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ERMA New Zealand
NGĀ KAIWHAKATŪPATO WHAKARARU TAIAO

Risk assessment of horizontal gene transfer from GM plants to bacteria and human cells

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Foreword

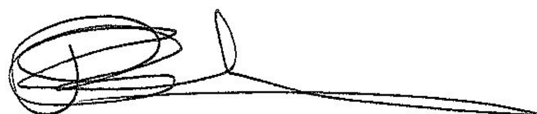
ERMA New Zealand has commissioned this report on horizontal gene transfer (HGT) in order to contribute to and further inform the discussion in New Zealand about the management of genetically modified organisms (GMOs).

The report explores issues around the potential for horizontal gene transfer, the current knowledge, research and scientific evidence, and the implications in terms of risk management. Its scope is limited to scientific evidence and research; it doesn't engage with issues of Māori cultural values or risk. As such, it contributes to one part of the debate about GMOs. Our intention is that the report progresses our understanding of issues associated with HGT, and makes a meaningful contribution to the ongoing discussion.

The material in the report is necessarily based on current scientific knowledge and evidence, which may change over time.

As a regulator, ERMA New Zealand is acutely aware of the wide range of views on GMOs. We wanted this report to fairly reflect as many of those views as possible. Consequently, the report was prepared and peer-reviewed by a number of people who hold a broad range of opinions.

It is my hope that this report will contribute to informed discussion and debate about genetic modification research in New Zealand. I encourage you to read it.



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Chief Executive
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Acknowledgments

A draft version of this report was reviewed by people chosen for their diverse perspectives and technical expertise, in accordance with procedures approved by ERMA New Zealand. The purpose of this independent review was to provide candid and critical comments which would assist ERMA New Zealand in making its published report as scientifically sound as possible. The review comments and draft manuscript are confidential, to protect the integrity of the deliberative process.

These reviewers provided many constructive comments and suggestions, but they were not asked to endorse the conclusions, nor did they see the final draft of the report before its release. Responsibility for its final content rests entirely with ERMA New Zealand.

ERMA New Zealand thanks Dr Philip Carter (Institute for Environmental Science and Research), Associate Professor Jack A Heinemann (University of Canterbury) and Professor Kaare M Nielsen (University of Tromso, Norway) for the roles they played in this process.

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Terms of reference

The objective is to produce a reference report for ERMA New Zealand that:

- reviews the current state of knowledge about horizontal gene transfer (HGT) related to genetically modified organisms (GMOs);
- evaluates the scientific evidence related to the potential for HGT occurring from GMOs;
- evaluates the scientific evidence related to the potential consequences of HGT occurring from GMOs;
- describes research occurring in New Zealand related to HGT; and
- identifies how the potential risks of HGT may be assessed under the current regulatory framework in New Zealand.

The work will include a review of the relevant scientific literature, recommendations of other regulatory agencies and informed inputs from the New Zealand research community, concerning potential risks and issues raised by the possibility of horizontal gene transfer.

The report will draw conclusions about the potential risks associated with horizontal gene transfer in the context of applications under Part V of the Hazardous Substances and New Organisms (HSNO) Act 1996. It will also try to identify any unresolved issues and the level of certainty/uncertainty that results from the current state of knowledge.

Preface

Most of the current scientific literature focuses on the potential for HGT to occur from genetically modified plants to other species. Therefore, the emphasis in this report is on HGT from GM plants, in particular GM food crops, rather than HGT occurring from other types of GMOs. The report focuses on the implications for risk assessment under the HSNO Act of HGT occurring from GMOs.

The qualitative descriptors used in this report and by ERMA New Zealand to describe the likelihood and magnitude of particular effects are included in Appendix 1.

1 Executive summary

HGT is defined as the transfer of genetic material from one organism to another organism outside the context of parent to offspring (ie vertical) reproduction. It is a common process for exchanging genetic material in the microbial world, and there are many non-GM examples of HGT that have occurred during the evolution of bacterial, plant and animal genomes. Public submissions on ERMA New Zealand applications frequently express concern over the potential environmental and human health impacts associated with HGT from GM crops and GM livestock.

ERMA New Zealand's approach to the risk assessment of effects resulting from HGT of transgenes is based on an assessment of the likelihood and magnitude (ie consequence) of an adverse effect occurring. If no credible exposure pathway or potential adverse effect can be demonstrated for HGT, then no further risk management measures are needed.

A number of biological factors act as a barrier to HGT and potentially reduce the likelihood of a gene transfer event. For example, DNA persistence and biological availability, bacterial competence, and presence of sequence homology affect the likelihood of HGT. A range of potential pathways that may permit HGT have been identified. Evidence to support the operation of some of these pathways is available at least in laboratory studies (eg GM plants to bacteria), while some others remain speculative (eg GM plants to invertebrates or viruses). The current literature suggests that the likelihood of detecting HGT of a transgene from GM plants to bacteria is highly improbable. To date, no instance of HGT of transgenes from GMOs to other organisms has been detected under field conditions.

However, estimates of the likelihood of HGT are highly context-dependent, and caution should be applied when extrapolating data from laboratory and field test studies to risk assessments. Assessment of the potential for HGT to cause ecological impacts in New Zealand is uncertain, because all published data are based on overseas environmental conditions. The Foundation for Research, Science and Technology has funded research on the factors that may affect HGT under New Zealand environmental conditions.

Also, current sampling methods are too insensitive for monitoring HGT of transgenes in the field. Experimental limitations on the amount of soil and bacterial colonies that can be realistically screened, and the fact that the majority of bacteria are unculturable, affect the ability of monitoring data to reliably inform risk assessment.

Very little work has been done on the potential consequences and adverse effects of HGT. In order to generate an effect, transferred genetic sequences need to be expressed in the recipient organism and selected for in the population. However, selective pressures and the impact of genetic modifications on fitness are poorly understood, and are the subject of ongoing research. We consider that novel transgenes without natural counterparts require particular attention in risk assessment of HGT.

2 Introduction

What is horizontal gene transfer?

Horizontal gene transfer (HGT) is defined as the transfer of genetic material from one organism to another outside the context of parent to offspring (ie vertical) reproduction (Heinemann, 2003).

HGT is a common event in bacterial evolution. This has been demonstrated by data from the sequencing of whole bacterial genomes (van den Eede et al, 2004; Smets and Barkey, 2005; Gogarten and Townsend, 2005). For example, the contribution of HGT to genome sequences has been estimated to vary between 0 percent and 17 percent of the genome with a mean of six percent in sequenced bacterial genomes (Ochman et al, 2000). HGT may facilitate rapid microbial adaptation to stress and new environments.

Mechanisms for horizontal gene transfer

There are three different mechanisms known for the horizontal transfer of genetic material (Nielsen et al 1998; Sorensen et al 2005):

- conjugation;
- transduction; and
- transformation.

These natural processes of gene transfer have also been adapted for use in modern biotechnology to introduce transgenes[1] into organisms.

Conjugation appears to be a common method of DNA transfer for some bacteria and involves "mating" (temporary connection of the cells). Transferable genes usually reside on extrachromosomal elements in the cell such as plasmids. The range of species with which bacteria may transfer genetic material varies; it appears to be very limited for some, but laboratory experiments indicate that under some circumstances conjugation can occur across different taxonomic kingdoms. For example, some laboratory experiments have demonstrated gene transfer between bacteria and yeast, or bacteria and mammalian cells (Heinemann and Sprague, 1989; Waters, 2001; Kunik et al, 2001). Inter-kingdom conjugation can occur outside of laboratory systems. For example, inter-kingdom gene transfer occurs naturally between *Agrobacterium* species and plant cells. Conjugation is unlikely to be a direct pathway for HGT from GM crops to other organisms because the inserted transgenic DNA is not usually present on a plasmid but is integrated into the plant genome.

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[1] A gene that is taken from the genome of one organism and introduced into the genome of another organism by artificial techniques.

Transduction involves the movement of genetic material by viruses. The accidental transfer of DNA between bacterial cells during bacteriophage[2] infections has been shown to facilitate HGT between bacterial populations residing on plant leaf surfaces, or in soil or water (Nielsen, 2003). Viruses that function in both plants and bacteria, and thereby possibly facilitate HGT from plants to bacteria, have not yet been identified (Nielsen et al, 1998).

Transformation involves uptake of free DNA from the environment by an organism. Natural transformation, resulting in new selectable phenotypes, such as antibiotic resistance, has been demonstrated in competent bacterial species exposed to bacterial DNA (Nielsen, 2003). Since some competent[3] bacterial species take up naked DNA[4] independently of its sequence, natural transformation may theoretically facilitate HGT from plants to bacteria (Nielsen et al, 1998). However, research has demonstrated that not all bacteria or other cells are equally likely to naturally take up foreign genetic material (see section 3). Transformation is considered to be the most likely potential mechanism by which HGT from GM crops to other organisms may occur. However, it is considered to occur much less frequently than bacteria-to-bacteria HGT events (see section 3).

Examples of naturally occurring horizontal gene transfer

Analysis of whole genome sequences has clearly demonstrated that HGT has contributed to prokaryotic evolution (Smets and Barkay, 2005; Gogarten and Townsend, 2005). There is also evidence that HGT has occurred naturally between Archaea and Bacteria and between Bacteria and Eukarya (van den Eede et al, 2004; Raymond and Blankenship, 2003). For example, transfer of DNA is well characterised from the bacteria *Agrobacterium* species to the plants that they associate with, and this property is exploited by plant biotechnologists to create GM plants (Miller and Conko, 2004). *Agrobacterium tumefaciens, A. rhizogenes, A. vitis* and *A. rubi* induce tumours in plants by transferring a portion of a large extrachromosomal DNA element (the tumour-inducing, or Ti plasmid) to its plant hosts. Under laboratory conditions, *Agrobacterium* can also genetically transform fungal and animal cells (Gelvin, 2005). Recently, several bacterial species outside of the genus *Agrobacterium* have been discovered that are also able to transfer genes to plants. *Rhizobium* species NGR234, *Sinorhizobium meliloti* and *Mesorhizobium loti* could be engineered to genetically transform several plant species, but the transformation efficiency was relatively low, ranging from one percent to almost 40 percent of that of *Agrobacterium*-mediated transformation (Broothaerts et al, 2005; Gelvin, 2005).

The ability of several bacterial species to transfer genes to plants reinforces the possibility that such HGT may have contributed to plant evolution. It is already known that the genomes of some plant species contain remnants of what were probably ancient transformation events by *A. rhizogenes*.

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[2] A virus which infects bacteria.

[3] Competent cells are bacteria which can accept extra-chromosomal DNA or plasmids.

[4] Naked DNA is histone-free DNA. Naked DNA is the genetic material that is passed from cell to cell during a gene transfer process called transformation.

HGT can also occur between parasites and their animal hosts. HGT has been identified from the parasitic *Wolbachia*[5] bacterium to its host, the adzuki beetle, *Callosobruchus chinensis* (Kondo et al, 2002). A large genome fragment containing a number of ORFs[6] from *Wolbachia* was found in the X chromosome of the beetle. It is unclear, however, whether the bacterial genes in the beetle were active and over what time scale the transfer event occurred.

Plant mitochondrial genes have been transferred from the parasitic flowering plants *Cuscuta* and *Bartsia* to their host flowering plants (*Plantago*), possibly as a result of direct physical contact between them (Mower et al, 2004). Parasitic plants penetrate host plants intracellularly as part of their normal life cycle. Transfer of mitochondrial genes in the opposite direction has also been documented from the host plant (*Tetrastigma*) to its endophytic parasite (Family: Rafflesiaceae; Davis and Wurdack, 2004). Recent studies have indicated that HGT may occur among flowering plants over an evolutionary timescale outside of the context of a parasitic relationship, but the potential mechanism is unknown (Bergthorsson et al, 2003; Bergthorsson et al, 2004).

There is also evidence to suggest that HGT of transposable elements has occurred between species of *Drosophila* flies (Sanchez-Gracia et al, 2005), and that HGT has occurred between algae during algal evolution (Archibald et al, 2003).

Why are there concerns about horizontal gene transfer?

As discussed above, horizontal transfer of genetic material between organisms occurs naturally in the environment and has played a role in the evolution of organism genomes. The increase over the last 50 years of human pathogenic bacteria that are resistant to multiple classes of antibiotics is an example of HGT occurring among bacterial populations and resulting in undesirable consequences (Smets and Barkay, 2005). The improper and excessive use of antibiotics, combined with the ability of bacteria to readily transfer resistance genes on plasmids and transposons amongst themselves, has promoted the widespread dissemination of these genes (see section 4).

The recent widespread cultivation of GM crops in many countries has raised concerns about potential effects arising from HGT of transgenes to other organisms, because of the novel combinations of genetic sequences (such as those encoding pharmaceuticals, biocontrol agents and industrial chemicals) being incorporated into some crop genomes. The likelihood and consequences of HGT occurring from GMOs will be addressed in sections 3 and 4 of this report.

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[5] Members of the genus *Wolbachia* are rickettsia-like endocellular bacteria associated with insects and other invertebrates.

[6] Open reading frames.

3 Assessments of the likelihood of HGT

Steps required for HGT to occur

There are several sequential steps required for HGT to occur and cause an effect:

1. **Exposure of the recipient organism to foreign DNA**
2. **Uptake of foreign DNA by the recipient organism**
3. **Integration of foreign DNA into the genome**[7]
4. Expression of foreign DNA in the organism
5. Selection of organism carrying foreign DNA

There can be confusion over which of the above steps represent gene transfer, but the term HGT is most commonly used to refer to both the transfer and integration of foreign sequences into a genome, rather than just the uptake of genetic material (which may subsequently be degraded). Steps 1 to 3 are addressed in this section as they affect the likelihood of HGT occurring. Steps 4 and 5 will be discussed in section 4 because they relate to the consequence or effect of HGT occurring.

The list above is not exhaustive in terms of all potential avenues for HGT to cause an effect.

Exposure to foreign DNA

For HGT to occur, DNA from the GMO must be accessible to other organisms (for example, free from cells), be of sufficient length and persist long enough for uptake by other organisms to take place. Published research indicates that DNA derived from dying plant cells is generally rapidly degraded but can survive in soils and aquatic environments for some time (Nielsen et al, 1998; Conner and Glare, 2003; Dunfield and Germida, 2004). PCR[8]-based data from field tests of GM sugar beet demonstrated long-term persistence over several months of transgenic plant DNA in the soil (Smalla et al, 1994). The persistence of plant DNA in the soil is related to a number of abiotic and biotic factors. For example, DNA persistence can be decreased by the presence of DNase in the soil, while DNA can be stabilised and protected from nuclease attack by clay minerals in soil. DNA integrity and function may also be reduced by mechanical forces, UV radiation or chemical modification occurring in the environment.

[7] This step relates to the integration of foreign DNA into genetic material which can be independently replicated and maintained within the organism, eg *both* the bacterial chromosome and plasmids, eukaryotic chromosomes.

[8] Polymerase chain reaction (PCR): A method for amplifying a DNA base sequence using a heat-stable polymerase and two 20-base primers, one complementary to the (+)-strand at one end of the sequence to be amplified and the other complementary to the (-)-strand at the other end. Because the newly synthesised DNA strands can subsequently serve as additional templates for the same primer sequences, successive rounds of primer annealing, strand elongation, and dissociation produce rapid and highly specific amplification of the desired sequence. PCR can also be used to detect the existence of the defined sequence in a DNA sample.

