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Proceedings of the Expert Panel Meeting on the Potential Risks Associated with Horizontal Gene Transfer from Novel Aquatic Organisms

January 11, 2006
West Vancouver, British Columbia

Jake Rice and Morven McLean, Co-chairs

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Canadian Science Advisory Secretariat
200 Kent Street
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SUMMARY

Under the *New Substances Notification Regulations (Organisms)* (NSNR, Organisms) of the *Canadian Environmental Protection Act, 1999* (CEPA 1999), research and development (R&D) involving animate products of biotechnology is exempt from regulatory notification if there is no release of the living organism, material from the organism involved in toxicity, or genetic material from the organism. This exemption is currently being reviewed by Environment Canada and Health Canada.

Fisheries and Oceans Canada (DFO) administers the NSNR for aquatic organisms with novel traits on behalf of Environment Canada and Health Canada. This includes the conduct of assessments of potential risks to the environment and human health of aquatic organisms with novel traits. In addition, DFO is developing new regulations specific to novel aquatic organisms. The potential risk associated with horizontal gene transfer (HGT) of genetic material from aquatic organisms with novel traits is an issue that requires consideration in the design of these regulations.

To address this, Fisheries and Oceans Canada commissioned a literature review and discussion document of the persistence of DNA in the environment, mechanisms for HGT, known occurrence of HGT, and potential risks associated with HGT. A scientific panel of experts was convened to address specific questions, based on the discussion document, on potential risks associated with HGT. This report summarizes the considerations and output from the expert panel and will be used to inform the regulatory amendment and the development of new regulations for novel aquatic organisms.

Horizontal gene transfer is a very rare event that is more likely between prokaryotes than between eukaryotes or eukaryotes and prokaryotes.

The potential consequences of HGT are important. Case-by-case evaluation of potential consequences of HGT from novel organisms will help to identify what further information may be required to complete an environmental risk assessment. This tiered approach will ensure that the allocation of risk management is in line with potential identified risks.

SOMMAIRE

En vertu du Règlement sur les renseignements concernant les substances nouvelles (organismes) (RRSN, organismes) de la Loi canadienne sur la protection de l'environnement, 1999 (LCPE 1999), la recherche et développement (R. et D.) touchant les produits vivants issus de la biotechnologie est exempte de déclaration réglementaire lorsqu'il n'y a aucun rejet dans l'environnement de l'organisme vivant, de matériel de l'organisme qui contribue à la toxicité ou de matériel génétique de l'organisme. Cette exemption est actuellement revue par Environnement Canada et Santé Canada.

Pêches et Océans Canada (MPO) administre le RRSN concernant les organismes aquatiques à caractères nouveaux au nom d'Environnement Canada et de Santé Canada. Cette administration comprend l'évaluation des risques potentiels que présentent les organismes aquatiques à caractères nouveaux pour l'environnement et la santé humaine. De plus, le MPO travaille actuellement à l'élaboration de nouveaux règlements propres aux organismes aquatiques à caractères nouveaux. Le risque potentiel lié à la transmission horizontale de gènes (THG) provenant d'organismes aquatiques à caractères nouveaux doit être pris en compte lors de l'élaboration de ces nouveaux règlements.

Pour traiter de cette question, Pêches et Océans Canada a commandé une revue de la littérature scientifique et un document de discussion sur la persistance de l'ADN dans l'environnement, les mécanismes de la THG, la fréquence connue de la THG et les risques potentiels liés à la THG. Un groupe d'experts scientifiques a été convoqué pour examiner des questions spécifiques du document de discussion concernant les risques potentiels liés à THG. Ce rapport résume les considérations et les réponses du groupe d'experts et sera utilisé pour étayer la modification réglementaire et l'élaboration de nouveaux règlements sur les organismes aquatiques à caractères nouveaux.

La THG est un phénomène très rare qui est davantage susceptible de se produire entre des procaryotes qu'entre des eucaryotes ou entre des eucaryotes et des procaryotes.

Les conséquences potentielles de la THG sont importantes. Une évaluation au cas par cas des conséquences potentielles de la THG d'organismes nouveaux permettra de déterminer l'information supplémentaire nécessaire à l'évaluation des risques pour l'environnement. Cette démarche par étapes assurera la concordance entre la gestion des risques

et les risques potentiels identifiés.

There could be differences among the transformation techniques utilized in their potential to influence the rate of HGT, but the uncertainty of the rate of each method is so high that the techniques or vectors cannot currently be ranked in respect of their relative potential to influence rate of HGT.

Les techniques de transformation utilisées peuvent différer quant à leur potentiel à influencer le taux de THG, mais l'incertitude du taux de chaque méthode est tellement grande que les techniques ou les vecteurs ne peuvent actuellement être classés en fonction de leur potentiel à influencer le taux de THG.

It was difficult to define categories of hazard due in part to the necessary consideration of whether a specific trait would constitute a risk in a specific receiving environment and the consideration of any selection pressures, which would also vary depending on the specific trait and the receiving environment.

Il a été difficile de définir les catégories de risque, en partie en raison de la nécessité de prendre en compte le risque que représente un caractère nouveau donné dans un milieu récepteur précis ainsi que toute pression sélective, laquelle peut également varier en fonction du caractère spécifique et du milieu récepteur considérés.

However, some possible HGT hazard and frequency considerations were identified. Hazards may be lower where the genes already exist in the release environment. In the case of risks to human health and endangered or rare species, potential effects should be considered at the individual level. In the case of environmental impact, effects should generally be considered at the population level.

Néanmoins, certains risques possibles de THG ont été identifiés, et on a retenu certaines considérations ayant trait à la fréquence de THG. Le risque peut être moins élevé lorsque les gènes existent déjà dans le milieu récepteur. Dans le cas des risques pour la santé humaine ou les espèces rares ou en péril, les effets potentiels doivent être considérés par rapport aux individus. Dans le cas de l'impact sur l'environnement, les effets doivent généralement être considérés par rapport aux populations.

The sequence of events leading to HGT was identified.

La séquence d'événements qui entraîne la THG a été déterminée.

Additional considerations with respect to risks associated with HGT from novel aquatic plants include their role as primary producers and the challenges associated with the containment of certain species.

Il faut également considérer les risques associés à la THG de plantes aquatiques nouvelles, sur le plan de leur rôle en tant que producteurs primaires et des difficultés de confinement de certaines espèces.

The potential for risk associated with the HGT from novel aquatic organisms should be considered in the context of environmental and human health risks posed by other aquatic organisms such as exotics.

Le risque potentiel lié à la THG d'organismes aquatiques à caractères nouveaux doit être évalué en fonction des risques que posent d'autres organismes aquatiques, tels les organismes exotiques, pour l'environnement et la santé humaine.

INTRODUCTION

Canada's regulatory framework was established through agreement among federal regulatory bodies and was announced in 1993. Under Canada's biotechnology regulatory regime, all organisms and food products, whether they are produced using conventional technologies or biotechnologies, are governed under the same legislation as their traditional counterparts. Depending on the type of product, the relevant piece of legislation is the *Seeds Act, Feeds Act, Fertilizers Act, Health of Animals Act*, or the *Canadian Environmental Protection Act* (CEPA).

In Canada, regulatory oversight is triggered by the novelty of traits expressed by the host organism or the novel attributes of foods or food ingredients, irrespective of the means by which the novel traits were introduced. This "product-based" approach to regulation has been validated by numerous scientific bodies and expert consultations. Because the scope of Canada's regulatory approach is broader than just genetically engineered organisms, Canadian regulators have adopted unique terminology and definitions, *e.g.* aquatic organisms with novel traits.

The importation or manufacture of novel aquatic organisms in Canada is currently regulated under the *New Substances Notification Regulations (Organisms)* (NSNR-Organisms; CEPA 1999). Fisheries and Oceans Canada is responsible for conducting risk assessments of aquatic organisms with novel traits with respect to potential adverse effects on the environment or human health. Subsection 2(4) of the NSNR (Organisms) provides for the exemption from notification of organisms other than micro-organisms where the organism is used in research and development and where it is "imported into" or "manufactured in" a contained facility from which there is no release into the environment of: a) the organism; b) the genetic material of the organism; or c) material from the organism involved in toxicity. A specific issue with respect to the release of genetic material into the environment is related to potential risks associated with HGT of genetic material from the carcasses of aquatic organisms with novel traits, their somatic cells or other biological waste that contains DNA to microorganisms or eukaryotic organisms in the environment.

INTRODUCTION

Annoncé en 1993, le cadre de réglementation du Canada a été établi à la suite d'une entente entre les organismes de réglementation fédéraux. En vertu du régime de réglementation de la biotechnologie du Canada, tous les organismes et produits alimentaires, qu'ils soient issus de technologies classiques ou de la biotechnologie, sont régis par la même législation que leurs homologues traditionnels. Selon le type de produit, la législation en question sera la *Loi sur les semences, la Loi relative aux aliments du bétail, la Loi sur les engrais, la Loi sur la santé des animaux* ou la *Loi canadienne sur la protection de l'environnement* (LCPE).

Au Canada, la surveillance réglementaire est mise en branle par la nouveauté des caractères exprimés par l'organisme hôte ou les nouveaux attributs des aliments ou des ingrédients alimentaires, indépendamment des moyens par lesquels les caractères nouveaux ont été introduits. Cette approche de réglementation « basée sur le produit » a été validée par de nombreux organismes scientifiques et conseillers experts. Puisque la portée de l'approche réglementaire canadienne ne se limite pas aux organismes génétiquement modifiés, les organismes de réglementation canadiens ont adopté une terminologie et des définitions uniques, tels que « organismes aquatiques à caractères nouveaux ».

L'importation ou la fabrication d'organismes aquatiques à caractères nouveaux au Canada est actuellement régie par le *Règlement sur les renseignements concernant les substances nouvelles (organismes)* (RRSN-organismes; LCPE 1999). Pêches et Océans Canada est tenu d'évaluer les risques que présentent les organismes aquatiques à caractères nouveaux pour l'environnement et la santé humaine. Le paragraphe 2(4) du RRSN- organismes prévoit l'exemption de déclaration des organismes autres que les microorganismes lorsque l'organisme est utilisé en recherche et développement, qu'il est « importé dans » ou « fabriqué dans » une installation à partir de laquelle il n'y a aucun rejet dans l'environnement de a) l'organisme; b) du matériel génétique de l'organisme; ou c) du matériel de l'organisme qui contribue à la toxicité. Le problème concernant le rejet de matériel génétique dans l'environnement est lié aux risques potentiels que présente la transmission horizontale de matériel génétique provenant de carcasses d'organismes aquatiques à caractères nouveaux, de leurs cellules somatiques ou d'autres résidus biologiques contenant de l'ADN à des microorganismes ou à des organismes eucaryotes dans l'environnement.

The exemption criteria specified in subsection 2(4) of the NSNR-Organisms are currently being reviewed by Environment Canada and Health Canada. In addition, Fisheries and Oceans Canada is considering the development of novel aquatic organism-specific regulations for the CEPA-equivalent regulatory oversight of novel aquatic organisms in lieu of the NSNR-Organisms regime.

In order to provide information and knowledge to inform these regulatory developments, Fisheries and Oceans Canada has undertaken a review of the current state of knowledge regarding the potential for, and risks associated with HGT of DNA from eukaryotic organisms, including aquatic organisms with novel traits and their associated biological waste.

In this context, an expert panel was asked to review the potential for genetic material from aquatic organisms with novel traits to adversely impact the environment, whether released from carcasses on landfill sites or in effluent from R&D facilities.

INPUT FROM THE EXPERT PANEL

1. Does the potential for HGT warrant the destruction of DNA from aquatic organisms with novel traits in research effluent and waste?

Horizontal gene transfer occurs at a very low rate, best measured in evolutionary timeframes. HGT from prokaryote (virus, bacterium) to prokaryote is more likely than from eukaryote (fungus, plant, animal) to prokaryote, which is more likely than HGT from eukaryote to eukaryote. Therefore, protocols for management of eukaryotic waste need not be more stringent than requirements for prokaryotes.

There are identifiable factors that can influence the rate of HGT, e.g. higher concentrations of DNA in contained waste than in the release environment. Based on the transient nature of RNA, risk assessment for HGT from novel aquatic organisms can focus on DNA.

It is the *consequences* of HGT from aquatic organisms with novel traits that need to be considered. This is best done on a case-by-case basis. In the absence of the potential for adverse consequence, treatment of DNA in waste tissues,

Les critères d'exemption précisés au paragraphe 2(4) du RRSN-organismes font actuellement l'objet d'un examen par Environnement Canada et Santé Canada. De plus, Pêches et Océans Canada étudie la possibilité d'élaborer des règlements sur les nouveaux organismes aquatiques en vue d'établir un régime de surveillance réglementaire équivalent à celui de la LCPE en remplacement de celui du RRSN-organismes.

Afin de recueillir de l'information et des connaissances pour étayer ces nouveaux règlements, Pêches et Océans Canada a entrepris un examen de l'état actuel des connaissances sur le potentiel et les risques que présente la THG d'ADN provenant d'organismes eucaryotes, notamment d'organismes aquatiques à caractères nouveaux et de leurs résidus biologiques.

Dans ce contexte, un groupe d'experts a été convié à l'examen des risques que présente le matériel génétique d'organismes aquatiques à caractères nouveaux pour l'environnement, qu'il provienne de carcasses rejetées dans un site d'enfouissement ou de l'effluent d'installations de R. et D.

