nordic region. *Mitigation and Adaptation Strategies for Global Change,* , pp. 1-16.

KLEINER, K.W., ELLIS, D.D., MCCOWN, B.H. and RAFFA, K.F., 2003. Leaf ontogeny influences leaf phenolics and the efficacy of genetically expressed Bacillus thuringiensis cry1A (a) d-endotoxin in hybrid poplar against gypsy moth. *Journal of chemical ecology,* 29(11), pp. 2585-2602.

KOHLI, A., MIRO, B. and TWYMAN, R.M., 2010. Transgene integration, expression and stability in plants: strategies for improvements. *Transgenic crop plants.* Springer, pp. 201-237.

KUMAR, S. and FLADUNG, M., 2001. Gene stability in transgenic aspen (Populus). II. Molecular characterization of variable expression of transgene in wild and hybrid aspen. *Planta,* 213(5), pp. 731-740.

LAMARCHE, J. and HAMELIN, R.C., 2007. No evidence of an impact on the rhizosphere diazotroph community by the expression of Bacillus thuringiensis Cry1Ab toxin by Bt white spruce. *Applied and Environmental Microbiology,* 73(20), pp. 6577-6583.

LEBLANC, P.M., HAMELIN, R.C. and FILION, M., 2007. Alteration of soil rhizosphere communities following genetic transformation of white spruce. *Applied and Environmental Microbiology,* 73(13), pp. 4128-4134.

LEROY, C.J., WHITHAM, T.G., KEIM, P. and MARKS, J.C., 2006. Plant genes link forests and streams. *Ecology,* 87(1), pp. 255-261.

LEROY, C.J., WHITHAM, T.G., WOOLEY, S.C. and MARKS, J.C., 2007. Within-species variation in foliar chemistry influences leaf-litter decomposition in a Utah river. *Journal of the North American Benthological Society,* 26(3), pp. 426-438.

LI, X., WENG, J. and CHAPPLE, C., 2008. Improvement of biomass through lignin modification. *The Plant Journal,* 54(4), pp. 569-581.

LINACRE, N.A. and ADES, P.K., 2004. Estimating isolation distances for genetically modified trees in plantation forestry. *Ecological Modelling,* 179(3), pp. 247-257.

LOTTMANN, J., O'CALLAGHAN, M., BAIRD, D. and WALTER, C., 2010. Bacterial and fungal communities in the rhizosphere of field-grown genetically modified pine trees (Pinus radiata D.). *Environmental biosafety research,* 9(01), pp. 25-40.

LU, N., WEI, B., SUN, Y., LIU, X., CHEN, S., ZHANG, W., ZHANG, Y. and LI, Y., 2014. Field Supervisory Test of DREB-Transgenic Populus: Salt Tolerance, Long-Term Gene Stability and Horizontal Gene Transfer. *Forests,* 5(5), pp. 1106-1121.

MEILAN, R., HAN, K., MA, C., DIFAZIO, S.P., EATON, J.A., HOIEN, E.A., STANTON, B.J., CROCKETT, R.P., TAYLOR, M.L. and JAMES, R.R., 2002. The CP4 transgene provides high levels of tolerance to Roundup® herbicide in field-grown hybrid poplars. *Canadian Journal of Forest Research,* 32(6), pp. 967-976.

MERKLE, S., ANDRADE, G., NAIRN, C., POWELL, W. and MAYNARD, C., 2007. Restoration of threatened species: a noble cause for transgenic trees. *Tree Genetics & Genomes,* 3(2), pp. 111-118.

NAM, K.J., NAM, K., PACK, I.S., PARK, J.H., JEONG, S., IM CHOI, Y., NOH, E.W. and KIM, C., 2014. Effects of transgenic poplars expressing increased levels of cellular cytokinin on rhizosphere microbial communities. *European Journal of Soil Biology,* 63, pp. 78-82.

NEWHOUSE, A.E., SCHRODT, F., LIANG, H., MAYNARD, C.A. and POWELL, W.A., 2007. Transgenic American elm shows reduced Dutch elm disease symptoms and normal mycorrhizal colonization. *Plant Cell Reports,* 26(7), pp. 977-987.

NICOTRA, A.B., ATKIN, O.K., BONSER, S.P., DAVIDSON, A.M., FINNEGAN, E., MATHESIUS, U., POOT, P., PURUGGANAN, M.D., RICHARDS, C. and VALLADARES, F., 2010. Plant phenotypic plasticity in a changing climate. *Trends in plant science,* 15(12), pp. 684-692.

NIELSEN, K.M. and TOWNSEND, J.P., 2004. Monitoring and modeling horizontal gene transfer. *Nature biotechnology,* 22(9), pp. 1110-1114.

NORWEGIAN MINISTRY OF THE ENVIRONMENT, 2012. *Norwegian climate policy: Report No. 21 (2011–2012) to the Storting (white paper) Summary.* 21. Norway: Norwegian Ministry of the Environment.

OGUCHI, T., KASHIMURA, Y., MIMURA, M., YU, X., MATSUNAGA, E., NANTO, K., SHIMADA, T., KIKUCHI, A. and WATANABE, K.N., 2014. A multi-year assessment of the environmental impact of transgenic Eucalyptus trees harboring a bacterial choline oxidase gene on biomass, precinct vegetation and the microbial community. *Transgenic research,* 23(5), pp. 767-777.

PASONEN, H., LU, J., NISKANEN, A., SEPPÄNEN, S., RYTKÖNEN, A., RAUNIO, J., PAPPINEN, A., KASANEN, R. and TIMONEN, S., 2009. Effects of sugar beet chitinase IV on root-associated fungal community of transgenic silver birch in a field trial. *Planta,* 230(5), pp. 973-983.

PILATE, G., GUINEY, E., HOLT, K., PETIT-CONIL, M., LAPIERRE, C., LEPLÉ, J., POLLET, B., MILA, I., WEBSTER, E.A. and MARSTORP, H.G., 2002. Field and pulping performances of transgenic trees with altered lignification. *Nature biotechnology,* 20(6), pp. 607-612.

POST, K. and PARRY, D., 2011. Non-target effects of transgenic blight-resistant American chestnut (Fagales: Fagaceae) on insect herbivores. *Environmental Entomology,* 40(4), pp. 955-963.

SEDJO, R., 2011. Transgenic trees for biomass: the effects of regulatory restrictions and court decisions on the pace of commercialization.

SÉGUIN, A., LACHANCE, D., DÉJARDIN, A., LEPLÉ, J. and PILATE, G., 2014. Scientific research related to genetically modified trees. *Challenges and Opportunities for the World's Forests in the 21st Century.* Springer, pp. 525-548.

SEVILLE, L.A., PATTERSON, A.J., SCOTT, K.P., MULLANY, P., QUAIL, M.A., PARKHILL, J., READY, D., WILSON, M., SPRATT, D. and ROBERTS, A.P., 2009. Distribution of tetracycline and erythromycin resistance genes among human oral and fecal metagenomic DNA. *Microbial Drug Resistance,* 15(3), pp. 159-166.

SLAVOV, G.T., LEONARDI, S., BURCZYK, J., ADAMS, W., STRAUSS, S. and DIFAZIO, S., 2009. Extensive pollen flow in two ecologically contrasting populations of Populus trichocarpa. *Molecular ecology,* 18(2), pp. 357-373.

STEFANI, F.O., MONCALVO, J.M., SEGUIN, A., BERUBE, J.A. and HAMELIN, R.C., 2009. Impact of an 8-year-old transgenic poplar plantation on the ectomycorrhizal fungal community. *Applied and Environmental Microbiology,* 75(23), pp. 7527-7536.

STEFANI, F.O., TANGUAY, P., PELLETIER, G., PICHE, Y. and HAMELIN, R.C., 2010. Impact of endochitinase-transformed white spruce on soil fungal biomass and ectendomycorrhizal symbiosis. *Applied and Environmental Microbiology,* 76(8), pp. 2607-2614.

STEWART, E.J., 2012. Growing unculturable bacteria. *Journal of Bacteriology,* 194(16), pp. 4151-4160.

STOUT, A.T., DAVIS, A.A., DOMEC, J., YANG, C., SHI, R. and KING, J.S., 2014. Growth under field conditions affects lignin content and productivity in transgenic Populus trichocarpa with altered lignin biosynthesis. *Biomass and Bioenergy,* 68, pp. 228-239.

STRAUSS, S.H., KERSHEN, D.L., BOUTON, J.H., REDICK, T.P., TAN, H. and SEDJO, R.A., 2010. Far-reaching deleterious impacts of regulations on research and environmental studies of recombinant DNA-modified perennial biofuel crops in the United States. *Bioscience,* 60(9), pp. 729-741.

TIAN, Y., ZHENG, J., YU, H., LIANG, H., LI, C. and WANG, J., 1999. Studies of transgenic hybrid poplar 741 carrying two insect-resistant genes. *Acta Botanica Sinica,* 42(3), pp. 263-268.

TIIMONEN, H., ARONEN, T., LAAKSO, T., SARANPÄÄ, P., CHIANG, V., YLIOJA, T., ROININEN, H. and HÄGGMAN, H., 2005. Does lignin modification affect feeding preference or growth performance of insect herbivores in transgenic silver birch (Betula pendula Roth)? *Planta,* 222(4), pp. 699-708.

TRØMBORG, E., BOLKESJØ, T.F. and SOLBERG, B., 2008. Biomass market and trade in Norway: Status and future prospects. *Biomass and Bioenergy,* 32(8), pp. 660-671.

TYSON, R.C., WILSON, J.B. and LANE, W.D., 2011. A mechanistic model to predict transgenic seed contamination in bee-pollinated crops validated in an apple orchard. *Ecological Modelling,* 222(13), pp. 2084-2092.

VAN ACKER, R., LEPLE, J.C., AERTS, D., STORME, V., GOEMINNE, G., IVENS, B., LEGEE, F., LAPIERRE, C., PIENS, K., VAN MONTAGU, M.C., SANTORO, N., FOSTER, C.E., RALPH, J., SOETAERT, W., PILATE, G. and BOERJAN, W., 2014. Improved saccharification and ethanol yield from field-grown transgenic poplar deficient in cinnamoyl-CoA reductase. *Proceedings of the National Academy of Sciences of the United States of America,* 111(2), pp. 845-850.

VAN DER HEIJDEN, MARCEL GA, BARDGETT, R.D. and VAN STRAALEN, N.M., 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters,* 11(3), pp. 296-310.

VANNOTE, R.L., MINSHALL, G.W., CUMMINS, K.W., SEDELL, J.R. and CUSHING, C.E., 1980. The river continuum concept. *Canadian Journal of Fisheries and Aquatic Sciences,* 37(1), pp. 130-137.

WAINHOUSE, D., CROSS, D. and HOWELL, R., 1990. The role of lignin as a defence against the spruce bark beetle Dendroctonus micans: effect on larvae and adults. *Oecologia,* 85(2), pp. 257-265.

WALTER, C., FLADUNG, M. and BOERJAN, W., 2010. The 20-year environmental safety record of GM trees. *Nature biotechnology,* 28(7), pp. 656-658.

WHITHAM, T.G., BAILEY, J.K., SCHWEITZER, J.A., SHUSTER, S.M., BANGERT, R.K., LEROY, C.J., LONSDORF, E.V., ALLAN, G.J., DIFAZIO, S.P. and POTTS, B.M., 2006. A framework for community and ecosystem genetics: from genes to ecosystems. *Nature Reviews Genetics,* 7(7), pp. 510-523.

ZHANG, B., CHEN, M., ZHANG, X., LUAN, H., TIAN, Y. and SU, X., 2011. Expression of Bt-Cry3A in transgenic Populus alba× P. glandulosa and its effects on target and non-target pests and the arthropod community. *Transgenic research,* 20(3), pp. 523-532.

ZHANG, C., HAMPP, R. and NEHLS, U., 2005. Investigation of horizontal gene transfer in poplar/Amanita muscaria ectomycorrhizas. *Environmental biosafety research,* 4(04), pp. 235-242.

5. GM salmon

5.1 Introduction

During the last three decades, aquaculture has expanded rapidly to meet the ever-growing demand for marine foods, a demand that cannot be met by commercial fisheries, as wild fish stocks are already under high pressure (Gillund et al., 2008, Bostock, 2011, Gjedrem et al., 2012). In Norway, Atlantic salmon (*Salmo salar*) has been intensively bred for more than forty years, and is established as the most important aquaculture species and a major export product. The Norwegian domesticated salmon is marketed throughout the world as a healthy, clean, environmentally-friendly and sustainable option for consumers.

The active selection of Atlantic salmon breeding stock for over four decades has significantly improved upon growth-related traits (such as growth and feed conversion rates) and disease resistance of the domesticated salmon (Thodesen and Gjedrem, 2006, Gjedrem, 2012, Gjedrem et al., 2012). However, traditional breeding has certain limitations with regards to the progress that can be made for a specific trait within a certain time frame. Genetically modified (GM) fish intended for food production has been under development for more than three decades (Fletcher et al., 2004, Mori and Devlin, 1999), among them the various salmonid species, tilapia and carp.

The Atlantic salmon genome has recently been mapped, and the scientific community is continuously gaining a better understanding of the roles that the various genes and their products play in different physiological processes. There are many potential genetic modifications that could benefit the aquaculture industry, both economically (for example increased growth rate) and with regards to animal welfare (for example disease resistance). However, most of the envisioned modifications will require years of research and optimization before they can be applied, if successful (see table 5.1 for a non-exhaustive list of potential genetic modifications to salmon). Currently, only one GM salmon seems to be approaching commercialization. This is the AquAdvantage salmon (see textbox 5.1), an Atlantic salmon which has been modified using a genetic construct consisting of anti-freeze protein regulatory genes and a Coho salmon growth hormone (GH) gene (Entis, 1998, Fletcher et al., 2004). Substantial literature already exists on the topic of genetic modification of salmonids using genetic constructs composed from various GH coding genes and a diversity of regulatory elements (Devlin et al., 2009a, Nam et al., 2008). The literature encompasses both the intended, as well as the unexpected or unintended effects on salmon growth, physiology and behaviour. A large body of literature also exists

Atlantic salmon
Photo: Dollarphotoclub_56242757/Alexander Raths

on the potential ecological impact of GH transgenic salmon (hereafter GH TG salmon), in particular focusing on the impact on wild salmon populations. However, much of this work has been done on species of Pacific salmon and trout (*Oncorhynchus* sp., *Salmo* sp.), rather than on Atlantic salmon.

There are many uncertainties, risks and knowledge gaps connected to the process of genetic modification, predicted and unpredicted impacts on the physiology, sensory perception, cognitive abilities (see review by Brown 2015) and behaviour of the modified organism and its offspring, and possible ecological impacts of GM organisms which are released or escape into the wild. Little work has been done on the wider impacts of GM salmon on the aquaculture industry and its stakeholders (including consumers), the environment (GM Atlantic salmon as an invasive species), or the society. According to the Norwegian Gene Technology Act, these latter considerations related to social science are just as important as the natural sciences when evaluating GM salmon in a Norwegian context. The purpose of the act is to "…ensure that the production and use of GM organisms and the production of cloned animals take place in an ethically justifiable and socially acceptable manner, in accordance with the principle of sustainable development and without adverse effects on health and the environment" (regjeringen.no).