Most studies investigating long-term DNA persistence in the soil have relied only on PCR detection and have not looked at whether the DNA is biologically available to organisms in the soil. It is therefore unclear whether the DNA present in the environment will be in a chemical or physical condition that makes it biologically available to transform competent bacteria. Studies of the biological activity of DNA in soil have shown that transforming activity persists for hours only and does not correspond with the observed prolonged physical presence of plant DNA fragments for months or years (Nielsen et al, 1997b; Nielsen, 2003).

Another factor to take into account when considering whether an organism will be exposed to transgenic DNA is that most microorganisms, plants and animals at a GMO field test site will be exposed to foreign DNA from other (non-modified) organisms (such as microbes and invertebrates) present at that site. However, this assumption may be challenged by the development of transplastomic plants, where the chloroplast genome is genetically modified. Transplastomic plants contain approximately 10,000 copies of a transgene per cell (within the chloroplast genome) compared to less than 10 copies of a transgene for a nuclear-modified transgenic plant. This may increase the likelihood that an organism is exposed to a transgenic sequence. Also, engineered plastid DNA is protected within chloroplasts, and it is unclear to what extent this DNA can resist degradation by cytoplasmic nuclease activity. Therefore, the stability and release of plastid DNA may differ from that of chromosomal DNA (Nielsen et al, 2001).

Uptake of foreign DNA

There is little information available on DNA uptake by organisms other than bacteria. Three factors are relevant in assessing the likelihood of uptake of foreign DNA by bacteria. These are:

- differences in competence of different bacteria to take up foreign DNA;
- degradation of DNA by restriction enzymes; and
- “hotspots” for HGT.

Differences in competence of bacteria to take up foreign DNA

Research has determined that many bacterial species need to be in a state of “competence” to take up foreign DNA (Nielsen, 1998; Nielsen et al, 1998; Nielsen et al, 2000b). Competence development in bacteria is a physiologically regulated process and is usually expressed in response to specific growth phases or environmental stimuli (Nielsen et al, 1997a; Thomas and Nielsen, 2005). The conditions necessary for this are unknown for many species of bacteria, and some growth stages and/or micro-habitats may be more or less likely to contain competent bacteria. In one study, *Acinetobacter* sp. was shown to develop competence during plant colonisation and take up a plant plastid gene *in situ* (Kay et al, 2002).

Only one to two percent of described microbial species exhibit natural competence (Jonas et al, 2001). Also, some bacteria can only take up DNA of their own species.

Degradation of DNA by restriction enzymes

Bacterial restriction modification systems could potentially act as defence mechanisms against foreign DNA invasion (Berndt et al, 2003). For example, a bacterium is able to destroy invading DNA from a bacteriophage by cleaving it with a restriction enzyme, while at the same time preventing the destruction of its own DNA (which is modified by methylation). The restriction modification system is effective against double-stranded DNA. However, most competent bacteria are believed to generate single-stranded DNA (ssDNA) during translocation of DNA into the cell. Usually sDNA is not affected by restriction enzymes. Therefore, the bacterial restriction enzyme system probably represents an insignificant barrier to HGT.

"Hotspots" for HGT

Studies of gene transfer from plants to bacteria have focused upon transfer in the plant rhizosphere (the area around plant roots), which is considered a "hotspot" for HGT because of the large numbers of bacteria in this habitat (Nielsen et al, 2001; Carter, 2003; Watson and Carter, 2003). Compounds exuded by roots into the rhizosphere of agriculturally grown plants are able to stimulate natural transformation of *Acinetobacter* in soil (Nielsen and van Elsas, 2001). In some cases, it is believed that exuded nutrients may affect HGT by allowing the bacteria to form localised dense aggregates, or microcolonies, where the likelihood of cell-to-cell contact is high (Sorensen et al, 2005).

Similar hotspots of HGT between bacteria are predicted to occur in animal digestive systems (Nielsen et al, 2000b; Carter, 2003; Nielsen, 2003; Watson and Carter, 2003). The digestive systems of protozoa, nematodes, insect larvae, and earthworms contain high numbers of bacteria. It should be noted, however, that such HGT hotspots are normally assessed on the basis of transfer of DNA between bacteria, rather than between higher organisms and bacteria. If bacteria did acquire and retain genetic material then it is likely, especially if the material is on a plasmid, that this material could be transferred to other bacteria by conjugation or transformation. This may be to other members of their species, or in some cases to different species.

Integration of the foreign DNA into the genome

For HGT to occur, the recipient organism must incorporate and maintain the incoming DNA. Recent work on HGT from plants to bacteria indicates that such integrations appear to occur at very low frequencies even under ideal conditions, and sequence similarity between donor and recipient sequences appears to be an important factor (de Vries et al, 2001; Nielsen et al, 2000a). Some sequences may be more likely to be transferred because of sequence similarities (via homologous recombination) to the recipient host or their innate ability to move independently (via site-specific recombination).

In addition, circularisation of linear DNA fragments inside the GM plant containing a bacterial origin of replication[9] remains a theoretical possibility that so far has not been found.

Recent research has also indicated that sub-populations of a bacterial species may differ in their abilities to acquire foreign genetic material. These differences in transformability may be due to some bacteria being deficient in DNA repair pathways. These bacteria may exhibit higher levels of mutation and have been called "mutators". Such mutator bacteria can be more likely to take up and integrate foreign DNA (Townsend et al, 2003; Nielsen and Townsend, 2001; Nielsen et al, 2001; Chopra et al, 2003; Thomas and Nielsen, 2005). A selective pressure may then increase the frequency of the bacteria that have been involved in HGT. It should be noted that mutator strains make up a higher proportion of natural populations of bacteria than expected from studies on laboratory strains (LeClerc et al, 1996; Oliver et al, 2000). However, continuous growth as a mutator has detrimental effects on the fitness of the bacterium (Funchain et al, 2001; Chopra et al, 2003). Also, as mentioned above, the soil environment is very likely to contain DNA from non-modified organisms as well as GMOs, so the chance of a mutator encountering or acquiring transgenic material from a GMO may still be improbable. Information on mutator strains in soil, however, is not well documented.

Laboratory studies have indicated that integration of foreign DNA is more likely if the transferred sequences contain regions of sequence similarity to the microbial genome, such as having part of the same gene (De Vries and Wackernagel, 1998; Nielsen et al, 2000a; Tepfer et al, 2003). This commonly occurs via homologous recombination. For example, in a marker-rescue approach involving artificially introduced stretches of sequence similarity (homology) in a recipient *Acinetobacter* strain, the kanamycin resistance gene from various GM plants was able to be transferred to bacteria, but in the absence of the artificially introduced homologous sequences, no HGT was detected (De Vries and Wackernagel, 1998). Under laboratory conditions, using recipient bacteria with no defined DNA sequence similarity to the plant transgene, no HGT was observed from GM plants to bacteria (Nielsen, 2003).

The length of DNA sequence similarity (or homology) needed for integration varies between bacteria and the specific composition of the recombining molecules. The minimal length of DNA homology required in *Escherichia coli* for integration of circular DNA molecules through a single recombination event is about 20bp. Short regions of homology can mediate recombination, including the incorporation of adjacent non-homologous sequences (Nielsen et al, 1998; Nielsen et al, 2001; Nielsen and Townsend, 2001). For linear DNA molecules to integrate in a bacterial genome, longer stretches of DNA similarity (such as 150-200bp or greater) are usually required (Thomas and Nielsen, 2005). Integration of foreign DNA into the genome is dependent upon the degree of sequence similarity between the incoming DNA and that of the bacterial recipient. The high level of

[9] The nucleotide sequence at which DNA synthesis is initiated. Origins of replication are necessary to prevent a plasmid from being lost by the cell harbouring it.

DNA sequence divergence present between wild type plant and bacterial DNA would therefore naturally limit the extent of successful horizontal gene transfers between them.

However, many GMOs will contain some sequences from viruses and bacteria (such as the promoter, T-DNA border sequences or the transgene) that may facilitate transfer by homologous recombination. The presence of bacterial or viral sequences in GM plants may potentially increase the likelihood of transfer, but this has not been studied in any depth. Recent studies have also demonstrated that many commonly occurring bacteria sequences have high sequence similarity with plant marker genes, suggesting that homologous recombination can occur (Bensasson et al, 2004).

The insertion of transgenes into the chloroplast genome could affect the likelihood of recombination of plant sequences (including the transgene) with bacterial DNA, because chloroplasts are genetically similar to bacteria (Nielsen et al, 2001). In addition, the presence of an anchor sequence, derived from the marker gene in transgenic plants, can facilitate HGT of adjacent larger heterologous fragments (>1kb) of plastid DNA to bacteria by homology-facilitated illegitimate recombination[10] (De Vries et al, 2004). Integration of DNA by illegitimate recombination does not require substantial DNA homology between the incoming DNA and the chromosome and occurs only at very low frequencies in bacteria (Nielsen and Townsend, 2001). None of the studies to date have demonstrated HGT from plants to bacteria in the absence of defined sequence similarity or homology.

The environment contains large quantities of naturally occuring bacterial and viral DNA. This DNA may, to a greater or lesser extent, contain sequences more similar to that in potential recipient organisms than that found in genetically modifies crops. This could make amplification of HGT-acquired transgenic sequences in a population less likely to have an effect, if it confers no selective advantage. There is uncertainty around this assumption because of the lack of experiments that have investigated such “competitive” effects.

The general requirements of sequence homology for integration can be circumvented by site-specific recombination (Nielsen, 1998). Such a mechanism is widely used by bacteriophages, integrons and transposons for their insertion into bacterial genomes (Bordenstein and Reznikoff, 2005). Transposable elements are nucleic acid sequences that have the ability to move to different sites in an organism’s genome. Unlike viruses, they do not have a proteinaceous coat and generally move within genomes rather than between genomes. Conjugative transposons are integrated DNA elements in bacterial genomes that excise themselves to form a covalently closed circular intermediate. This circular intermediate can either reintegrate in the same cell (intracellular transposition) or transfer by conjugation to a recipient and integrate into the recipient's genome (intercellular transposition). Conjugative transposons have a surprisingly broad host range, and they probably contribute as much as plasmids to the spread of antibiotic resistance genes in

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[10] Genetic recombination between two segments of DNA which are not homologous, and which occurs at random locations within their nucleotide sequences. Such a recombination is a rare event.

some genera of disease-causing bacteria. Resistance genes need not be carried on the conjugative transposon to be transferred. Many conjugative transposons can mobilise co-resident plasmids, and the *Bacteroides* conjugative transposons can even excise and mobilise unlinked integrated elements (Salyers et al, 1995). In addition, some antibiotic resistance genes are known to be gene cassettes which could be mobilised by integrons.

If foreign genetic material integrated into a transposable element within the recipient genome, it would have the potential to be mobilised in the genome along with the transposable element. There would be uncertainty over whether transposable elements that contain foreign genetic material would remain viable and able to move within genomes. This would depend upon the type of transposable element and whether it can accommodate additional sequences and remain functional. Transposable elements have been used as vectors to introduce foreign genetic material into cells, so there is the potential for them to acquire and transfer foreign genetic material. GMOs developed for commercialisation usually have identified sites of transgene insertion. This may help determine whether the sequences have inserted into or adjacent to transposable elements, and may, therefore, be more likely to move.

Evidence from laboratory and field studies of HGT

HGT via transformation from GM crops might occur by the following potential pathways:

- from transgenic plants to soil bacteria (particularly in the rhizosphere);
- from transgenic plants to pathogens that infect the plant (such as viruses or fungi);
- from transgenic plants to invertebrates that may feed on the plants; and/or
- from ingested transgenic plants to mammalian gut bacteria or mammalian cells.

The scientific literature to support each of these pathways is described in this section. If the genetic material transfers to another organism then there is also the potential for it to be subsequently transferred to other organisms.

HGT from transgenic plants to soil bacteria

The rhizosphere, aerial plant surfaces and plant tissues are all heavily colonised with bacteria. These bacteria could be exposed to transgenic DNA released from the GM plant during decomposition, cell lysis induced by plant pathogens or after mechanical disruption of plant tissue caused by pest activity or harvesting (Nielsen, 2003).

HGT from non-transgenic plants to bacteria has been suggested based on DNA sequence comparisons, eg transfer of a plant leghemoglobin gene to *Vitreoscilla* or transfer of a plant glucose-6-phopshate isomerase gene to *E. coli* (Nielsen et al, 1998). However, the comparison of DNA sequences to obtain evidence of HGT is still regarded as controversial, as it requires extensive knowledge of the DNA sequences evaluated, as well as other genes in both related and unrelated organisms (Kurland et al, 2003).

HGT from transgenic plants to bacteria has been difficult to demonstrate both experimentally (Broer et al, 1996; Schluter et al, 1995) and in field trials of transgenic plants (Smalla et al, 1994; Gebhard & Smalla, 1999; Paget et al, 1998). However, a soil bacterium,

Acinetobacter sp., has been shown experimentally to be transformed by DNA present in homogenised leaf tissue from transgenic sugar beet under optimised *in vitro* conditions (Gebhard and Smalla, 1998). HGT of DNA extracted from transgenic sugar beets to *Acinetobacter* sp. has also been demonstrated using sterile soil microcosms[11] (Nielsen et al, 2000a). However, studies have not been able to demonstrate HGT from transgenic plants to naturally occurring (ie non-laboratory adapted strains) bacteria (Paget et al, 1998; Gebhard and Smalla, 1999). These studies also show that HGT does not occur within the limits of detection of the experiment unless the experimenter has already artificially manipulated the bacterial strain to contain sequences homologous to the incoming transgenic DNA.

To date, HGT from transgenic plants to natural populations of soil microorganisms has not been demonstrated under field conditions (Nielsen, 2003; Heinemann and Traavik, 2004; Nielsen and Townsend, 2004). Studies on bacteria from soil samples obtained from field trials of transgenic sugar beet were unable to detect HGT of the transgene to culturable soil bacteria (Smalla et al, 1994; Gebhard and Smalla, 1999). A number of different conclusions may be drawn from the failure to demonstrate HGT in a field test study: 1) HGT did not occur; 2) transfer frequencies and expression were too low to be detected; 3) rare transformants were not amplified in the population; or 4) the techniques applied were not appropriate for the detection of such HGT. Nielsen and Townsend (2004) estimated that field trials to date have studied potential HGT events occurring in less than two grams of sample material. Thus, the low sample size analysed in these studies is insufficient to provide a basis for determining HGT frequency in agricultural fields. However, it can be concluded that as HGT from transgenic plants was not detected, it is unlikely to occur in agricultural fields at frequencies as high as those encountered in laboratory experiments.

Therefore, HGT from transgenic plants to bacteria is theoretically possible, but, based on studies in the laboratory and in the field, the likelihood of detecting such an event using current techniques is highly improbable.

HGT from transgenic plants to pathogens that infect the plant

Crop plants can be infected by a number of pathogens. Plant pathogens that lyse plant tissue may gain direct access to a plant's DNA. Therefore, there is potential for HGT to occur from a GM crop to the pathogen infecting it. A number of studies have tried to detect HGT between a plant and its associated pathogen. Laboratory studies to model *Erwinia chrysanthemi* infection of transgenic potato (*Solanum tuberosum*) tubers failed to detect transformation of the bacterial pathogen with the transgene from the potato (Schluter et al, 1995). In another model system, to address possible gene transfer from a plant to a pathogenic fungus, transgenic *Brassica* plants were incubated together with *Aspergillus niger* in a microcosm (Hoffmann et al, 1994). Some fungi acquired antibiotic resistance during co-cultivation, but there was no evidence of stable integration or inheritance of the transferred sequence.