RÉPONSES DU GROUPE D'EXPERTS

1. Est-ce que le potentiel de THG justifie la destruction de l'ADN des organismes aquatiques à caractères nouveaux présents dans l'effluent et les déchets des installations de recherche?

La transmission horizontale de gènes se produit à un taux très bas, plus facilement mesurable sur des périodes évolutives. La THG de procaryote (virus, bactérie) à procaryote est plus probable que la THG d'eucaryote (champignon, plante, animal) à procaryote, laquelle est plus probable que la THG d'eucaryote à eucaryote. Par conséquent, il n'est pas nécessaire que les protocoles de gestion des résidus d'eucaryotes soient plus rigoureux que les exigences relatives aux procaryotes.

Il existe des facteurs identifiables qui peuvent influencer le taux de THG, comme une plus forte concentration d'ADN dans les déchets confinés que dans l'environnement récepteur. Étant donné la nature transitoire de l'ARN, l'évaluation des risques de THG d'organismes aquatiques à caractères nouveaux peut être concentrée sur l'ADN.

Ce sont les *conséquences* de la THG d'organismes aquatiques à caractères nouveaux qui doivent être examinées, et il est préférable de le faire au cas par cas. S'il n'y a pas de risque de conséquences négatives, le traitement de l'ADN provenant des

carcasses and effluent would not be necessary.

2. Are there transformation techniques and vectors that could enhance the rate of HGT from carcasses, somatic cells or other waste associated with aquatic organisms with novel traits?

Transformation techniques can influence the site of insertion and copy number, which may influence HGT. Certain vectors or sequences used in transformation may have a greater impact on the potential for HGT, e.g. pantropic vectors; extraneous sequences which have homology for recombination events; mobilizable elements.

There could be differences among transgenic products generated using different transformation techniques in their potential to influence the rate of HGT, but the uncertainty of the rate of each method is so high, that, with current knowledge, we cannot rank which techniques or vectors are more likely to result in higher rates of HGT from waste DNA.

There are no data to determine whether the risk of HGT in aquatic environments is greater than in other environments. While habitat interconnectivity may be greater in aquatic systems than terrestrial environments, one still finds distinct populations in specific areas.

Not all contained experimentation leads to products that will be released into the environment. Much research is conducted in contained facilities to gathers basic data and so the transformation technique and the vector do not need to be optimized for minimal environmental impact.

Basic research could be severely hindered by regulations that focus on environmental release rather than containment. While minimizing extraneous DNA in organisms destined for release may simplify risk assessment, this is seldom necessary for contained research

Release of DNA to the environment occurs on a continuous basis from all organisms, including from genetically-engineered aquatic organisms. Release is

tissus, des carcasses et de l'effluent rejetés n'est pas nécessaire.

2. Existe-t-il des techniques ou des vecteurs de transformation pouvant accroître le taux de THG des carcasses, des cellules somatiques ou d'autres résidus d'organismes aquatiques à caractères nouveaux?

Les techniques de transformation peuvent influencer le point d'insertion et le nombre de copies et ainsi influencer la THG. Certains vecteurs ou séquences utilisés dans la transformation peuvent avoir plus d'effet sur le potentiel de THG, par exemple les vecteurs pantropes, les séquences étrangères qui ont une homologie pour les événements de recombinaison et les éléments mobilisables.

Les produits transgéniques issus de différentes techniques de transformation peuvent varier quant à leur potentiel à influencer le taux de THG, mais l'incertitude concernant le taux de chaque méthode est tellement élevée que les connaissances actuelles ne permettent pas de déterminer les techniques ou les vecteurs qui sont les plus susceptibles de générer des taux de THG élevés à partir de l'ADN rejeté.

Aucune donnée ne permet de déterminer si le risque de THG en milieu aquatique est plus important que dans les autres milieux. Bien que l'interconnectivité des habitats puisse être plus grande dans un système aquatique que dans les milieux terrestres, on trouve quand même des populations distinctes dans des zones spécifiques.

Ce ne sont pas toutes les expériences en milieu confiné qui entraînent le rejet de produits dans l'environnement. Bon nombre des recherches effectuées dans des installations confinées visent à recueillir des données de base; par conséquent il n'est pas nécessaire d'optimiser les techniques et les vecteurs de transformation en vue d'en réduire l'impact sur l'environnement.

La recherche fondamentale risque d'être gravement entravée par des règlements qui portent davantage sur les rejets dans l'environnement que sur le confinement. Bien que le fait de réduire au minimum l'ADN étranger dans les organismes destinés à être disséminés dans l'environnement puisse simplifier l'évaluation des risques, cette procédure est rarement nécessaire dans le cadre de recherches en milieu confiné.

La libération d'ADN dans l'environnement se fait sur une base continue pour tous les organismes, y compris les organismes aquatiques génétiquement

anticipated from lysed cells which have sloughed off or released from internal (e.g. gut, gonads) or external (e.g. gill, mucosal surfaces) tissues of the organism or have been released following death of the organism. Thus, complete containment of organism DNA would require extremely stringent control of all waste materials, body parts, and gametes present in effluent and aerosol waters, as well as filtration of all air from the culture area to capture DNA in airborne particles which have flaked away from dried water droplets on the sides of tanks. Further, during the preparation of a gene construct using normal laboratory procedures, the gene construct has been released to the environment in the form of aerosols from bacterial cultures and laboratory manipulations. Thus, stringency applied to the rearing of aquatic organisms should be implemented in balance with existing standards in place to control the release of recombinant DNA molecules to the environment from research activities in microbiology laboratories.

modifiés. Par exemple, l'ADN est libéré de cellules lysées qui proviennent de tissus internes (par ex. intestin, gonades) ou externes (par ex. branchies, surface des muqueuses) de l'organisme ou il est libéré des cellules après la mort de l'organisme. Par conséquent, le confinement complet de l'ADN de l'organisme nécessiterait un contrôle extrêmement rigoureux de tous les résidus, parties du corps et gamètes présents dans l'effluent ou les aérosols d'eau ainsi qu'une filtration de l'air dans la zone de culture afin de capter la matière particulaire en suspension dans l'air qui pourrait contenir de l'ADN (par exemple, des particules provenant de gouttelettes d'eau séchées sur les côtés des réservoirs). De plus, lors de la préparation d'un gène hybride à l'aide des techniques de laboratoire habituelles, l'ADN peut être rejeté dans l'environnement sous forme d'aérosol provenant des cultures bactériennes ou résultant des manipulations de laboratoire effectuées. Par conséquent, la rigueur apportée à l'alevinage d'organismes aquatiques doit être appliquée en fonction des normes existantes visant à limiter le rejet, dans l'environnement, de molécules d'ADN recombinant par les laboratoires de recherche en microbiologie.

3. *Can categories of hazard be applied to different classes of gene arrangements?*

3. *Peut-on associer des catégories de risque peuvent à différentes catégories de dispositions de gènes?*

The NIH guidelines[1] have categorized risks of transgenic microorganisms.

Les normes NIH[1] classifient les risques que présentent les microorganismes transgéniques.

It is difficult to define categories of hazardous traits for aquatic organisms due in part to the necessary consideration of whether a specific trait would constitute a risk in a specific receiving environment as well as the consideration of any selection pressures, which would also vary depending on the specific trait and the specific receiving environment. The panel recognized that the specifics of the sequence structure, including the organization of a gene construct, the hazardous characteristics of the gene products (nature of biological functions), the expression of the transgenes in anticipated recipient organisms and the release environment will influence risk and will need to be taken into consideration in case-by-case risk assessments.

Il est difficile de définir des catégories de caractères dangereux pour les organismes aquatiques, d'une part en raison de la nécessité de considérer le risque potentiel d'un caractère spécifique dans un environnement récepteur spécifique et, d'autre part, à cause des pressions sélectives qui varient également en fonction du caractère et de l'environnement récepteur. Le groupe d'experts reconnaît que les détails de la structure séquentielle, notamment l'organisation d'un gène hybride, les caractéristiques dangereuses des produits géniques (nature des fonctions biologiques), l'expression des transgènes dans des organismes récepteurs prévus et le rejet dans l'environnement influencent le risque et doivent être prises en compte dans les évaluations de risque sur une base de cas par cas.

The panel considered the categorization of gene arrangements and associated risk of mobility from lowest to highest. This categorization was further delineated after the meeting and agreed upon by all panel members (see Appendix 1).

Le groupe d'experts a entrepris la classification des différentes dispositions de gènes et des risques de mobilité qui leur sont associés, depuis le risque le plus faible jusqu'au plus élevé. Cette classification a été délimitée de façon plus approfondie après la

[1] NIH Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines) April 2002 **http://www4.od.nih.gov/oba**
Normes NIH sur les recherches touchant les molécules d'ADN recombinant (normes NIH), avril 2002 **http://www4.od.nih.gov/oba**

réunion et a été approuvée par tous les membres du groupe d'experts (se reporter à l'Annexe 1).

Risk tolerance is a cultural issue and decisions will need to take the Canadian culture into consideration.

La tolérance à l'égard du risque est une question d'ordre culturel, et la prise de décisions doit tenir compte de la culture canadienne.

4. Can there be types of novel aquatic organisms that pose no new hazard to the environment?

4. Est-il possible que certains types d'organismes aquatiques à caractères nouveaux ne posent aucun nouveau risque pour l'environnement?

When novel aquatic organisms contain transgenes that already exist in the release environment, there may be less associated risk. Natural genes and transgenes enter the aquatic ecosystem in effluent and by the sloughing off and lysis of cells from living organisms and from the lysis of cells from dead carcasses. If these genes already exist in the release environment, regulators need to consider:

Lorsque de nouveaux organismes aquatiques contiennent des transgènes qui existent déjà dans le milieu récepteur, le risque est moindre. Les gènes naturels et les transgènes entrent dans l'écosystème aquatique par l'effluent lors du détachement et de la lyse de cellules d'organismes vivants et de carcasses. Si ces gènes existent déjà dans le milieu récepteur, les organismes de réglementation doivent tenir compte :

1) the concentration of the transgene relative to that of the source genes in nature and whether exposure is increased;

1) De la concentration du transgène par rapport à celle des gènes source dans la nature et de l'augmentation potentielle d'exposition;

2) the likelihood of a gene modifying on excision or reinsertion;

2) De la probabilité de modification du gène à l'excision ou à la réinsertion;

3) the distribution and abundance of the gene in the release environment; and

3) De la distribution et de l'abondance du gène dans le milieu récepteur;

4) the origin and potential recipients of HGT in the aquatic environment.

4) De l'origine et des récepteurs potentiels de la THG dans le milieu aquatique.

5) Are there additional considerations that must be considered with the disposal of effluent from research and development with novel aquatic plants?

5) Doit-on tenir compte d'autres aspects dans le rejet de l'effluent des installations de recherche et développement concernant les nouvelles plantes aquatiques?

Additional considerations with respect to HGT are not anticipated for novel aquatic plants. However, the containment of novel aquatic plants may be more difficult than animals or even terrestrial plants. Sloughed off cells may become new plants.

On ne s'attend pas à e qu'il y ait d'autres facteurs à prendre en compte relativement à la THG de plantes aquatiques à caractères nouveaux. Cependant, le confinement des plantes aquatiques à caractères nouveaux peut s'avérer plus difficile que celui des animaux et même des plantes terrestres. Des cellules détachées peuvent devenir de nouvelles plantes.

A change in aquatic plant populations may trigger large changes in the environment, as observed with terrestrial plants, because they are primary producers.

Un changement dans les populations de plantes aquatiques peut entraîner de grands changements dans l'environnement, comme cela a été observé pour les plantes terrestres, car il s'agit de producteurs primaires.

However, adverse consequences of inadequate containment are more likely through gamete or seed dispersal, outcrossing or vegetative spread than HGT. The spread of some genes through

Toutefois, le confinement inadéquat risque plus d'avoir des conséquences négatives par le biais de la dispersion de gamètes ou de graines, du croisement hétérogène ou de la multiplication végétative que par

outcrossing, like herbicide tolerance, may impact on the use of herbicides to control aquatic invasive plant species.

6. Can guidance be provided in assessing the potential risks associated with HGT from novel aquatic organisms?

The panel reiterated the importance of determining the potential consequences of HGT when assessing risk. They detailed a 'chain of events' outlining the steps needed for successful HGT and gene expression. Scientifically supportable probabilities of HGT and the factors that would affect these might be a useful addition to the chain of events, but should be used illustratively, not embedded in regulations. The list of events required for HGT from novel aquatic organisms is:

- Transgene constructed
- DNA transferred to organism
- DNA integrated to organism
- DNA released to environment
- DNA persistence[2]
- DNA taken up by recipient
- DNA integrated into reproductive genome
- DNA confers a phenotypic effect
- Organism persists / is selected for

Consequences take into account the potential for the gene to spread in the population, the effect the gene might have on the population and effects these population changes may have on the ecosystem. Indirect effects need to be considered. In the case of human health and endangered or rare species, potential effects should be considered at the individual level. In the case of environmental impact, effects are considered at the population level.

l'intermédiaire de la THG. La propagation de certains gènes, comme les gènes de tolérance aux herbicides, par croisement hétérogène, peut avoir des répercussions sur l'utilisation d'herbicides pour lutter contre les plantes aquatiques envahissantes.