Norway is at the forefront in research on wild Atlantic salmon, aquaculture, and the impact of escaped salmon on wild populations and the environment. This research can provide important information with regards to how GM salmon will interact and behave in the wild. However, a thorough characterization of the GM salmon beyond the added trait will be essential to understand the full effect of the modification on the organism, and for predicting possible ecological impacts the GM salmon might have. As such, each new GM salmon opens up a myriad of quantitative and qualitative epistemological knowledge gaps and ontological uncertainties to be covered with regards to both the impact on the salmon itself (product quality, health, behaviour etc.) and the potential impacts of escaped GM salmon on wild salmon populations, biodiversity and the environment.

This chapter will address knowledge gaps, uncertainties and questions surrounding GM salmon, and among the issues raised are:

- effects of construct composition, site of integration and gene dosage on expression and effectiveness of the transgene
- effects of genetic background and the impact of pleiotropic and epigenetic effects on the resulting phenotype
- the importance of comparative studies

A discussion about questions related to ecological impact, and the potential containment of GM salmon is also included, focusing on:

- current knowledge about escape and survival of domestic salmon
- potential impacts on wild salmon populations
- other ecosystem impacts (on species with similar niches, potential prey, trophic interactions etc.)
- potential for physical and reproductive containment of GM salmon

Table 5.1: A non-exhaustive overview of genetic modifications that could be considered useful by the aquaculture industry

Trait	Approaches	Status	References
Growth enhancement	Growth hormone	• Various GH genetic constructs have successfully increased growth in several salmonid species • Specific effects seem to depend on the specific salmonid line and genetic construct used. • An Atlantic salmon containing such a construct is currently undergoing the approval process for commercialization in the USA. • Many pleiotropic effects on both physiology and behavior have been observed.	Devlin, Raven et al. (2009a); Hallerman (2007); Eales, Devlin et al. (2004); Rise, Douglas et al. (2006); Mori et al. (2007); Abrahams and Sutterlin (1999); Devlin, Johnsson et al. (1999); Sundström, Devlin et al. (2003); Sundström, Lõhmus et al. (2004)
	Insulin-like growth factor I (IGF-I)	• So far attempts at using IGF-I in genetic constructs for growth enhancement has failed due to highly negative impacts on the modified organism.	Devlin, Raven et al. (2009a)
	Others	• It is likely that with more knowledge of the processes regulating growth in salmon other genes could also be used in genetic constructs for growth enhancement.	
Temperature tolerance	Antifreeze proteins	• Increasing cold tolerance • Anti-freeze constructs have been inserted into salmon; however there seem to be some issues reaching a sufficient level of gene expression and protein production.	Zbikowska (2003); Entis (1998)
	Others	• Increased heath tolerance • No actual attempts so far, but genes involved in temperature tolerance are being mapped and characterized.	Perry et al. (2001); Somorjai et al. (2003)
Disease resistance	Over-expression of immune-related genes	• Overexpression of immune-related genes show promising results for increased disease resistance and lower mortality.	Devlin, Raven et al. (2009a)
	DNA vaccines	• DNA vaccines for preventing infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia (VHS) and more are in	Traxler, Anderson et al. (1999); Lorenzen, Lorenzen et al. (2001);

Trait	Approaches	Status	References
		the process of commercialization or development.	Evensen and Leong (2013)
	Others	• Gene editing could be a potential tool for changing alleles etc.	McMahon, Rahdar et al. (2012); Gaj, Gersbach et al. (2013)
Vitamin Independency	L-ascorbic acid synthetic pathway	• Teleost fish cannot synthesize vitamin C (L-ascorbic acid), and thus this must be added to their captive diet. • Attempts were made in the 90's to introduce the gene coding for L-gulono-gamma-lactone oxidase, (enzyme essential to the synthesis of vitamin C) to medaka and rainbow trout.	Toyohara, Nakata et al. (1996); Krasnov, Reinisalo et al. (1998)
Metabolism	Carbohydrates	• Salmonids are not effective utilizers of carbohydrates • Engineering alternative pathways for carbohydrate metabolism	Krasnov, Pitkänen et al. (1999); Pitkänen, Krasnov et al. (1999)
	Lipids	• Salmon only produce docosahexaenoic and eicosapentaenoic acid (DHA, EPA) endogenously to satisfy its own needs, the surplus that consumers of salmon rely on mainly comes from the salmon's marine food resources. • Modifying the salmon to produce DHA and EPA in excess would relieve the aquaculture industry of its dependence on marine feed sources for product quality. • Successful modification of zebrafish	Devlin, Raven et al. (2009a); Alimuddin, Yoshizaki et al. (2005)
Late/No maturation (sterility)	Interference with physiological pathways to delay or prevent maturation "Terminator"-technology Triploidy	• Maturation reduces animal welfare in sea pens and decreases the palatability of salmon. • Fast growth has already reduced time to maturation, and concerns have been raised that increasing sea temperatures could further influence this. • Avoiding hybridization with wild fish	Wong and Van Eenennaam (2008)

Trait	Approaches	Status	References
Flesh character-istics/product quality	Delaying tenderization process	• Tenderization of muscle tissue was delayed in medaka by inserting a genetic construct expressing an inhibitor of matrix metallo-proteinase.	Toyohara et al. (2005); Devlin, Raven et al. (2009a)
	Myostatin regulation	• Myostatin regulates muscle development, by regulating it muscle conformation and visual appeal could be altered in accordance to consumer acceptability. • Mice and zebrafish studies	Devlin, Raven et al. (2009a); Reisz-Porszasz et al. (2003a, Reisz-Porszasz et al., 2003b); Yang et al. (2006); Acosta et al. (2005); Xu et al. (2003)

5.2 Molecular Characterization

5.2.1 Transformation methods

There are three commonly used methods for introducing genetic constructs[9] to animals:

1. Transformation of embryonic stem cells.
2. Electroporation; the use of electric impulses to make cell membranes more permeable to macromolecules.
3. Transformation by microinjection where the desired gene construct is injected into a fertilized egg.

The latter is the preferred and most effective method (Wang et al., 2007, Devlin et al., 2009a) for transforming fish. However there are some issues related to the microinjection technology. Because this transformation process is random, and does not necessarily take place at the one-cell stage of embryo development, a chimeric fish, where only a portion of the cells contain the transgene, is often the results of the transformation. Due to the randomness of the insertion these chimeric fish might exhibit very different phenotypes, and the transgene might show different heritability between individuals. In order to establish a fixed genotype for study and characterization (and possibly commercialization) one must breed the modified fish over generations in order to establish a stable GM line. The initial spectre of genotypes and expressed phenotypes allows for broad starting point when selecting for the applicable pheno- and genotype for the intended purposes of the experiment.

[9] Genetic constructs can be i) Transgenic, that is the genes introduced are from species different from and not sexually compatible with the receiving organisms; ii) cisgenic, that is the genes introduced come from the organism itself or from a sexually compatible species including its original regulatory elements; iii) intragenic, that is the introduced genetic construct originated from the organism itself or a sexually compatible species, but elements can be combined form different genes, both full and partial, in any order of combinations.

Box 5.1: The AquAdvantage Atlantic salmon

The AquAdvantage salmon is a GM Atlantic salmon carrying a genetic construct consisting of the regulatory components (promotor, terminator) of the Ocean Pout (Zoarces americanus) anti-freeze gene, and a gene coding for the Chinook salmon (Oncorhynchus tshawytscha) growth hormone. There are several differences between this exogenous growth hormone genetic construct and the endogenous growth hormone gene, both in expression and regulation. The endogenous GH gene, primarily expressed in the pituitary, is regulated by a negative feedback system, and heavily down-regulated over winter when food access and water temperature is usually low. However, due to the nature of the anti-freeze promoter the exogenous gene is continuously expressed throughout the year, in a multitude of tissue types, thus keeping GH plasma levels high throughout the year and thereby increasing growth.

The development of the AquAdvantage started in the late 1980's with general research into the development of GM salmon at the Memorial University of Newfoundland. In 1989 the GH TG genetic construct mentioned above was created and the founder animal of the AquAdvantage line was engineered. During the subsequent years the AquAdvantage line was established, and the American Food and Drug Administration (FDA) was informed by A/F Protein of the intention to develop a GM line of salmon for commercial purposes in 1995. The GH TG salmon will be regulated as a new animal drug, and thus subject to the approval criteria that exist for animal drugs. In 2000 A/F Protein divided into A/F Proteins and AquaBounty Farms, AquaBounty retains the AquAdvantage technology. A few years later the name was changed to AquaBounty Technologies.

The first regulatory study of the GH TG salmon was filed with the FDA nearly a decade after the establishment of the GH TG line, and the final regulatory study was delivered to the FDA in 2009. In 2010 both the FDA and the Veterinary Medicine Advisory Committee concluded that the AquAdvantage salmon is indistinguishable from Atlantic salmon, safe to eat, and that it poses no threat to the environment under its condition for use (breeding and production of eyed-eggs in Canada, land-based production in closed facilities at inhospitable locations for the salmon in Panama, processing in Panama, and sale of processed salmon in the USA). In 2011 the National Marine Fisheries Service of the National Oceanic and Atmospheric Administration (NOAA) and the U.S. Fish and Wildlife Service concurred with the FDA in their finding of no effect on, nor threat, to the environment. In 2013 the Canadian government gave AquaBounty permission to produce AquAdvantage eggs for commercial purposes at the company's facilities on Prince Edwards Island. At the beginning of 2015 the FDA had not yet given its final approval to the import and sale of food products from the AquAdvantage salmon due to several issues related to the studies and environmental assessment of the AquAdvantage salmon raised by different organizations and interest groups representing both consumers, scientists and environmentalist during the public commentary period in 2012-2013.

5.2.2 Construct composition, site of integration, stability, gene dosage and expression

The composition of the genetic construct is important for effect on the GM organism. In fish, it has been observed that constructs consisting of promoters and/or genes closely related or homologous to promoters and genes native to the modified species are more successful than constructs consisting of non-homologous elements (Nam et al., 2008). However, it is important to consider that homologous gene constructs could be subjected to the endogenous regulatory system if they are 100% homologous and thus might not be effective at all (Nam et al., 2008). When using non-homologous genes the problem could be the opposite, i.e. too little interaction with the native

system of the organism. Thus, it is important to evaluate the promotors and genes selected for a construct thoroughly, focusing on how well it would interact with the endogenous system of the target organism.

Different modification events, using the same gene construct and species, but having individual impacts on the modified organisms indicate that the site of integration of the genetic construct could affect gene expression and thus the effect of the introduced construct on the GM organism (Devlin et al., 2004a).

The gene dosage, i.e. number of insertions of the construct into the genome, is another important aspect of the genetic modification of an organism. The number of integrations will affect the expression level of the transgene, and through that, affect systemic regulation as well. It can be expected that a plateau will be reached for the expression, production, and utilization of the transgene and its product, regardless of its dosage as all reactions are dependent on a limited amount of interacting reagents (f. ex. limitations in receptors or enzymatic activity). However, there could be potential to raise the plateau via genetic engineering affecting the levels of involved reagents (would require a sophisticated level of knowledge). Devlin et al. (2014) found that transgene dosage affected growth rate and plasma levels of GH and IGF-1. Transgene dosage effect was found to be dependent on developmental stage, and ploidy (both diploid and triploid individuals were used in the study). It has also been suggested that the transgene expression could be influenced by competition with endogenous genes and promoters for transcriptional regulators (micro-competition) (Rollo, 2014), which might set off a compensatory mechanism that could cause indirect pleiotropic effects on the organism itself.

It is important to establish whether the genetic construct is inherited in a stable and predictable manner over multiple generations, with the genetic integrity of the inserted construct remaining intact, and conferring the expected phenotype. The GM line should ideally be tested under varying environmental conditions and with different stressors to make sure the intended effect of the introduced construct on the phenotype does not shift.

5.2.3 Genetic Background and Pleiotropic and Epigenetic Effects

The genetic background into which a gene construct is introduced can have a large impact on how effectively the construct affects the modified organism. It is to be expected that the domestication process of salmon has led to genetic and epigenetic adaptation, both through target directed breeding, but also due to changes in the habitat and feed offered the farmed salmon. For example, Devlin et al. (2001) found that a GH construct had a much larger impact on growth of trout with a wild type genetic background than on trout with a domesticated genetic background, compared to non-modified counterparts. In another study comparing gene expression between wild type, domestic type and GH TG strains, Devlin et al. (2009b) observed that there was a tendency for the same genes to be up- and down-regulated in GH TG salmon and domesticated salmon as when compared to wild type salmon. This could indicate that similar changes are introduced at the genome level when breeding for rapid growth and when introducing additional GH genes. These two studies highlight the importance of knowing the genetic background and the potential of the transgene to influence other genes and their expression and vice versa.

Pleiotropic effects can arise directly when the product of a gene influences several biological mechanisms and traits, or indirectly through the impact of the gene's presence (interactions include up- or down regulation and blocking of expression). Some pleiotropic effects might only be observable during certain life stages, or in certain environments. Pleiotropic effects thus complicate the evaluation and risk assessment of GM organisms, as it is impossible to perform a comprehensive mapping of all pleiotropic effects caused by the insertion of a genetic construct. Box 5.2 presents several studies investigating pleiotropic effects in GH TG salmon.

A case-by-case evaluation and post-approval monitoring would be instrumental in uncovering pleiotropic effects, as each event is unique in relation to the species, transgene and insertion site(s). As illustrated by Devlin et al. (2009b), the insertion of a transgene may subsequently affect the transcription of a multitude of other genes, with further impacts on the organism. Pleiotropic effects not only affect the morphology, physiology and systemic regulation of an organism; they might also affect the behaviour of the organism. This is important to remember when assessing GM organisms, particularly so when the organism is intended for environmental release, or when accidental release might occur. Several studies have evaluated behavioural changes in GH TG fish (Bessey et al., 2004, Fredrik Sundström et al., 2007, Hallerman et al., 2007, Devlin et al., 2009a, Fredrik Sundström et al., 2003, Fredrik Sundström et al., 2004), as it is critical for the risk assessment to evaluate such information when considering risk and potential impacts of escape from contained facilities.

Salmon is a highly plastic species and environmental conditions are expected to influence its development and adaptation through genotype by environment interactions (see textbox 5.3). Pleiotropic effects can be influenced by environmental circumstances, e.g. what types of stress the salmon is exposed to. The ability to cope with stress is an important factor for animal welfare and product quality in aquaculture, as stress is known to affect general immune functions and have negative health implications. However, studies so far have been indecisive with regards to if there is any impacts on stress tolerance in GH TG salmonid fish (Deitch et al., 2006, Jhingan et al., 2003, Leggatt et al., 2007).