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[11] A laboratory system that approximates soil conditions.

Viruses are known to transfer nucleic acid sequences between species, and this is well documented in bacteria (Lawrence and Ochman, 1998). The role of viruses in transferring non-viral genes between plants has been less well investigated, but may also occur in some circumstances. Viral infection of plants creates the potential for the foreign genetic material to be acquired and transferred from the plant genome to another plant by a viral-mediated pathway, or for new viruses to be formed by recombination (Conner and Glare, 2003). The likelihood of this is uncertain, due to limited information on this topic, but viral recombinations are known to occur in plants.

Recombination is a common event for some viruses, although incorporation of additional material in a virus can impair viral replication and/or infectivity (Logg et al, 2001), making the likelihood of transfer very low. However, some viruses can remain infective when they contain additional sequences, and selective pressures may enable a defective virus to acquire the missing or new viral sequences (Chenault and Melcher, 1994; Wintermantel and Schoelz, 1996). These cases of viruses acquiring host genetic material involved DNA viruses with large genomes. Many plant viruses have only a few (<10) genes and are single-stranded RNA viruses. Even under experimental conditions, most plant viruses tend to be quite unstable when additional genes are added.

As noted earlier, the introduced foreign genetic material will represent only a small proportion of the GM plant's genome. Consequently, the foreign genetic sequences would need to be similar to the virus in order for those sequences to be preferentially acquired by the virus via homologous recombination. Many transgenic crops include promoter sequences derived from plant viruses. The likelihood of viral recombination with such short sequences of viral origin is difficult to determine. The potential role of transposons and non-homologous recombination mechanisms in viral acquisition of transgenic sequences is also unclear. It is uncertain whether such recombinations would lead to escape of foreign genetic material from genetically modified plants, since the viability of the resulting virus could only be determined after the event.

Therefore, HGT from GM plants to pathogenic microorganisms (such as fungi and viruses) is theoretically possible, but, based on previous studies in the laboratory and in the field, such an event is highly improbable.

HGT from transgenic plants to invertebrates that may feed on the plants

There has been less research on the ability of organisms other than microorganisms to take up foreign genetic material. There are many invertebrate pests that may feed on GM crops. As already discussed, the insect gut is a potential "hotspot" for HGT. For example, conjugative transfer between bacteria of a marker-carrying plasmid occurred at a higher frequency within the insect gut than in culture conditions (Vilas-Boas et al, 1998). HGT by conjugation has also occurred in the digestive system of a nematode, *Rhabditis* sp., a protozoan, *Tetrahymena* sp., and in the gut of the soil insect *Folsomia candida* (Nielsen et al, 2001). However, in all these cases, HGT occurred between bacteria, and not to invertebrate cells.

No studies have reported HGT from plants to plant-associated invertebrates such as nematodes and insects.

HGT from ingested transgenic plants to mammalian gut bacteria or mammalian cells

There is potential for transgenes to migrate from GM plants to microorganisms in the guts of animals or humans that consume them or to migrate to animal or human tissues (Heritage, 2005). It has been estimated that humans ingest between 0.1 and one gram of DNA in their food per day, and that cows ingest 60 grams of DNA per day (Nielsen et al, 2005; GM Science Review Panel, 2003). The epithelial lining of the mammalian gastrointestinal tract presents a large surface area for contact with foreign DNA and/or DNA-protein complexes. A potential pathway for uptake of foreign DNA could be from the gut contents through the epithelial cells, M cells and Peyer patches in the intestinal wall to peripheral white blood cells, to the spleen and liver.

GM foods and feeds are not a homogenous group. They range from plant material that is consumed fresh and unprocessed, to pure chemical compounds, such as sucrose, that are derived from GM plants (Heritage, 2005). Clearly, purified and highly processed foods such as canola oil are unlikely to contain detectable transgenic DNA, and so the potential for gene transfer will be highly improbable. Mechanical shear forces, temperature, pH, ionic strength, chemical agents and enzymes involved in food processing could affect the primary structure of DNA by causing hydrolysis, oxidation and deamination that damage DNA (see Table 1). Studies have shown that both high temperature and chemical or mechanical treatments reduce the survival of DNA in soymilk[12], tofu[13] and corn masa[14], and that these effects are additive (Kharazmi et al, 2003; Heritage, 2005). Less processed GM food products are more likely to contain detectable transgenic DNA, which could be potentially transferred to cells in the gut if it is not completely degraded by the digestion process.

Conditions in the mammalian gut, such as low pH conditions and enzymes including pancreatic DNases, have a degradative effect on ingested DNA (van den Eede et al, 2004). As well as the DNA degrading activities of the host, the microflora of the gut are also involved in DNA degradation. For example, some ruminal *Prevotella* species produce abundant extracellular DNase activity.

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[12] Steps involved in the production of soymilk include grinding soybeans and boiling the soymilk.

[13] Steps involved in tofu production include grinding the soybeans and the precipitation of proteins with acid or $CaCl_2$.

[14] Corn masa is produced by alkaline boiling of corn and is used to produce Mexican corn-based foods such as tortillas, corn chips and taco shells.

Table 1: Factors affecting the integrity of DNA molecules in the environment and food (adapted from van den Eede et al, 2004).

Factor	Importance in food	Site of action on DNA
Shear forces	Varies according to amount of processing	Double-strand breaks in sugar-phosphate backbone
Enzymes	Potentially high in fermented or raw products	Double-strand breaks, hydrolysis of base sugar bonds
pH	Can vary according to processing	Hydrolysis, strand breaks, deamination of bases
Temperature	If thermal processes are used can exceed 80°C	Denaturation of DNA
Chemical agents (eg bisulphite, nitrite, alkylating agents)	Can be high when additives, curing agents or storage under air is used	Chemical modification and loss of bases
Protective interactions	Variable influences by absorption of DNA to particles. Some food ingredients prevent enzymatic attack	Protection against various damaging factors including shear forces, enzymes and heat

For risk assessment, it is important to know the quantity and integrity of DNA from GM plants that reaches the various intestinal compartments. The lower part of the gastrointestinal tract has the highest density and diversity of bacteria; the likelihood of transformation occurring is generally believed to be most favourable. Several factors make it difficult to estimate the exposure of an intestinal epithelial cell or bacterium to transgenic DNA from diet (van den Eede et al, 2004):

- competition with a large surplus of non-recombinant DNA in a diet as well as from endogenous sources, such as the intestinal microbial population or shed epithelial cells;
- the effect of diet on DNA degradation by binding to or inclusion into compartments of particles that protect DNA from enzymatic attack; and
- the effect of food processing and food storage on the amounts of DNA present and DNA integrity.

In animals fed on unprocessed food, DNA is released during the digestive process. The oral cavity is the first site of contact between DNA released from food, the resident microflora and transient colonisers. There is also potential for gene transfer to occur in ruminants (such as cattle and sheep) where the rumen is not preceded by an acidic barrier. Duggan et al (2000) investigated the transfer of bacterial plasmid DNA to bacteria in sheep saliva or in rumen fluid under laboratory conditions. They found that free DNA was rapidly (within one minute) degraded in rumen fluid, and that exposure to rumen fluid "markedly inhibited" the transformation ability of plasmid DNA. However, in sheep saliva, free DNA was not rapidly degraded and the plasmid DNA retained an ability to

transform a laboratory strain of *E. coli*. However, this study only investigated the transforming potential of plasmid DNA, not chromosomal DNA (chromosomal DNA is the form of DNA likely to be released from a transgenic plant). Plasmids are known to be frequently passed between bacteria.

Degradation of DNA and RNA in the mammalian gut is not always complete, and is not instantaneous. Feeding studies using bacteriophage DNA indicated that foreign DNA is not always completely degraded in the gastrointestinal tract of mice (Schubbert et al, 1997). In these studies, very small quantities of the bacteriophage genetic construct were detected in gut epithelial cells, Peyer's patches, liver cells, in lymphocytes and macrophages found in the spleen. These results were confirmed by more recent studies where ingested DNA sequences (*gfp* gene, bacteriophage M13 or adenovirus type 2 DNA) were taken up by cells of the gastrointestinal tract of mice, and a high-fibre diet sped up the clearance of foreign DNA from the gut (Palka-Santini et al, 2003). Schubbert et al (1998) also demonstrated that ingested foreign DNA can be transmitted via the placenta to the offspring of mice.

Studies of pigs also suggest that DNA is not completely degraded in the gut. Fragments of the *cryIAb* gene were detected by PCR in the gastrointestinal contents of pigs fed Bt11 corn, but not in the control pigs (Chowdhury et al, 2003). This study did not investigate whether or not the uptaken DNA was expressed or conferred an effect. In other studies, DNA recovered from rat intestinal contents was shown to still be biologically active because it retained the ability to transform electro-competent[15] *E. coli* (Wilcks et al, 2004). Interestingly, in this study, the plasmid DNA failed to transform *E. coli* MS15979 in mono-associated rats. This may indicate a difference in competence development within the gut. It is important for risk assessment to determine whether non-degraded DNA is biologically active, because the ability to transform cells is crucial for HGT to exert an effect. This property is lost before the ability to detect target DNA by PCR from samples is lost. It should also be noted that most experiments done to examine the stability of DNA are qualitative in nature, and few have provided quantitative data.

Several other studies have investigated the potential of ingested DNA to persist and enter animal cells (Heritage, 2002). Hohlweg and Doerfler (2001) investigated the fate of gene sequences from soybean leaves eaten by mice. They found that plant-associated DNA may be more stable in the digestive system than naked DNA, with fragments still detectable after many hours. They detected plant gene sequences (ribulose bisphosphate carboxylase/oxygenase) in spleen and liver cells but did not find any evidence that these sequences were expressed or that germline transmission of the ingested DNA occurred.

Einspanier et al (2001) investigated the fate of ingested plant DNA in cattle (fed maize silage) and chickens (fed maize grains). They found that a short piece of non-transgenic chloroplast DNA (199 base pairs) was detectable in cattle lymphocytes and milk, and in a range of tissues in chickens, but not in eggs. The results did not report whether the maize DNA was integrated into the animal chromosomes. Sequences of the *cryIAb* transgene from genetically modified maize were not detected in cattle or chickens fed GM maize.

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[15] Able to be transformed with foreign DNA following treatment with a pulsed electrical field.

However, the authors did not report the minimum copy number required for detection in their system. They considered that the presence of non-transgenic chloroplast DNA but absence of the *cryIAb* gene in animal cells might be due to the much higher amount of the chloroplast sequence in the food (the chloroplast genome is normally present in multiple copies within a single cell) compared to the Cry sequence (present as a single copy). The use of plastid transformation systems that allow expression of multiple copies of transgenes may increase the likelihood that transgenic DNA sequences will be detected in the tissues of animals or humans who consume GM plants.

In another study, the *epsps* transgene could not be detected in milk from cows receiving up to 26 percent of their diet as glyphosate-tolerant soybean meal (Phipps and Beever, 2002).

Human studies

Data from simulated digestion studies demonstrate that transgenes from GM food may survive passage through the human small intestine (Martin-Orue et al, 2002). Bt maize and Roundup Ready soya were incubated in *in vitro* simulation models of human digestion conditions in the stomach and small intestine. Transgenic DNA was not completely degraded in the stomach or small-intestine simulations and it is likely that the food matrix provided some protection for the DNA from digestion.

Netherwood et al (2004) have shown that microorganisms found in the small bowel of people with ileostomies (resection of the terminal ileum and diversion of digesta to a colostomy bag) are capable of acquiring and harbouring DNA sequences from GM plants. In this study, 12 healthy volunteers and seven ileostomists were given a meal of GM soya containing the *epsps* gene. In the healthy subjects, DNA from the transgenic plant material was degraded completely after passage through the colon; in the ileostomist patients, it was incompletely degraded. Therefore, DNA can survive passage through the human stomach in patients with a dysfunctional gastrointestinal system. There was also evidence that transgenic DNA was transferred to a small proportion of the intestinal microflora for three out of the seven ileostomist patients before the study was conducted. Great care was taken to avoid sample contamination. Although contamination cannot be ruled out, the pattern of results makes this explanation unlikely (Heritage, 2004). The transferred DNA only contained a fragment of the *epsps* gene and the full-length gene could not be detected in the intestinal microflora. The transgene fragment was only detected when the microbial population had been amplified, suggesting that the DNA was stably maintained in the bacteria and thus had integrated either into the microbial genome or into a stable extrachromosomal element. Netherwood et al did not study whether the transferred DNA sequences were expressed in the recipient bacteria or identify the site of transgene insertion in the genome. In addition, the transformed organism was never isolated, so the evidence is indirect. These findings indicate that gene transfer from GM plants to bacteria in the human gut may not be as rare as initially expected, especially for humans with severe gastrointestinal disease. However, for healthy humans with an intact gut, gene transfer from GM plants was not detected in this study.

Summary of animal and human studies

In studies which did not detect food-derived DNA, it is unclear whether this was due to its absence in the tissues of humans or animals who had consumed it, or to the insensitivity of the current assay systems for DNA sequences which are typically present in low copy

number. Another limitation of current methods is that over 90 percent of the microorganisms in the gastrointestinal tract are unculturable.

The transfer of foreign genetic material to animal cells following ingestion is theoretically possible in situations where a significant quantity of that DNA is ingested repeatedly over a period of time. It is important to note that non-transgenic conventional DNA sequences derived from non-GM food have also been detected in blood samples of humans, and that the uptake of DNA derived from food is a natural process that relates, at least in part, to the copy number[16] of the DNA sequence that is detected (Heritage, 2005). In any food or feed, conventional plant-derived sequences will be present in vast excess of the DNA inserted into transgenic plant cells (Heritage, 2002). Since the transgenic genetic material would represent only a small fraction of the ingested DNA, it is highly improbable that it would be transferred and incorporated into cells of animals or humans consuming it unless it contained sequence homology that facilitated its preferential integration into the host genome. Furthermore, there is no credible hypothesis to suggest that the transgenic DNA would behave differently from non-transgenic DNA following ingestion.

The implications that can be taken from these studies are that:

- unprocessed food crops are a better source of donor DNA than highly processed foods;
- differences observed in DNA degradation may reflect species-specific differences in the physiology of the gastrointestinal tract between birds, humans, ruminants and rodents;
- DNA is not completely broken down by the mammalian digestive system;
- some ingested DNA may be taken up by a range of cells;
- the DNA is likely to be short and may not code for a complete gene or be functional;
- consequently, the material taken up may not be expressed or lead to a functional effect on the recipient organism;
- the number of copies of a particular sequence may influence the likelihood of it either remaining in cells or being detected; and
- such a process is not unique to genetically modified material and must have been occurring with all genetic material throughout evolutionary history.

More recently, the European Commission-funded ENTRANSFOOD network established a working group to assess HGT in the context of food and feed safety. The results of this working group were published in a review by van den Eede et al (2004). This group did not look at potential consequences or effects of HGT apart from the transfer of antibiotic resistance genes. They concluded that:

[16] The number of copies of a particular sequence of DNA, a particular gene, or a particular set of genes, in the haploid genome of an organism.