6. Peut-on fournir des lignes directrices pour l'évaluation des risques potentiels liés à la THG d'organismes aquatiques à caractères nouveaux?

Le groupe d'experts a réitéré l'importance de déterminer les conséquences potentielles de la THG lors de l'évaluation des risques. Il a présenté une « chaîne d'événements détaillée » comprenant les étapes nécessaires à la THG et à l'expression des gènes. Les probabilités scientifiques de THG ainsi que les facteurs qui influencent ces probabilités constitueraient un ajout utile à la chaîne d'événements, mais ceux-ci ne doivent être utilisés qu'à des fins d'illustration et ils ne doivent pas être incorporés dans la réglementation. La liste des événements nécessaires à la THG d'organismes aquatiques à caractères nouveaux se présente comme suit :

- Construction du transgène
- Transfert de l'ADN à l'organisme
- Intégration de l'ADN dans l'organisme
- Libération de l'ADN dans l'environnement
- Persistance de l'ADN[2]
- Captation de l'ADN par un organisme récepteur
- Intégration de l'ADN dans un génome reproducteur
- Production d'un effet phénotypique
- Persistance/sélection de l'organisme

Les conséquences tiennent compte du potentiel de propagation du gène dans la population, de l'effet que le gène peut avoir sur la population et des effets que ces changements de population peuvent avoir sur l'écosystème. Les effets indirects doivent être pris en compte. Dans le cas des risques pour la santé humaine ou pour les espèces rares ou en péril, les effets potentiels doivent être considérés par rapport aux individus. Dans le cas de l'impact sur l'environnement, les effets doivent être considérés par rapport aux populations.

[2]It is recognized that modifications to extracellular DNA in the environment may occur through physical, chemical and/or biological mechanisms. These modifications are equally likely to occur to naturally occurring DNA , as to occur in recombinant DNA. Similarly, after HGT has occurred, modifications (i.e. mutations) may occur to DNA in the new cellular environment over generations. Naturally occurring DNA would be vulnerable to these types of modifications/mutations as would transgenic DNA.
Les modifications de l'ADN extracellulaire dans l'environnement peuvent se produire par le biais de mécanismes physiques, chimiques et/ou biologiques. Ces modifications sont aussi probables pour l'ADN d'origine naturelle que pour l'ADN recombinant. De même après la THG, des modifications (c.-à-d. mutations) de l'ADN peuvent se produire dans le nouvel environnement cellulaire au fil du temps (sur plusieurs générations). L'ADN d'origine naturelle est vulnérable à ces types de modifications ou mutations, comme le serait l'ADN transgénique.

Appendix 1: Delineation of Categorization of Gene Arrangements

CATEGORIZATION OF GENE ARRANGEMENTS AND ASSOCIATED MOBILITY RISK GRADIENT

During the meeting, panel members considered the categorization of gene arrangements (the proximity of open reading frames to regulatory elements and other genes) and associated risk of mobility. This categorization of gene arrangements was further delineated after the meeting and agreed upon by all panel members in order of lowest to highest risk of mobility as follows:

- Un-integrated (i.e. transient, single-generation presence) eukaryotic or prokaryotic DNA normally found in the environment and not rearranged with other sequences. [3]

- Un-rearranged sequences taken from an organism and reintegrated into the genome of the same species or closely related species (e.g. same genus).
- Eukaryotic DNA which has been re-arranged with other eukaryotic DNA sequences not known to be mobile or facilitate replication or recombination.
- Prokaryotic DNA which has been re-arranged with eukaryotic DNA sequences not known to be mobile or facilitate replication or recombination.

- Eukaryotic sequences juxtaposed with functional eukaryotic non-viral mobilization elements or eukaryotic non-viral sequences that facilitate replication or recombination.

- Prokaryotic sequences juxtaposed with known eukaryotic non-viral mobilization elements or other eukaryotic non-viral sequences that

Annexe 1 : Délimitation des catégories de disposition de gènes

CLASSIFICATION DES DISPOSITIONS DE GÈNES ET GRADIENT DU RISQUE DE MOBILITÉ ASSOCIÉ

Lors de la réunion, les membres du groupe d'experts ont entrepris la classification des dispositions des gènes (la proximité des cadres de lecture ouverts par rapport aux éléments de régulation et aux autres gènes) et des risques de mobilité associés à ces arrangements. Cette classification des dispositions des gènes s'est poursuivie après la réunion, a été approuvée par tous les membres du groupe d'experts et se présente comme suit, du risque de mobilité le moins élevé au plus élevé :

- De l'ADN eucaryote ou procaryote non intégré (c.-à-d. transitoire, présence chez une seule génération) normalement présent dans l'environnement et non réarrangé avec d'autres séquences[4].
- Des séquences non réarrangées prélevées d'un organisme et réintégrées dans le génome d'un organisme d'une même espèce ou d'une espèce étroitement (par ex. même genre).
- De l'ADN eucaryote réarrangé avec d'autres séquences d'ADN eucaryote qui ne sont pas mobiles ou qui ne facilitent pas la réplication ou la recombinaison.
- De l'ADN procaryote réarrangé avec des séquences d'ADN eucaryote qui ne sont pas mobiles ou qui ne facilitent pas la réplication ou la recombinaison.
- Des séquences eucaryotes juxtaposées à des éléments de mobilisation eucaryotes fonctionnels et non viraux ou à des séquences eucaryotes non virales qui facilitent la réplication ou la recombinaison.
- Des séquences procaryotes juxtaposées à des éléments de mobilisation eucaryotes non viraux connus ou à d'autres séquences eucaryotes non

[3] While integration of eukaryotic DNA into prokaryotes may occur relatively frequently, retention of these sequences is anticipated to be remote unless specific functions have been provided to the prokaryotic organism. Evidence available to date suggests that uptake of un-rearranged DNA into eukaryotes from environmental sources is very rare (i.e. evolutionary time scales).

[4]While integration of eukaryotic DNA into prokaryotes may occur relatively frequently, retention of these sequences is anticipated to be remote unless specific functions have been provided to the prokaryotic organism. Evidence available to date suggests that uptake of un-rearranged DNA into eukaryotes from environmental sources is very rare (i.e. evolutionary time scales).

Bien que l'intégration d'ADN eucaryote dans des procaryotes puisse se produire assez fréquemment, on ne s'attend pas à ce que ces séquences soient retenues à moins que des fonctions spécifiques ne soient ainsi fournies au procaryote récepteur. Les données disponibles jusqu'ici donnent à penser que la captation par des eucaryotes d'ADN non réarrangé provenant de sources environnementales est très rare (échelle temporelle de l'évolution).

facilitate replication or recombination.

- Eukaryotic DNA juxtaposed with known prokaryotic non-viral mobilization sequences or non-viral sequences that facilitate replication or recombination in prokaryotes.

- Prokaryotic DNA juxtaposed with known non-viral prokaryotic mobilization sequences or non-viral sequences that facilitate replication or recombination in prokaryotes.

- Prokaryotic or eukaryotic sequences fused to sequences from eukaryotic or prokaryotic viruses which confer mobility, recombination, or replication.

virales qui facilitent la réplication ou la recombinaison.

- De l'ADN eucaryote juxtaposé à des séquences de mobilisation procaryotes non virales connues ou à des séquences non virales qui facilitent la réplication ou la recombinaison dans des procaryotes.
- De l'ADN procaryote juxtaposé à des séquences de mobilisation procaryotes non virales connues ou à des séquences non virales qui facilitent la réplication ou la recombinaison dans des procaryotes.
- Des séquences procaryotes ou eucaryotes fusionnées à des séquences de virus d'eucaryotes ou de procaryotes qui confèrent des propriétés de mobilité, de recombinaison ou de réplication.

Appendix 2: Discussion Document

SUMMARY OF THE REVIEW OF POTENTIAL RISKS ASSOCIATED WITH HORIZONTAL GENE TRANSFER FROM NOVEL AQUATIC ORGANISMS

In order to provide information and knowledge to inform regulatory developments, Fisheries and Oceans Canada undertook a review of the current state of knowledge regarding the potential for, and risks associated with, horizontal gene transfer (HGT) of DNA from eukaryotic organisms, including novel aquatic organisms and their associated biological waste. This review was divided along four thematic areas dealing with: persistence of DNA in the natural environment; mechanisms of horizontal gene transfer; approaches to producing transgenic aquatic organisms; and considerations for risk assessment and risk management.

A comprehensive search of the scientific literature was undertaken for information regarding the potential for horizontal gene transfer from eukaryotic plants and animals, including novel aquatic organisms. The search utilized existing bibliographic databases, and the primary and secondary information derived from these.

Persistence of DNA in the Natural Environment

DNA is a double stranded polymer with a long, flexuous structure that is vulnerable to physical forces and active degradation by chemicals or enzymes. In the living cell DNA is protected by structural supports such as chromatin and active repair mechanisms that quickly fix damaged nucleotides.

At cell death, DNA, along with the other components of the cell, is quickly degraded by enzymatic activity. However, instances have occurred after cell death where DNA is protected from rapid degradation by an environment that limits enzymatic activity and supports the flexuous structure of the molecules. Degradation of DNA during decay would occur equally for conventional and transgenic organisms unless the transgenic DNA sequences were engineered to be more resistant to nuclease degradation.

Exposed DNA is hydrolyzed at substantial rates in

Annexe 2 : Document de discussion

RÉSUMÉ DE L'EXAMEN DES RISQUES POTENTIELS LIÉS À LA TRANSMISSION HORIZONTALE DE GÈNES D'ORGANISMES AQUATIQUES À CARACTÈRES NOUVEAUX

Afin de recueillir de l'information et des connaissances pour étayer une nouvelle réglementation, Pêches et Océans Canada a entrepris un examen de l'état actuel des connaissances sur le potentiel et les risques que présente la transmission horizontale de gènes (THG) d'ADN provenant d'organismes eucaryotes, notamment d'organismes aquatiques à caractères nouveaux et de leurs résidus biologiques. Cet examen est divisé en quatre catégories thématiques : la persistance de l'ADN en milieu naturel; les mécanismes de transmission horizontale de gènes; les méthodes de production d'organismes aquatiques transgéniques et les aspects à considérer dans l'évaluation et la gestion des risques.

Un examen approfondi de la littérature scientifique a été entrepris en vue d'obtenir de l'information sur le potentiel de transmission horizontale de gènes de plantes ou d'animaux eucaryotes, notamment d'organismes aquatiques à caractères nouveaux. Les bases de données bibliographiques existantes et l'information primaire et secondaire qui en découle ont été utilisées dans le cadre de cette recherche.

Persistance de l'ADN en milieu naturel

L'ADN est un polymère à double brin doté d'une longue structure flexueuse sensible aux éléments physiques et à la dégradation par des produits chimiques ou des enzymes. Dans une cellule vivante, l'ADN est protégé par ses supports structurels, tels que la chromatine et des mécanismes de réparation actifs qui restaurent rapidement les nucléotides endommagés.

À la mort de la cellule, l'ADN, tout comme les autres composantes de la cellule, est rapidement dégradé par des enzymes. Cependant, il peut arriver qu'après la mort de la cellule, l'ADN soit protégé contre une dégradation rapide dans un environnement qui limite l'activité enzymatique et supporte la structure flexueuse des molécules. La dégradation de l'ADN lors de la décomposition de la matière organique se produit de façon similaire pour les organismes classiques et transgéniques, à moins que les séquences d'ADN transgéniques n'aient été rendues plus résistantes à la dégradation par les nucléases.

L'ADN exposé est hydrolysé à des taux

soil, seawater, freshwater, wastewater and marine sediments, but can remain detectable at low levels for many weeks. In aquatic environments sediments are a more likely niche for natural transformation than water columns. DNA consumed by animals is rapidly degraded in the digestive system.

considérables dans le sol, l'eau de mer, l'eau douce, les eaux usées et les sédiments marins, mais il peut être détecté à de faibles concentrations pendant de nombreuses semaines. Dans les milieux aquatiques, les sédiments constituent une niche de transformation naturelle plus probable que les colonnes d'eau. L'ADN consommé par les animaux est rapidement dégradé dans l'appareil digestif.

Autoclaving, incineration and alkaline hydrolysis are effective measures for ensuring organic degradation of waste materials. The first two are commonly used to dispose of waste from novel organisms.

L'autoclavage, l'incinération et l'hydrolyse alcaline sont des mesures efficaces pour assurer la décomposition organique des matières résiduelles. Les deux premières méthodes sont couramment utilisées pour l'élimination des résidus d'organismes nouveaux.

Mechanisms of horizontal gene transfer

Horizontal gene transfer is the non-sexual transfer of genetic material between organisms belonging to the same or different species. It is a naturally occurring phenomenon that facilitates bacterial adaptation to a changing environment by expressing genetic information that has evolved in other cells. The three possible mechanisms of HGT are transformation, transduction or conjugation.

Mécanismes de transmission horizontale de gènes

La transmission horizontale de gènes est un transfert non sexuel de matériel génétique entre des organismes appartenant à la même espèce ou à une espèce différente. Ce phénomène naturel facilite l'adaptation bactérienne à un environnement changeant et permet l'expression d'information génétique ayant évolué à l'intérieur d'autres cellules. Les trois mécanismes de THG possibles sont la transformation, la transduction et la conjugaison.