There are known differences in the spawning success of wild vs. domesticated salmon (Naylor et al., 2005, Fleming et al., 2000). For any GM salmon, it will be important to have a good understanding of its reproductive potential and fitness in order to conduct a thorough risk assessment. It is of particular importance that potential effects of the GM trait on reproduction is evaluated on a case-by-case basis. For the GH TG fish, it is suspected that it will have poorer reproductive success than observed in escaped domesticated fish today, however faster growth and earlier maturation, might still give a selective advantage (Naylor et al., 2005, Bessey et al., 2004). It is hypothesized that poor mating success among escaped domestic salmon is due to culture conditions rather than genetic effects alone (Bessey et al., 2004). That indicates that a second generation of feral salmon could have a mating success similar to that of wild salmon, however genetic change would still influence this to some degree. Such genotype by environment interactions makes it very difficult to predict what the potential impact of GM fish would be if interaction between GM and wild type fish would occur (Devlin et al., 2006, Sundström et al., 2007). Bessey et al. (2004) found that GH TG salmon females produced more, but smaller eggs, than their non-GM comparator, which could have implications for the survival of eggs and fry. However, by producing more eggs more offspring could survive (depending on environmental conditions and predation pressure) to compete against the wild salmon.

Box 5.2: Pleiotropic effect in GH TG salmon

GH is a high level regulatory hormone involved in the regulation of many important physiological processes, and thus the introduction of a gene coding for additional GH to be produced is expected to cause a myriad of effects on the GH TG organism.

The natural expression and production of piscine GH takes place in the pituitary from where it enters the blood plasma, and is subject to negative feedback regulation. The introduced GH is produced by cells throughout the body, and through increasing blood plasma levels of non-native GH, the pituitary's own GH production is down-regulated (Devlin et al., 2009a, Mori and Devlin, 1999).

The increased growth rate (Cook et al., 2000), with an elevated feed intake and utilization rate (Hallerman et al., 2007, Tibbetts et al., 2013) is considered direct and intended effects of the insertion of a GH construct into salmonid fish species. However, many additional effects of this modification have been observed and scientifically documented:

- Cascading effects on hormones influenced by GH (Mori et al., 2007, Rise et al., 2006, Devlin et al., 2009a, Eales et al., 2004, Jhingan et al., 2003).
- Reaches an appropriate size for smoltification and actually initiates and completes the smoltification process earlier in life than the non-GM salmon (Saunders et al., 1998).
- Changes in oxygen consumption and metabolic rates, which could affect their rearing requirements (Stevens et al., 1998, Leggatt et al., 2003, Deitch et al., 2006, Sundt-Hansen et al., 2007, Devlin et al., 2009a).
- The swimming ability of the GH TG fish is most likely affected through effects of the transgene on muscle tissue and oxygen uptake and transportation (Stevens et al., 1998, Deitch et al., 2006, Devlin et al., 2009a).
- Lower levels of proteins and lipids and more whole body moisture (Cook et al., 2000).
- Changed morphology and malformations due to the excessive growth of bone and cartilage (Hallerman et al., 2007, Devlin et al., 2009a, Devlin et al., 1995, Deitch et al., 2006, Nam et al., 2008)

Some pleiotropic effects manifest as behavioural changes such as increased foraging and feeding motivation, active feeding throughout the year, increased willingness to try new prey items, increased aggression, more risk taking when foraging (however, though increased activity increases predation risk, a larger size could potentially reduce the number of potential predators) and increased explorative behaviour (Hallerman et al., 2007, Devlin et al., 2009a, Fredrik Sundström et al., 2003, Fredrik Sundström et al., 2004, Fredrik Sundström et al., 2007, Abrahams and Sutterlin, 1999, Vandersteen Tymchuk et al., 2005, Devlin et al., 1999, Devlin et al., 2004a). A recent study performed with GH TG Coho (Oncorhynchus kisutch) in a contained stream-environment found that the impact of stream reared GM salmon on a wild-type population was similar to that of non-GM salmon, however if given the chance to outgrow its wild conspecific first, the invading GM salmon significantly reduced survival and growth of the wild-type population as compared to the non-GM invaders (Sundström et al., 2014). This difference could be related to the observed behavioural shifts, which provides an advantage as size differences increases (size is connected to dominance). Behavioural shifts such as increased aggression, could be detrimental to fish welfare in the context of aquaculture, but preventative measures such as feeding to satiation might alleviate such welfare issues (Devlin et al., 2004b, Devlin et al., 2009a, Fredrik Sundström et al., 2003).

Another important aspect of behaviour includes whether the GM salmon show changed tendencies towards migration and dispersal if escaped (Devlin et al., 2009a). Though some prediction can be made on the basis of what is seen in domesticated escaped salmon (Hansen, 2006), the impact of the transgene on this behaviour must still be considered (Fredrik Sundström et al., 2007).

Box 5.3: Genotype by Environment Interactions and plasticity

The phenotype derived from a specific genotype can be influenced by environmental input such as temperature and other climatic factors, or through exposure or lack of exposure to certain chemical compounds. How this expressed phenotype shifts under varying environmental conditions is called a reaction norm. These shifts could occur during an organism's lifetime, or between generations experiencing different environmental conditions during development. This capacity of genotypes to vary in their phenotypic expression conditioned by environmental input is termed plasticity. The genotype by environment interaction can make an organism show plasticity throughout all aspects of life, including but not limited to morphology, physiology, behavior, mating strategy and other life history and fitness traits. Salmon is considered a highly plastic species, where variations in phenotype can be observed among siblings raised in different environments (Chittenden et al., 2010). Studies have already shown that the effect of the GH TG in salmon is dependent on environment (Sundström et al., 2005, Sundström et al., 2007), indicating that a thorough evaluation of the effects that environment has on the expression of the transgene and further pleiotropic effects due to differential expressions of the transgene should be undertaken.

5.2.4 Comparative Analysis

Essential to the approval of GM organisms is the comparison with its unmodified counterpart. This is done to show that the genetic modification is working as intended and has no adverse effects on the modified organism or consumers of the organism. Ideally the organism should be tested during different life stages, varying environmental conditions and during different forms of physiological and mental stress (conditions that the GM salmon will meet in rearing and grow-out facilities are particularly relevant), as these factors might impact the organism's interaction with the introduced transgene and its product (e.g. to account for genotype by environment and pleiotropic effects). Depending on the organism, one should be able to identify several traits and biological compounds of particular interest based on animal health, toxicological and nutritional viewpoints. In the case of fish, which is an important nutritional source for marine fatty acids, it is only natural that special attention should be paid to the levels of these in GM salmon. Table 5.2 provides a short overview of the important points in a comparative analysis of transgene animals.

A phenotypic comparison between the GM animal and the non-GM animal is done in order to show that the intended effect of the introduced construct is achieved without adverse effects on the physiology, health, behaviour, sensory perception and cognitive skills of the animal. It is important to use an appropriate comparator which has been subject to the same circumstances and treatments as the GM animal. Only then can it be inferred that any dissimilarity is due to effects of the introduced construct. Importantly, any containment methods used to limit the animals' interactions with the natural environment and wild conspecifics, such as landlocked rearing facilities or induced triploidy

should be included in the comparative study. This because it is know that certain proposed containment methods such as triploidy also have health and welfare implications for the fish which could interact with the effects of the genetic modification (Rasmussen and Morrissey, 2007, Devlin et al., 2014).

Table 5.2: Simplified overview of elements that should be included in a comparative assessment of GM organisms.

Phenotypic comparison
•Animal health •Animal welfare •Behavior and function of the organism •Pleiotropic effects
Compositional comparison
•Nutrients and anti-nutrients •General composition (lipids, protein, ash, water, etc.) •Allergenicity •Toxins

The compositional analysis is used to establish substantial equivalence between the GM organism and its comparator for levels of bioactive compounds such as key nutrients, anti-nutrients, minerals, fatty acids and potential toxins. When performing a compositional analysis, it is important to consider any effects that the transgene could have on the accumulation and metabolism of potentially toxic or otherwise harmful compounds such as allergens. Increased levels of anti-nutrients or toxins could have negative impacts on consumer health. For GH TG fish it would for example, be relevant to test the level of GH, omega-3 fatty acids, and levels of metals and agricultural herbicides and pesticides which the salmon might be exposed to through its feed.

Though there are many independent studies comparing the phenotype, composition and behaviour of GH TG salmon, very few of these studies are working with the same construct and species as the AquAdvantage. Studies of similar, but not identical strains, might provide results that are not consistent with the actual event intended for release due to the impact of integration site on the effect of the transgene construct. This highlights the importance of good and thorough studies being accessible from the developers of GM organisms. However, a comparative assessment might be subject to qualitative uncertainties due to different views and ambiguity when interpreting data and results on what would be an acceptable deviation or not. There is thus uncertainty connected to the studies being performed by the developers themselves; which together with the lack of independent research and analysis of specific strains developed for commercialization, gives rise to possible interpretation and judgement bias.

The comparative analysis is a necessary tool for gaining information about the GM organisms regarding how the genetic construct is functioning in the modified organism. However, the comparative analysis also represents our biggest knowledge gap as it can never be exhaustive, but subject to the pre-existing knowledge and capacity of the developers, regulators, assessors and

independent researchers to make appropriate decisions on what should be the focus of such a comparative assessment. This lack of a complete knowledge base to draw information from highlights the need for continued monitoring and research even after approval of a GM organism. This is especially in the case of fish, such as salmon, which have potential for escape and survival (including interaction with wild salmon and sympatric species in the wild.

In box 5.4 a brief description of the FDA's assessment of the AquAdvanatge salmon is given, and some of the feedback provided by independent researcher and various non-governmental organizations during the process.

5.2.5 Summary of Uncertainties and Knowledge Gaps

The cliché "one gene, one protein, one function" has been debunked for some time now, and it is widely accepted that genes interact with each other, and commonly have more than one function. Thus it can be asserted that with any genetic modification there must exist knowledge gaps on how the organism is affected. Much of this knowledge gap can be attributed to quantitative uncertainties which are reducible through research. This would be the case of GM salmon. Much of the physiological and behavioural impact of an introduced construct on salmon can be investigated and observed. However, what research choices are made will depend on the pre-existing knowledge of salmon physiology and behaviour. Thus we have uncertainty arising from ignorance, i.e. we can only investigate the potential effects that we are able to imagine. This ignorance is also important to consider when evaluating uncertainty and knowledge gaps in relation to GM salmon, and highlights the need for continued surveillance and research even after a GM organism has been approved for commercialization or release. Another form of uncertainty that is very prominent when it comes to GM organisms is the ambiguous interpretation of the research and data available to developers, governmental bodies, independent researchers and non-profit organizations. This is a form of uncertainty where an open and transparent approach becomes important to create both scientific and public engagement and debate.

Major sources of knowledge gaps and uncertainties that currently apply to the development and characterization of GM salmon:

- Gaps in understanding of mechanisms behind insertion of the transgene into the organisms DNA.
- The understanding of genetic interaction, and the multiple roles of genes is lacking
- Regulatory pathways and systems affected by the transgene are not fully understood nor mapped, leading to unforeseen consequences on the organism
- Limitations on the scale of the comparative analysis leaves us ignorant to potential, as of yet undetected, effects of the genetic modification.

- Poor cooperation and knowledge sharing between developers and independent research limits the total body of scientific studies performed on any particular event, leading to ontological uncertainties (i.e. in the case of GH TG salmon because a great diversity of events involving different species, constructs and rearing methods are being used).

Though it is unlikely that all knowledge gaps and uncertainties will ever be resolved it is better to be aware and consider the potential consequences and risks they entail, rather than to remain ignorant of their presence.

Box 5.4: The American Food and Drug Administrations' Evaluation of the AquAdvantage salmon

The American Food and Drug Administration (FDA) regulates GM animals under the New Animal Drug Provision of the Federal Food, Drug and Cosmetic Act, and the National Environmental Policy Act. It is the inserted genomic construct rather than the animal itself that is considered the drug. The FDA uses a hierarchical risk-based approach, drawing on data from i) controlled studies of the specific modification event in question; ii) other non-controlled studies of the event in question; iii) historical records and data from these animals; and iv) per-reviewed studies on the event in question or similar modification events, putting most weight on the from the first source in a weight-of-evidence evaluation. The hierarchical process consists of seven steps;

1. Product definition
2. Molecular characterization of the construct
3. Molecular characterization of the GM animal
4. Phenotypic characterization of the GM animal
5. Durability and stability of the genetic construct (consistent inheritance of the genotype and the expressed phenotype
6.
    A) Food and feed safety
    B) Environmental safety
7. Claim validation

In their evaluation of the AquAdvantage salmon the FDA did not identify any hazards in relation to the molecular characterization of the construct or the GM salmon. They also found that the construct was stable, and consistently inherited in the transgenic (TG) lineage. In their evaluation of the phenotypic characterization of the GM salmon the FDA found that no unique adverse outcomes were the result of the incorporation of the AquAdvantage construct, and that the reported adverse outcomes were also present in comparators or described in the literature in relation to non-GM rapid growth phenotypes, or the induction of triploidy. However, the FDA is concerned by the heavy culling of early-life stage fish at the brood stock facility (due to space limitations), and that this might cause bias since one would expect this culling to be performed in a manner that would lead to improvement of the brood stock. The FDA is worried that the data provided for the phenotypic characterization may not reflect the actual nature or frequency of abnormalities in the population, and recommends a post-market surveillance program that will address issues related to abnormalities. Due to certain metabolic demands (feed, oxygen saturation) of the rapidly growing AquAdvantage the FDA also recommends labelling to ensure that animals are properly kept. The FDA concluded in their evaluation that no direct nor indirect food consumption hazards had been identified, and that the triploid AquAdvantage salmon is safe to eat. It follows that it is also safe as animal feed.

The FDA also concludes based on the Environmental Assessment provided by AquaBounty technologies that the AquAdvantage salmon poses no risk to the environment as long as it is breed and raised under the described conditions of physical, biological and geographic/geophysical confinements present at the hatchery facility and the grow-out facilities.

Finally, the FDA finds that the claim put forward by AquaBounty Technologies that when compared to unmodified salmon i) the AquAdvantage Salmon grow to a mean body weight of at least 100 g within 2700 degree-days of first-feeding, and (2) a greater proportion of AquAdvantage Salmon grow to at least 100 g within 2700 degree-days after first-feeding under normal commercial aquaculture conditions is supported by the data presented by AquaBounty Technologies.

However, the findings by the FDA presented in this briefing package has been heavily criticized during the public hearing process due to lack of independently acquired data, small sample sizes in the data provided by AquaBounty Technologies, studies describing phenotype where not performed under realistic production scenarios, and many respondents also expressed concern in relation the culling practice at the AquaBounty facilities and how this might have affected the data presented regarding phenotype and abnormalities.

The publicly available documents from which this short summary was made are all accessible here: http://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/VeterinaryMedicineAdvisoryCommittee/ucm201810.htm

5.3 Ecological impacts

A major concern in relation to salmon aquaculture is the potential impact that escaped salmon might have on the many wild populations. Wild populations, homing to different rivers and even different sections of larger river systems have been shown to have distinct genetic backgrounds with adaptations specific to each population (Verspoor et al., 2008, Dillane et al., 2008, Ozerov et al., 2012, Fraser et al., 2011, Garcia de Leaniz et al., 2007, Taylor, 1991). The domesticated salmon used in aquaculture has become differentiated from its wild counterpart through decades of breeding for economically important traits, and general adaptation to the captive environment. This intensive breeding has led to the development of domesticated and highly homogenous salmon strains (Taranger et al., 2014, Skaala et al., 2004, Karlsson et al., 2011). It is feared that introduction of the domesticated salmon to the wild populations through repeated escape events will affect the degree of local adaptation and lead to a homogenization of salmon populations across river systems, followed by negative fitness and survival impacts (Fraser et al., 2011, Verspoor et al., 2006, Fleming et al., 2000, Skaala et al., 2012, Muir and Howard, 1999, Muir and Howard, 2002). Norwegian salmon populations are already vulnerable due to small population sizes, pollution, disease, habitat and environmental changes (Aas et al., 2010). The ecological impact of GM salmon could be expected to be similar to that of non-GM domesticated salmon, however, how the presence of the introduced genetic constructs might influence this impact is difficult to predict due to both epistemological and ontological uncertainties. It is to be expected that the various transgenes that might be introduced to domestic strains have different impacts on the physiology and behavior of the salmon. It follows that the impact of escaped GM salmon on river ecosystem and wild salmon populations could vary greatly, depending on the nature of their modification. Additionally, once established against a different genetic background (wild salmon) and with different environmental input the impact

becomes unpredictable due to the great variability of ecosystems and the high plasticity of salmon (Devlin et al., 2006, Sundström et al., 2007).