- DNA, once it has been introduced into the recipient organism, is indistinguishable from the host DNA in its physical and chemical properties and behaves identically;
- HGT is a fundamental process in creating genetic variation, and there is very little reason to assume that consumption of transgenic food or feed adds any particular generalised risk;
- the impact of HGT events will depend upon a number of environmental factors, the selective advantage for the bacterial population being the most important; and
- although uptake of ingested DNA by mammalian somatic cells has been demonstrated, there is so far no evidence that such DNA may end up in germline cells as a consequence of the consumption of food.

Although many studies have examined the fate of transgenic DNA from GM plant material in its passage through the gastrointestinal tract, most have concentrated on the detecting a transfer event rather than on the effect of such a transfer on the organism. The consequences of such a transfer event will be discussed in section 4 of this report.

Transfer to and between gut microflora

Bacillus subtilis and *E. coli* are examples of food- or intestinal-associated bacteria that could potentially be transformed by foreign DNA sequences in food. As discussed in this section, the likelihood that a DNA sequence will become integrated into the bacterial genome is dependent on the size of the donor DNA molecule and the presence of sequence homology between the donor and recipient DNA. For example, in *B. subtilis*, transformation is optimal when donor DNA fragments are greater than 5 kb with a minimal region of homology of 70 bp (Kharazmi et al, 2003).

Conjugative plasmids and transposons, as described in section 2, are an important mechanism by which HGT can occur between bacteria in the natural environment. Although GM bacteria intended for environmental release are modified to prevent mobilisation of the inserted construct (ie elimination of helper or transposase functions), this ability could be recovered by complementation by natural helper plasmids and transposons. Gnotobiotic or germ-free mice mono-associated with the recipient bacteria *Enterococcus faecalis* were fed *Lactococcus lactis* genetically modified with constructs favourable for gene transfer, to create the worst-case scenario by which HGT might occur (Alpert et al, 2003). The *E. faecalis* which pick up the mobilisable plasmid were found to be stable in mono-associated gnotobiotic mice, suggesting that the transconjugants have no ecological disadvantage. No transfer to *E. faecalis* was observed for the GM bacteria carrying a mini-transposon. It is difficult to predict whether gene transfer will be reduced or enhanced by the presence of complex microflora in the mammalian gut, as opposed to the single species used in this study.

Genetically modified probiotic bacteria are being investigated for nutritional and medical applications. These applications could potentially allow transfer of transgenes to gut microflora. The ability of GM probiotic bacteria to transfer plasmids to intestinal microflora has been studied. In probiotic feeding studies of broiler chicks, gene transfer to endogenous avian microflora was not detected, but transfer was observed between probiotic strains in the avian gut (Netherwood et al, 1999).

Transformed bacteria could potentially transfer genetic sequences to intestinal epithelial cells. It is known that some bacteria can adhere to the gastrointestinal epithelia, invade intestinal enterocytes[17] and transfer genes into mammalian cells. Evidence to support this potential exposure pathway comes from studies using bacteria in gene therapy strategies designed to deliver DNA into mammalian cells, and experiments showing functional gene transfer from bacteria to mammalian cell lines (Grillot-Corvalin et al, 1998). Co-culture experiments with transformed bacteria and an intestinal epithelial Caco-2 cell line suggest that gene transfer from transformed bacteria to the intestinal epithelium is unlikely (Netherwood et al, 2004). However, negative data from *in vitro* co-culture models are difficult to interpret in this case, because the complex environment of the intestinal mucosa and lumen cannot be simulated perfectly *in vitro*.

HGT during fermentation of food

High concentrations of bacteria (>10^9 cells per gram) are in contact with plant material during food fermentation processes such as the production of sauerkraut, fermented olives, tomatoes, cucumbers, beer, sourdough, and many Asian foods based on soy, beans, peanuts, cereals or coconut (van den Eede et al, 2004). These bacteria include lactic acid bacteria, acetic acid bacteria and enterobacteria. Many species from these groups have been shown to be transformable with DNA. Therefore, the fermentation of foods provides a potential pathway by which HGT could occur from plant material to bacteria, and then to the gut cells or microflora of humans who consume those products.

HGT during silage production

Maize is often used to make silage for livestock feed. Silage is a product of mixed microbial fermentation, in which lactic acid bacteria form the predominant flora. Many of the bacteria are naturally competent for genetic transformation. In these flora there are enterococci, which might act as opportunistic pathogens in animals or humans. High molecular weight DNA survives the silage-making process, and whole gene sequences are present in maize silage. For example, the single copy gene *cryIAb* from Bt176 maize could still be detected in silage (Einspanier et al, 2004). Therefore, silage provides an environment in which high molecular weight DNA from GM plants is in close proximity to a diverse population of bacteria, some of which are both competent for natural transformation and opportunistic human pathogens (Heritage, 2005). Silage is then consumed by livestock, providing a pathway by which recombinant bacteria could be transferred to the gut of cattle. The potential for HGT during ensilage has not been well studied, and it is unknown what the consequences of such events would be.

Assessment of the likelihood of HGT

The discussion above identifies a range of potential pathways that may permit horizontal transfer of genetic material. Evidence for the operation of some of these pathways is available at least in laboratory studies, while others remain speculative. Features of the

[17] Enterocytes are a type of epithelial cells of the superficial layer of the small and large intestine tissue. These cells can help breaking up molecules and transport them into the tissue.

genetic sequences can increase to some degree the likelihood of their being integrated into bacterial or viral genomes. In most cases the frequency of transfer observed is very low, but some of the sequences in the proposed genetic constructs may facilitate acquisition by other organisms. The likelihood of this occurring and involving GM plants is uncertain, because of a lack of scientific studies using these constructs and information on how similar sequences need to be to facilitate transfer.

The likelihood of HGT for a transgene of bacterial origin from a GM plant to a bacterium is much lower than the likelihood of acquiring the same gene from another bacterial species in the environment. It is estimated that there are two billion microorganisms per gram of top soil and 10^{11} bacteria in the human gut. The total global count of bacteria is 5×10^{30}, with an average turnover of three years (Heinemann and Traavik, 2004). The probability of a gene transfer from transgenic plants to bacteria has been estimated to be 2×10^{-11} to 1.3×10^{-21} per bacterium and that of a gene transfer by conjugation between soil and enterobacteria is 10^{-1} to 10^{-8} per donor cell (van den Eede et al, 2004). The estimates of frequency of HGT are highly context-dependent, and caution should be applied when extrapolating data from laboratory and field test studies to risk assessments.

However, frequency of transfer is a secondary issue to the consequence of transfer. If a sequence will confer a selective advantage to the recipient bacteria, then that sequence may be selected for and become more common in the population. There is uncertainty concerning assessment of the potential for HGT to cause ecological impacts in New Zealand, because all published data are based on overseas environmental conditions. Consequences of HGT are discussed in the next section.

4 Potential consequences of HGT

Steps required for HGT to have an effect

As described in section 3, there are several sequential steps required for HGT to occur and cause an effect:

1. Exposure of the recipient organism to foreign DNA
2. Uptake of foreign DNA by the recipient organism
3. Integration of foreign DNA into the genome
4. **Expression of foreign DNA in the organism**
5. **Selection of organism carrying foreign DNA**

Steps 4 and 5 will be discussed in this section because they relate to the consequence or effect of HGT occurring.

Expression of the transferred foreign DNA

For risk assessment, one significant factor to be considered is the potential for HGT to cause adverse effects. In comparison to discussions on the frequency of HGT, there has been very little published on the potential consequences or adverse effects of such a transfer event with transgenes (Nielsen et al, 1998; Nielsen and Townsend, 2004).

After transfer and establishment barriers, failure to express the transferred genes represents a strong barrier to HGT from distantly related organisms. The transferred sequence requires appropriate transcriptional control elements, such as promoter and termination sequences, in order for it to be expressed in the recipient organism (see Table 2). Eukaryotic sequences may not be expressed in bacteria if they are under the control of eukaryotic promoters, enhancers or contain introns. However, there is evidence that some eukaryotic sequences (such as genomic sequences derived from yeast) are capable of initiating gene expression in bacteria (Lewin et al, 2004). Also, the use of prokaryotic promoters in transgenic plants may increase the likelihood of the transgene being expressed if it is transferred to bacteria. The use of plant-derived tissue-specific promoters will pose a barrier to this. Horizontally transferred plant genes are unlikely to be positively selected in bacteria, due to constraints on expression caused by introns, altered codon usage, lack of promoter recognition and potential loss of functionality due to altered cytoplasmic conditions and requirements for post-translational modifications (Nielsen and Townsend, 2001).

The inclusion of other plant-derived regulatory sequences such as leader, 3' untranslated and chloroplast transit peptide sequences in the GM plants may reduce the likelihood of gene expression following transfer to bacteria.

Table 2: Some characteristics of genes inserted in GM plants (adapted from Nielsen et al, 1998)

Characteristics	Examples	Possible consequences if the genes are translocated into the bacterial cytoplasm
Prokaryotic DNA sequences	Bacterial vector sequences, T-DNA border sequence, promoters, genes	Integration into bacterial genome and potentially gene expression
Bacterial origins of replication	*oriV* sites from plasmids	Plasmid recircularisation and autonomous replication
Lack of introns	cDNA cloned genes	Gene expression if a bacterial promoter is present and active
Altered regulation and genetic background	Chimeric genes with constitutive promoters	Unknown, the expressed gene product may interfere with bacterial metabolism

The likelihood of gene expression following genetic transfer is uncertain, as it depends on whether the bacteria that integrate the sequence are able to activate that promoter. Many transgenic crops include promoters from plant viruses, so there is the potential for eukaryotic sequences attached to such promoters to be expressed in bacteria. For example, the 35S promoter from cauliflower mosaic virus (CaMV) can drive gene expression in *E. coli* (Assad and Signer, 1990). Inserted plant and viral promoters may also act as regions for homologous recombination, thus bringing together the transformed part with unintended sequences that subsequently replicate in other genomic backgrounds. In addition, strong promoters may affect gene expression in downstream regions of the genome, which may lead to unintended effects.

Synthetic plant-preferred versions of bacterial genes are often used to modify crops. This is to allow expression of bacterial genes in plants, because plant cells often use different codons and polyadenylation sites from bacteria. Use of synthetic plant-preferred transgenes could potentially mean that the transgene will not be expressed in bacteria. However, in a recent study, the plant-preferred version of the bacterial *epsps* gene was isolated from GM soybeans and back-cloned into *E. coli* (where it was placed under the control of bacterial regulatory elements). It was still able to be expressed at high levels in the bacteria (Chang et al, 2003). Therefore, random insertion of plant-preferred versions of bacterial genes into existing regulatory units in the bacterial genome could potentially allow transgene expression after HGT from plants. In addition, the cytoplasm of the chloroplast is biochemically similar to that of the bacterial cell, so transplastomic plants are likely to contain engineered genes optimised for expression (regulatory sequences) and functionality (codon usage) in a bacterial cytoplasm (Nielsen et al, 2001).

The site of insertion of the foreign DNA in the recipient organism's genome has implications for its expression and on the phenotype of the organism. If the DNA is

integrated such that it affects the function of important genes, the host cell may be seriously damaged or die. If the DNA is inserted into a region of the host genome with normally suppressed expression, such as areas of heterochromatin in eukaryotes, then the expression of the insert will also be suppressed. In addition, novel chimeric proteins could be produced by the juxtaposition of DNA sequences from the transgene and host DNA.

Therefore, potential exists for the transgene from GM crops to be expressed in recipient organisms following HGT. However, there is considerable uncertainty about whether the conditions required for gene expression will be fulfilled, and the potential outcome on the phenotype of the recipient organism.

Selective pressure and HGT

Selection for the transgene in the new host may be negative, neutral or positive. Even if transfer frequency is very low, if there is a selective advantage conferred by the acquired genetic material then bacteria, viruses, or other organisms containing this material are likely to become more common. In contrast, if positive selection for the genetic material transferred does not occur, the very short generation times and large population sizes of bacteria may decrease the frequency and impact of that gene in a population (Nielsen and Townsend, 2001; Kurland et al, 2003). Neutrally selected traits may become fixed in a population if the horizontal gene transfer frequency is sufficiently high. Neutral mutations, while having no benefit to the host, can provide a substrate for natural selection to occur over the time (Gogarten and Townsend, 2005). However, positive selection on a rare transformant is presumed to be the most likely scenario that would result in an ecological impact.

A key question for risk assessment is to identify which transgenes would experience positive selection if they were transferred to other organisms, such as bacteria. Unfortunately, selective pressures are poorly known, so it is not always possible to predict consequences of transfer (Nielsen, 2003; Heinemann and Traavik, 2004). There is likely to be significant temporal and spatial variation in selection pressures in complex environments, including soil. In some cases, the emergent phenotype may be seen only when the environment changes or the microorganism changes environments.

In addition, the potential for positive selection is not the only factor to consider during risk assessment. Even bacteria that have acquired DNA and are initially less fit than the wild type can, after a number of generations, regain fitness via compensatory mutations (Schrag et al, 1997). They may even out-compete the wild type form which has been subject to continuous growth in the environment (Johnsen et al, 2002). It has also been shown that, in the absence of the selective agent, carriage of an additional gene essentially imposes no burden on the organisms (Nguyen et al, 1989). Therefore, to consider the possibility of positive selection alone may seriously underestimate the ability of the transformed bacteria to survive and evolve.

The effects on host fitness of a gene cassette inserted into *Pseudomonas fluorescens* were investigated by Lilley et al (2003) in a model microcosm system. Bacteria carrying the gene cassette inserted into the chromosome achieved significantly higher population densities than the control, despite the fact that the manipulated strain appeared, prior to release, to perform identically to the unmodified strain in laboratory assays. The scientists concluded that 1) the “random” insertion of a gene cassette into the chromosome can lead to

enhanced fitness; 2) simple assays may fail to detect such fitness increases; and 3) microcosm studies may be of value in identifying them.

Studies on the persistence of genetically modified probiotic bacteria in the gut of broiler chicks also suggest that in some cases a GMO may have a selective advantage over the unmodified strain (Netherwood et al, 1999). In these studies, *Enterococcus faecium* was genetically modified with the *Ruminococcus flavefaciens* β-1,4-glucanase gene involved in cellulase production. The carriage rate of the GMO probiotic strain increased, but the carriage rate of the non-GM strain did not, in the gut contents of broiler chicks. This difference may be due to the production of cellulase by the GM bacteria which provided an accessible energy source for the microorganism and the host.

Antibiotic resistance genes have attracted considerable attention in regard to discussions of the consequence of horizontal gene transfer, because they have clearly identifiable advantages in the presence of a selection pressure (ie presence of the relevant antibiotic) and are an issue commonly raised in public submissions for ERMA New Zealand applications (see discussion in next section).

HGT and food safety

DNA is ubiquitous in food and the environment. DNA sequences of various origins (plant, animal, bacteria, and virus) have been present in human food and animal feed throughout history. Therefore, over time some sequences will have entered the mammalian gut. The significance of the uptake of DNA sequences from an unlimited variety of natural sources in food is unclear. Insertion of foreign DNA sequences (both GM and non-GM sources) into the mammalian genome could potentially alter gene expression patterns in the recipient cells or be oncogenic. Recent attempts at gene therapy in humans have shown that non-specific insertion of foreign DNA into the human genome can have serious consequences (Hacein-Bey-Abina et al, 2003). In addition, further assessment is needed of DNA sequences encoding traits that, if transferred, have the potential to harm health. There is concern about the inclusion of DNA sequences that encode increased bacterial virulence, pharmaceuticals or antibiotic resistance into GM crops.