The most likely mechanism of bacterial uptake and incorporation of free, extracellular DNA is via the natural transformation of competent bacteria with DNA released into the environment or digestive tract of humans or animals. In this process, a number of events must occur sequentially, the likelihood of which depend on the availability of the right kind of DNA (exposure), the type of bacteria and the ability of these bacteria to take up DNA (uptake) and be transformed by that DNA (stability), expression of incorporated sequences (expression), and the competitiveness of the transformed bacteria (selection). Each of these sequential events represents a potential barrier to successful HGT.

Le mécanisme de captation d'ADN par les bactéries et d'incorporation d'ADN extracellulaire libre le plus probable est la transformation naturelle de bactéries compétentes avec de l'ADN présent dans l'environnement ou le tube digestif d'un humain ou d'un animal. Dans ce processus, un certain nombre d'événements doivent se produire de façon séquentielle. La probabilité de ces événements dépend de la disponibilité du bon type d'ADN (exposition), du type de bactéries, de la capacité de ces bactéries à incorporer l'ADN (incorporation) et à être transformées par cet ADN (stabilité), de l'expression des séquences incorporées (expression) et de la compétitivité des bactéries transformées (sélection). Chacun de ces événements séquentiels représente une barrière potentielle à la THG.

The existing scientific data, while not excluding the possibility of natural transformation of bacteria, do indicate that, if it occurs, it is a very low frequency event. The chance of acquiring the same genes from another bacterial species in the environment is much greater. From a risk assessment perspective, it is the combination of transfer frequency and selective advantage conferred to the successful transformant that is significant, which must be evaluated on a case-by-case basis.

Les données scientifiques existantes n'excluent pas la possibilité d'une transformation naturelle des bactéries, mais indiquent qu'il s'agit d'un événement très peu fréquent. La possibilité d'acquérir les mêmes gènes d'une autre espèce bactérienne dans l'environnement est beaucoup plus grande. Du point de vue de l'évaluation des risques, ce sont la fréquence de transfert et l'avantage sélectif conféré au transformant qui sont importants et qui doivent être évalués au cas par cas.

Approaches to producing transgenic aquatic organisms

Fish are generally considered to be more amenable than other vertebrate species to transgenic modification as females produce an abundance of eggs which are easily manipulated, and embryos develop outside of the mother. Methods for gene transfer include microinjection, electroporation, transposon-, retrovirus-, sperm- and liposome-mediated transfer, particle bombardment, stem cell transfection and transient gene expression.

The NRC Committee on Defining Science-based Concerns Associated with Products of Animal Biotechnology specifically identified transposons and viral vectors as being of particular concern with respect to the potential for HGT of the novel gene. The Committee members speculated that HGT via transposition among highly diverse hosts could be possible if these sequences were mobilized by the constructs used to transfer *mariner*-like elements into the germline, where their insertion into genes could result in unexpected genetic damage. It was suggested that this potential avenue to HGT could be minimized or eliminated by expressing the transposase in the *trans* configuration and deleting the gene for these enzymes from the transgene construct, so that once inserted into the host genome the element is immobilized.

Considerations for risk assessment and risk management

Scientific risk assessment is the cornerstone of biotechnology regulatory systems and public policy decisions related to the safety and acceptability of genetically modified organisms (GMOs). Even in countries that have incorporated structures and mechanisms for including non-safety (i.e., socioeconomic) issues in the decision-making process, a strong scientific capacity and knowledge base is viewed as key to identifying hazards, and assessing their impacts and likelihood. The focus of risk assessment should be on asking empirical questions about probabilistic or hypothetical (possible) risks, not speculative (scientifically indefensible) risks.

The Office of Laboratory Security (OLS) of the

Méthodes de production d'organismes aquatiques transgéniques

Les poissons sont généralement considérés comme des organismes qui se prêtent mieux aux modifications transgéniques que les espèces vertébrées, car la femelle produit beaucoup d'œufs, lesquels peuvent être manipulés facilement, et parce que les embryons se développent à l'extérieur de la mère. Les méthodes de transfert génétique comprennent la micro-injection, l'électroporation, le transfert au moyen de transposons, de rétrovirus, de spermatozoïdes et de liposomes, le bombardement de particules, la transfection de cellules souches et l'expression de gènes transitoires.

Le comité du CNRC qui travaille à définir les préoccupations scientifiquement fondées associées aux produits de biotechnologie animale a identifié de façon spécifique les transposons et les vecteurs viraux comme des éléments de préoccupation en ce qui a trait au potentiel de THG des gènes nouveaux. Les membres du comité ont convenu que la THG par transposition chez des hôtes très diversifiés était possible si les séquences géniques étaient mobilisées par les gènes hybrides utilisés pour transférer des éléments de type *mariner* dans une cellule germinale, où leur insertion dans des gènes pourrait entraîner des dommages génétiques imprévus. On a fait observer que cette avenue possible de la THG pourrait être minimisée, voire éliminée en exprimant la transposase dans la *trans*configuration et en supprimant le gène codant les enzymes du transgène, de manière à immobiliser l'élément une fois qu'il est inséré dans le génome hôte.

Aspects à considérer dans l'évaluation et la gestion des risques

L'évaluation scientifique des risques est la pierre angulaire des systèmes de réglementation de la biotechnologie et des décisions de politique générale sur la sécurité et l'acceptabilité des organismes génétiquement modifiés. Même dans les pays ayant implanté des structures et des mécanismes visant à inclure des questions non liées à la sécurité (c.-à-d. socio-économiques) dans le processus décisionnel, une grande capacité scientifique et des connaissances scientifiques solides constituent la clé pour l'identification des risques, l'évaluation de leurs impacts et l'estimation de leur probabilité. L'évaluation des risques doit s'appuyer sur des questions empiriques concernant les risques probables et hypothétiques (possibles) et non les risques spéculatifs (scientifiquement indéfendables).

Le Bureau de la sécurité des laboratoires (BSL) de

Public Health Agency of Canada provides guidance to researchers about the use, including decontamination and disposition, of biohazardous material in laboratory settings through its Laboratory Safety Guidelines. While the Guidelines emphasize biosafety as related to human pathogens, they are used as a source of guidance for public and private sector laboratories undertaking any research that requires biological containment. The OLS Guidelines provide a classification structure for determining the level of risk associated with biological agents. Risk is assigned according to the potential for harm to human, environmental and animal health with the emphasis placed on human health protection. Bearing in mind that HGT is very rare and more likely to occur between prokaryotes than between eukaryotes or eukaryotes and prokaryotes, the containment and disposal measures for human pathogens are likely to be sufficient for aquatic organisms with novel traits.

l'agence de santé publique du Canada fournit des conseils aux chercheurs quant à l'utilisation de matériel biologique dangereux, notamment la décontamination et l'élimination de ce type de matériel, par le biais de directives sur la sécurité en laboratoire. Bien que ces directives mettent l'accent sur la biosécurité par rapport aux agents pathogènes pour les humains, ces directives sont utilisées à titre de référence dans les laboratoires des secteurs public et privé qui effectuent des recherches pour lesquelles un confinement biologique est nécessaire. Les directives du BSL offrent une structure de classification permettant de déterminer le niveau de risque associé aux agents biologiques. Le risque varie en fonction du danger potentiel pour la santé humaine, animale et environnementale, l'accent portant sur la protection de la santé humaine. En gardant à l'esprit que la THG est très rare et plus susceptible de se produire entre des procaryotes qu'entre des eucaryotes ou entre des eucaryotes et des procaryotes, il est probable que les mesures de confinement et d'élimination des agents pathogènes pour les humains soient également suffisantes en ce qui a trait aux organismes aquatiques à caractères nouveaux.

Considerations for the Expert Panel

The members of the Expert Panel are requested to:

- evaluate, comment on and supplement the information provided in this document,
- provide insight into the possible nature, magnitude and source of risk, if any, to the environment or human health that may be presented by the HGT of free DNA or DNA associated with waste from novel aquatic organisms including somatic cells in waste effluent,
- consider possible frameworks for categorizing hazards associated with transgenes,
- address the circumstances under which such hazards may result in significant risk to the environment or human health, and
- provide science-based regulatory options to contain and mitigate risks related to HGT of DNA from waste biomass from novel aquatic organisms and effluent.

Mandat du groupe d'experts

Les membres du groupe d'experts ont pour mandat :

- D'évaluer, de commenter et de compléter l'information présentée dans ce document.
- De fournir un aperçu de la nature, de l'ampleur et de la source possibles des risques pour l'environnement et la santé humaine, que peut présenter la THG d'ADN libre ou d'ADN associé à des résidus d'organismes aquatiques à caractères nouveaux, notamment les cellules somatiques présentes dans l'effluent de déchets.
- De tenter d'établir un cadre de classification des risques liés aux transgènes.
- D'examiner les circonstances dans lesquelles de tels risques seraient considérables pour l'environnement et la santé humaine.
- De fournir des options de réglementation scientifiquement fondées visant à contenir et à atténuer les risques liés au THG d'ADN provenant de la biomasse résiduelle d'organismes aquatiques à caractères nouveaux et d'effluents.

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REVIEW OF POTENTIAL RISKS ASSOCIATED WITH HORIZONTAL GENE TRANSFER FROM NOVEL AQUATIC ORGANISMS

Draft discussion document

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1. INTRODUCTION

In Canada, biotechnology is defined under the federal framework for regulating biotechnology products as "the application of science and engineering in the direct or indirect use of living organisms or parts or products of living organisms in their natural or modified forms." This broad definition covers all organisms, their parts and products. Both traditionally developed products and those developed through techniques such as genetic engineering are included.

Canada's regulatory framework was established through agreement among federal regulatory bodies and was announced in 1993. The need for an investment in this regulatory strategy to meet new challenges was recognized when the Canadian Biotechnology Strategy was renewed in 1998. The principles of this strategy, which are still in place, include: reflecting Canadian values; engaging Canadians in open, ongoing, dialogue; promoting sustainable development, competitiveness, public health, scientific excellence, and an innovative economy; and ensuring responsible action and cooperation domestically and internationally. These principles established that the practical benefits of biotechnology products and processes would be balanced with the need to protect health, safety, and the environment.

Aside from the approach taken by U.S. Food and Drug Administration towards bioengineered foods, Canada is the only country where regulatory oversight is triggered solely by the novelty of traits expressed by the host organism or the novel attributes of foods or food ingredients, irrespective of the means by which the novel traits were introduced. This "product-based" approach to regulation has been validated by numerous scientific bodies and expert consultations. Because the scope of Canada's regulatory approach is broader than just genetically engineered organisms, Canadian regulators have adopted unique terminology and definitions. For example, Fisheries and Oceans Canada refers to aquatic organisms with novel traits.

Under Canada's biotechnology regulatory regime, all organisms and food products, whether they are produced using conventional technologies or biotechnologies, are governed under the same acts. Depending on the type of product, the relevant piece of legislation is the Seeds Act, Feeds Act, Fertilizers Act, Plant Protection Act, Food and Drugs Act, Health of Animals Act, or the Canadian Environmental Protection Act (CEPA).

The importation or manufacture of novel aquatic organisms in Canada is currently regulated under the *New Substances Notification Regulations* (NSNR; CEPA 1999). Fisheries and Oceans Canada is responsible for conducting risk assessments on novel aquatic organisms with respect to potential adverse effects on the environment or human health. Section 29.16 of the NSNR provide for exemption from regulatory oversight of organisms other than micro-organisms where the organism is used in research and development and where it is "imported into" or "manufactured in" a contained facility from which there is no release into the environment of: a) the organism; b) the genetic material of the organism; or c) material from the organism involved in toxicity. A specific concern with respect to the release of genetic material into the environment is related to potential risks associated with horizontal gene transfer (*i.e.*, non-sexual transfer) of genetic material from the carcasses of novel aquatic organisms, their somatic cells or other biological waste that contains DNA to microorganisms or eukaryotic organisms in the environment.

The regulatory exemption criteria specified in section 29.16 of the NSNR are currently being reviewed by Environment Canada and Health Canada. In addition, Fisheries and Oceans Canada is considering the development of novel aquatic organism-specific regulations for the CEPA-equivalent regulatory oversight of novel aquatic organisms *in lieu* of the NSNR regime.

In order to provide information and knowledge to inform both of these regulatory developments, Fisheries and Oceans Canada has undertaken a review of the current state of knowledge regarding the potential for, and risks associated with, horizontal gene transfer of DNA from eukaryotic organisms, including novel aquatic organisms and their associated biological waste. This review will be considered by an expert panel that Fisheries and Oceans Canada will convene to evaluate, comment on and supplement the information provided, and to provide science-based regulatory options to contain and mitigate risks related to the horizontal gene transfer of DNA from waste biomass from novel aquatic organisms . The knowledge gained from the expert panel and the scientific review will be used to inform and design science-based regulatory approaches to containment and disposal of novel aquatic organism carcasses, including somatic cells in effluents and other biological waste that contains DNA.

This review has been broadly divided along four thematic areas dealing with: persistence of DNA in the natural environment; mechanisms of horizontal gene transfer; approaches to producing transgenic aquatic organisms; and

considerations for risk assessment and risk management.