5.3.1 Escape and survival of domestic salmon

Fish farm
Photo: istockphoto_000019170114/Howard Oates

The Norwegian Institute for Marine Research estimated in their risk evaluation for Norwegian Aquaculture 2013 that the number of escaped fish is grossly underestimated (Taranger et al., 2014). Between 2006 and 2013 an average of 315 741 salmon were reported as escaped each year, and close to 80% of these were reported to be adult salmon. The adjusted estimate, believed to provide a more accurate number of escapees, is 4-5 times as high at between 1 million and 1.5 million escaped salmon every year (Taranger et al., 2014). This large discrepancy is thought to partly be due to parr and smolt escapes often being undetected and thus underreported from the industry (Taranger et al., 2014). Most of the salmon that escape are immature at the point of escape, and will swim out to sea shortly after escaping. However, this can vary depending on geography and the specific life stage (Taranger et al., 2014, Skilbrei et al., 2015). The salmon usually stays out at sea until they mature, and then seek the coastline and swim upriver. Both wild and domesticated salmon have poor survival at sea (Taranger et al., 2014). The tendency to swim out to sea also depends on season, with more of the escaped salmon swimming out to sea in spring than in autumn (Taranger et al., 2014). Photo manipulation[10], used to rush or delay salmon development, could also affect migration tendencies independently of season (Skilbrei et al., 2014). Some individuals might be in a mature state at escape, these are likely to seek upriver quite soon after escape. It seems that the younger the individuals are at escape, the easier it is to adapt to the wild environment, and their behavior becomes more similar to the wild salmon, as opposed to domestic salmon that escape as adults (Taranger et al., 2014).

In the case that GM salmon is allowed in sea pens it can be assumed that similar numbers of escapes will be observed, unless more effective precautionary measures are put in place. However, complete elimination of escapes while holding salmon in sea pens seems unlikely.

[10] The use of artificial dark:light cycles in order to give the organism a skewed perception of the seasons and passing of time. Used to rush or delay development of an organism, or to provoke or inhibit seasonally timed events.

5.3.2 Impacts on Wild Salmon Populations

When escaped salmon matures it will be attracted to rivers, and attempt to participate in the mating of the local salmon strains it encounters upriver. It is clear that when this repeats over several generations in a river, the genetic compositions of the salmon population inhabiting that river will be affected (Hindar et al., 2006). Other risks include disease transfer and resource competition (Naylor et al., 2005, Jonsson and Jonsson, 2006). Glover et al. (2013) found that at least five salmon rivers out of the twenty investigated had become significantly more similar to farmed fish over time when comparing genetic markers between historical and contemporary samples from wild and farmed salmon. The degree of introgression depends on many factors such as wild salmon population size (the smaller the population the higher the introgression of escaped salmon), frequency of escaped salmon, and the competitive ability off the domestic salmon in mate competition (Taranger et al., 2014). In general, studies show that domestic salmon have lower mating success than wild salmon in nature, but that this is variable and dependent upon factors such as sex, age at escape and maturation, and the specific strains involved in the interaction (Fleming, 1996a, Garant et al., 2003, Weir et al., 2004, Weir et al., 2005). Offspring survival also seems to be lower for farm genotypes (Skaala et al., 2012). A study by Fleming et al. (2000) found a total negative impact on smolt production in a river invaded by farmed salmon. It is hypothesized that this 28% reduction compared to what was expected based on egg production is due to fluctuating selection of offspring type, the level of competition between the different offspring types, and maladaptation of the farmed and hybrid offspring (Fleming et al., 2000). Data suggests that farmed and hybrid offspring grow and develop faster than their wild counterpart, and thus might have an competitive advantage at certain life stages (Fleming et al., 2000, McGinnity et al., 2003).

When it comes to the potential of transgene salmon to influence wild populations, three scenarios have been described: the Purge, the Spread and the Trojan Horse, and many attempts have been made to model these scenarios using different models and parameters (Muir and Howard, 1999, Muir and Howard, 2002, Devlin et al., 2006, Howard et al., 2004, Aikio et al., 2008). In the Purge scenario, the transgene confers lower fitness compared to the wild type and is weeded out of the population. The opposite is expected to happen in the Spread hypothesis, where the transgene is of a nature that confers higher fitness to its carriers, and thus the gene spreads throughout the population. In The Trojan Horse hypothesis, the transgene provides a mating benefit, but also affects survival negatively. According to a deterministic model used by Hedrick (2001) the transgene will, under such circumstances, increase in frequency in 66.7% of the possible combinations of mating and viability parameters, and in 50% of these combinations the transgene would go to fixation within the populations, drastically reducing the viability and resilience of the populations, and possibly causing extinction. However, in a different model created by Aikio et al. (2008) extinction does not occur because the lower viability introduced through the transgene is balanced out by the effect of population decrease on density dependence. These examples illustrate how models can differ greatly in their interpretation of these scenarios, and thus do not provide any definitive answers. A fourth scenario could be that of a neutral transgene not affecting fitness in the wild with the potential of random fixation through genetic drift. However, this scenario seems unlikely considering the proposed modifications for salmon (table 1).

Since salmon females preferably mate with the larger males (Fleming, 1996b), a salmon carrying genes increasing growth rate and size is likely to have an advantage in competition for mates. Increased growth rate could also mean earlier smoltification (Saunders et al., 1998) and maturation (Devlin et al., 2004a) shortening the generation time for those carrying the transgene and providing further advantage. However, research on the domesticated strains show that they have a lower survival in the wild, indicating a fitness disadvantage (Skaala et al., 2012, McGinnity et al., 2003, Fleming et al., 2000). The assumed mating advantage due to size combined with lower survival would put the invaded population at risk for a Trojan Horse scenario (Hedrick, 2001). A recent study (Moreau et al., 2011) comparing the mating success of male GH TG salmon with that of male wild salmon for both mature adults and mature parr found that the non-GM individuals performed better than the GH TG individuals. However, the study also demonstrated that the GH TG salmon could mate successfully, i.e. expected impacts of escaped GH TG salmon could be similar to what is seen today from escaped farmed salmon.

Studies of escaped, domestic salmon have shown that they generally have low mating success compared with the wild salmon (Fleming et al., 2000), but that repeated invasions are still likely to negatively affect the general fitness of the population through outbreeding depression (McGinnity et al., 2003). So, even if the criteria for a Trojan scenario are not present (mating advantage), the repetitiveness of invasion events could still have severe negative impacts on population productivity and genetic diversity (Fleming et al., 2000, Jonsson and Jonsson, 2006, Skaala et al., 2012, Glover et al., 2013, Hindar et al., 2006), and cause introgression of the transgene into the wild population.

Several studies have investigated the interactions between non-GM and GH TG Coho salmon. In these studies they have found that the GH TG salmon is both more explorative and more competitive, with higher feeding motivation and increased aggressiveness when compared with non-GM salmon (Fredrik Sundström et al., 2003, Fredrik Sundström et al., 2004, Fredrik Sundström et al., 2007, Devlin et al., 2004a, Devlin et al., 1999, Hallerman et al., 2007). This could be an indication that the interactions between wild salmon and GM salmon will be different than the interactions seen between today's domestic salmon and its wild conspecifics. Their studies indicate that if food availability is sufficient to sustain growth and activity early in life (i.e. under farm conditions before escape) the GH TG salmon could be a strong competitor. Except for the genetic impact through mating, there are several other modes of interaction which could impact the wild salmon.

Competition for habitat and prey, cannibalism of smaller individuals (particularly relevant for GH TG salmon), nest disturbance and disease transfer are all examples of potential interactions which could impact the native salmon population if exposed to GM salmon. The introduction of disease resistance through genetic modification could potentially lead to escaped farmed fish behaving as disease reservoirs, spreading severe illnesses mainly seen in connection to intensive aquaculture into wild populations, and at the same time providing a significant fitness advantage to their descendants in the wild. Changes to the metabolism of salmon which do not affect them negatively in the farmed context could prove to have severe fitness implications in the wild, negatively affecting hybrid offspring and the wild population. These examples are just a few of the impacts that can be envisioned pending on the specific nature of the genetic modification.

5.3.3 Other Ecosystem Impacts

The wild salmon populations are not the only species that would be impacted by escaped GM salmon. It is to be expected that other salmonids species such as trout and char, which are often found in the same habitats as salmon, would be affected through competition for resources such as prey and territory. Certain introduced GM traits could prove to be beneficial in competition with other species inhabiting a similar ecosystem niche as salmon. The high growth rate could, for example, provide the GH TG salmon with the opportunity to prey on conspecifics (particularly juveniles) and sympatric species (particularly juveniles) earlier in its life due to its larger size. There is also a chance that the GM salmon could hybridize with salmonids other than Atlantic salmon (Oke et al., 2013, Solem et al., 2014).

It can be suggested that the disturbance would be greatest if the GM salmon were to establish in river systems not already inhabited by salmon, as this would cause greater perturbations to the local ecosystems by introducing a new species, and not just a novel variation of a species (Moreau, 2014). Increasing temperature tolerance might allow the salmon to establish in new ecosystems outside of its natural range, encountering species with no previous history of coexistence with salmon. This has been observed in New Zealand where brown trout became an invasive species, and had large impacts on the native river ecosystems (Townsend, 1996). Though farmed salmon has shown little invasiveness outside the salmon's natural range, this need not be the case for a GM salmon, which could have other competitive qualities. Not only will the GM salmon compete with possible sympatric species, it could also be preying upon them, due to a size advantage.

Prey species would also be affected by the introduction of GM salmon. The GH TG salmon grows at a higher rate than its wild conspecifics and hunts more actively with increased motivation for trying new prey items (Fredrik Sundström et al., 2003, Fredrik Sundström et al., 2004). This could cause predation pressure on natural prey species to increase. Additionally, species not previously preyed upon might become subject to predation by the GH TG salmon.

Impacts as described above are likely to influence tropic interactions and energy flow through an ecosystem new to the salmon species, affecting its structure and resilience and making it more vulnerable to other disturbances.

5.3.4 Summary of Uncertainties and Knowledge Gaps

There are many epistemological and ontological uncertainties to how a river ecosystem would respond to the introduction of a GM salmon. It is very difficult to predict how a GM salmon would behave and survive in the wild because of the many factors influencing behaviour and survival. Even though studies made in semi-natural experiments, and information on how domestic non-GM salmon perform can be used to make predictions, these predictions can only be considered as qualified guesses. The impact of GM salmon would also depend on the frequency of escapes (stochastic events), the number of escaped salmon, and the nature of the genetic modification and its pleiotropic effects. The more GM salmon in the wild, the larger the potential impact, and particularly on sympatric and prey species in ecosystems not naturally inhabited by salmon. Many of the potential genetic modifications that could be beneficial for the aquaculture industry (such as

increased temperature tolerance or disease resistance) have been given limited attention in an ecological context, and there is little available literature. It is important that modifications are thoroughly evaluated to better be able to predict their potential impact on wild salmon populations and both native and non-native ecosystems.

It is inherently difficult to compare life history traits between GM and non-GM salmon within contained research facilities as conditions (population densities, access to feed, tropic interactions, climatic conditions etc.) will differ from farm or natural environment. Though some studies set in semi-natural environments already exist, there is a need for studies set in farm or semi-natural environments looking at and comparing development, life history traits and interactions. Though such studies can never be comprehensive enough, they might provide valuable information on the possible impacts of GM salmon on various ecosystem types.

According to Moreau (2014) the knowledge gaps and uncertainties connected to the escape and invasion of GM salmon into natural ecosystems can be summarized as

- Knowledge not sufficient to predict the ecological consequences of invasion.
- The understanding of the eco-evolutionary dynamics associated with rapid environmental change is insufficient.
- Lack of understanding of molecular, biochemical and physiological dynamics associated with modulating influential genes in different environments.

There is also a lack of research on how GM salmon could impact fjord and sea ecosystems, little is also understood in general about the salmons life at sea.

The bullet points above both have elements of epistemological uncertainty, which can be diminished through further research, and ontological uncertainty, which is related to the inherent variability and diversity in the entity subject to research. The latter form of uncertainty can be perceived as much more complex than the former because it subject to both indeterminacy in that exhaustive studies cannot be performed, and ambiguity with regards to the interpretation of data (ambiguity also relevant in relation to epistemological uncertainty).

The lack of complete knowledge (ignorance), and the uncertainties connected to what we know highlights the importance of continued research, risk evaluation and a continuous monitoring (post-approval/post-market) and evaluation process of the possible impacts and the not yet perceived impacts that GM salmon could have on wild salmon and native and non-native ecosystems.

5.4 Containment Methods

So far the only application for commercial production of a GM fish for food purposes claims that the production will be contained by multiple safety and containment measures. The term containment measure when used in relation to GM fish is any form of barrier that prevents the GM fish from interacting with the environment (physical/mechanical/chemical barriers), from mating with compatible wild species (reproductive barrier), or from survival in the wild (dependency on a specific input only accessible while in captivity). The method of containment should be intrinsically linked to

the evaluation and risk assessment of the GM organism. Changing the containment regime should inevitably lead to the need for a new application and evaluation process. This because the various methods of containment might have different risks and environmental impacts linked to them. Without evaluation of these methods and their implication for risk and impact, we will experience major quantitative uncertainties. In table 5.3 some examples of possible containment methods are discussed. These are measures frequently discussed in the scientific literature, both by proponents and opponents of GM fish.

Table 5.3: Overview of in-use and potential future containment methods for GM salmon.