One of the most commonly used promoters in GM plants is the 35S CaMV promoter, derived from the cauliflower mosaic virus (CaMV). Ho et al (1999) suggest that the CaMV promoter may recombine with dormant endogenous viruses in mammals, which in turn may result in new infectious viruses. CaMV is commonly found in temperate countries where it infects cauliflower, cabbage, oilseed rape, mustard and other brassicas and some solanaceaous species. In a survey, up to 10 percent of cauliflowers and 50 percent of cabbages on local markets in the UK were infected with CaMV (Nielsen, 2003). Thus, it is expected that humans are already exposed to CaMV DNA from infected plants. Despite intensive research, the CaMV promoter has never been found to generate new viruses by recombination, or to cause adverse effects in humans (van den Eede et al, 2004).

Uptake of DNA sequences from food (as discussed in section 3) is more likely for somatic cells (such as gut and immune cells) than for germ line cells. This may account for the almost complete lack of evidence for sequences of plant origin in mammalian genomes. Somatic cells of the gut lining have a rapid turnover, such that the most likely fate of any

cells that take up foreign DNA is to be excreted. These considerations make the likelihood of deleterious consequences from HGT during consumption of food highly improbable.

Antibiotic resistance

Antibiotic resistance in human and animal pathogens is a public health problem of increasing global importance (World Health Organization, 2002). Infections caused by antibiotic-resistant bacteria are difficult to treat with first line antibiotics and are often associated with increased morbidity[18], increased time in hospital and higher mortality rates[19]. Some important examples are vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus*, multi-resistant salmonellae, penicillin-resistant *Streptococcus pneumoniae*, and multi-resistant *Mycobacterium tuberculosis*. Resistance to antibiotics[20] is a natural biological phenomenon that can be amplified or accelerated by a variety of factors, including human practices. The use of an antibiotic for any infection, real or feared, in any dose and over any time period, exerts selective pressure on bacterial populations for mutants that are resistant to the antimicrobial agent.

Bacteria are particularly efficient at enhancing the effects of resistance because they rapidly multiply and can also transfer their resistance genes to other bacteria. Therefore, resistance to a single drug can spread rapidly through a bacterial population. Using antibiotics incorrectly (eg for too short a time, at too low a dose, at inadequate potency, or for the wrong disease) greatly increases the likelihood that bacterial populations will adapt to develop resistance and replicate, rather than be killed.

According to the World Health Organization (2002), much evidence supports the view that total consumption of antibiotics is the critical factor in selecting for resistance. Examples of overuse of antibiotics include the routine use of antibiotics as growth promoters or preventative agents in food-producing animals and poultry flocks[21], self-medication[22] with antibiotics, and over-prescription of broad spectrum antibiotics by doctors and veterinarians.

A recent issue has emerged concerning antibiotic resistance genes in genetically modified organisms that are released into the environment. Antibiotic resistance genes are used during the development phase of most GMOs (bacteria, plants and animals) in order to facilitate the identification and selection for successful transformation events. There is some concern that the antibiotic resistance genes might find their way from GMOs via horizontal gene transfer into gut or soil bacteria, and from there into disease-causing bacteria, leading to reduced therapeutic options. Most antibiotic resistance genes used in

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[18] Incidence of disease in a population, including both fatal and nonfatal cases.

[19] The death rate; the number of people who die of a certain disease compared with the total number of people.

[20] Intrinsic and transferable resistance

[21] In North America and Europe, an estimated 50 percent in tonnage of all antimicrobial production is used in food-producing animals and poultry (WHO 2002).

[22] Many antibiotics can be easily obtained in pharmacies and markets without a prescription. A wide variety of household products that contain antimicrobial ingredients are being marketed to consumers.

genetic modification were originally derived from naturally occurring microorganisms. Therefore, GMOs containing antibiotic resistance genes may not necessarily introduce new antibiotic resistance genes into a particular environment. However, GMO introduction may change the environmental persistence, the concentrations of antibiotic resistance genes exposed to microorganisms, and the frequency and locations of such genes (Nielsen et al, 2005). This potential risk is most relevant to the release (or conditional release) of a GMO into the environment, rather than a GMO kept in containment, due to the absence of a relevant risk pathway if containment is maintained. However, an alternative exposure pathway could be presented by GMOs in containment if waste from microbial fermentation plants (potentially containing transgenic DNA) is used as crop fertiliser.

The most frequently used antibiotic resistance genes in GMOs are the ampicillin resistance gene (*bla*$_{TEM-1}$[23]) for bacterial transformation and the kanamycin resistance gene (*nptII*[24]) for plant transformation. The quantities of antibiotics used in the development of GMOs are much lower than the quantities used in medicine. Read (2000) noted that only very small quantities of antibiotics (eg <1kg of aminoglycosides[25]) are used annually in molecular biology laboratories, in contrast to the large quantities used in veterinary and human medicine (eg >2000kg aminoglycosides) within New Zealand.

The Norwegian Scientific Committee for Food Safety considers that "our current understanding of resistance development in bacterial pathogens is more descriptive than predictive in nature" (Nielsen et al, 2005). Although we can describe retrospectively how resistance has spread in bacterial populations, the initial horizontal transfer events, environmental location and conditions that produced the first generation of resistant bacteria remain largely unknown. This limits the ability to accurately predict further resistance development through HGT.

Risk assessment of HGT of antibiotic resistance genes from GM food to gut bacteria needs to be viewed in relation to: 1) the presence of already existing antibiotic resistance genes in the environment, including the human gut; 2) the therapeutic relevance of the corresponding antibiotic; and 3) the selective pressure on bacteria carrying the antibiotic resistance genes. These criteria enable a comparative assessment of the relative contribution of antibiotic resistance genes from GMOs to the overall antibiotic resistance gene reservoir in the environment, and to resolve whether rare bacterial transformants that have acquired antibiotic resistance genes are likely to undergo positive selection leading to clinically troublesome populations (Nielsen et al, 2005). The European Food Safety Authority (2004) has used these criteria extensively in its risk assessments.

Antibiotic resistance genes already present in the environment may be carried by mobile elements such as plasmids and transposons, so the likelihood of gene transfer may be higher for natural bacteria-to-bacteria rather than for GM plant-to-bacteria. There are also

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[23] *bla* is also referred to as *amp* and encodes a beta-lactamase enzyme.

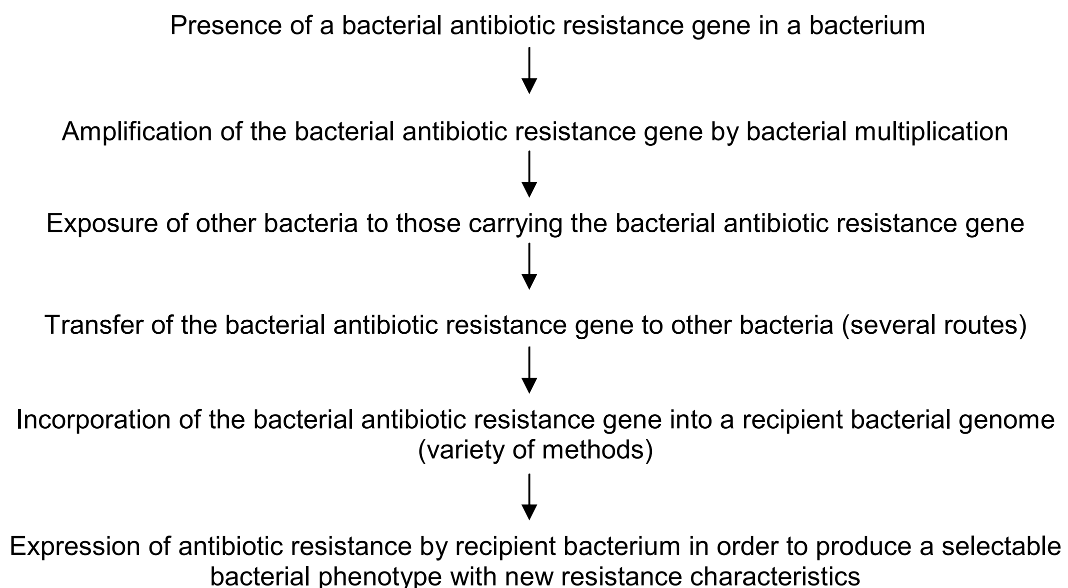
[24] *nptII* encodes neomycin phosphotransferase II and is also referred to as *aph(3)-II.*

[25] Aminoglycosides include the antibiotics kanamycin and neomycin.

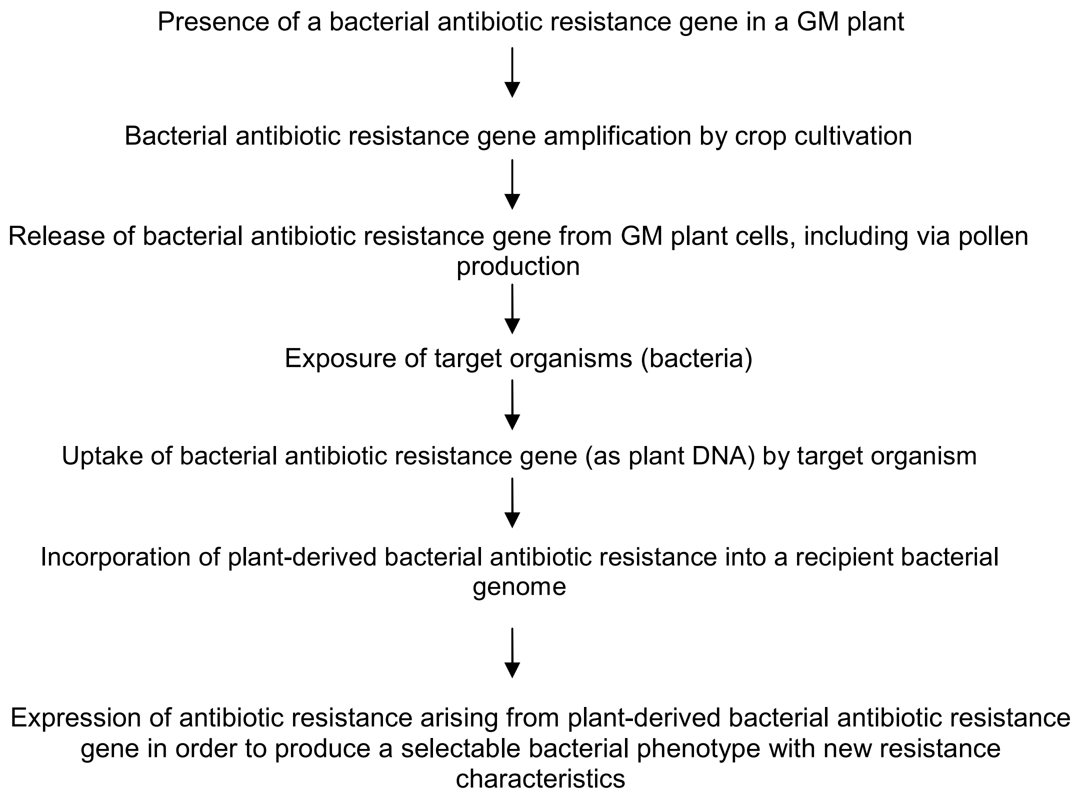
differences between countries in bacterial resistance levels and usage levels of antibiotics, so case-by-case assessment is critical.

Very recently, a plant gene (*Atwbc19*) that confers resistance to the antibiotic kanamycin has been identified. An antibiotic resistance gene derived from plants would be less likely to be transferred and expressed by bacteria for a number of reasons, including its plant codon usage (Stewart and Mentewab, 2005).

The Report of the Working Party of the British Society for Antimicrobial Chemotherapy (2004) identified two risk pathways for bacterial antibiotic resistance gene transfer in the environment. See below and over the page.



Risk pathway 2: gene transfer from plants to bacteria



If an antibiotic resistance gene was present in a viable GM bacterium released into the environment, then risk pathway 1 would be the most likely route for dissemination. Bacteria have several well-documented pathways for DNA transfer[26] between bacteria and incorporation (via recombination) of foreign DNA. The likelihood of transfer of antibiotic resistance to a pathogen would depend on the environment into which the GM bacteria were released. Transfer from a viable GM food microorganism would be considered more likely to occur as a result of gene transfer to gut microorganisms (including pathogens) in the human or animal gut than transfer from a GM microorganism used in industrial processes where the organism would not necessarily come into contact with pathogenic microorganisms.

The magnitude of the potential adverse health effect from such a transfer event would depend on whether the antibiotic resistance gene was already common (ie a significant proportion of bacteria carrying the gene) among bacterial pathogens. The magnitude of the adverse effect from transfer of antibiotic resistance genes that are already common in bacterial pathogens would not be as serious as that arising from transfer of bacterial antibiotic resistance genes that are uncommon (or are not present) in bacterial pathogens

[26] Conjugation, transduction and transformation.

and confer resistance to the drug used to treat it. The consequence of gene transfer would also depend on whether there was selection for, and a fitness advantage conferred by, the antibiotic resistance gene in the recipient bacterium. Therefore, the overall risk associated with the use of antibiotic resistance genes in GM bacteria released into the environment should be considered using a case-by-case approach, because the likelihood and magnitude of such an adverse event occurring depends on the nature and proposed use of the GM microorganism, the existing distribution of naturally occurring homologues of the antibiotic resistance gene in the environment, and the effect of selection. This assessment requires knowledge of the numbers of antibiotic resistance genes naturally present in antibiotic-exposed environments, in order to estimate the relative contribution of antibiotic resistance genes from introduced GMOs to the total number of homologous antibiotic resistance genes in the environment (Nielsen et al, 2005). Data are needed on the specific usage of relevant antibiotics and corresponding resistance patterns in New Zealand, to improve the robustness of risk assessments for GMOs containing antibiotic resistance genes.

If an antibiotic resistance gene was present in a viable GM plant released into the environment, then risk pathway 2 would be the most likely route for dissemination. Risk pathway 2 is a currently hypothetical mechanism for the transfer of resistance genes from transgenic plants to bacteria, via the mechanism of horizontal gene transfer. For an adverse effect to occur, the antibiotic resistance gene would then have to transfer from the soil bacterium to a pathogenic bacterium. This would probably occur via well-established mechanisms, described in pathway 1. Based on a review of the scientific literature, which indicates that horizontal gene transfer events from transgenic plants to bacteria occur at extremely low frequencies, the likelihood of this series of events is considered highly improbable, and has not yet been detected under field conditions (see section 3).

The magnitude of such an adverse event occurring is again dependent on whether the antibiotic resistance gene is uncommon in bacterial pathogens, whether any further spread would be undesirable, and whether positive selection occurs. It is widely accepted that the present problems in human and veterinary medicine, resulting from the selective pressure posed on microbial communities, were created by the unrestricted use of antibiotics in medicine and animal husbandries, and not by transgenic crops carrying antibiotic resistance genes (Gay and Gillespie, 2005; Nielsen et al, 2005).

However, the administration of antibiotics to humans and animals might have a profound effect on the microflora of the gut and provide a selective pressure for resistance to antibiotics among bacteria exposed to them (Heritage, 2005). It is not known whether antibiotic use will select resistant transformants in human and animals that consume GM foods. The impact of HGT-acquired antibiotic resistance genes in bacteria also needs to be considered in relation to the potential for those rare bacterial transformants to be amplified selectively in the larger population.