2. METHODOLOGY

A comprehensive search of the scientific literature for information regarding the potential for horizontal gene transfer (HGT) from eukaryotic plants and animals, including novel aquatic organisms, was conducted. Utilizing existing primary and secondary information, a bibliographic search of the following databases was undertaken:

Academic databases

- PubMed
- Ingenta
- Agricola
- CABI
- Aquatic Sciences and Fisheries Abstracts

Biotechnology-specific databases

- AGBIOS' Bibliography Database
- AgSymbion
- Biosafety Clearing-House
- ICGEB's Biosafety Bibliographic Database

Additionally, relevant publications prepared by regulatory agencies (*e.g.*, EFSA, DEFRA, OGTR, EPA, USDA-APHIS); science councils and national academies of science (*e.g.* USNAS, UK Royal Society), international organizations with a biotechnology/biosafety mandate (*e.g.*, FAO, UNEP, IUCN), and non-governmental (*e.g.*, Pew Initiative on Food and Biotechnology) and private sector companies/organizations (*e.g.*, EuropaBio, BIO) were reviewed.

A database of citations (*e.g.*, EndNote) and available documents in electronic (Adobe Acrobat PDF) or printed form was prepared.

Based on the scientific literature review, a discussion document outlining key issues, questions and points of consideration relevant to the potential risks associated with HGT and regulatory options for containment and disposal of waste from novel aquatic organisms was prepared for deliberation by the Fisheries and Oceans Canada.

3. PERSISTENCE OF DNA IN THE NATURAL ENVIRONMENT

3.1. INTRODUCTION

Deoxyribonucleic acid (DNA) is the more stable of the two commonly occurring nucleic acids, DNA and RNA. It forms the genetic material of most living organisms and is present in most living cells. Within cells, DNA is found as a single circular chromosome in prokaryotes or as a cluster of chromosomes in eukaryotes. In eukaryotic cells DNA also is found in plastids such as mitochondria and chloroplasts. DNA is a double stranded polymer of nucleotides with two covalently linked sugar phosphate backbones and hydrogen bonds between corresponding nucleotides on each strand. This gives DNA a long, flexuous structure that is vulnerable to physical forces and active degradation by chemicals or enzymes.

In the living cell DNA is protected by structural supports such as chromatin and active repair mechanisms that quickly fix damaged nucleotides. At cell death, DNA, along with the other components of the cell, is quickly degraded by enzymatic activity. However, instances have occurred after cell death where DNA is protected from rapid degradation by an environment that limits enzymatic activity and supports the flexuous structure of the molecules. For example, antiquated pollen grains and seeds are frequently identified in archaeological digs. Some

of these have been able to provide fragments of DNA for analysis. In addition, ancient remains of hard animal tissues like bones and teeth have protected small fragments of DNA over thousands of years (Powledge and Rose, 1996). These have been isolated and analysed to reveal short sections of nucleic acid sequence. However, this ancient DNA has not been suitable for cloning, as it is occurs in very small quantities and is highly degraded.

Davison (2004) stated that viral, microbial, plant, animal and human DNA are common and "rather stable" in the environment. When dead microbial, plant and animal tissues degrade to their molecular building blocks there are instances where fragments of DNA might survive the degradation process inside dead cells or as naked molecules in the environment. DNA is not toxic or allergenic (Jonas *et al.*, 2001); it is consumed in gram quantities on a daily basis and quickly degrades in the digestive system of animals. In assessing the risk that un-degraded DNA fragments pose to the environment it is important to assess what the likelihood is that this DNA will remain intact, how available it is to other living organisms and, most importantly, what consequences might result should the DNA be taken up and provide new traits in the recipient organisms. This section reviews the first two points: the likelihood that extracellular DNA will remain intact in the environment in a form amenable to transformation and subsequent expression, and the availability of naked DNA for uptake by living organisms.

3.2. DEGRADATION OF DNA

Free DNA is hydrolyzed at substantial rates in soil, seawater, freshwater, wastewater and marine sediments, but can remain detectable at low levels for many weeks. This hydrolysis is due to the ubiquitous presence of free or cell-bound DNases, which are produced by microorganisms that use free DNA as a growth substrate. The DNase-producing microorganisms can account for up to 90 percent of the heterotrophic bacteria isolated from soils and water systems. There is a positive correlation between increasing viable counts of microorganisms and increasing levels of DNase activity. Studies with selective antimicrobial compounds have shown that the DNase-producers are prokaryotes (Dröge *et al.*, 1999). Studies with seeded DNA have shown that the rates of degradation vary considerable between different habitats, but are generally higher in aquatic than terrestrial environments. In addition, the availability of adsorbed DNA for transformation appears to vary depending on the substrate, concentrations of DNA, the environmental conditions and the competence of the host (Dröge *et al.*, 1999).

3.3. EXTRACELLULAR DNA IN SOIL

Extracellular DNA in soil results from passive release by dead microbial, plant and animal cells or active release by living cells. Different abiotic and biotic factors seem to affect the persistence of free DNA in soil (Table 1; Ceccherini *et al.*, 2003). The content and type of clay minerals influence the extent to which free DNA is adsorbed to mineral surfaces and protected from degradation by nucleases (Ogram *et al*., 1988; Romanowski *et al*., 1991; Paget *et al*., 1992; Khanna and Stotzky, 1992; Paget and Simonet, 1994; Gallori *et al*., 1994; Poly *et al*., 2000). Other abiotic factors influencing the adsorption of nucleic acids to soil particles are pH and the availability of bivalent ions. Demaneche *et al.* (2001) were able to confirm that adsorption of nucleases to clay minerals protects adsorbed DNA by restricting the availability of the enzymes to access and degrade DNA. They also demonstrated that a small portion of adsorbed DNA in the studied soils was biologically available for bacterial transformation. Stotsky (2000) confirmed that soil-bound DNA could be used to transform bacteria whether the DNA was bound to clay, humic acid or clay-humic complexes. He suggested that one end of a strand of DNA can bind to clay in soil, leaving the other end free to interact with bacterial cell walls to initiate natural transformation.

It has recently been shown that DNA is also well protected in dead cells in soil and that this DNA is still available for transformation (Nielsen *et al*., 2000b). Nielsen *et al*. (2000b) could show that cell lysates of *Pseudomonas fluorescens*, *Burkholderia cepacia* and *Acinetobacter* spp. were available as a source of transforming DNA for *Acinetobacter* sp. populations in sterile and nonsterile soil for a few days and that cell debris protects DNA from inactivation in soil. Cell walls could play an important role in protecting DNA after cell death (Paget and Simonet, 1997). Contact between dead or intact donor and competent recipient cell membranes might facilitate binding and take-up of the DNA (Paul *et al*., 1992; Paget and Simonet, 1997). Degand *et al.* (2002) showed that DNA from conventional and transgenic crops was detectable in soil 25 to 50 days after harvest, with no difference in DNA persistence between the conventional and transgenic crops of the same species. The range in persistence over time related to the varying degradation rates of plant residue from the different crops studied.

Studies on the persistence of free DNA in soil have often been performed in rather artificial soil model systems such as sand or sterile soil. Only recently have reports on the persistence of DNA in nonsterile soil been published (Widmer *et al*., 1996; Nielsen *et al*., 1997; Blum *et al*., 1997; Gebhard and Smalla, 1999). Microbial

activity was pinpointed as an important biotic factor affecting the persistence of free DNA in soil (Blum *et al*., 1997). A more rapid decrease of free DNA was observed at higher soil humidity and temperature, both of which would be expected to contribute to a higher microbial activity, and thus higher DNase activity, in soil (Widmer *et al*., 1996; Blum *et al*., 1997). In investigating long-term persistence of bacterial DNA, Willerslev *et al.* (2004) confirmed that cold conditions are critical to long-term DNA survival. Under sub-zero conditions small fragments (120 to 600 bp) of bacterial DNA that were thousands of years old were isolated from permafrost. These data suggest that DNA persistence in temperate climates may be extended by cold winter temperatures.

Table 1: Abiotic and biotic factors that influence the persistence of DNA in the soil[1]

Abiotic	Biotic
Content and type of clay minerals	Microbial activity
Temperature	- DNase activity
Osmotic potential (affects cell lysis)	Concentration of DNA
Water activity	Length of DNA fragments
Presence of anti-microbial compounds	Presence of mobilizable elements
Nutrient levels (C, N, P)[2]	
Optimum salts for cell competence (e.g. Fe, Ca, Mo)	
pH	

1. Adapted from Gebhard and Smalla, (1999) and Singh (2002).
2. Although Paul *et al*., (1989) found that DNA degradation rates were independent of trophic status in sub-tropical environments, nutrient levels influence microbial activity, which, in turn, influences levels of DNase activity.

Table 2: Half-life of DNA in various environments[1]

Environment	Half-life (h)
Wastewater	0.017 – 0.23
Freshwater	4.2 – 5.5
Estuarine	3.4 – 5.5
Ocean surface	4.5 – 83.0
Marine sediment	140 – 235
Soil	9.1 – 28.2

1. Adapted from Lorenz and Wackernagel (1994).

Given this complex combination of abiotic and biotic factors there appears to be no general rate of DNA degradation in soils. However, it seems clear that extracellular DNA in soil is quickly degraded within a matter of days (Table 2), while DNA that becomes bound to clay particles remains physically protected from nuclease degradation for longer periods

(Demaneche *et al.*, 2001) and DNA that is taken up by soil bacteria, may persist indefinitely in the soil system under conditions of positive selection for genes provided by the DNA fragment (Gebhard and Smalla, 1999). This is emphasized by the UK Advisory Committee on Releases to the Environment (ACRE) in their review of Gebhard and Smalla's 1999 paper where they state *"ACRE's view on horizontal gene transfer is that there will always be the opportunity for DNA to be taken up by bacteria in the soil and it is not possible to say that integration will not happen at very low frequency. What is also clear however, is that unless there is very strong selection for the gene that is transferred, it will remain at a low frequency in the population at large. Selection pressure is key."* (ACRE, 2000).

3.4. EXTRACELLULAR DNA IN LEACHATE WATER

Recent laboratory studies with transgenic soybean and corn demonstrated that DNA was released from plants into soil, most probably from growing roots and during decomposition, and subsequently washed away by water (Gulden *et al*., 2005). The half life of the DNA was affected by the level of microbial activity in the soil and by temperature; higher microbial activity and higher temperatures both increased the rate at which plant DNA was degraded in leachate. Microbial activity was believed to increase the concentration of DNase in the water and temperature increased the growth rate of microbes and the activity of enzymes.

3.5. EXTRACELLULAR DNA IN AQUATIC ENVIRONMENTS

Data from the 1980s and early 1990s give estimations of extracellular DNA in aquatic environments (Table 3). More recent studies of aquatic sediments using improved sampling methods showed higher concentrations of DNA. The DNA in these sediments was mostly extracellular and bound to inorganic and organic particles that protected against degradation (Corinaldesi *et al.*, 2005). It is believed that the residence time of DNA in the sediments was considerably longer than that measured in a water column and that potential for horizontal gene transfer may be greater in sediments where microbial populations flourish. The studies of Corinaldesi *et al*. (2005) concluded that extracellular DNA fragments in the sediments were long enough (up to 10 kb) to contain viable gene sequences but that bound DNA was probably less available for uptake than free (unbound) DNA, which constitutes about 5 percent of total extracellular DNA in aquatic sediments. Stewart and Sinigalliano (1990) found that marine sediments facilitate the uptake and expression of exogenous DNA by transformable marine bacteria. These sediments are a more likely niche for natural transformation than water columns in marine environments.

Table 3: Reported incidence of extracellular DNA in freshwater and marine environments[1]

Habitat	Concentration of extracellular DNA ($\mu g\ l^{-1}$)	Sample source
Freshwater		
Oligotrophic	1.43 ± 1.1	Crystal River Springs, Florida
	1.74	
Eutrophic	12.1 ± 1.2	Medard reservoir, Florida
	11.9 ± 8.9	
	6.97	
Swamp	7.8	Boyd Hill Nature Park, Florida
Sediment	1.0 $\mu g\ g^{-1}$	Fort Loudon, Tennessee
Marine water		
Estuarine	9.4 – 11.6	Bayboro, Florida
	6 – 44	
Coastal	2.0 – 7.0	Gulf of Trieste
	5.0 – 15.0	Gulf of Mexico
Offshore	17.1 ± 12.7	Baltic Sea
	4.6	Gulf of Mexico
	0.5 – 5.0	Gulf of Mexico
Deep Sea (500-1500m)	0.2 – 0.5	Gulf of Mexico

1. Adapted from Dröge *et al*., (1999)

In his review, Singh (2002) summarizes the half life of free DNA in the environment to range from 1 minute to 235 hours in aquatic environments and 9 minutes to 28 hours in terrestrial environments. Recent field studies in forest aquatic microcosms detected the presence of spiked baculovirus DNA for up to 24 hours (England *et al.*, 2005). The researchers believed the DNA may have persisted for a longer period but were unable to detect it at concentrations below the 13.5 pg detection limit of the polymerase chain reaction (PCR) amplification system.

3.6. FATE OF DNA IN DECOMPOSITION, COMPOSTING AND INCINERATION

Studies by Ceccherini *et al.* (2003) indicated that while most of the DNA in decomposing plants was degraded inside plant cells, sufficient DNA persisted to be released into the soil. During plant senescence active nucleases degrade DNA and this continues during subsequent decay because the nucleases remain active after plant cell death. Additionally, plant DNA is degraded by nucleases from microorganisms that contribute to the decomposition of plant material. Fragments of DNA that enter the soil during decay are available in the interface between the decaying material and the soil; a biologically active area that provides a potential hot spot for horizontal gene transfer (Ceccherini *et al.*, 2003). Degradation of DNA during decay would occur equally for conventional and transgenic plants unless the transgenic plants contained DNA sequences that were more resistant to nuclease degradation.