	Containment method	Pros	Cons	References
Physical containment	Landlocked production including: -physical barriers -mechanical barriers -chemical barriers	• Contributes towards alleviating many issues related to aquaculture (better control of waste, easier to contain disease, better input and output control, etc.) • Little risk of escape (but theft and natural disasters like flooding could be a risk)	• Use of valuable land near rivers and the sea • Questions whether the technology is good enough to maintain animal welfare in large-scale facilities • Profitability is questionable	Wong and van Eenennaam (2008) Norwegian Ministry of Trade, Industry and Fisheries report "Laks på land" (2015) Nofima report 32, Iversen et al. (2013)
Biological containment	Polyploidy:	• Fish are sterile. • Current, in use, technology	• Not 100% effective • Welfare concerns connected to welfare, health and cognitive abilities • Still allows for interaction and disturbance (ecological impacts)	Wong and van Eenennaam (2008) Devlin, Raven et al. (2009a) Benfey (2001) Dunham (2004) Rasmussen and Morrissey (2007) Gjedrem and Baranski (2009) Fraser, Fjelldal, Hansen et al. (2012a) Fraser, Fjelldal, Skjaeraasen et al. (2012b)
Biological containment	Genetic modification:	• Can be introduced with primary modification	• Process of maturation and gonad development not yet fully understood in fish. • Still allows for interaction and disturbance	Wong and Van Eenennaam (2008)
	-induced	• Once established	• Risk of pleiotropic effects	

	Containment method	Pros	Cons	References
	sterility	sterility would be 100%. • Potentially reversible for breeding purposes		
	-"Terminator technology"	• Leaves fertilized eggs non-viable without activation of inducible promotor and gene. • Reversible for breeding purposes	• Not yet successfully (commercialized) in plants • What trigger to choose for the inducible gene that would not be present in the environment? • Still allows for interaction and disturbance (maybe to an even higher degree as all potential offspring from such a mating would be non-viable, potential to severely impact population size?) • Risk of pleiotropic effects	
	-containment of transgene (gonad-specific excision of transgene)	• Transgene function kept in the organism for its full lifetime, but not transferred to potential offspring	• Also requires excision of the recombinase gene behind the excisions of the transgene. • Very preliminary research stage • Still allows for interaction and disturbance (ecological impacts) • Risk of pleiotropic effects	
Biological containment	Synthetic biology:	• Containment based on redesign of essential enzymes and dependency on non-standard amino acids for survival	• Still allows for interaction and disturbance (ecological impact) • Developing technology, not fully risk assessed	Mandell, Lajoie et al. (2015)
Biological containment	DNA vaccines/Gene therapy (affecting reproductive systems):	• Can be distributed with other vaccines	• Still allows for interaction and disturbance (ecological impacts) • Far away from realization	

With regards to the biologically based containment methods the main concern is the extent of ecological impact sterile individuals may have on wild salmon populations and the river and ocean ecosystems. This will probably vary depending on the transgenic construct, containment method, the sex of the fish, and whether or not the escaped individuals stay in the fjord, or wander out to sea or upriver. Another aspect to consider here is the size of the potential impact. Few and far between escapees are less likely to have any meaningful impact (changes in wild salmon, other fish and potential prey populations, ecosystem perturbations), compared to many and regularly arriving escapees. A disease-carrying but resistant GM salmon moving upriver during the mating season could be a great risk to the wild salmon population of that river. However, it might take many and repeated invasions of fast-growing GM salmon to affect prey populations in a fjord or river to the extent that the prey population and other species are impacted.

The developers and producers of the AquAdvantage salmon are implementing several strategies in order to contain their GH TG salmon. Firstly, all production occurs in land-locked facilities and the salmon intended for food production will even be raised in land-locked facilities outside the natural range of salmon. Secondly, all salmon intended for commercial use are triploid and female. Such a combination of strategies might be complicated to achieve, but could be much more effective overall placing different types of barriers between the GM salmon and the ecosystem and between the GM salmon and the wild type salmon. However, containment methods should be thoroughly assessed with regards to impacts on animal health and welfare, and stringency.

5.5 New and emerging technologies

The last decade the development in gene technology has moved fast, and there are now several methods available for inducing targeted genetic mutations (targeted mutagenesis technology), such as the zing-finger, TALENs and CRISPR/CAS technologies. These methodologies have all been used experimentally on fish in recent years (Doyon et al., 2008, Meng et al., 2008, Huang et al., 2011, Edvardsen et al., 2014). Firstly, this technology will allow for easier and more precise manipulations of the salmon genome for research purposes (establishing the roles and regulation of genes in various physiological functions). Secondly, these technologies might allow for the manipulation of genotypes by inducing changes in the different alleles of genes so they shift into the appropriate genotype (in the context of aquaculture), without breeding on different strains trying to converge the preferable alleles and weed out the less valued genotypes. However, it is uncertain how useful this approach will be as salmon is a very plastic species where environmental input greatly affects the expressed phenotype of the individual (i.e. environmental changes such as temperature differences or shifts in food access could led to a different phenotype, despite the genotype being identical).

5.6 Directions for further studies

As of yet it is not clear what kind of genetic modifications that might be effectively applied to farmed salmon, except for in the case of the GH TG AquAdvantage salmon. However, what is clear is that there are large knowledge gaps and much uncertainty connected to the use and possible impact of GM salmon. It is likely that further development of GM salmon will make us aware of much more specific knowledge gaps that are much more quantitative in nature than the ones we are currently facing, and thus easier to resolve through further research. However the major epistemological and ontological knowledge gaps are expected to remain as reminders that risk cannot always be calculated, and that there will always be uncertainty due to the complexity of living organisms and their interaction with the environment.

However, to conclude, we have chosen to highlight some of the current uncertainties and knowledge gaps in relation to GM salmon that require further investigation and experimental studies:

- The mechanisms behind genetic interactions causing pleiotropic effects
- Studies furthering our understanding of the salmon's physiological functions and mechanisms, its plasticity, and how the environmental input influences this (for example through epigenetics or other mechanisms influencing gene expression).
- Studies focusing on ecological relevance of GM salmon, including all trophic levels of the riverine ecosystems, and the potential for the GM salmon to establish outside the native range of wild salmon (i.e. potential as an invasive species).
- Studies improving on the understanding of behaviour and interactions of salmon in marine ecosystems.
- The impact of containment methods on the general health and welfare of the salmon, particularly biological containment methods such as additional genetic modifications, triploidy or the use of synthetic biology.

Additionally it can be expected that providing independent researchers (without economical or other interests regarding the commercialization of GM salmon products) access to the actual events intended for commercialization would i) increase the amount of scientifically based knowledge gathered on these events, and the rate at which this information would be acquired; ii) increase the public's confidence in the scientific data; and iii) give the opportunity to investigate a broader set of research questions in relation the GM salmon events than what is asked as a minimum in the standard evaluations and assessments required by law.

References

AAS, Ø., KLEMETSEN, A., EINUM, S. & SKURDAL, J. 2010. *Atlantic Salmon Ecology*.

ABRAHAMS, M. V. & SUTTERLIN, A. 1999. The foraging and antipredator behaviour of growth-enhanced transgenic Atlantic salmon. *Animal Behaviour,* 58**,** 933-942.

ACOSTA, J., CARPIO, Y., BORROTO, I., GONZALEZ, O. & ESTRADA, M. P. 2005. Myostatin gene silenced by RNAi show a zebrafish giant phenotype. *Journal of Biotechnology,* 119**,** 324-331.

AIKIO, S., VALOSAARI, K.-R. & KAITALA, V. 2008. Mating preference in the invasion of growth enhanced fish. *Oikos,* 117**,** 406-414.

ALIMUDDIN, YOSHIZAKI, G., KIRON, V., SATOH, S. & TAKEUCHI, T. 2005. Enhancement of EPA and DHA biosynthesis by over-expression of masu salmon Δ6-desaturase-like gene in zebrafish. *Transgenic Research,* 14**,** 159-165.

BENFEY, T. J. 2001. Use of sterile triploid Atlantic salmon (Salmo salar L.) for aquaculture in New Brunswick, Canada. *Ices Journal of Marine Science,* 58**,** 525-529.

BESSEY, C., DEVLIN, R. H., LILEY, N. R. & BIAGI, C. A. 2004. Reproductive Performance of Growth-Enhanced Transgenic Coho Salmon. *Transactions of the American Fisheries Society,* 133**,** 1205-1220.

BOSTOCK, J. 2011. The application of science and technology development in shaping current and future aquaculture production systems. *J Agric Sci,* 149**,** 133-141.

BROWN, C. 2015. Fish intelligence, sentience and ethics. *Animal Cognition,* 18**,** 1-17.

CHITTENDEN, C. M., BIAGI, C. A., DAVIDSEN, J. G., DAVIDSEN, A. G., KONDO, H., MCKNIGHT, A., PEDERSEN, O.-P., RAVEN, P. A., RIKARDSEN, A. H., SHRIMPTON, J. M., ZUEHLKE, B., MCKINLEY, R. S. & DEVLIN, R. H. 2010. Genetic versus Rearing-Environment Effects on Phenotype: Hatchery and Natural Rearing Effects on Hatchery- and Wild-Born Coho Salmon. *PLoS ONE,* 5**,** e12261.

COOK, J. T., MCNIVEN, M. A., RICHARDSON, G. F. & SUTTERLIN, A. M. 2000. Growth rate, body composition and feed digestibility/conversion of growth-enhanced transgenic Atlantic salmon (Salmo salar). *Aquaculture,* 188**,** 15-32.

DEITCH, E. J., FLETCHER, G. L., PETERSEN, L. H., COSTA, I. A. S. F., SHEARS, M. A., DRIEDZIC, W. R. & GAMPERL, A. K. 2006. Cardiorespiratory modifications, and limitations, in post-smolt growth hormone transgenic Atlantic salmon Salmo salar. *Journal of Experimental Biology,* 209**,** 1310-1325.

DEVLIN, R. H., BIAGI, C. A. & YESAKI, T. Y. 2004a. Growth, viability and genetic characteristics of GH transgenic coho salmon strains. *Aquaculture,* 236**,** 607-632.

DEVLIN, R. H., BIAGI, C. A., YESAKI, T. Y., SMAILUS, D. E. & BYATT, J. C. 2001. Growth of domesticated transgenic fish - A growth-hormone transgene boosts the size of wild but not domesticated trout. *Nature,* 409**,** 781-782.

DEVLIN, R. H., D'ANDRADE, M., UH, M. & BIAGI, C. A. 2004b. Population effects of growth hormone transgenic coho salmon depend on food availability and genotype by environment interactions. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 9303-9308.

DEVLIN, R. H., JOHNSSON, J. I., SMAILUS, D. E., BIAGI, C. A., JÖNSSON, E. & BJÖRNSSON, B. T. 1999. Increased ability to compete for food by growth hormone-transgenic coho salmon Oncorhynchus kisutch (Walbaum). *Aquaculture Research,* 30**,** 479-482.

DEVLIN, R. H., RAVEN, P. A., SUNDSTRÖM, L. F. & UH, M. 2009a. Issues and Methodology for Development of Transgenic Fish for Aquaculture with a Focus on Growth Enhancement. *Molecular Research in Aquaculture.* Wiley-Blackwell.

DEVLIN, R. H., SAKHRANI, D., BIAGI, C. A., SMITH, J. L., FUJIMOTO, T. & BECKMAN, B. 2014. Growth and endocrine effect of growth hormone transgene dosage in diploid and triploid coho salmon. *General and Comparative Endocrinology,* 196**,** 112-122.

DEVLIN, R. H., SAKHRANI, D., TYMCHUK, W. E., RISE, M. L. & GOH, B. 2009b. Domestication and growth hormone transgenesis cause similar changes in gene expression in coho salmon (Oncorhynchus kisutch). *Proceedings of the National Academy of Sciences,* 106**,** 3047-3052.

DEVLIN, R. H., SUNDSTRÖM, L. F. & MUIR, W. M. 2006. Interface of biotechnology and ecology for environmental risk assessments of transgenic fish. *Trends in Biotechnology,* 24**,** 89-97.

DEVLIN, R. H., YESAKI, T. Y., DONALDSON, E. M. & HEW, C. L. 1995. Transmission and phenotypic effects of an antifreeze GH gene construct in coho salmon (Oncorhynchus kisutch). *Aquaculture,* 137**,** 161-169.

DILLANE, E., MCGINNITY, P., COUGHLAN, J. P., CROSS, M. C., DE EYTO, E., KENCHINGTON, E., PRODÖHL, P. & CROSS, T. F. 2008. Demographics and landscape features determine intrariver population structure in Atlantic salmon (Salmo salar L.): the case of the River Moy in Ireland. *Molecular Ecology,* 17**,** 4786-4800.

DOYON, Y., MCCAMMON, J. M., MILLER, J. C., FARAJI, F., NGO, C., KATIBAH, G. E., AMORA, R., HOCKING, T. D., ZHANG, L., REBAR, E. J., GREGORY, P. D., URNOV, F. D. & AMACHER, S. L. 2008. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotech,* 26**,** 702-708.

DUNHAM, R. A. 2004. *Aquaculture and fisheries biotechnology : genetic approaches,* Wallingford, CABI Publ.

EALES, J. G., DEVLIN, R., HIGGS, D. A., MCLEESE, J. M., OAKES, J. D. & PLOHMAN, J. 2004. Thyroid function in growth-hormone-transgenic coho salmon (Oncorhynchus kisutch). *Canadian Journal of Zoology,* 82**,** 1225-1229.

EDVARDSEN, R. B., LEININGER, S., KLEPPE, L., SKAFTNESMO, K. O. & WARGELIUS, A. 2014. Targeted Mutagenesis in Atlantic Salmon (<italic>Salmo salar</italic> L.) Using the CRISPR/Cas9 System Induces Complete Knockout Individuals in the F0 Generation. *PLoS ONE,* 9**,** e108622.

ENTIS, E. 1998. Aquadvantage salmon: A case study in transgenic food. *Animal Biotechnology,* 9**,** 165-170.

EVENSEN, Ø. & LEONG, J.-A. C. 2013. DNA vaccines against viral diseases of farmed fish. *Fish & Shellfish Immunology,* 35**,** 1751-1758.

FLEMING, I. 1996a. Reproductive strategies of Atlantic salmon: ecology and evolution. *Reviews in Fish Biology and Fisheries,* 6**,** 379-416.

FLEMING, I. A. 1996b. Reproductive strategies of Atlantic salmon: Ecology and evolution. *Reviews in Fish Biology and Fisheries,* 6**,** 379-416.

FLEMING, I. A., HINDAR, K., MJØLNERØD, I. B., JONSSON, B., BALSTAD, T. & LAMBERG, A. 2000. *Lifetime success and interactions of farm salmon invading a native population*.

FLETCHER, G. L., SHEARS, M. A., YASKOWIAK, E. S., KING, M. J. & GODDARD, S. V. 2004. Gene transfer: potential to enhance the genome of Atlantic salmon for aquaculture. *Australian Journal of Experimental Agriculture,* 44**,** 1095-1100.

FRASER, D. J., WEIR, L. K., BERNATCHEZ, L., HANSEN, M. M. & TAYLOR, E. B. 2011. Extent and scale of local adaptation in salmonid fishes: review and meta-analysis. *Heredity,* 106**,** 404-420.

FRASER, T. W. K., FJELLDAL, P. G., HANSEN, T. & MAYER, I. 2012a. Welfare Considerations of Triploid Fish. *Reviews in Fisheries Science,* 20**,** 192-211.

FRASER, T. W. K., FJELLDAL, P. G., SKJAERAASEN, J. E., HANSEN, T. & MAYER, I. 2012b. Triploidy alters brain morphology in pre-smolt Atlantic salmon Salmo salar: possible implications for behaviour. *Journal of Fish Biology,* 81**,** 2199-2212.

FREDRIK SUNDSTRÖM, L., DEVLIN, R. H., JOHNSSON, J. I. & BIAGI, C. A. 2003. Vertical Position Reflects Increased Feeding Motivation in Growth Hormone Transgenic Coho Salmon (Oncorhynchus kisutch). *Ethology,* 109**,** 701-712.