5 Consideration of HGT under the HSNO Act

ERMA New Zealand's approach to risk assessment of HGT

ERMA New Zealand's approach to risk assessment of HGT for GMO applications is based on evaluation of the available scientific information. Risk assessment is a combination of the likelihood and the magnitude (ie the consequence) of an effect occurring (ERMA New Zealand, 2004). Assumption-based reasoning and a variety of information sources are used in the risk assessment of effects arising from HGT. The basis for the assumptions made, and the information sources, include: 1) direct test results submitted by the GMO developers; 2) experimental data available in the peer-reviewed literature; 3) published and/or communicated historical and comparative experiences, observations and inference from similar biological systems; and 4) submitted or conducted expert evaluations of the outcomes of possible worst-case scenarios (Nielsen et al, 2005). Also, identifying and clearly communicating the level and nature of uncertainties is a major component of the risk assessment.

Section 44A(2)(c) of the HSNO Act states that for outdoor developments and field tests of GMOs, the Authority must take into account:

"any effects resulting from the transfer of any genetic elements to other organisms in or around the site of the development or field test".

Section 44A(2)(c) is considered to apply to horizontal gene transfer (HGT), and not to vertical gene transfer, which is addressed elsewhere in the Act. The intention of this section of the Act is to ensure that the occurrence and consequence of HGT is considered explicitly, irrespective of its likelihood. In GM field test and outdoor development applications to ERMA New Zealand, applicants are expected to consider and address the likelihood and the magnitude of effects that may arise from HGT. Any effects that may arise from HGT will then be considered in the normal course of the consideration process (ERMA New Zealand, 2005). Previous ERMA New Zealand approvals of GM field tests and outdoor developments have included controls requiring research on HGT from the GMO to surrounding soil microorganisms. However, technology to reliably detect HGT events in the field is yet to be developed. Therefore, any monitoring using the current technology is likely to have limited benefits.

If the Authority considers that the overall adverse effects of an application outweigh the benefits, they will decline the application. In addition, all release applications are judged against the minimum standards in section 36 of the HSNO Act. These criteria include the ability to displace native species, cause habitat deterioration, adversely affect New Zealand's genetic diversity or cause disease. These represent the "environmental bottom line" of the legislation. If an application fails to meet these standards then it must be declined.

Examples of risk assessment questions

There are a number of questions that can be used to structure a risk assessment for the potential effects of HGT. These might include:

- What are the key pathways by which HGT could occur from a GMO?
    - Is the GMO in containment or to be released into the environment?
    - Will the GMO be consumed by humans or animals?
    - What are the potential recipient organisms for a gene transfer event?

- How likely is HGT to occur?
    - Is there a credible exposure pathway for gene transfer?
    - How many copies of the transgene are present in the GMO?
    - Will the transgenic DNA be biologically available and persist in the environment?
    - What is the degree of sequence similarity of the donor sequence to the genetic material of potential recipient organisms?
    - Are potential recipient organisms competent to take up DNA?

- What would be the consequences and magnitude of a potential effect if HGT did occur?
    - Will the genetic material be integrated and expressed in the recipient organism?
    - Will the transferred genetic material confer an effect on the recipient organisms? Will it confer a selective advantage?
    - Is the transferred genetic material toxic or hazardous to the environment or human health?
    - For GM constructs, is the transferred genetic material identical to genetic sequences already being released into the environment by non-GMOs?

If no credible exposure pathway and potential adverse effect can be identified for HGT, no further risk management measures are needed.

HGT research in New Zealand

When data on HGT specific to New Zealand environmental conditions are available, this will aid the risk assessment of GMOs in New Zealand. Research into the nature and effects of HGT was one area identified by the Royal Commission on Genetic Modification (2001) as requiring greater investment.

In 2001/02, the Foundation for Research, Science and Technology (FRST) reprioritised funds to address this. Scientists at Environmental Science and Research (ESR), AgResearch and HortResearch were funded by FRST as part of the GM Impacts Research Programme to study the specific processes and effects of HGT from GM plants to soil bacteria under conditions extant in New Zealand (Carter, 2003; Watson and Carter, 2003). This research is aimed at understanding components of HGT in order to predict the risks of ecological consequences arising from field use of GMOs in New Zealand.

The programme's objectives are to examine:

- the persistence of transgenic plant DNA in soil and in animal guts;
- the role of soil factors in HGT;
- potential HGT hotspots;
- occurrence of competence-associated genes in New Zealand soil bacteria;
- whether transgenes transfer more frequently than natural genes;
- occurrence of antibiotic resistance genes in environmental bacteria;
- selection of bacterial transformants in soil; and
- modelling and prediction of the spread of transgenes in soil bacterial communities.

This research team is also collaborating with Dr Kaare Nielsen, University of Tromso, Norway, an expert on the transfer of transgenes from plants to soil bacteria.

AgResearch is required to monitor for HGT as a condition of their approval for the outdoor development of GM cattle[27]. They have not yet commenced the outdoor phase of the development, but have started developing assay systems for detecting HGT. They have also identified that a high proportion of bacteria present in soil are already naturally resistant to antibiotics used in the development of GMOs (AgResearch, 2004).

The New Zealand Institute of Gene Ecology[28] (NZIGE) also has an interest in HGT. It was established in December 2001, and its membership includes natural scientists, social scientists, and philosophers within and outside academia. The NZIGE works in cooperation with, the Norwegian Institute of Gene Ecology (GENØK). Dr Jack Heinemann is the Founding Director of NZIGE, and has published a number of reviews on HGT and research papers on HGT occurring by conjugation and phage transduction (Heinemann and Traavik, 2004; Heinemann, 2003; Heinemann and Sprague, 1989).

It is important to continue to support HGT-focused research in New Zealand, in order to build capacity for the assessment of future releases of GMOs, and in particular to generate data that are applicable to understanding New Zealand conditions.

Outstanding issues for risk assessment

Current methods of environmental sampling to capture genes or traits in a recombinant are too insensitive for monitoring HGT. Techniques being used to monitor HGT in soil have sampling limits of about one recombinant bacterium in 10^8-10^{11}. These experiments uniformly yield no detectable recombinants unless special conditions are applied (Heinemann and Traavik, 2004). Extrapolating additional assumptions concerning barriers to HGT has led to estimates of HGT frequency many orders of magnitude below that of

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[27] ERMA approval for application GMD02028.

www.ermanz.govt.nz/appfiles/execsumm/pdf/GMD02028-005.pdf Accessed 9 September 2005.

[28] www.nzige.canterbury.ac.nz/about.shtml Accessed 1 September 2005.

the sampling limits, to less than one event in 10^{16}-10^{17}. Therefore, studies for monitoring HGT events in the field would need to have features that permitted detection of recombinants at HGT frequencies $<10^{-17}$. No published methods have demonstrated this degree of sensitivity of detection.

Some scientists consider that experimental limitations preclude measuring HGT with the sensitivity necessary to dismiss environmental harm. Heinemann and Traavik (2004) used a model for the evolution of penicillin resistance in *Streptococcus pneumoniae* to demonstrate the frequency of HGT events implicated in causing harm (ie spread of antibiotic resistance) and the sensitivity required for detection of such rare events. They estimated that 10^{25}-10^{37} bacteria would have to be screened for penicillin resistance. This would require a minimum of 10^{15}-10^{27} Petri dishes (if 10^{10} bacteria could be assayed per dish) in order to detect one recombinant arising *de novo* in the field trial. The current methodology for detecting HGT events also relies on individual analysis of bacterial colonies, which is time-consuming, expensive and generally limited to processing, at most, several thousand samples (Nielsen and Townsend, 2004).

The number of potential recombinants arising from HGT can be calculated from HGT frequencies and the size of the microbial population. For example, a transmission frequency of 10^{-24} would be expected to result in 5000 recombinants in the estimated 11.4 million hectares worldwide planted with Bt corn. In order to detect one of these recombinants, 500 million metric tons of soil would have to be sampled (Heinemann and Traavik, 2004). No time scale was indicated by the authors for the estimates of transmission frequencies. The large number of bacteria present in most natural environments quickly saturates sampling efforts and also makes monitoring of HGT very difficult (Nielsen and Townsend, 2004; see section 3).

In addition, the majority of bacteria in soil and in the mammalian gut are nonculturable, which severely limits the ability to identify and characterise bacterial recombinants arising from HGT (Nielsen and Townsend, 2004; Sorensen et al, 2005). To detect potential HGT events occurring in the nonculturable fraction of soil bacteria, researchers isolate DNA from the bacterial fraction of soil and use PCR to assay for the transgene. However, a successful PCR amplification of DNA extracted from soil typically requires >50 copies of the transgene per reaction, which is higher than experimentally measured rates of HGT in soil (Nielsen and Townsend, 2004). DNA extraction is also biased by a number of factors, including biased enrichment, fragmentation, uneven cell lysis and the presence of DNA from fungi (a major component of soil biomass) in DNA samples extracted directly from soil. This bias results in a 10- to 100-fold reduction of the sensitivity of the PCR analysis, effectively limiting detection to only those HGT events generating $>2 \times 10^{-5}$ to 2×10^{-4} transformants per total number of bacterial genomes analysed. Increasing the number of samples analysed by PCR will not overcome this problem if transgene copy number is below the limit required for successful PCR amplification. Even if positive results are obtained, it is difficult to prove that the PCR signal comes from a bacterial transformant and not from decaying plant residues.

The fact that it is difficult to predict the lag time between the possible environmental outcome and an HGT event altering the phenotype of a bacterium is another complicating factor. Published monitoring studies have relied on detecting bacterial transformants within their first generations, immediately following transgene exposure. Many bacteria have large

population sizes and relatively long generation times in nutrient-limited soil environments, so it may take years or several decades for the transformants to reproduce and out-compete non-transformed cells of the population at a scale that is detectable by monitoring programmes. Therefore, the time of sampling is crucial to the useful implementation of monitoring. For example, an early sampling time combined with a weakly selected transgene makes virtually any sampling effort futile (Nielsen and Townsend, 2004).

For monitoring data to be informative for risk assessment, they will need to clearly communicate the inherent limitations in the methods and experimental design used, such as the detection limit, the sensitivity of the methods used, and the sampling size and time necessary to resolve the hypotheses presented. There are also a number of assumptions underlying current risk assessments that need to be verified, eg whether transgenes would confer the same fitness to the host as the gene would in the natural host from which it was originally derived.

Based on the current limitations in estimating the frequency of HGT events and the inability to rule out the possibility of HGT occurring from GMOs to other organisms, the cautious option for risk assessment is to assume that HGT will occur and determine the possible consequences of such an event. Novel transgenes that do not have natural counterparts require particular attention in risk assessment of HGT, eg transgenic constructs that contain novel combinations of regulatory elements and toxin protein domains that may differ substantially from those arising by natural evolutionary processes.

6 Conclusions

Horizontal gene transfer is a naturally occurring process for the exchange of genetic material between organisms, irrespective of whether or not they are genetically modified. In addition, there is a significant amount of non-GM DNA sequences naturally present in the environment that are also subject to HGT. The likelihood that a particular transgenic DNA sequence will be subject to HGT depends on a number of factors, including the biological availability of the sequence, the presence of a recipient organism competent to take up the DNA, and the presence of sufficient sequence homology to allow integration into the recipient genome. Based on these potential barriers and current evidence from field and laboratory studies, the likelihood of detecting HGT of a transgene occurring from GM crops to bacteria is highly improbable.

However, the current evidence concerning the frequency of HGT from GMOs to other organisms is affected by experimental limitations for detecting these events. Based on these current limitations, and the inability to rule out the possibility of HGT from GMOs to other organisms, the cautious option for risk assessment is to determine the possible consequences of such an event. The magnitude of any adverse effect arising from HGT will depend on whether the transgene is expressed by the recipient organism and confers an effect on that organism which is subject to positive selection. In many cases, a foreign DNA sequence is expected to confer no effect, or a deleterious effect, on the recipient organism, and thus will be rapidly lost from the population. Assumptions about the effects of specific transgenes on fitness and selection need to be verified by further research.

Antibiotic resistance genes have attracted considerable attention in discussions about the potential consequence of HGT because they have clearly identifiable adverse effects if they spread to pathogenic bacteria. Risk assessment of HGT of antibiotic resistance genes in GMOs needs to be viewed in relation to: 1) the presence of already existing antibiotic resistance genes in the environment; 2) the therapeutic relevance of the corresponding antibiotic; and 3) the selective pressure on bacteria carrying the resistance genes. In many cases, the contribution of antibiotic resistance genes via HGT of GM plants to bacteria is believed to be insignificant compared to the selection imposed by unrestricted use of antibiotics in medicine and animal husbandries in some nations.

However, we consider that novel transgenes without natural counterparts require particular attention in risk assessment of HGT, for example transgenic constructs that contain novel combinations of regulatory elements, and toxin protein domains that may differ substantially from those arising by natural evolutionary processes.

ERMA New Zealand conducts risk assessment of effects arising from HGT on a case-by-case basis, in accordance with the requirements of the Hazardous Substances and New Organisms Act 1996.

References

AgResearch 2004. *Annual Report to ERMA New Zealand*. 31 December 2004.

Alpert, C-A, Mater, DDG, Muller, M-C, Ouriet, M-F, Duval-Iflah, Y, Corthier, G 2003. Worst-case scenarios for horizontal gene transfer from *Lactococcus lactis* carrying heterologous genes to *Enterococcus faecalis* in the digestive tract of gnotobiotic mice. *Environmental Biosafety Research* 3: 173-180.

Archibald, JM, Rogers, MB, Toop, M, Ishida, K, Keeling, PJ 2003. Lateral gene transfer and the evolution of plastid-targeted proteins in the secondary plastid-containing alga *Bigelowiella natans*. *Proceedings of the National Academy of Sciences USA* 100: 7678-7683

Assad, FF, Signer, ER 1990. Cauliflower mosaic virus P35S promoter activity in *Escherichia coli*. *Molecular and General Genetics* 223: 517-520.

Bennett, PM, Livesey, CT, Nathwani, D, Reeves, DS, Saunders, JR, 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* 53(3): 418-431.

Bensasson, D, Boore, JL, Nielsen, KM 2004. Genes without frontiers? *Heredity* 92(6):483-489.

Bergthorsson, U, Adams, KL, Thomason, B, Palmer, JD 2003. Widespread horizontal transfer of mitochondrial genes in flowering plants. *Nature* 424: 197-201.

Bergthorsson, U, Richardson, AO, Young, GJ, Goertzen, LR, Palmer, JD 2004. Massive horizontal transfer of mitochondrial genes from diverse land plant donors to the basal angiosperm *Amborella*. *Proceedings of the National Academy of Sciences USA* 101(51): 17747-17752.

Berndt, C, Meier, P, Wackernagel, W 2003. DNA restriction is a barrier to natural transformation in *Pseudomonas stutzeri* JM300. *Microbiology* 149: 895-901.

Bordenstein, SR, Reznikoff, WS 2005. Mobile DNA in obligate intracellular bacteria. *Nature Reviews Microbiology* 3: 688-699.