Studies of leaf decay in transgenic poplar field trial sites have monitored the persistence of tree DNA in the decomposing leaf material suspended above the soil, on the soil and below the soil (Hay *et al.*, 2002). The authors reported that a 742 bp fragment of the *nptII* marker gene could not be detected as early as four months after exposing poplar leaves to external environmental conditions but that smaller *nptII* PCR products were visible in

samples taken one month after that. Degradation of DNA in leaf material suspended above the ground was lowest and this was attributed to lower moisture and lower microbial activity. Hay *et al*. (2002) concluded that the rapid degradation of plant DNA makes transgenic plant waste an unlikely source of DNA for horizontal gene transfer.

In composting studies, Guan *et al.* (2005) have shown that degradation of shredded transgenic corn material is rapid. They detected transgenic DNA only on days 0 and 7, but not on day 14 or at any sampling time after that for the 12 month duration of their study. Degradation of composted corn seed was assayed only once, but after 12 months no transgenic DNA was detected. The composting procedure provided elevated microbial counts and high temperatures (40 to 60ºC). The former may have accelerated DNase production and the latter may have accelerated enzyme activity in and around the plant material. Guan *et al*. (2005) proposed composting as an efficient way to dispose of transgenic plant material to eliminate the possibility of DNA persistence in the environment.

Alkaline hydrolysis has been used as an alternative to incineration or autoclaving to decontaminate animal carcasses and biological waste. In this process alkaline solutions of metal hydroxides and heat are combined to reduce biological molecules to their building blocks by hydrolyzing the bonds between molecules (Kaye *et al.*, 2004). During this treatment the phosphodiester bonds of nucleic acids are rapidly hydrolyzed, completely destroying RNA and DNA in the materials. As such, waste management of material derived from transgenic organisms with this system is not likely to release small DNA fragments into the environment. Thus, alkaline hydrolysis may be an option for reducing high-risk transgenic waste to low-risk where potential consequences of horizontal gene transfer are unacceptable.

Incineration, commonly used in biological waste treatment, is generally at temperatures well over 250ºC where DNA has been postulated to have a half life of 3.5 milliseconds (White, 1984). This very short time frame has made experimental confirmation difficult, but all detectable DNA disappeared quickly under these conditions. Assuming that incineration is complete and no organic material is left un-degraded, incineration offers a proven option for treatment of high-risk biological waste.

3.7. FATE OF DNA IN DIGESTIVE SYSTEMS

DNA stability in the gut of animals and humans directly influences the ability of microflora or animal cells to take up naked DNA. Data from the EU's GMOBILITY studies (2002) indicated that naked and GM-potato DNA were rapidly digested in animal rumen models. Under these conditions free plasmid DNA lost its capability to transform *Escherichia coli* with an observed half-life period of about one minute. DNA present in ground transgenic potato material was degraded to below the limit of PCR detection within 2 h. The GMOBILITY studies showed that rumen conditions destroyed the transformation potential of plasmid DNA.

Alexander *et al.* (2002) studied the fate of endogenous and recombinant canola DNA after incubation in rumen fluid. The authors reported that plant DNA was not detected in the supernatant, but only in pellets containing plant debris, suggesting that that the presence of plant DNA in ruminant systems is directly related to intact plant cells. The authors equated the disappearance of plant DNA fragments after *in vitro* incubation in rumen fluid with the digestion of plant cells in the rumen, proposing this as the limiting step for transformation of rumen bacteria because once DNA is released into the ruminal environment it is degraded almost immediately.

Studies with mice have shown that ingested bacteriophage M13 DNA can persist long enough to cross the intestinal epithelium and reach leukocytes, spleen and liver cells and the foetus of the host (Schubbert, *et al.*, 1997; Schubbert *et al.*, 1998). However, the apparently high rate of uptake and persistence of DNA in leukocytes and even in foetal tissues may be related to the un-methylated CpG sequences in the *E. coli* DNA used (Beever and Kemp, 2000). Small fragments of plant DNA have been found in mice, chickens and cattle (Hohlweg and Doerfler, 2001; Einspanier *et al.*, 2001). Van den Eede *et al.* (2004), in their review of the safety of food and feed from transgenic plants, concluded that whereas uptake of ingested DNA by mammalian somatic cells has been demonstrated, there is so far no evidence that such DNA may end up in germ line cells as a consequence of the consumption of food.

In human digestion, the initial breakdown of foods occurs in the acidic environment of the stomach. This is followed by further digestion in the small intestine. Here a slightly alkaline environment aided by digestive enzymes enables alkaline hydrolysis of organic material. In this process complex molecules are broken down into their building blocks by the insertion of water ions between atoms of covalently bonded molecules (Kaye *et al.*, 2004). Action by pancreatic nucleases in the intestine cleaves nucleic acids into nucleotides and these are cleaved into nucleosides and phosphoric acid by enzymes found on the luminal surfaces of the mucosal cells (Jonas *et al.*,

2001). This effectively destroys polymers such as DNA, although some DNA may persist to excretion, probably through protection from digestion in undigested materials or by adsorption to inorganic matter.

In a comprehensive review of the safety of DNA in foods, Jonas *et al.*, (2001) concluded that the breakdown of DNA during food processing and passage through the gastrointestinal tract reduced the likelihood that intact genes capable of encoding foreign proteins will be transferred to gut microflora. They indicated that the likelihood of transfer and functional integration of DNA from ingested food by gut microflora or human cells is minimal.

3.8. FATE OF DNA IN FOOD AND FEED PROCESSING

The integrity of DNA in food or during processing is affected by a number of factors, including temperature, degradative enzymes, pH, shear forces, water activity and presence of reactive or protective substances (Table 4). In a study of the stability of DNA in food it was found that DNA in purchased soy products remained detectable over a long period of time, with arginine, polyamines, and biogenic amines (positively charged molecules) exerting a protective effect on DNA against nuclease attack (GMOBILITY, 2002). During food processing conditions, *e.g.*, heat (85°C) and/or low pH (pH 4), maize DNA and plasmid DNA were rapidly degraded. Alkaline processing of instant maize products degraded the maize DNA to fragments of less than 500 bp. Heat treatment (100°C, 30 to 60 min) of soymilk products decreased the concentration of DNA, but fragments up to 1.7 kb in length remained detectable for up to 30 min of processing. The production of chips, flakes and dried potato sticks from the transgenic potato lead to degradation of the plant DNA. In addition, adsorption of bacterial DNA to the food matrix decreased DNA availability for microbial transformation within the processed food.

Table 4: Factors affecting the integrity of DNA molecules in the environment and food[1]

Factor (effect)	Importance in food	Site of action on DNA
Shear forces	Variable between minimal to high during processing of foods	Sugar-phosphate backbone (double-strand breaks)
Enzymes	*e.g.* DNases; potentially high in foods originating from tissues of plant or animal origin or subjected to microbial activities	Sugar-phosphate backbone, nucleobases (modification, hydrolysis)
pH	Most foods are in the neutral pH or weak acid range, then weak effects; during processing pH may vary between 1 to ~12, then strong effects	Base-sugar bonds at low pH (hydrolysis) with secondary effects on sugar-phosphate backbone (strand breaks), bases (deamination)
Temperature	When foods are subjected to thermal processes (>80ºC), then strong effects	Denaturation of DNA, various hydrolysis and deamination reactions, Q_{10}=2–3
Water activity	Low in dried foods; high at high water activity	All reactions leading to damage
Chemical agents (*e.g.*, reactive oxygen species, bisulfite, nitrite, alkylating agents)	Partially high when SO2 is used as additive, curing agents are employed or when stored under air	Nucleobases (chemical modification, loss of bases)
Protective interactions	In certain food matrices highly important; variable influences by adsorption of DNA to particles, inclusion into compartments, or interaction with polyamines, cations etc.	Protection against various damaging factors including shear forces, enzymes, heat etc.

1. Reproduced from van den Eede *et al*. (2004)

In his review, Singh (2002) reported on the degradation of DNA during specific food processing steps: during steeping, wet milling and processing of maize, DNA was detected in maize kernels throughout the steep process, but was not found in steep water. After wet milling DNA was found in starch, germ, coarse fibre, wet gluten fractions but not in the fine fibre fraction. When dried by heating at 135°C for 2 hours, DNA was not detected in the gluten fraction and dehydrated kernels nor was DNA detected in feed pellets, starch, dextrose, sorbitol or high-fructose maize syrup derived from the wet-milled substrate. DNA from transgenic starter cultures in sausage fermentation was not detectable after 9 weeks of incubation. In sugar beet processing, nucleases were shown to be active during the first extraction step, carried out at 70°C. DNA degradation continued in raw juice and through alkaline hydrolysis. DNA was removed also by irreversible adsorption to sludge, precipitation and exclusion in the final crystallisation steps so that no DNA is detectable in refined sugar.

Feed preparation through the silage process provides a harsh environment for plant DNA. Chopping disrupts cell walls and membranes, which exposes plant DNA to endogenous plant and microbial nucleases. The pH drop as a result of lactic acid fermentation accelerates DNA degradation. As such, detection of DNA fragments up to 1914 bp was possible for up to 5 days only after initiating the silage process, however smaller fragments of 226 bp were

detectable after 100 days of the silage process (Jonas *et al.*, 2001). These authors concluded that the presence of intact, functional plant genes in maize after an extended time of ensilage is highly unlikely. In dry feed processing the plant material is subjected to dehydration, shearing forces and temperature changes that also enhance DNA degradation. Heat treatment of maize flour resulted in a rapid decrease in detectable plant DNA (Jonas *et al.*, 2001)

Kharazmi *et al.*, (2003) investigated the degradation of DNA when soymilk, tofu, corn masa, and cooked potato were processed from transgenic raw materials. The major degrading factors for soymilk and tofu were the mechanical treatment of soaked soybeans and for corn masa and cooked potatoes, the thermal treatment. After processing, no DNA fragments greater than 1.1 kb were detected in any of the foods. Marker rescue studies were undertaken by transformation of *B. subtilis* LTH 5466, which has a deleted neomycin phosphotransferase (*nptII*) gene, with fragments of DNA isolated from cooked potato and with linearized plasmid, both containing a complete *nptII* gene. These data indicated that the length of DNA, the amount of homologous sequence and the concentration of DNA fragments isolated from cooked potato all affected the efficiency of marker rescue. Based on the data, the hypothetical frequency of transformation of transgenic DNA from cooked potatoes to *B. subtilis* was calculated to be 8.5×10^{-19} and 1.2×10^{-27} for homologous and illegitimate recombination, respectively. These data were extrapolated to roughly estimate the time after which a person (108 years) or the world population (15 days) is exposed to one transformant generated by homologous recombination, when the daily consumption per person is 130 g of cooked potatoes (Kharazmi *et al.*, 2003).

Salts are known to protect DNA against thermodegradation. Marguet and Forterre (1997) showed the 2M KCl protected 60 percent of double-stranded plasmid DNA from degradation for over 1 hour at 107ºC. The addition of mM amounts of KCl or MgCl2 significantly inhibited depurination of plasmid DNA held at 75ºC for 10 minutes and longer. In the absence of the salts, the plasmid DNA degraded in 10 minutes at 75ºC. When depurinated DNA was transferred to 95ºC for 30 minutes, KCl or MgCl2 provided little protection against cleavage of the depurinated plasmid DNA. Their studies indicate that the salts directly protect the N-glycosidic bonds of the plasmid DNA against depurination, rather than increase the stability of the double helix, as had been previously proposed. These data suggest that salt concentrations in some environments, such as food industry waste, may increase the stability of extracellular DNA.

3.9. FATE OF DNA IN AUTOCLAVES

It is well documented that the transforming activity of DNA is destroyed following autoclaving at 135ºC for 20 minutes, which is the standard sterilization cycle. However, Masters *et al.*, (1998) have shown that substantial protection against the loss of transforming ability during heating was provided by concentrations of NaCl. In the presence of 0.5 to 2.0 M NaCl the transforming capacity of plasmid DNA was not destroyed in a typical heat sterilisation treatment at 121ºC for 15 minutes in a pressure cooker, or in a media preparation cycle of autoclaving at 116ºC for 30 minutes. These data suggest that researchers need to be cognisant of the salt concentrations of waste material and to adjust the sterilization conditions to address the protective effects of salts on DNA degradation.

4. MECHANISMS OF HORIZONTAL GENE TRANSFER

4.1. INTRODUCTION

Horizontal gene transfer (HGT) is the non-sexual exchange of genetic material between organisms belonging to the same, or different species. It is a naturally occurring phenomenon that was first demonstrated to occur between bacteria (Veal *et al*. 1992; Wellington and van Elsas, 1992; Nielsen *et al*. 1998) and facilitates bacterial adaptation to a changing environment by expressing genetic information that has evolved in other cells. HGT is at the origin of biological diversity and the impact of HGT events will depend on a number of environmental factors, chief among these being the potential selective advantage conferred by newly acquired genes. And importantly, 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.