FREDRIK SUNDSTRÖM, L., LÕHMUS, M., DEVLIN, R. H., JOHNSSON, J. I., BIAGI, C. A. & BOHLIN, T. 2004. Feeding on Profitable and Unprofitable Prey: Comparing Behaviour of Growth-Enhanced Transgenic and Normal Coho Salmon (Oncorhynchus kisutch). *Ethology,* 110**,** 381-396.

FREDRIK SUNDSTRÖM, L., LÕHMUS, M., JOHNSSON, J. I. & DEVLIN, R. H. 2007. Dispersal Potential is Affected by Growth-Hormone Transgenesis in Coho Salmon (Oncorhynchus kisutch). *Ethology,* 113**,** 403-410.

GAJ, T., GERSBACH, C. A. & BARBAS, C. F., 3RD 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol,* 31**,** 397-405.

GARANT, D., FLEMING, I. A., EINUM, S. & BERNATCHEZ, L. 2003. Alternative male life-history tactics as potential vehicles for speeding introgression of farm salmon traits into wild populations. *Ecology Letters,* 6**,** 541-549.

GARCIA DE LEANIZ, C., FLEMING, I. A., EINUM, S., VERSPOOR, E., JORDAN, W. C., CONSUEGRA, S., AUBIN-HORTH, N., LAJUS, D., LETCHER, B. H., YOUNGSON, A. F., WEBB, J. H., VOLLESTAD, L. A., VILLANUEVA, B., FERGUSON, A. & QUINN, T. P. 2007. A critical review of adaptive genetic variation in Atlantic salmon: implications for conservation. *Biol Rev Camb Philos Soc,* 82**,** 173-211.

GILLUND, F., DALMO, R., TONHEIM, T. C., SETERNES, T. & MYHR, A. I. 2008. DNA vaccination in aquaculture — Expert judgments of impacts on environment and fish health. *Aquaculture,* 284**,** 25-34.

GJEDREM, T. 2012. Genetic improvement for the development of efficient global aquaculture: A personal opinion review. *Aquaculture,* 344**,** 12-22.

GJEDREM, T. & BARANSKI, M. 2009. Selective Breeding in Aquaculture: An Introduction. Springer Netherlands.

GJEDREM, T., ROBINSON, N. & RYE, M. 2012. The importance of selective breeding in aquaculture to meet future demands for animal protein: A review. *Aquaculture,* 350**,** 117-129.

GLOVER, K., PERTOLDI, C., BESNIER, F., WENNEVIK, V., KENT, M. & SKAALA, O. 2013. Atlantic salmon populations invaded by farmed escapees: quantifying genetic introgression with a Bayesian approach and SNPs. *BMC Genetics,* 14**,** 74.

HALLERMAN, E. M., MCLEAN, E. & FLEMING, I. A. 2007. Effects of growth hormone transgenes on the behavior and welfare of aquacultured fishes: A review identifying research needs. *Applied Animal Behaviour Science,* 104**,** 265-294.

HANSEN, L. P. 2006. Migration and survival of farmed Atlantic salmon (Salmo salar L.) released from two Norwegian fish farms. *ICES Journal of Marine Science: Journal du Conseil,* 63**,** 1211-1217.

HEDRICK, P. W. 2001. Invasion of transgenes from salmon or other genetically modified organisms into natural populations. *Canadian Journal of Fisheries and Aquatic Sciences,* 58**,** 841-844.

HINDAR, K., FLEMING, I. A., MCGINNITY, P. & DISERUD, O. 2006. Genetic and ecological effects of salmon farming on wild salmon: modelling from experimental results. *ICES Journal of Marine Science: Journal du Conseil,* 63**,** 1234-1247.

HOLM, J. C. 2015. Fisk på land -en utredning om egne tillatelser til landbasert matfiskeoppdrett av laks, ørret og regnbueørret med bruk av sjøvann. *In:* FISKERIDEPARTMENTET, N. O. (ed.).

HOWARD, R. D., DEWOODY, J. A. & MUIR, W. M. 2004. Transgenic male mating advantage provides opportunity for Trojan gene effect in a fish. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 2934-2938.

HUANG, P., XIAO, A., ZHOU, M., ZHU, Z., LIN, S. & ZHANG, B. 2011. Heritable gene targeting in zebrafish using customized TALENs. *Nat Biotech,* 29**,** 699-700.

IVERSEN, A., ANDREASSEN, O., HERMANSEN, Ø., LARSEN, T. A. & TERJESEN, B. F. 2013. Oppdrettsteknologi og konkurranseposisjon. *Nofima rapport.* Nofima.

JHINGAN, E., DEVLIN, R. H. & IWAMA, G. K. 2003. Disease resistance, stress response and effects of triploidy in growth hormone transgenic coho salmon. *Journal of Fish Biology,* 63**,** 806-823.

JONSSON, B. & JONSSON, N. 2006. Cultured Atlantic salmon in nature: a review of their ecology and interaction with wild fish. *Ices Journal of Marine Science,* 63**,** 1162-1181.

KARLSSON, S., MOEN, T., LIEN, S., GLOVER, K. A. & HINDAR, K. 2011. Generic genetic differences between farmed and wild Atlantic salmon identified from a 7K SNP-chip. *Molecular Ecology Resources,* 11**,** 247-253.

KRASNOV, A., PITKÄNEN, T. I. & MÖLSÄ, H. 1999. Gene transfer for targeted modification of salmonid fish metabolism. *Genetic Analysis: Biomolecular Engineering,* 15**,** 115-119.

KRASNOV, A., REINISALO, M., PITKÄNEN, T. I., NISHIKIMI, M. & MÖLSÄ, H. 1998. Expression of rat gene for l-gulono-γ-lactone oxidase, the key enzyme of l-ascorbic acid biosynthesis, in guinea pig cells and in teleost fish rainbow trout (Oncorhynchus mykiss). *Biochimica et Biophysica Acta (BBA) - General Subjects,* 1381**,** 241-248.

LEGGATT, R., BRAUNER, C., IWAMA, G. & DEVLIN, R. 2007. The glutathione antioxidant system is enhanced in growth hormone transgenic coho salmon (Oncorhynchus kisutch). *Journal of Comparative Physiology B,* 177**,** 413-422.

LEGGATT, R. A., DEVLIN, R. H., FARRELL, A. P. & RANDALL, D. J. 2003. Oxygen uptake of growth hormone transgenic coho salmon during starvation and feeding. *Journal of Fish Biology,* 62**,** 1053-1066.

LORENZEN, N., LORENZEN, E. & EINER-JENSEN, K. 2001. Immunity to viral haemorrhagic septicaemia (VHS) following DNA vaccination of rainbow trout at an early life-stage. *Fish Shellfish Immunol,* 11**,** 585-91.

MANDELL, D. J., LAJOIE, M. J., MEE, M. T., TAKEUCHI, R., KUZNETSOV, G., NORVILLE, J. E., GREGG, C. J., STODDARD, B. L. & CHURCH, G. M. 2015. Biocontainment of genetically modified organisms by synthetic protein design. *Nature,* 518**,** 55-+.

MCGINNITY, P., PRODÖHL, P., FERGUSON, A., HYNES, R., MAOILÉIDIGH, N. Ó., BAKER, N., COTTER, D., O'HEA, B., COOKE, D., ROGAN, G., TAGGART, J. & CROSS, T. 2003. Fitness reduction and potential extinction of wild populations of Atlantic salmon, Salmo salar, as a result of interactions with escaped farm salmon. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 270**,** 2443-2450.

MCMAHON, M. A., RAHDAR, M. & PORTEUS, M. 2012. Gene editing: not just for translation anymore. *Nat Meth,* 9**,** 28-31.

MENG, X., NOYES, M. B., ZHU, L. J., LAWSON, N. D. & WOLFE, S. A. 2008. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat Biotech,* 26**,** 695-701.

MOREAU, D. T. R. 2014. Ecological Risk Analysis and Genetically Modified Salmon: Management in the Face of Uncertainty. *Annual Review of Animal Biosciences,* 2**,** 515-533.

MOREAU, D. T. R., CONWAY, C. & FLEMING, I. A. 2011. Reproductive performance of alternative male phenotypes of growth hormone transgenic Atlantic salmon (Salmo salar). *Evolutionary Applications,* 4**,** 736-748.

MORI, T. & DEVLIN, R. H. 1999. Transgene and host growth hormone gene expression in pituitary and nonpituitary tissues of normal and growth hormone transgenic salmon. *Molecular and Cellular Endocrinology,* 149**,** 129-139.

MORI, T., HIRAKA, I., KURATA, Y., KAWACHI, H., MANO, N., DEVLIN, R. H., NAGOYA, H. & ARAKI, K. 2007. Changes in hepatic gene expression related to innate immunity, growth and iron metabolism in GH-transgenic amago salmon (Oncorhynchus masou) by cDNA subtraction and microarray analysis, and serum lysozyme activity. *General and Comparative Endocrinology,* 151**,** 42-54.

MUIR, W. & HOWARD, R. 2002. Assessment of Possible Ecological Risks and Hazards of Transgenic Fish with Implications for Other Sexually Reproducing Organisms. *Transgenic Research,* 11**,** 101-114.

MUIR, W. M. & HOWARD, R. D. 1999. Possible ecological risks of transgenic organism release when transgenes affect mating success: Sexual selection and the Trojan gene hypothesis. *Proceedings of the National Academy of Sciences,* 96**,** 13853-13856.

NAM, Y. K., MACLEAN, N., HWANG, G. & KIM, D. S. 2008. Autotransgenic and allotransgenic manipulation of growth traits in fish for aquaculture: a review. *Journal of Fish Biology,* 72**,** 1-26.

NAYLOR, R., HINDAR, K., FLEMING, I. A., GOLDBURG, R., WILLIAMS, S., VOLPE, J., WHORISKEY, F., EAGLE, J., KELSO, D. & MANGEL, M. 2005. Fugitive Salmon: Assessing the Risks of Escaped Fish from Net-Pen Aquaculture. *BioScience,* 55**,** 427-437.

OKE, K. B., WESTLEY, P. A. H., MOREAU, D. T. R. & FLEMING, I. A. 2013. *Hybridization between genetically modified Atlantic salmon and wild brown trout reveals novel ecological interactions.*

OZEROV, M. Y., VESELOV, ALEXEY E., LUMME, J. & PRIMMER, CRAIG R. 2012. “Riverscape” genetics: river characteristics influence the genetic structure and diversity of anadromous and freshwater Atlantic salmon (Salmo salar) populations in northwest Russia. *Canadian Journal of Fisheries and Aquatic Sciences,* 69**,** 1947-1958.

PERRY, G. M. L., DANZMANN, R. G., FERGUSON, M. M. & GIBSON, J. P. 2001. Quantitative trait loci for upper thermal tolerance inoutbred strains of rainbow trout (Oncorhynchus mykiss). *Heredity,* 86**,** 333-341.

PITKÄNEN, T. I., KRASNOV, A., REINISALO, M. & MÖLSÄ, H. 1999. Transfer and expression of glucose transporter and hexokinase genes in salmonid fish. *Aquaculture,* 173**,** 319-332.

RASMUSSEN, R. S. & MORRISSEY, M. T. 2007. Biotechnology in Aquaculture: Transgenics and Polyploidy. *Comprehensive Reviews in Food Science and Food Safety,* 6**,** 2-16.

REGJERINGEN.NO. Ministry of Climate and Environment. Available: https://www.regjeringen.no/en/dokumenter/gene-technology-act/id173031/ [Accessed 23.03.2015 2015].

REISZ-PORSZASZ, BHASIN, S., ARTAZA, J. N., SHEN, R., SINHA-HIKIM, I., HOGUE, A. & GONZALEZ-CADAVID, N. F. 2003a. Decreased skeletal muscle mass in transgenic mice with muscle-specific hyper-expression of myostatins. *Journal of Investigative Medicine,* 51**,** S117-S117.

REISZ-PORSZASZ, S., BHASIN, S., ARTAZA, J. N., SHEN, R. Q., SINHA-HIKIM, I., HOGUE, A., FIELDER, T. J. & GONZALEZ-CADAVID, N. F. 2003b. Lower skeletal muscle mass in male transgenic mice with muscle-specific overexpression of myostatin. *American Journal of Physiology-Endocrinology and Metabolism,* 285**,** E876-E888.

RISE, M. L., DOUGLAS, S. E., SAKHRANI, D., WILLIAMS, J., EWART, K. V., RISE, M., DAVIDSON, W. S., KOOP, B. F. & DEVLIN, R. H. 2006. Multiple microarray platforms utilized for hepatic gene expression profiling of GH transgenic coho salmon with and without ration restriction. *Journal of Molecular Endocrinology,* 37**,** 259-282.

ROLLO, C. D. 2014. Trojan Genes and Transparent Genomes: Sexual Selection, Regulatory Evolution and the Real Hopeful Monsters. *Evolutionary Biology,* 41**,** 367-387.

SAUNDERS, R. L., FLETCHER, G. L. & HEW, C. L. 1998. Smolt development in growth hormone transgenic Atlantic salmon. *Aquaculture,* 168**,** 177-193.

SKAALA, Ø., GLOVER, KEVIN A., BARLAUP, BJØRN T., SVÅSAND, T., BESNIER, F., HANSEN, MICHAEL M. & BORGSTRØM, R. 2012. Performance of farmed, hybrid, and wild Atlantic salmon (Salmo salar) families in a natural river environment. *Canadian Journal of Fisheries and Aquatic Sciences,* 69**,** 1994-2006.

SKAALA, Ø., HØYHEIM, B., GLOVER, K. & DAHLE, G. 2004. Microsatellite analysis in domesticated and wild Atlantic salmon (Salmo salar L.): allelic diversity and identification of individuals. *Aquaculture,* 240**,** 131-143.

SKILBREI, O. T., HEINO, M. & SVÅSAND, T. 2015. Using simulated escape events to assess the annual numbers and destinies of escaped farmed Atlantic salmon of different life stages from farm sites in Norway. *ICES Journal of Marine Science: Journal du Conseil,* 72**,** 670-685.

SKILBREI, O. T., SKULSTAD, O. F. & HANSEN, T. 2014. The production regime influences the migratory behaviour of escaped farmed Atlantic salmon. *Aquaculture,* 424–425**,** 146-150.

SOLEM, Ø., BERG, O. K., VERSPOOR, E., HINDAR, K., KARLSSON, S. O., KOKSVIK, J., RØNNING, L., KJÆRSTAD, G. & ARNEKLEIV, J. V. 2014. Morphological and genetic comparison between naturally produced smolts of Atlantic salmon, brown trout and their hybrids. *Fisheries Management and Ecology,* 21**,** 357-365.

SOMORJAI, I. M. L., DANZMANN, R. G. & FERGUSON, M. M. 2003. Distribution of Temperature Tolerance Quantitative Trait Loci in Arctic Charr (Salvelinus alpinus) and Inferred Homologies in Rainbow Trout (Oncorhynchus mykiss). *Genetics,* 165**,** 1443-1456.

STEVENS, E. D., SUTTERLIN, A. & COOK, T. 1998. Respiratory metabolism and swimming performance in growth hormone transgenic Atlantic salmon. *Canadian Journal of Fisheries and Aquatic Sciences,* 55**,** 2028-2035.