Broer, I, Droge-Laser, W, Gerke, M 1996. Examination of the putative horizontal gene transfer from transgenic plants to agrobacteria, p67-70. *In*: Schmidt, ER, Hankeln, T (ed.) *Transgenic organisms and biosafety, horizontal gene transfer, stability of DNA and expression of transgenes*. Springer Verlag: Heidelberg, Germany.

Broothaerts, W, Mitchell, HJ, Weir, B, Kaines, S, Smith, LMA, Yang, W, Mayer, JE, Roa-Rodriguez, C, Jefferson, RA 2005. Gene transfer to plants by diverse species of bacteria. *Nature* 433: 629-633.

Carter, P 2003. Horizontal gene transfer in the New Zealand environment. *NZ BioScience* (May): 29-31.

Chang, HS, Kim, NH, Park, MJ, Lim, SK, Kim, SC, Kim, JY, Kim, JA, Oh, HY, Lee, CH, Huh, K, Jeong, TC, Nam, DH 2003. The 5-enolpyruvylshikimate-3-phosphate synthase of glyphosate-tolerant soybean expressed in *Escherichia coli* shows no severe allergenicity. *Molecular Cells* 15: 20-26.

Chenault, KD, Melcher, U 1994. Phylogenetic relationships reveal recombination among isolates of cauliflower mosaic virus. *Journal of Molecular Evolution* 39: 496-505.

Chopra, I, O'Neill, AJ, Miller, K 2003. The role of mutators in the emergence of antibiotic-resistant bacteria. *Drug Resistance Updates* 6: 137-145.

Chowdhury, EH, Kuribara, H, Hino, A, Sultana, P, Mikami, O, Simada, N, Guruge, KS, Saito, M, Nakajima, Y 2003. Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. *Journal of Animal Science* 81: 2546-2551.

Conner, A, Glare, TR 2003. Popular Summary of: The release of genetically modified crops into the environment II. Overview of ecological risk assessment. [A condensed version of a paper published in The Plant Journal in January 2003.]

Davis, CC, Wurdack, KJ 2004. Host-to-parasite gene transfer in flowering plants: phylogenetic evidence from malpighiales. *Science* 305: 676-678.

De Vries, J, Wackernagel, W 1998. Detection of *nptII* (kanamycin resistance) genes in genomes of transgenic plants by marker-rescue transformation. *Molecular and General Genetics* 257(6): 606-613.

De Vries, J, Meier, P, Wackernagel, W 2001. The natural transformation of the soil bacteria *Pseudomonas stuzeri* and *Acinetobacter* sp. by transgenic plant DNA strictly depends on homologous sequences in the recipient cells. *FEMS Microbiology Letters* 195: 211-215.

De Vries, J, Herzfeld, T, Wackernagel, W 2004. Transfer of plastid DNA from tobacco to the soil bacterium *Acinetobacter* sp. by natural transformation. *Molecular Microbiology* 53(1): 323-334.

Duggan, PS, Chambers, PA, Heritage, J, Forbes, JM 2000. Survival of free DNA encoding antibiotic resistance from transgenic maize and the transformation activity of DNA in ovine saliva, ovine rumen fluid and silage effluent. *FEMS Microbiology Letters* 191: 71-77.

Dunfield, KE, Germida, JJ 2004. Impact of genetically modified crops on soil- and plant-associated microbial communities. *Journal of Environmental Quality* 33: 806-815.

Einspanier R, Klotz, A, Kraft, J, Aulrich, K, Poser, R, Schwagele, F, Jahreis, G, Flachowsky, G 2001. The fate of forage plant DNA in farm animals: a collaborative case-study investigating cattle and chicken fed recombinant plant material. *European Food Research and Technology* 212: 129-134.

Einspanier, R, Lutz, B, Rief, S, Berezina, O, Zverlov, V, Schwarz, W, Myer, J 2004. Tracing residual recombinant feed molecules during digestion and rumen bacterial diversity in cattle fed transgene maize. *European Food Research and Technology* 218: 269-273.

ERMA New Zealand 2004. *Decision Making: A Technical Guide to Identifying, Assessing and Evaluating Risks, Costs and Benefits*. ERMA New Zealand, Wellington. http://www.ermanz.govt.nz/resources/publications/pdfs/ER-TG-05-01.pdf Retrieved 2 September 2005.

ERMA New Zealand 2005. *Interpretations and Explanations of Key Concepts*. ERMA New Zealand, Wellington. Protocol 3 ER-PR-03-14 08/05. http://www.ermanz.govt.nz/resources/publications/pdfs/ER-PR-03-14.pdf Retrieved 2 September 2005.

European Food Safety Authority 2004. Opinion of the Scientific Panel on Genetically Modified Organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants. *The EFSA Journal* 48: 1-18.

Ferretti, JJ, McShan, WM, Ajdic, D, Savic, DJ, Savic, G, Lyon, K, Primeaux, C, Sezate, S, Suvorov, AN, Kenton, S, Lai, HS, Lin, SP, Qian, Y, Jia, HG, Najar, FZ, Ren, Q, Zhu, H, Song, L, White, J, Yuan, X, Clifton, SW, Roe, BA, McLaughlin, R 2001. Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. *Proceedings of the National Academy of Sciences USA* 98: 4658-4663.

Funchain, P, Yeung, A, Stewart, J, Clendenin, WM, Miller, JH 2001. Amplification of mutator cells in a population as a result of horizontal transfer. *Journal of Bacteriology* 183: 3737-3741.

Gay, PB, Gillespie, SH 2005. Antibiotic resistance markers in genetically modified plants: a risk to human health? *Lancet Infectious Diseases* 5: 637-646.

Gebhard, F, Smalla, K 1998. Transformation of *Acinetobacter* sp. strain BD413 by transgenic sugar beet DNA. *Applied Environmental Microbiology* 64: 1550-1554.

Gebhard, F, Smalla, K 1999. Monitoring field releases of genetically modified sugar beets for persistence of transgenic plant DNA and horizontal gene transfer. *FEMS Microbiological Ecology* 28: 261-272.

Gelvin, SB 2005. Gene exchange by design. *Nature* 433: 583-584.

GM Science Review Panel 2003. *GM Science Review: first report*. GM Science Review, Britain. http://www.gmsciencedebate.org.uk/report/pdf/gmsci-report1-full.pdf Retrieved 8 September 2005.

Gogarten, JP, Townsend, JP 2005. Horizontal gene transfer, genome innovation and evolution. *Nature Reviews Microbiology* 3: 679-687.

Grillot-Courvalin, C, Goussard, S, Huetz, F, Ojcius, DM, Courvalin, P 1998. Functional gene transfer from intracellular bacteria to mammalian cells. *Nature Biotechnology* 16: 862-866.

Hacein-Bey-Abina, S, Von Kalle, C, Schmidt, M, McCormack, MP, Wulffraat, N, Leboulch, P, Lim, A, Osborne, CS, Pawliuk, R, Morillon, E, Sorensen, R, Forster, A, Fraser, P, Cohen, JI, de Saint Basile, G, Alexander, I, Wintergerst, U, Frebourg, T, Aurias, A, Stoppa-Lyonnet, D, Romana, S, Radford-Weiss, I, Gross, F, Valensi, F, Delabesse, E, Macintyre, E, Sigaux, F, Soulier, J, Leiva, LE, Wissler, M, Prinz, C, Rabbitts, TH, Le Deist, F, Fischer, A, Cavazzana-Calvo, M 2003. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302: 415-419.

Heinemann, JA 2003. Is horizontal gene transfer the Cinderella of genetics? *NZ BioScience* (May): 18-20.

Heinemann, JA, Sprague, GF 1989. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. *Nature* 340**:** 205-209.

Heinemann, JA, Traavik, T 2004. Problems in monitoring horizontal gene transfer in field trials of transgenic plants. *Nature Biotechnology* 22: 1105-1109.

Heritage, J 2002. Degradation of transgenic DNA from genetically modified soyabean and maize in human intestinal simulations. *British Journal of Nutrition* 87: 529-531.

Heritage, J 2004. The fate of transgenes in the human gut. *Nature Biotechnology* 22: 170-172.

Heritage, J 2005. Transgenes for tea? *Trends in Biotechnology* 23: 17-21.

Ho, MW, Ryan, A, Cummins, J 1999. Cauliflower mosaic virtual promoter- a recipe for disaster? *Microbial Ecology in Health and Disease* 11: 194-197.

Hoffmann, T, Golz, C, Schneider, O 1994. Foreign DNA sequences are received by a wild-type strain of *Aspergillus niger* after co-culture with transgenic higher plants. *Current Genetics* 27: 70-76.

Hohlweg, U, Doerfler, W 2001. On the fate of plant or other foreign genes upon the uptake in food or after intramuscular injection in mice. *Molecular Genetics and Genomics* 265: 225-233.

Johnsen, PJ, Simonsen, GS, Olsvik, O, Midtvedt, T, Sundsfjord, A 2002. Stability, persistence, and evolution of plasmid-encoded VanA glycopeptide resistance in enterococci in the absence of antibiotic selection *in vitro* and in gnotobiotic mice. *Microbial Drug Resistance* 8(3):161-170.

Jonas, DA, Elmadfa, I, Engel, KH, Heller, KJ, Kozianowski, G, Konig, A, Muller, D, Narbonne, JF, Wackernagel, W, Kleiner, J 2001. Safety considerations of DNA in food. *Annals of Nutrition and Metabolism* 45(6): 235-254.

Kay, E, Vogel, TM, Bertolla, F, Nalin, R, Simonet, P 2002. *In situ* transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. *Applied and Environmental Microbiology* 68(7): 3345-3351.

Kharazmi, M, Bauer, T, Hammes, WP, Hertel, C, 2003. Effect of food processing on the fate of DNA with regard to degradation and transformation capability in *Bacillus subtilis*. *Systematic and Applied Microbiology* 26: 495-501.

Kondo, N, Nikoh, N, Ijichi, N, Shimada, M, Fukatsu, T 2002. Genome fragment of *Wolbachia* endosymbiont transferred to X chromosome of host insect. *Proceedings of the National Academy of Sciences USA* 99: 14280-14285.

Kunik, T, Tzfira, T, Kapulnik, Y, Gafni, Y, Dingwall, C, Citovsky, V 2001. Genetic transformation of HeLa cells by *Agrobacterium*. *Proceedings of the National Academy of Sciences USA* 98: 1871-1876.

Kurland, CG, Canback, B, Berg, OG 2003. Horizontal gene transfer: a critical view. *Proceedings of the National Academy of Sciences USA* 100: 9658-9662.

Lawrence, JG, Ochman, H 1998. Molecular archaeology of the *Escherichia coli* genome. *Proceedings of the National Academy of Sciences USA* 95: 9413-9417.

LeClerc, JE, Li, B, Payne, WL, Cebula, TA 1996. High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. *Science* 274: 1208-1211.

Lewin, A, Tran, TT, Jacob, D, Mayer, M, Freytag, B, Appel, B 2004. Yeast DNA sequences initiating gene expression in *Escherichia coli*. *Microbiological Research* 159: 19-28.

Lilley, AK, Bailey, MJ, Barr, M, Kilshaw, K, Timms-Wilson, TM, Day, MJ, Norris, SJ, Jones, TH, Godfray, HCJ 2003. Population dynamics and gene transfer in genetically modified bacteria in a model microcosm. *Molecular Ecology* 12: 3097-3107.

Logg, CR, Logg, A, Tai, CK, Cannon, PM, Kasahara, N 2001. Genomic Stability of Murine Leukemia Viruses Containing Insertions at the Env-3' Untranslated Region Boundary. *Journal of Virology* 75: 6989-6998.

Martin-Orue, SM, O'Donnell, AG, Arino, J, Netherwood, T, Gilbert, HJ, Mathers JC 2002. Degradation of transgenic DNA from genetically modified soya and maize in human intestinal simulations. *British Journal of Nutrition* 87: 533-542.

Miller, HI, Conko, G 2004. Chasing 'transgenic' shadows. *Nature Biotechnology* 22: 654-655.

Mower, JP, Stefanovic, S, Young, GJ, Palmer, JD 2004. Gene transfer from parasitic to host plants. *Nature* 432: 165-166.

Netherwood, T, Bowden, R, Harrison, P, O'Donnell, AG, Parker, DS, Gilbert, HJ 1999. Gene transfer in the gastrointestinal tract. *Applied and Environmental Microbiology* 65: 5139-5141.

Netherwood, T, Martin-Orue, SM, O'Donnell, AGO, Gockling, S, Graham, J, Mathers, JC, Gilbert, HJ 2004. Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. *Nature Biotechnology* 22: 204-209.

Nguyen, TN, Phan, QG, Duong, LP, Bertrand, KP, Lenski, RE 1989. Effects of carriage and expression of the Tn10 tetracycline-resistance operon on the fitness of *Escherichia coli* K12. *Molecular Biology and Evolution* 6: 213-225.

Nielsen, KM 1998. Barriers to horizontal gene transfer by natural transformation in soil bacteria. *Acta Pathologica Microbiologica et Immunologica Scandanavia* 106: 77-84.

Nielsen, KM 2003. An assessment of factors affecting the likelihood of horizontal transfer of a recombinant plant DNA to bacterial recipients in the soil and phytosphere. In: *Collection of Biosafety Reviews* (ICGEB, Italy) 1: 96-149.

Nielsen, KM, Bones, AM, van Elsas, JD 1997a. Induced natural transformation of *Acinetobacter calcoaceticus* in soil microcosms. *Applied and Environmental Microbiology* 63: 3972-3977.

Nielsen, KM, van Weerelt, MDM, Berg, TN, Bones, AM, Hagler, AN, van Elsas, JD 1997b. Natural transformation and availability of transforming DNA to *Acinetobacter calcoaceticus* in soil microcosms. *Applied and Environmental Microbiology* 63: 1945-1952.

Nielsen, KM, Bones, AM, Smalla, K, van Elsas, JD 1998. Horizontal gene transfer from transgenic plants to terrestrial bacteria- a rare event? *FEMS Microbiology Reviews* 22:79-103.

Nielsen, KM, van Elsas, JD, Smalla, K 2000a. Transformation of *Acinetobacter* sp. strain BD413(pFG4Δ*nptII*) with transgenic plant DNA in soil microcosms and effects of kanamycin on selection of transformants. *Applied and Environmental Microbiology* 66: 1237-1242.

Nielsen, KM, van Elsas, JD, Smalla, K 2000b. *Safety issues in antibiotic resistance marker genes in transgenic crops.* Proceedings of the 6th International Feed Production Conference, Piacenza, 27-28 November 2000.

Nielsen, KM, van Elsas, JD, Smalla, K 2001. Dynamics, horizontal transfer and selection of novel DNA in bacterial populations in the phytosphere of transgenic plants. *Annals of Microbiology* 51: 79-94.

Nielsen, KM, Berdal, KG, Kruse, H, Sundsfjord, A, Mikalsen, A, Yazdankhah, S, Nes, I 2005. *An assessment of potential long –term health effects caused by antibiotic resistance marker genes in genetically modified organisms based on antibiotic usage and resistance patterns in Norway*. VKM-Report, Norwegian Scientific Committee for Food safety, Oslo, Norway.

Nielsen, KM, Townsend, JP 2001. Environmental exposure, horizontal transfer and selection of transgenes in bacterial populations. *In: Enhancing biocontrol agents and handling risks* (Vurro *et al* ed.). IOS Press, The Netherlands.

Nielsen, KM, Townsend, JP 2004. Monitoring and modelling horizontal gene transfer. *Nature Biotechnology* 22: 1110-1114.