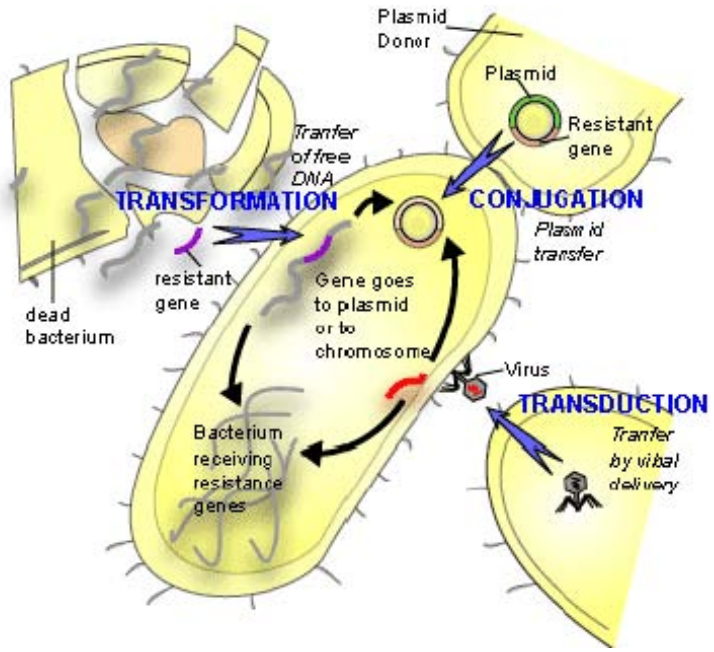

Figure 1. Mechanisms of horizontal gene transfer to and between prokaryotes. The three possible mechanisms of HGT are transformation, transduction or conjugation. Transformation is a process whereby free DNA is taken up by the bacteria from the external environment. Transduction occurs when bacteria-specific viruses or bacteriophages transfer DNA between two closely related bacteria, and conjugation occurs when there is direct cell-to-cell contact between two bacteria (which need not be closely related) and transfer of plasmid DNA (*Illustration courtesy of the University of British Columbia BioTeach Project*).

This section will focus on the evolutionary evidence for HGT and basic mechanisms of HGT, especially with regard to bacterial transformation, which is the only known way that prokaryotes can take up DNA from the environment. Once bacteria have been transformed with exogenous DNA, there are other mechanisms (*e.g.*, transduction and conjugation) that can efficiently transfer DNA to other bacteria (Figure 1). The involvement of mobile genetic elements (MGEs) —bacteriophages, plasmids and transposons— in the latter two processes is widely accepted (Thomas, 2000) and MGEs represent the main routes of gene transfer between bacteria (Wilkins, 1995). Direct transfer of DNA from the environment to eukaryotes, or from prokaryotes to eukaryotes, will also be discussed.

4.2. EVOLUTIONARY EVIDENCE FOR HGT

The importance of HGT in prokaryotic genome evolution has been largely inferred from phylogenetic analyses (*e.g.* Ponting *et al*., 2000; Lawrence and Ochman, 1998; Nelson *et al*., 1999; Syvanen and Kado, 1998; Jain *et al*., 1999; Lower *et al*., 1996), which have demonstrated for a number of genes that the sequence-based phylogenetic tree does not correspond to phylogenetic trees generated using other methods based on vertical gene transfer [*e.g.*, ribosomal DNA (rDNA) based trees] (Woese, 2000). Other studies have looked at codon usage patters for specific genes in comparison with codon usage patterns typical for a given phylum. For example, tetracycline antibiotic resistance genes often show nearly identical sequences across a range of hosts that are only distantly related based on rDNA sequence similarity and that show quite different percent G+C content (Barbosa *et al*., 1999). Considering that, on an evolutionary time scale, base composition of acquired genes would approach the base composition of the recipient organism, the implication is that HGT mechanisms are responsible.

Gene sequence comparisons between different species of bacteria (*e.g.*, *Escherichia coli* and *Neisseria*) have indicated that gene acquisition through HGT may account for up to 17 percent of the genome (Lawrence and Ochman, 1998; Lawrence, 1999; Ochman *et al*., 2000) and may also have occurred between Archaeabacteria and Bacteria (Nelson *et al*., 1999).

Naturally occurring HGT is not limited to bacteria. Fungal genes, transferred on group I introns, which are mobile self-splicing genetic elements found principally in organellar genomes and nuclear ribosomal RNA genes, have been identified as being widespread in many flowering plants (Cho *et al*., 1998). There is also strong evidence for the movement of genes on transposable elements between different species of insects (Robertson and Lampe,

1995), and between bacteria and insects (Hawtin *et al*., 1995).

The HGT of DNA from prokaryotes to eukaryotes via the transfer of plasmid DNA from *Agrobacterium* spp. to the plants they infect is well established and there is also evidence that tobacco plants acquired "hairy root" genes from *A. rhizogenes* (Aoki and Syno, 1999). *Agrobacterium tumefaciens* has also been demonstrated to transfer its T-DNA efficiently to the filamentous fungus *Aspergillus awamori* under laboratory conditions (de Groot *et al*., 1998; Gouka *et al*., 1999), with the implication that since *Agrobacterium* and many fungi are cohabitants in the soil, there may be natural routes of HGT between the bacterium and fungi.

Other than the early evolution of prokaryotic-derived organelles (*e.g.*, mitochondria, chloroplasts) within eukaryotes and the subsequent transfer of organellar genomes into the eukaryotic nucleus (Gray, 1999), there are no definitive data demonstrating the transfer of bacterial genes into mammalian germ line cells. An early analysis of the human genome (International Human Genome Sequencing Consortium, 2001) suggested evidence for over 200 bacterial genes being transferred from bacteria to humans (Lander *et al*., 2001), however, later re-analysis of the data did not support this conclusion (Salzberg *et al*., 2001; Roelofs and Haastert, 2001; Stanhope *et al*., 2001). While a few examples remain where the evolutionary history is unclear, other biological explanations, such as gene loss in non-vertebrates, are more likely.

4.3. HGT TO BACTERIA THROUGH NATURAL TRANSFORMATION

The most likely mechanism of bacterial uptake and incorporation of free extracellular DNA is via the natural transformation of competent bacteria with DNA released into the environment or digestive tract of humans or animals (Nielsen *et al*., 1998; Bertolla and Simonet, 1999). In this process, a number of events must occur sequentially, the likelihood of which depend on the availability of the right kind of DNA (exposure), the type of bacteria and the ability of these bacteria to take up DNA (uptake) and be transformed by that DNA (stability), expression of incorporated sequences (expression), and the competitiveness of the transformed bacteria (selection). Each of these sequential events represents a potential barrier to successful HGT, and is briefly discussed below.

4.3.1. EXPOSURE

Naturally transformable bacteria may, in principle, be transformed by any type of double-stranded DNA to which they are exposed, and this will be limited both by the quality and stability of free DNA in the environment and its temporal availability to the bacteria. If DNA from a donor organism is rapidly degraded by nucleases, availability to transformable bacteria would be very limited; however, its availability would be prolonged if DNA was able to become stabilized, either within the decomposing donor or in the surrounding environment (DNA persistence in the environment was discussed at length in Section 3.).

4.3.2. UPTAKE

The uptake of DNA by transformation is a genetically and environmentally controlled process. Bacterial cells that are transformable enter a physiologically regulated state of competence for the uptake of exogenous DNA (Solomon and Grossman, 1996). To date, about 80 bacterial strains from more than 30 species have been found to be naturally transformable and among these are several with special relevance to food production or spoilage, including *Bacillus subtilis*, *Acinetobacter* sp., *Lactobacillus lactis*, *Campylobacter* sp., *Helicobacter pylori*, and Streptococci. In addition, bacterial genome sequencing data have indicated the presence of sequences homologous to competence genes in many bacteria, including those regarded as non-competent such as *Lactococcus lactis* (Bolotin *et al*., 2001; Håvarstein, 1998). Although these genes may function other than to direct competence development, there is the clear indication that the capacity to become competent may be more widespread than earlier believed.

As competence is usually not constitutively expressed by bacteria (the only known exception is *Neisseria gonorrhoeae*), the development of competence remains a major limiting factor in the process of natural transformation and knowledge of the environmental factors regulating bacterial competence is scarce (Nielsen *et al*., 1997). For *Streptococcus pneumoniae* and *B. subtilis*, competence development involves the transient expression and secretion of a small polypeptide (competence factor) (Lorenz and Wackernagel, 1994).

The process of DNA uptake has been extensively reviewed by Dubnau (1999) and will not be discussed at length

here. In some species (*e.g.*, *Haemophilus influenza*), DNA uptake is dependent on the presence of specific recognition sequences while in others (*e.g.*, *Acinetobacter calcoaceticus* and *B. subtilis*) it is sequence independent (Palmen *et al*., 1993). The efficacy of natural transformation by chromosomal DNA can sometimes be reduced by restriction endonucleases (*e.g.*, *Pseudomonas stutzeri*; Berndt *et al*., 2003) but not in all cases (*e.g.*, *B. subtilis* and *S. pneumoniae*; Majewski, 2001). In order for newly acquired DNA to be heritably transmitted it must be integrated into the host genome through recombination or maintained as an autonomously replicating unit.

In most studies on transformation, competent bacteria have been inoculated into the soil system under study (Gallori *et al*., 1994; Nielsen *et al*., 1997; Sikorski *et al*., 1998) and only recently has it been shown that competence could be stimulated in non-competent soil bacteria following the addition of specific nutrients (Nielsen *et al*., 2000b). The system employed in this latter study consisted of *Acinetobacter* sp. BD413 pFG4nptII containing a 317 bp deletion in the *nptII* gene. Restoration of this deletion following homologous recombination with transgenic plant DNA containing the *nptII* gene would be detected under kanamycin selection. Using this system, transformation with transgenic sugar beet DNA was detected in sterile soil but not in non-sterile soil. The estimated threshold of detection was 10^{-10} to 10^{-11}, implying an even lower frequency of transformation in non-sterile soil. Under more realistic conditions, transformation would not proceed via homologous recombination (as above) but through illegitimate recombination, a much more infrequent occurrence (see 4.3.3).

There have been no reports of naturally transformable bacteria in the predominant species of rumen or intestinal bacteria, although numerous attempts have been made to detect natural transformation of them (Salyers, 1997). Under the EU supported GMOBILITY project, gene transfer by natural transformation has been extensively studied *in vivo* in the intestinal tracts of germ-free animals using different recipient species (*e.g.*, enterococci, *E. coli*, *Acinetobacter*, *B. subtilis*, *S. gordonii*) and resultant transformants have not been detected (Midtvedt *et al*., unpublished results; Schön *et al*., unpublished results; Wilcks and Jacobsen, unpublished results in the framework of the GMOBILITY project, as cited in van den Eede *et al*., 2004).

4.3.3. STABILITY

An environmental consequence of the uptake of heterologous DNA would only be expected if DNA is able to replicate in recipient bacteria, which requires linkage to an origin of replication, i.e., the bacterial chromosome or a plasmid. Transferred DNA containing replication functions, like plasmids, may not be dependent upon the host genome for heritable incorporation. However, if the transferred DNA does not contain sequences that can mediate autonomous replication, stabilization of DNA will depend on its ability to integrate by recombination with an existing replicating unit. Recombination depends on sequence similarities and usually occurs between homologous stretches of DNA with only few sequence differences (*i.e.*, homologous recombination). The general requirement for homologous recombination is the most significant barrier against HGT by natural transformation. However, examples of illegitimate recombination exist, illustrated by studies of *E. coli* and *B. subtilis mmr*$^{-}$ mutants (with defects in the methyl-directed mismatch DNA repair system, MMR) where recombination with DNA displaying up to near 20 percent divergence proceeded (Zawadzki *et al*., 1995; Vulic *et al*., 1997), as compared to wild types (non-mutators), which generally abort recombination events where DNA divergence exceeds 1-2 percent (Rayssiguier *et al*., 1989). Thus, HGT and recombination with diverged chromosomal DNA can occur in bacteria, albeit at a frequency that is about 1000-fold less than the frequency of homologous recombination, which occurs at rates of *ca*. 10^{-5} (Rayssiguier *et al*., 1989).

When assessing the likelihood of recombination and HGT, it is important to evaluate potential sequence homologies to putative recipients in sequences surrounding the transgene as well as the transgene itself. If the sequences that surround the transgene have homology to bacterial recipients, these flanking sequences can facilitate the additive integration of the transgene in bacterial recipients. Studies of recombination in *E. coli* and *B. subtilis* have reported that the minimal size of homology required for recombination to initiate in these bacteria is 20-25 bp (Majewski and Cohan, 1999). Once initiated, recombination initiated from short stretches of high sequence homology can proceed into more heterogeneous DNA regions and facilitate integration of non-homologous DNA (transgenes). As reported by Nielsen *et al*. (2000b), interspecies transfer of non-homologous chromosomal DNA from inactivated bacterial cells in soil occurred at frequencies approaching those for wholly homologous recombination when flanking homology was present. The horizontal transfer of plant-inserted transgenes to bacteria has also been shown to occur when flanking DNA sequence homology is present, both *in vitro* (Gebhard and Smalla, 1998; de Vries and Wackernagel, 1998; de Vries *et al*., 2001) and in sterile soil (Nielsen *et al*., 2000a).