SUNDSTRÖM, L. F., LÕHMUS, M., TYMCHUK, W. E. & DEVLIN, R. H. 2007. Gene–environment interactions influence ecological consequences of transgenic animals. *Proceedings of the National Academy of Sciences,* 104**,** 3889-3894.

SUNDSTRÖM, L. F., LÖHMUS, M. & DEVLIN, R. H. 2005. SELECTION ON INCREASED INTRINSIC GROWTH RATES IN COHO SALMON, ONCORHYNCHUS KISUTCH. *Evolution,* 59**,** 1560-1569.

SUNDSTRÖM, L. F., VANDERSTEEN, W. E., LÕHMUS, M. & DEVLIN, R. H. 2014. Growth-enhanced coho salmon invading other salmon species populations: effects on early survival and growth. *Journal of Applied Ecology,* 51**,** 82-89.

SUNDT-HANSEN, L., SUNDSTRÖM, L. F., EINUM, S., HINDAR, K., FLEMING, I. A. & DEVLIN, R. H. 2007. *Genetically enhanced growth causes increased mortality in hypoxic environments*.

TARANGER, G. L., SVÅSAND, L., KVAMME, B. O., KRISTIANSEN, T. & KROON BOXASPEN, K. 2014. Risikovurdering av norsk fiskeoppdrett 2013. *Fisken og havet.* Norwegian Institute of Marine Research.

TAYLOR, E. B. 1991. A review of local adaptation in Salmonidac, with particular reference to Pacific and Atlantic salmon. *Aquaculture,* 98**,** 185-207.

THODESEN, J. & GJEDREM, T. 2006. Breeding programs on Atlantic salmon in Norway -lessons learned. WorldFish Center Conference, 2006.

TIBBETTS, S. M., WALL, C. L., BARBOSA-SOLOMIEU, V., BRYENTON, M. D., PLOUFFE, D. A., BUCHANAN, J. T. & LALL, S. P. 2013. Effects of combined 'all-fish' growth hormone transgenics and triploidy on growth and nutrient utilization of Atlantic salmon (Salmo salar L.) fed a practical grower diet of known composition. *Aquaculture,* 406–407**,** 141-152.

TOWNSEND, C. R. 1996. Invasion biology and ecological impacts of brown trout Salmo trutta in New Zealand. *Biological Conservation,* 78**,** 13-22.

TOYOHARA, H., NAKATA, T., TOUHATA, K., HASHIMOTO, H., KINOSHITA, M., SAKAGUCHI, M., NISHIKIMI, M., YAGI, K., WAKAMATSU, Y. & OZATO, K. 1996. Transgenic Expression ofl-Gulono-γ-lactone Oxidase in Medaka (Oryzias latipes), a Teleost Fish That Lacks This Enzyme Necessary forl-Ascorbic Acid Biosynthesis. *Biochemical and Biophysical Research Communications,* 223**,** 650-653.

TOYOHARA, H., TAKAGI, M., KINOSHITA, M., TOUCHATA, K., KUBOTA, S. & KATO, K. Improvement of fish meat quality by transgenic technology. International Marine Biotechnology Conference, June 7-12 2005 St. John's, New Foundland, Canada. 71.

TRAXLER, G. S., ANDERSON, E., LAPATRA, S. E., RICHARD, J., SHEWMAKER, B. & KURATH, G. 1999. Naked DNA vaccination of Atlantic salmon Salmo salar against IHNV. *Diseases of aquatic organisms,* 38**,** 183-190.

VANDERSTEEN TYMCHUK, W. E., ABRAHAMS, M. V. & DEVLIN, R. H. 2005. Competitive Ability and Mortality of Growth-Enhanced Transgenic Coho Salmon Fry and Parr When Foraging for Food. *Transactions of the American Fisheries Society,* 134**,** 381-389.

VERSPOOR, E., DONAGHY, M. & KNOX, D. 2006. The disruption of small scale genetic structuring of Atlantic salmon within a river by farm escapes. *Journal of Fish Biology,* 69**,** 246-246.

VERSPOOR, E., STRADMEYER, L. & NIELSEN, J. L. 2008. *The Atlantic salmon: genetics, conservation and management*, John Wiley & Sons.

WANG, W., LIU, X., GELINAS, D., CIRUNA, B. & SUN, Y. 2007. A Fully Automated Robotic System for Microinjection of Zebrafish Embryos. *PLoS ONE,* 2**,** e862.

WEIR, L. K., HUTCHINGS, J. A., FLEMING, I. A. & EINUM, S. 2004. Dominance relationships and behavioural correlates of individual spawning success in farmed and wild male Atlantic salmon, Salmo salar. *Journal of Animal Ecology,* 73**,** 1069-1079.

WEIR, L. K., HUTCHINGS, J. A., FLEMING, I. A. & EINUM, S. 2005. Spawning behaviour and success of mature male Atlantic salmon (Salmo salar) parr of farmed and wild origin. *Canadian Journal of Fisheries and Aquatic Sciences,* 62**,** 1153-1160.

WONG, A. C. & VAN EENENNAAM, A. L. 2008. Transgenic approaches for the reproductive containment of genetically engineered fish. *Aquaculture,* 275**,** 1-12.

XU, C., WU, G., ZOHAR, Y. & DU, S. J. 2003. Analysis of myostatin gene structure, expression and function in zebrafish. *Journal of Experimental Biology,* 206**,** 4067-4079.

YANG, J. Z. & ZHAO, B. P. 2006. Postnatal expression of myostatin propeptide cDNA maintained high muscle growth and normal adipose tissue mass in transgenic mice fed a high-fat diet. *Molecular Reproduction and Development,* 73**,** 462-469.

ZBIKOWSKA, H. 2003. Fish can be First – Advances in Fish Transgenesis for Commercial Applications. *Transgenic Research,* 12**,** 379-389.

Glossary

A

Active domain (active sites): the part of a protein that must be maintained in a specific shape if the protein is to be functional, e.g. the part to which the substrate binds in an enzyme.

Adaptation: evolutionary process involving genetic change by which a population becomes fitted to its prevailing environment.

Adenine: a purine base that pairs with thymine in the DNA double helix.

Adenovirus: an icosahedral (20-sided) virus that contains DNA (as opposed to RNA).

Allele: a different version of the same gene (found at the same locus but in homologous chromosomes or in different individuals) that may produce different phenotypes.

Allergen: a substance which causes an allergic reaction.

Allopatric: having separate and mutually exclusive areas of geographical distribution.

Amino acid: any member of a class of 20 molecules, which are combined to form proteins in living things. The sequence of amino acids in a protein is determined by the genetic code, and different sequences indicate different functions.

Amplification: an increase in the number of copies of a specific DNA fragment; can be *in vivo* or *in vitro*.

Antibiotic: organic compound that is naturally formed and secreted by various species of microorganisms and plants. It has a defensive function and is often toxic to other bacterial species, e.g. penicillin, originally produced by bread mould, is toxic to numerous human pathogens.

Antibiotic resistance: usually refers to microorganisms, it is the ability of an organism to withstand the effects of an antibiotic. Antibiotic resistance can be a serious problem in a clinical setting, but it can also be induced in genetically modified organisms in order to select successfully transformed individuals (see antibiotic resistance marker gene).

Antibiotic resistance marker gene (ARM gene): marker genes which are inserted along with transgenes during genetic modification, in order to determine whether transformation was successful. ARM genes allow antibiotics to be used to detect the organisms which have been successfully transformed, since the ARM gene confers resistance to the antibiotic which the recipient organism would only have if insertion was successful.

Antibody: a protein produced by the B-lymphocytes (white blood cells) of the immune system. It binds to a particular antigen (foreign substance) to inactivate it or mark it for destruction by other immune system cells. Also used in molecular biology to detect particular proteins in biological samples (see Western Blot and ELISA).

Anti-freeze protein: glycoprotein found in the blood of some polar fish, and which depress the freezing point of the blood by enveloping small ice crystals that would otherwise form ice nuclei and cause the blood to freeze.

B

***Bacillus thuringiensis* (Bt):** rod-shaped soil bacteria that produce toxins, called the Bt toxin or 'cry' (crystal) proteins, which are usually specific to insects from the orders Lepidoptera, Coleoptera or Diptera. The genes which code for these toxins are known as Bt genes, and are used in some genetically modified crops (Bt crops) to confer insect resistance.

Biodiversity: a broad term referring to the variety of life in a given area. It includes ecosystem diversity, species diversity and genetic diversity. It also involves the countless, complex ways in which living things function and interact.

Biodistribution: the dissemination, into different body organs, of gene therapy (GT) vectors, GM vaccines/vectors, and/or products arising from these. Biodistribution studies are necessary for safety evaluation of novel GT products and GM vaccines.

Biofuels: fuels obtained from biomass (such as trees, algae etc.) which has not undergone fossilisation.

Biomolecule: any organic molecule that is an essential part of a living organism.

Bt gene: a gene coding for a Bt toxin, see *Bacillus thuringiensis*.

Bt crop: a crop containing one or several gene(s) coding for a Bt toxin, see *Bacillus thuringiensis*.

Bt toxin/'cry' protein: insecticidal crystal proteins originally isolated from the bacterium *Bacillus thuringiensis*, see *Bacillus thuringiensis*.

C

CaMV: Cauliflower Mosaic Virus**.** A member of the caulimoviruses, one of the six genera in the Caulimoviridae family pararetroviruses that infect plants. The 35S CaMV promoter of this virus is important in genetic engineering, and is frequently used in transgene constructs.

Cartagena Protocol: the Cartagena Protocol on Biosafety to the Convention on Biological Diversity is an international agreement which concerns the safe transfer, handling and use of living modified organisms (LMO – similar meaning to GMO) resulting from modern biotechnology, that may have adverse effects on the conservation and sustainable use of biodiversity, taking also into account risks to human health, and specifically focusing on transboundary movements (see CBD).

CBD: Convention on Biological Diversity. The CBD is a global, comprehensive agreement addressing all aspects of biological diversity: genetic resources, species, and ecosystems.

cDNA: Complementary DNA. cDNA is single-stranded DNA made from a messenger RNA template under the aegis of the enzyme reverse transcriptase. This form of DNA is often used as a probe in the physical mapping of a chromosome.

Centre of origin: the geographical region in which a species developed its characteristic properties.

Chimaera: an animal or plant consisting of some cells with one genetic constitution and some with another, i.e. composed of cells of two different genotypes.

Chromosome: the self-replicating genetic structure of cells containing the cellular DNA that bears in its nucleotide sequence the linear array of genes.

Clone: A group of cells derived from a single ancestor or parent.

Cloning: 1) making many copies (amplification) of a section of DNA in a microorganism such as *Escherichia coli*; 2) Production of a DNA identical series of organisms such as through natural cell division or *in vitro* manipulation.

Codon: A block of three nucleotide residues (three bases) that corresponds with one unique amino acid. There can be many different codons for each amino acid, but only one amino acid for each codon.

Conjugation: A form of DNA transfer involving cell-to-cell contact, usually refers to the transfer of mobile genetic elements such as plasmids between cells.

Conspecific: Belonging to the same species.

Contained use: An activity in which genetically modified organisms are developed, cultured, stored, transported, destroyed, disposed of, or used in any other way, and for which physical, chemical or biological barriers, or any combination of such barriers, are used to limit their contact with, and to provide a high level of protection for, humans and the environment.

D

Deliberate release: no specific containment measures are taken to prevent interaction with the general population and the environment.

DHA: Docosahexaenoic acid. Nutritionally important omega-3 fatty acid.

Differentiation: the process by which a group of unspecialized cells (e.g. stem cells) become specialized to perform particular functions.

Diploid: organisms whose cells (apart from the gametes) have two sets of chromosomes, and therefore two copies of the basic genetic complement of the species.

Dispersal: the tendency of an organism to move away, either from its birthplace (natal dispersal) or breeding site (breeding dispersal). Rates of regional dispersal depend on the interaction of several factors, notably the size and

shape of the source area, the dispersal ability of the organism, and the influence of other environmental factors such as winds or ocean currents.

DNA: Deoxyribonucleic acid (DNA) is a macromolecule made up of two polynucleotide chains in a double helix, which is responsible for carrying the genetic information of cells and some viruses.

DNA polymerase: an enzyme that catalyzes the synthesis of DNA from deoxyribonucleotides in a 5 prime to 3 prime direction alongside a DNA template strand. DNA polymerases cannot initiate the synthesis of new DNA chains, but can only extend chains from pre-existing 3' hydroxyl termini.

DNA replication: the process by which cells make an identical copy of a section of double-stranded DNA, using existing DNA as a template in the assembly of the nucleotides into a new strand. The two resulting copies are identical in sequence but not origin with each of the new products composed of one newly synthesized strand.

DNA vaccine: a technique for protecting an organism against disease by injecting it with naked DNA to produce an immunological response.

DNA virus: a virus with a DNA genome.

Domestication: the selective breeding by humans of plant and animal species in order to accommodate human needs.

Dominant allele: an allele associated with a phenotype that is expressed when the allele is either heterozygous or homozygous.

E

***E. coli*:** *Escherichia coli*. A species of bacteria which inhabits the lower intestines of mammals, which has also been used extensively in biotechnology as a model organism.

Ecological niche: the role of an organism in a community in terms of the habitat it occupies, its interactions with other organisms and its effect on the environment. A given niche may be occupied by different species in different ecosystems and different parts of the world.

Ecosystem: A discrete unit consisting of living and non-living parts, interacting to form a stable system.

ELISA: Enzyme-Linked ImmunoSorbent Assay. Highly sensitive technique for detecting and measuring antigens or antibodies in a solution. The solution is run over a surface to which immobilized antibodies specific to the substance have been attached, and if the substance is present, it will bind to the antibody layer, and its presence is verified and visualized with an application of antibodies that have been tagged in some way.

Embryo: an early stage of development in a multi-celled organism, between fertilization and the point at which it becomes free-living.

Endogenous: originating within the organism, cell or system being studied.

Enzyme: a protein that acts as a catalyst in biochemical reactions.

EPA: Eicosapentaenoic acid. Nutritionally important omega-3 fatty acid.

Epigenetics: Basis of inheritance by which modifications in gene function can be inherited without a change in DNA sequence. Such modifications include DNA methylation, heterochromatin formation, genomic inactivation, paramutation, X-chromosome inactivation.

Epitope: the portion (determinant) of an antigen which allows it to be recognized by the immune system.

ERA: Environmental risk assessment. The process of determining whether a substance or activity may be at risk of causing adverse effects on the environment.

Eukaryote: organism composed of one or more cells with a distinct nucleus and cytoplasm. It includes all forms of life except viruses and prokaryotes (Bacteria and Archea).

Exotic: not native to a given area; either intentionally transplanted from another region or introduced accidentally.

Expression system: the genetic and cellular components needed for the expression of a selected gene, including structural genes, control sequences, inducers etc. Although expression systems traditionally include both prokaryote and eukaryote cell types, cell-free systems for the use in laboratories also exist.

Expression cassette: a genetic construct which includes regulatory sequences as well as the transgene(s) of interest, which directs cellular machinery in the production of RNA and proteins.

Exogenous: Originating outside the organism, cell or system being studied.