Nielsen, KM, van Elsas, JD 2001. Stimulatory effects of compounds present in the rhizosphere on natural transformation of *Acinetobacter* sp. BD413 in soil. *Soil Biology and Biochemistry* 33: 345-357.

Ochman, H, Lawrence, JG, Groisman, EA 2000. Lateral gene transfer and the nature of bacterial innovation. *Nature* 405: 299-304.

Oliver, A, Canton, R, Campo, P, Baquero, F, Blazquez, J 2000. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 288: 1251-1253.

Paget, E, Lebrun, M, Freyssinet, G, and Simonet, P 1998. The fate of recombinant plant DNA in soil. *European Journal of Soil Biology*. 34: 81-88.

Palka-Santini, M, Schwarz-Herzke, B, Hōsel, M, Renz, D, Auerochs, S, Brondke, H, Doerfler, W 2003. The gastrointestinal tract as the portal of entry for foreign macromolecules: fate of DNA and proteins. *Molecular Genetics and Genomics* 270: 201-215.

Perna, NT, Plunkett, G, Burland, V, Mau, B, Glasner, JD, Rose, DJ, Mayhew, GF, Evans, PS, Gregor, J, Kirkpatrick, HA, Posfai, G, Hackett, J, Klink, S, Boutin, A, Shao, Y, Miller, L, Grotbeck, EJ, Davis, NW, Lim, A, Dimalanta, ET, Potamousis, KD, Apodaca, J, Anantharaman, TS, Lin, J, Yen, G, Schwartz, DC, Welch, RA, Blattner, FR 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* 409: 529-533.

Phipps, RH, Beever, DE 2002. Detection of transgenic DNA in milk from cows receiving herbicide tolerant (CP4EPSPS) soyabean meal. *Livestock Production Science* 74: 269-273.

Raymond, J, Blankenship, RE 2003. Horizontal gene transfer in eukaryotic algal evolution. *Proceedings of the National Academy of Sciences USA* 100: 7419-7420.

Read, D 2000. *ERMA Generic Issues Report: Use of Antibiotic Resistance Marker Genes in Genetically Modified Organisms*. ERMA New Zealand, Wellington. http://www.ermanz.govt.nz/resources/publications/pdfs/ER-GI-01-1.pdf Retrieved 26 August 2005.

Royal Commission on Genetic Modification, 2001. Report of the Royal Commission on Genetic Modification. http://www.mfe.govt.nz/publications/organisms/royal-commission-gm/index.html Retrieved 8 September 2005.

Salyers, AA, Shoemaker, NB, Stevens, AM, Li, LY 1995. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiological Reviews* 59(4): 579-590.

Sanchez-Gracia, A, Maside, X, Charlesworth, B 2005. High rate of horizontal transfer of transposable elements in *Drosophila*. *Trends in Genetics* 21: 200-203.

Schluter, K, Futterer, 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. *Bio/Technology* 13: 1094-1098.

Schrag, SJ, Perrot, V, Levin, BR 1997. Adaptation to the fitness costs of antibiotic resistance in *Escherichia coli. Proceedings Biological Sciences The Royal Society London* 264: 1287-1291.

Schubbert, R, Renz, D, Schmitz, B, Doerfler, W 1997. Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. *Proceedings of the National Academy of Sciences USA* 94: 961-966.

Schubbert, R, Hohlweg, U, Renz, D, Doerfler, W 1998. On the fate of orally ingested foreign DNA in mice: chromosomal association and placental transmission to the fetus. *Molecular General Genetics* 259: 569-576.

Smalla, K, Gebhard, F, van Elsas, JD, Matzk, A, Schiemann, J 1994. Bacterial communities influenced by transgenic plants. *In:* Jones, DD (ed) *Proceedings of the 3rd international symposium on the biosafety results of field tests of genetically modified plants and microorganisms*, University of California, Oakland, 157-167.

Smets, BF, Barkay, T 2005. Horizontal gene transfer: perspectives at a crossroads of scientific disciplines. *Nature Reviews Microbiology* 3: 675-678.

Sorensen, SJ, Bailey, M, Hansen, LH, Kroer, N, Wuertz, S 2005. Studying plasmid horizontal transfer *in situ*: a critical review. *Nature Reviews Microbiology* 3: 700-710.

Stewart, CN, Mentewab, A 2005. Horizontal gene transfer: plant vs. bacterial genes for antibiotic resistance scenarios – what's the difference? *ISB News Report October 2005*. http://www.isb.vt.edu/news/2005/news05.Oct.htm Retrieved 10 October 2005.

Tepfer, D, Garcia-Gonzales, R, Mansouri, H, Seruga, M, Message, B, Leach, F, Perica, MC, 2003. Homology-dependent DNA transfer from plants to soil bacterium under laboratory conditions: implications in evolution and horizontal gene transfer. *Transgenic Research* 12: 425-437.

Thomas, CM, Nielsen, KM 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature Reviews Microbiology* 3: 711-721.

Timmis, JN, Ayliffe, MA, Huang, CY, Martin, W 2004. Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. *Nature Reviews Genetics* 5: 123-135.

Townsend, JP, Nielsen, KM, Fisher, DS, Hartl, DL 2003. Horizontal acquisition of divergent chromosomal DNA in bacteria: effects of mutator phenotypes. *Genetics* 164:13-21.

van den Eede, G, Aarts, H, Buhk, HJ, Corthier, G, Flint, HJ, Hammes, W, Jacobsen, B, Midtvedt, T, van der Vossen, J, von Wright, A, Wackernagel, W, Wilcks, A 2004. The

relevance of gene transfer to the safety of food and feed derived from genetically modified (GM) plants. *Food and Chemical Toxicology* 42: 1127-1156.

Vilas-Boas, GFLT, Vilas-Boas, LA, Lereclus, D, Arantes, OMN 1998. *Bacillus thuringiensis* conjugation under environmental conditions. *FEMS Microbiology Ecology* 25: 369-374.

Waters, VL 2001. Conjugation between bacterial and mammalian cells. *Nature Genetics* 29:375-376.

Watson, SK, Carter, PE 2003. Horizontal gene transfer in the New Zealand environment. *Pacific Ecologist* 6: 29-31.

Wilcks, A, van Hoek, AHAM, Joosten, RG, Jacobsen, BBL, Aarts, HJM 2004. Persistence of DNA studied in different ex vivo and in vivo rat models simulating the human gut situation. *Food and Chemical Toxicology* 42: 493-502.

Wintermantel, WM, Schoelz, JE 1996. Isolation of recombinant viruses between cauliflower mosaic virus and a viral gene in transgenic plants under conditions of moderate selection pressure. *Virology* 223: 156-164.

World Health Organization 2002. *Antimicrobial Resistance. Fact Sheet No. 194* http://www.who.int/mediacentre/factsheets/fs194/en/ Retrieved 29 April 2004.

Appendix 1
ERMA New Zealand qualitative scales for describing adverse effects

Qualitative risk assessment

Risks and benefits are assessed by estimating the magnitude of the possible effects and the likelihood of their occurrence. For each effect, the combination of these two components determines the level of that effect, which is a two-dimensional concept. Risk assessment may be qualitative or quantitative. Qualitative assessment is informed by quantitative data where this is available.

Qualitative matrices are used to prioritise risks (and benefits), and to identify any risks that are unacceptable. The measure of the level of risk (combination of magnitude and likelihood) is specific to the application therefore measures of level of risk should not be compared between applications. However, the measures (descriptors) for different types of risk (human health, ecological etc) should be established so that they represent relative orders of magnitude.

Magnitude of effect

The magnitude must be a measure of the endpoint (specified by the Act and the Methodology), and is described in terms of the element that might be affected. The magnitude of the effect is not the same as the effect itself. The qualitative descriptors for magnitude of effect are surrogate measures that should be used to gauge the end effect or the 'what if' element.

Tables 1 and 2 contain generic descriptors for magnitude of adverse effects (risks and costs) and beneficial effects (benefits). These descriptors are examples only, and their generic nature means that it may be difficult to use them in some particular circumstances. They are included here simply to illustrate how qualitative tables may be used to represent levels of risk.

Table 1 Magnitude of adverse effect

Descriptor	Examples of descriptions
Minimal	• Mild reversible short-term adverse health effects to individuals in highly localised area • Highly localised and contained environmental impact, affecting a few (less than 10) individual members of communities of flora or fauna, no discernible ecosystem impact • Low dollar cost of containment/cleanup/repair (<$5,000) • No social disruption[29]
Minor	• Mild reversible short-term adverse health effects to identified and isolated groups[30] • Localised and contained reversible environmental impact, some local plant or animal communities temporarily damaged, no discernible ecosystem impact or species damage • Dollar cost of containment/cleanup/repair in order of $5,000-$50,000 • Potential social disruption (community placed on alert)
Moderate	• Minor irreversible health effects to individuals and/or reversible medium-term adverse health effects to larger (but surrounding) community (requiring hospitalisation) • Measurable long-term damage to local plant and animal communities, but no obvious spread beyond defined boundaries, medium-term individual ecosystem damage, no species damage • Dollar cost of containment/cleanup/repair in order of $50,000-$500,000, • Some social disruption (eg people delayed)

[29] The concept of social disruption includes both physical disruption, and perceptions leading to psychological disruption. For example, some chemicals may have nuisance effects (through odour) that result in communities feeling threatened.

[30] Note that the reference to 'groups' and 'communities' in the context of human health effects includes the notion of groups defined by health status.

Major	• Significant irreversible adverse health effects affecting individuals and requiring hospitalisation and/or reversible adverse health effects reaching beyond the immediate community • Long-term/irreversible damage to localised ecosystem but no species loss • Dollar cost of containment/cleanup/repair in order of $500,000-$5,000,000 • Social disruption to surrounding community, including some evacuations
Massive	• Significant irreversible adverse health effects reaching beyond the immediate community, and/or deaths • Extensive irreversible ecosystem damage, including species loss • Dollar cost of containment/cleanup/repair greater than $5,000,000 • Major social disruption with entire surrounding area evacuated and impacts on wider community

The economic effects category has been given a surrogate magnitude. This is for demonstration as a means of illustrating the type of magnitudes that might be encountered.

Table 2 Magnitude of beneficial effect

Descriptor	Examples of descriptions
Minimal	• Mild short-term positive health effects to individuals in highly localised area • Highly localised and contained environmental impact, affecting a few (less than 10) individual members of communities of flora or fauna, no discernible ecosystem impact • Low dollar benefit (<$5,000) • No social effect
Minor	• Mild short-term beneficial health effects to identified and isolated groups • Localised and contained beneficial environmental impact, no discernible ecosystem impact or species damage • Dollar benefit in order of $5,000-$50,000 • Minor localised community benefit

Moderate	• Minor health benefits to individuals and/or medium term health impacts on larger (but surrounding) community and health status groups • Measurable benefit to localised plant and animal communities expected to pertain to medium term. • Dollar benefit in order of $50,000-$500,000, • Local community and some individuals beyond immediate community receive social benefit.
Major	• Significant beneficial health effects to localised community and specific groups in wider community • Long-term benefit to localised ecosystem(s) • Dollar benefit in order of $500,000-$5,000,000 • Substantial social benefit to surrounding community, and individuals in wider community.
Massive	• Significant long-term beneficial health effects to the wider community • Long-term, widespread benefits to species and/or ecosystems • Dollar benefit greater than $5,000,000 • Major social benefit affecting wider community

Likelihood of effect occurring

Likelihood in this context applies to the composite likelihood of the end effect, and not either to the initiating event, or any one of the intermediary events. It includes:

- the concept of an initiating event (triggering the hazard); and
- the exposure pathway that links the source (hazard) and the area of impact (public health, environment, economy, or community).

The likelihood term applies specifically to the resulting effect or the final event in the chain, and will be a combination of the likelihood of the initiating event and several intermediary likelihoods[31]. The frequency or probability solely of the initial incident or hazard event should not be used (as it sometimes is in the safety discipline).

The best way to determine the likelihood is to specify and analyse the complete pathway of the "chain of events" from source to the final environmental impact or effect. Each event in the chain is dependent upon the previous event occurring in the first place.

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31 Qualitative event tree analysis may be a useful way of ensuring that all aspects are included.

Likelihood may be expressed as a frequency or a probability. While frequency is often expressed as a number of events within a given time period, it may also be expressed as the number of events per head of (exposed) population. As a probability the likelihood is dimensionless and refers to the number of events of interest divided by the total number of events (range 0-1).

Table 3 Likelihood (adverse effect)

	Descriptor	Description
1	Highly improbable	Almost certainly not occurring but cannot be totally ruled out
2	Improbable (remote)	Only occurring in very exceptional circumstances
3	Very unlikely	Considered only to occur in very unusual circumstances
4	Unlikely (occasional)	Could occur, but is not expected to occur under normal operating conditions
5	Likely	A good chance that it may occur under normal operating conditions
6	Very likely	Expected to occur if all conditions met
7	Extremely likely	Almost certain

Table 3 provides an example of a set of generic likelihood descriptors for adverse and beneficial effect. Note that when estimating these likelihoods, the impact of default controls should be taken into account.

The table is not symmetrical. This is to allow for classification of very low probability adverse effects.

In practical terms, where the exposure pathway is complex, it may be conceptually difficult to condense all the information into a single likelihood. For any risk where the likelihood is other than "highly improbable" or "improbable", then an analysis of the pathway should include identifying the "critical points": the aspects that are the most vulnerable, and the elements where controls might be used to "cut" the pathway.

Calculating the level of risk

Using these qualitative descriptors for magnitude of effect and likelihood of the event occurring, an additional two-way table representing a level of risk (combined likelihood and measure of effect) can be constructed as shown in Table 4, where six levels of effect are allocated: A, B, C, D, E and F. These terms have been used to emphasise that the matrix is a device for determining which risks (benefits) require further analysis to determine their significance in the decision making process. Avoiding labels such as "low", "medium", and "high" removes the aspect of perception.

The lowest level (A) may be deemed to be equivalent to "insignificant". In this table "A" is given to three combinations; minimal impact and an occurrence of improbable or highly improbable, and minor impact with a highly improbable occurrence. In some cases where there is high uncertainty it may be preferable to split this category into A1 and A2, where only A1 is deemed to equate as insignificant.

For negative effects, the levels are used to show how risks can be reduced by the application of additional controls. Where the table is used for positive effects it may also be possible for controls to be applied to ensure that a particular level of benefit is achieved, but this is not a common approach.

Table 4 Calculating the level of risk (benefit)

	Magnitude of effect				
Likelihood	**Minimal**	**Minor**	**Moderate**	**Major**	**Massive**
Highly improbable	A	A	B	C	D
Improbable	A	B	C	D	E
Very unlikely	B	C	D	E	E
Unlikely	C	D	E	E	F
Likely	D	E	E	F	F
Very likely	E	E	F	F	G
Extremely likely	E	F	F	G	G

The table presented here is symmetrical around an axis from highly improbable and minimal to massive and extremely likely. However, this will not necessarily be the case in all applications.

Impact of uncertainty in estimates

Uncertainty may be taken into account in two ways. Firstly, when describing a risk a range of descriptors may be used. For example, a risk may be allocated a range of very unlikely – improbable, and minor – major. This would put the range of the risk as B through E. Alternatively, the level of risk (or benefit) may be adjusted *after* it has been estimated on the grounds of uncertainty.