F

Field trials: an experimental trial involving GMOs grown at a specific site for the purposes of conducting research.

Fitness: the fitness of an individual is defined as the relative contribution of its genotype to the next generation relative to the contributions of other genotypes. Its fitness is determined by the number of offspring it manages to produce and rear successfully.

Fixation: loss of all alleles of a gene but one in a population.

Food webs: networks which graphically represent the feeding relationships and flow of energy and nutrients between species in a biotic community.

Foundation species: a species that structures the local community and modulates fundamental ecosystem processes.

Fry: newly hatched fish.

G

Gene: a unit of heredity, made up of DNA. Genes are made up of DNA sequences which are transcribed into RNA, which either functions directly or is translated into a polypeptide chain which could form part of a protein.

Gene ecology: the study of interactions between hereditary materials and their surrounding environment in the broadest sense. This includes the organization, function and transmission of genes and nucleic acid fragments in different ecosystems and in the context of varying ecological parameters. Hence, gene ecological research should be based on knowledge from functional genomics and other modern biosciences, combined with ecology, evolutionary sciences, bioethics, social sciences, and the philosophy of science. Since human-made changes crucially influence the ecosystems, social scientific approaches should be included in the working hypotheses, research designs and impact analyses of gene ecology.

Gene expression: the process by which a gene is regulated and its product synthesized. This is most often associated with the process by which genetic information in DNA is converted to functional proteins within a cell, but could also comprise RNA as an end product.

Gene dosage: the number of copies of a particular gene in a nucleus or cell.

Gene flow: the transmission of genes between compatible populations.

Gene silencing: interruption or suppression of the expression of a gene at transcriptional or translational levels.

Gene therapy: techniques that use genetic material to treat or prevent disease.

Genetic diversity: usually expressed in terms of percentage of genes that are polymorphic and/or are heterozygous.

Genetic drift: (1) Random changes in gene frequency in small isolated populations owing to factors other than natural selection, such as sampling of only a small number of gametes in each generation; (2) Random nucleotide changes in genes not subject to natural selection.

Genetic engineering: a variety of techniques used to intentionally change the genes in a living cell or organism.

Genetically engineered organism: see GMO.

Genetic material: DNA and/or RNA, which store the genetic information of an organic life form.

Genetic modification: the deliberate alteration of the genetic constitution of a living organism or cell by artificial means (i.e. not by conventional breeding, but using a number of scientific gene modifying techniques), such as the introduction of a gene from another species or the introduction of a mutation in a specific gene.

Genetically modified organism (GMO): any organism which has had its genetic material altered by genetic modification techniques (i.e. not through mating and/or natural recombination). See Genetic modification.

Genetically modified microorganism (GMM): a micro-organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination, but through the use of certain

biotechnological techniques.

Genetically Recoded Organism (GRO): an organism which has been modified to be dependent on synthetic metabolites, such as non-canonical amino acids, thereby losing the ability to survive outside of controlled conditions.

Genome/Genomic DNA: the total complement of genetic material (DNA or RNA) contained in a cell or virus, including genes and non-coding sequences, but excluding other genetic elements such as plasmids.

Genomics: The study of an organism's genome, including the organization of nucleotide sequences of the genes contained in the genome.

Genomic plasticity: a change in genome structure or organization associated with environmental signal, possibly due to the effects of mutational hotspots, genome expansion, transposable elements or somatic recombination.

Genotype: the precise genetic constitution of an organism. For a diploid organism, it refers to the particular pair of alleles present for any given gene.

Growth hormone (GH): a growth-promoting protein hormone produced by the pituitary, also stimulates the production of several other important hormones involved in a variety of processes.

H

Habitat: the environment within which an organism is normally found. A habitat is characterized by the physical characteristics of the environment and/or the dominant vegetation or other stable biotic characteristics.

Harmful Algal Blooms (HABs): an increase in the population of a single species of algae within a given area, designated as harmful when they directly or indirectly detrimentally affect other organisms or ecosystem functions.

Herbicide: a toxic substance used to destroy unwanted vegetation (weeds).

Heterologous: (1) Of different origin; (2) Derived from different species; (3) Differing morphologically.

Homologous: (1) DNA or protein sequences that have some degree of similarity to each other because they have been derived from a common ancestral sequence by divergent evolution; (2) Structures or other attributes in different species that resemble each other because of origin by common descent; (3) Chromosomes in a diploid organism which contain the same sequence of genes but are derived from different parents, and which pair with each other at meiosis; (4) Structures having the same phylogenetic origin but not necessarily the same final structure or function.

Horizontal Gene Transfer (HGT): all forms of gene transfer which do not involve parent-to-offspring transfer.

Hybrid: progeny of a cross between parents of different genotype.

Hybridization: (1) Formation of a hybrid. (2) The specific re-association of complementary strands of nucleic acids.

I

Immunogenic/immune response: how the body recognizes and defends itself against bacteria, viruses and substances that are foreign and identified as harmful to it. **Intracellular:** inside the cell.

Introgression: the gradual diffusion of genes from the gene pool of one species into another when there is some hybridization between the two species as a result of incomplete genetic isolation.

Invasive species: an organism that is not indigenous to a given habitat, which has been accidentally or deliberately introduced by human activity.

Iridoviruses: A family of viruses all with dsDNA genomes.

Isogenic control: the parental line of organisms used to develop GMOs, which thus have the same genetic background as the GMO and can be used to assess changes brought about due to genetic modification.

L

Living modified organism: see GMO.

Locus (pl. loci): the location of a gene on a chromosome.

M

Marine fatty acids: long chained organic acids of the general formula $CH_3(C_nH_x)COOH$, of marine origin.

Marker genes: a marker gene is used to determine whether a transgene has been successfully inserted into a recipient organism.

Maturation: (1) ripening; (2) the process of becoming mature and fully functional; (3)automatic development of a behavior pattern which becomes progressively more complex as the animal matures and which does not involve learning.

Metabolic rate: the rate at which an organism uses energy to sustain essential life processes such has respiration, growth, reproduction, and in animals, processes such as blood circulation, muscle tone and activity. A measure of the rate of metabolic activity in a living organism.

Metagenomics: the direct genetic analysis of genomes contained with an environmental sample, usually applying to microorganisms. Metagenomics applies a suite of genomic technologies and bioinformatics tools to access the genetic content of entire communities of organisms.

Microbiome: the collective genomes of the microbes (composed of bacteria, bacteriophage, fungi, protozoa and

viruses) that live in/on a particular environment, such as the human body.

Migration: (1) the seasonal journey to a different location made by many animal species, usually in response to changes in seasonal climate and food supply, often traveling very long distances along predetermined routes; (2) the movement of plants into new areas.

Micro-competition: a hypothesis suggesting that regulatory disturbances can arise from competition among endogenous promotors and foreign DNA for a limited pool of transcriptional regulators.

Monitoring: regular data collection, often over long time intervals, to look for changes in selected variables such as environmental status, the amount of GM material in food or feed products, etc. **MON810:** a type of Bt maize which expresses the cry1Ab gene, conferring resistance to Lepidopteran insects to the plant. As one of the first generation of GM crops, MON810 is the best characterized and one of the very few GM crops for which the transgene sequence is publicly known.

Morphology: (1) The form and structure of an organism; (2) The study of form and structure.

mRNA: Messenger Ribonucleic Acid (mRNA) is transcribed from a DNA template, and carries coding information to the ribosomes, where it is translated into a polypeptide chain (protein).

Mutagenesis: The process by which a gene undergoes a heritable alteration; also, the treatment of a cell or organism with a known mutagen.

Mutation: A change in the sequence of the base pairs in a DNA molecule.

N

Next-Generation Sequencing (NGS): non-Sanger-based high-throughput DNA sequencing technologies in which millions or billions of DNA strands can be sequenced in parallel.

O

Oncogenic: any tumour-forming process.

P

Parr: juvenile salmon after the fry stage, named for the characteristic black 'parr' marks which develop at this time on the sides of their bodies.

PCR: polymerase chain reaction. A technique in which a specific section of DNA is copied (amplified) many times using DNA polymerase.

Phenotype: the sum of characteristics (physical, not genetic characteristics) of an organism (see Genotype).

Phenotypic plasticity: the range of phenotypes a single genotype can express as a function of its environment.

Plasmids: a type of circular mobile genetic element common in bacteria.

Piscine: adjective used to describe something originating from/pertaining to fish (pisces).

Pituitary: in vertebrates, an endocrine gland attached to the undersurface of the brain below the hypothalamus by a short stalk. It secretes a number of important hormones.

Plasticity: the capacity for change under the influence of stimuli.

Pleiotropy: the capacity which certain genes have, whereby they are able to influence multiple phenotypic traits.

Polyploidy: the situation in which the organism has more than two (2n) sets of chromosomes.

Post-translational modification: modifications that occur on a protein, catalysed by enzymes, after its translation by ribosomes is complete.

Primers: short oligonucleotide sequences used during PCR, which, when annealed to a complementary template strand, provides a starting point for replication of the region of DNA between the annealed primers.

Prokaryote: an organism that lacks a nucleus (Bacteria, Archaea).

Promoter: initial binding site for RNA polymerase in the process of gene expression. First transcription factors bind to the promoter which is located 5' to the transcription initiation site in a gene. General and tissue/cell-specific promoters stimulate the expression of a gene under the control of enhancers.

Promoter: a DNA sequence at which RNA polymerase binds and initiates transcription.

Protein: a biomolecule composed of one or more polypeptide chains.

Proteome: the entire complement of proteins in a given organism.

R

Receptor: in the context of this report, receptor refers to a protein molecule which is located in the cell membrane, which interacts with stimuli outside of the cell (such as the protein-binding receptors on the midgut epithelial cells of Lepidopteran insects which render them susceptible to cry toxins).

Recombinant DNA techniques: techniques involving the formation of genetic constructs by combining sections of DNA produced outside an organism, which is then inserted (usually via a vector such as a plasmid or virus) into the genome of a recipient organism.

Recombination: exchange of sections of DNA between different DNA molecules. Examples include rearrangement of a transgenic construct as a result of its insertion into the genome of a recipient organism; or viral recombination between two or more viruses co-infect the same cell.

Regulatory genes/elements: genes that direct the production of proteins that regulate the activity of other genes, or which represent control sites in DNA at which gene expression is regulated.

Resilience: ability of a living system to restore itself to its original condition after being disturbed.

Retrovirus: any virus belonging to the viral family Retroviridae. They are enveloped viruses possessing a RNA genome, and replicate via a DNA intermediate.

Ribosome: a small cytoplasmic organelle that is the site of mRNA translation, thus protein synthesis.

RNA: Ribonucleic acid. A type of nucleic acid in which ribose is the sugar constituent (in contrast to DNA, in which the sugar component is deoxyribose). In some viruses, RNA and not DNA is the repository of hereditary information. In most organisms, where DNA contains the hereditary information, RNA has several other functions, including involvement in transcription and translation of genetic material. Messenger RNA **(mRNA)**, for example, has a sequence complementary to DNA, and is synthesized during transcription in order to convey this information to the ribosomes, where it is used to specify the amino acid sequence needed to build polypeptide chains.

RNA editing: changes made to an RNA molecule which render it no longer complementary to the DNA molecule it was modelled from.

RNA-mediated interference (RNAi): a post-transcriptional modification mechanism in which double-stranded RNA identical to a section of a given gene interferes with gene expression.

Roundup: glyphosate-based herbicide produced by Monsanto Company.

Roundup Ready: crops which are genetically modified to be tolerant to the herbicide Roundup. The active ingredient in Roundup is glyphosate. This modification allows farmers to spray Roundup on fields to kill weeds, without harming the crop plants.

S

Secondary pest development: the subsequent outbreak of a different pest species, following the reduction in target pest densities due to the action of a pesticide.

Site of integration: the position into which the introduced transgenes settles on the genome.

Shedding: the release of newly-synthesized virus particles from a cell. My also refer to release from one body part into another, or from the body into the environment.

Smoltification: The physiological and behavioral adaptation to sea water by juvenile salmon.

Specificity: in the context of this report, specificity refers to the property that a toxin or GMO affect only target organisms (or those very closely related to them), and to leave other organisms unaffected. For example, the Bt toxin Cry1Ab is referred to as being specific because it activity is mostly restricted to insects of the order Lepidoptera.

Stacked events: crops which contain more than one transgene are known as stacked or pyramided crops. Presently, this usually refers to a crop which contains more than one Bt gene, and/or one or more herbicide tolerance genes.

Susceptibility: the property which an organism has, which causes it to be vulnerable to a substance (Bt toxin, herbicide, antibiotic etc.)

Sympatric: Species inhabiting the same or overlapping geographical areas.

Smolt: Fully silvered juvenile salmon migrating or about to migrate to sea.

Synthetic biology (SynBio): the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in living organisms (European Commission Opinion on Synthetic Biology).

T

Teleost: Group of fish including all modern bony fishes except lungfishes, holosteans and crossopterygians. They have thin bony scales covered by an epidermis, a homocercal tail, a hydrostatic air bladder (swinbladder), no spiracle and no spiral valve in the gut.

Transcription: The process through which a DNA sequence is enzymatically copied by an RNA polymerase to produce a complementary RNA. In other words, transcription is the transfer of genetic information from DNA into RNA.

Transduction: the process by which DNA is transferred between prokaryote cells due to the action of viruses known as bacteriophages. Transduction may also refer to the introduction of genetic material into a cell, through the action of a virus vector.

Transformation: may refer to (1) a type of horizontal gene transfer in which cells (usually bacteria) take up naked DNA from their surroundings; or (2) techniques used to introduce DNA (transgenic constructs) into cells. The most common of these techniques used in making transgenic organisms are particle bombardment (a particle accelerator (gene gun), that delivers DNA-coated micro particles into cells), and *Agrobacterium*-mediated transformation (insertion of DNA into cells is facilitated by the bacterium *Agrobacterium*).

Transgenic: an organism into which novel or recombinant DNA has been incorporated.

Transgene: any genetic construct which is transferred into a recipient organism via the techniques of genetic engineering. See GMO.

Translation: the process through which amino acids are assembled into a polypeptide chain according to the sequence of an mRNA molecule.

Triploidy: having three sets of chromosomes in the somatic cells.

Trophic interactions: interactions between the different trophic levels of a food web. The trophic level is a step in the transfer of food or energy within a food chain. There may be several trophic levels within a system, for example producers (e.g. plants), primary consumers (e.g. herbivores), secondary consumers (e.g. predators), tertiary and quaternary consumers (also predators).

Trophic level: the position that an organism occupies in a food chain – what it eats and what eats it.

Type I error: a 'false positive': the error of rejecting a null hypothesis when it is actually true.

Type II error: a 'false negative': the error of not rejecting a null hypothesis when the alternative hypothesis is true.

U/V/W

Vector: in the context of this report, a vector is a vehicle which is used to introduce DNA into the cells of a recipient organism. Such vectors may be viruses, plasmids etc.

Viability: capability of living, developing and surviving to parturition (delivering offspring).

Virus vaccine: a preparation containing dead, inactivated or attenuated virus particles, administered for the purpose of developing an immune response (antibody production) against such agents.

Western Blot: a technique which is used to detect specific proteins within a sample containing many proteins. Characterization of the protein in terms of size, as well as amount expressed, can also be done with Western blots.