The general requirements of sequence homology for integration can be circumvented by site-specific recombination, which is widely used by bacteriophages, transposons and integrons for their insertion into bacterial genomes (discussed below in Section 4.4).

In summary, in the absence of significant DNA homology (>70 percent) between the transgene, or its flanking sequences, and bacterial recipients, horizontal transfer into bacteria by natural transformation is extremely unlikely.

4.3.4. EXPRESSION

Evolutionary successful HGT may depend on the expression of the novel genetic material to generate significantly altered phenotypes for selection. The transfer of small DNA fragments (parts of coding or non-coding sequences) into bacterial genomes would mainly result in mosaic genes within existing gene expression units. Such transfer would not require any co-transfer of DNA transcriptional regulatory sequences. The transfer of larger DNA fragments containing complete coding sequences would, however, generally require the co-transfer of regulatory elements in order to ensure functional expression. This requirement for the presence and appropriate arrangement of regulatory sequences, such as promoters, represents an additional strong barrier to effective HGT. As a rule, the promoter sequences used to drive transgene expression in eukaryotes have very low activity in prokaryotic hosts. One exception is the cauliflower mosaic virus 35S promoter, often used in plant transformation, which is active in *E. coli* (Assad and Signer, 1990). Furthermore, the introduction of whole plasmids into transgenic plants via biolistics could theoretically lead to the presence of bacterially expressible sequences, such as the ampicillin resistance gene. Or alternatively, the circularization of linear DNA fragments containing a bacterial origin of replication within a transformed cell is also a theoretical possibility. Neither of these theoretical possibilities has yet been observed.

4.3.5. SELECTION

Ultimately, it is the effect of natural selection that will determine the stability and environmental impact of HGT to bacterial populations (Nielsen and Townsend, 2001). The important question is not whether HGT can occur, at some low frequency, but, rather, whether there is sufficient selective advantage to maintain these transgenes over other endogenous genes found in the environment. Concerns about environmental impacts of HGT are germane only in those instances where the transgene is expressed and spreads through the population. Most transgenes that are expressed in a transformed bacterium will have a fitness effect on the reproductive potential of the bacterium in which they reside. Due to the very low frequency of HGT events likely to occur in the open environment, negatively selected traits (and transformant bacteria bearing these traits) will be lost from the population over time (Ohta, 1973). Scott *et al*. (2000) transformed *Lactococcus* with a green fluorescent protein (GFP) expression cassette that was linked to 450 bp sequence that is homologous to sequences in many bacteria (to promote homologous recombination). Transformed *Lactococcus* cultured in a simulated human gut environment inoculated with human fecal flora had impaired survival relative to non-transformed enteric bacteria. Therefore, HGT was not completely successful because the gene did not persist in the population even though it was taken up and integrated into the bacterial host genome. Thus, only those traits that are neutral or positively selected would raise concerns related to HGT.

If HGT and transgene introgression occurs in isolated bacterial subpopulations, then geographical structuring could, in principle, limit the spatial dissemination of the transgene further into the bacterial gene pool (Nielsen and Townsend, 2001). Geographical structuring and low rates of migration have been reported in populations of the root-nodulating bacterium *Rhizobium leguminosarum*. However, other common soil-borne bacteria such as

B. subtilis are less spatially structured and a large proportion of the global diversity can be detected locally (Roberts and Cohan, 1995). Likewise, for many human-associated bacteria, such as *Neisseria* spp. *Streptococcus* sp. and *E. coli* little geographical structuring has been found (Maynard Smith *et al*., 2000). Thus, the geographical isolation of putative bacterial transformants carrying the transgene will depend strongly on the persistence and ecology of the specific organism.

4.3.6. SUMMARY

The existing scientific data, while not excluding the possibility of natural transformation of bacteria under natural conditions, do indicate that, if it occurs, it is a very low frequency event and the chance of acquiring the same genes from another bacterial species in the environment is much greater. For example, the likelihood of gene transfer from transgenic plants to bacteria is thought to be 2×10^{-11} to 1.3×10^{-21} per bacterium as compared to the 10^{1} to 10^{-8} probability of gene transfer by conjugation between soil and enterobacteria (Dröge *et al*., 1998). From a risk assessment perspective, it is the combination of transfer frequency and selective advantage conferred to the successful transformant that is significant, which must be evaluated on a case-by-case basis. *"Thus, during assessment of the potential for gene transfer, efforts should be made to understand the selective forces acting upon the genetic material which is 'likely' to be transferred. Only a clear prediction of the selective factors present will allow a sound assessment of the potential of gene transfer and dissemination"* (Nielsen, 1998).

4.4. DNA TRANSFER BETWEEN BACTERIA

4.4.1. TRANSDUCTION

Transduction is the bacteriophage-mediated horizontal transfer of DNA between different prokaryotic cells and is due to errors in lysogenic phage integration into and/or excision from the chromosome of their host, or erratic packaging of nonphage DNA into phage particles in the lytic cycle. While the host range of most bacteriophages is quite narrow, generally restricted to a single species, some phages have a broad host range (Sayre and Miller, 1991). Bacteriophages are ubiquitous in both terrestrial and aquatic environments (Jiang and Paul, 1998; Miller, 1998; Paul, 1999) and are also found in great numbers in the mammalian intestinal tract.

The genes encoding many bacterial toxins from both Gram-negative and Gram-positive bacterial pathogens are carried by phages, which can transfer these between bacteria in the gut microflora through lysogenic conversion (i.e., gene transfer mediated by incorporation of the phage genome into the bacterial host; Cheetham and Katz, 1995). For example, lysogens of Shiga toxin (Stx)-converting phages were capable of transducing *E. coli* within the mouse intestinal tract (Acheson *et al*., 1998). Cholera toxin from *Vibrio cholerae* is encoded by the filamentous phage CTXΦ, whose lysogenic conversion has been shown to be more efficient within the gastrointestinal tract of mice than under laboratory conditions (Waldor and Mekalanos, 1996). The implication from such studies is that the gastrointestinal tract may be a "hot-spot" for transduction-mediated HGT, perhaps because it provides the environmental signals necessary for expression of important proteins mediating interactions between the phages and their host bacterium.

Additionally, plasmids can be carried from one bacterial cell to a new host by transduction, because linear plasmid replication intermediates are packaged and form a circular entity in the new host by recombination (Ripp and Miller, 1995).

4.4.2. CONJUGATION

Bacterial conjugation requires cell-to-cell contact between the donor and recipient requiring a complex set of trans- and cis-acting factors within the donor cell, and is mediated by large conjugative plasmids or conjugative transposons in the donor cell. Besides being self-transmissible, conjugative plasmids and transposons are often capable of mobilizing smaller non-conjugative plasmids and chromosomal DNA (Jonas *et al*., 2001). Because there are fewer requirements for similarity between donor and recipient, conjugation is considered more promiscuous than transformation and transduction.

Data from the EU supported GMOBILITY project have demonstrated horizontal transfer via conjugation *in vivo* using rodent models. In these studies, a recipient *Enterococcus faecalis* strain in the intestines of germfree mice acquired the conjugative plasmid pIL205, and/or the mobilizable plasmid, pCAC, from a donor, *Lactococcus lactis,* and the orientation of the lux:ery^{R} gene-cassette relative to the *mob* gene influenced the efficiency of transfer, which in the case of transfer of plasmid pIL205 was observed in *E. faecalis* transconjugants with a frequency of 10^{4} CFU/g feces. In dixenic mice with a flora from human feces, the transfer to

E. faecalis was repressed to about 5 log below that seen when only the donor and recipient strains were present, confirming that gene transfer is less detectible in more complex and competitive microbial populations.

Van den Eede *et al*. (2004) postulated that random insertion of transgenes into transposon sequences may lead to spread and multiplication of the genes in the plant population, thereby increasing their availability for horizontal gene transfer.

Andrews *et al*. (2004) have shown that survival of fecal enterococci from animal waste in soils was affected by the natural soil microbial populations. In sterile soils the fecal enterococci populations remained stable over many weeks, but in natural soils the populations quickly decreased to an undetectable level, probably due to competition from other microbes. When they investigated the level of conjugative transposons in the soils, using detection for Tn916, they discovered that while the inoculant enterococci decreased, the level of Tn916 remained at about 20 percent of the initial inoculation. They concluded that the conjugative transposons transferred to natural soil microbes and remained in the soil after the degradation of the fecal waste and the associated fecal enterococci. Thus, conjugative transposons from the Tn916 family provide a mechanism for transfer of genes from animal waste to soil populations. This family of transposons has a wide host range that includes both Gram-positive and Gram-negative species and they are known to facilitate not only self-transfer, but also the transfer of genetic information from host chromosomal and plasmid origin.

The observation that conjugative transposons increase the incidence of wide transfers of genetic material between microbes may have implications in particular transformation systems and/or the design of vectors and inserted gene sequences for transgenic development.

4.5. DNA UPTAKE INTO MAMMALIAN CELLS

The work of Schubbert *et al*. (1997, 1998) showed that following feeding of naked M13 bacteriophage DNA replicative form (dsDNA circular) to mice, small DNA fragments could be detected not only in gut contents but also in cells of the intestinal wall, liver and B and T cells. Evidence was produced for covalent linkage ofM13 DNA to mammalian chromosomal DNA (Schubbert *et al*., 1997) and for transplacental transfer of the ingested DNA in pregnant mice. Mucosal cells of the gastrointestinal tract are constantly exposed to DNA fragments derived from food and, in the case of cattle and poultry, uptake of maize chloroplast DNA has been demonstrated after feeding with maize (Einspanier *et al*., 2001; Klotz *et al*., 2002). However, the significance of such DNA uptake with respect to expression of any new genes has yet to be demonstrated. To date there is no evidence for germline transfer of orally administered DNA and results from studies where mice were fed daily with green fluorescent protein (GFP) DNA for 8 generations with no detectable GFP DNA in DNA isolated from tail tips and internal organs by PCR argue against this possibility (Hohlweg and Doerfler, 2001).

4.6. HGT INVOLVING EUKARYOTES

Compared to HGT among prokaryotes, HGT involving eukaryotes is even more rare, especially when it involves the movement of genes between organisms in different kingdoms. HGT from eukaryotes to prokaryotes has never been shown experimentally under non-laboratory conditions (Bertolla and Simonet, 1999). However, evolutionary studies comparing genomic sequences obtained from prokaryotes and eukaryotes provide growing evidence that gene transfer has occurred (in both directions) over a geological time frame (Mazodier and Davies, 1991; Smith *et al*., 1992; Syvanen, 1994; Woese, 1998; Jain *et al*., 1999). Recent research results suggest that more recent within the evolutionary timeframe, the adzuki bean beetle (*Callosobruchus chinensis*) has acquired a genome fragment from a bacterium, its natural endosymbiont *Wolbachia* (Kondo *et al*., 2002). Gene transfer from bacteria to mammalian cell lines has been demonstrated in the laboratory from *Agrobacterium* to HeLa cells (Courvalin *et al*., 1995) and *E. coli* to CHO K1 cells (Waters, 2001).

5. APPROACHES TO PRODUCING TRANSGENIC AQUATIC SPECIES

5.1. INTRODUCTION

Fish are generally considered to be more amenable than other vertebrate species to transgenic modification as females produce an abundance of eggs which are easily manipulated, and embryos develop outside of the mother. Fish species such as medaka, tilapia, catfish and striped bass are used as experimental models for the study of cell and developmental biology in vertebrates and so have been used for the development of advanced molecular techniques that have then been adapted for use in other fin species.

Table 5: Teleost, mollusk, crustacean and echinoderm species used in transgenic research[1]

Common name	Latin name
Teleost	
Tilapia	*Oreochromis niloticus*
African catfish	*Clarias gariepinus*
Channel catfish	*Ictalurus punctatus*
Northern pike	*Esox lucius*
Medaka	*Oryzias latipes*
Zebrafish	*Danio rerio*
Loach	*Misgurnus anguillicaudatus*
Goldfish	*Carassius auratus*
Red crucian carp	*Carassius carassius auratus*
Common carp	*Cyprinus carpio*
Brown trout	*Salmo trutta*
Atlantic salmon	*Salmo salar*
Rainbow trout	*Oncorhynchus mykiss*
Cutthroat trout	*Oncorhynchus clarkii*
Coho salmon	*Oncorhynchus kisutch*
Chinook salmon	*Oncorhynchus tshawytscha*
Black sea bream	*Acanthopagrus schlegeli*
Red sea bream	*Chrysophrys major*
Blunt snout bream	*Megalobrama amblycephala*
Nigorobuna	*Carrassius auratus grandoculis*
Walleye	*Stizostedion vitreum*
Mollusc	
Abalone	*Haliotis rufescens*; *Haliotis iris*
Oyster	
Surfclams	*Mulinia lateralis*
Crustacean	
Brine shrimp	*Artemia franciscana*
Crayfish	
Karuma prawns	*Penaeus japonicus*
Echinoderm	
Sea urchin	*Arbacia lixula*; *Hemicentrotus pulcherrimus*; *Paracentrotus lividus*; *Strongylocentrotus purpuratus*; *Temnopleurus toreumaticus*
Marine plants	
Spirulina	*Spirulina platensis*
Seaweed	*Laminaria japonica; Undaria pinnatifida*
Algae	*Chlamydomonas*

1. Adapted from Sin (1997).

Transgenic fish were first reported in 1985 with the transformation of goldfish (*Carassius auratus* L.) with a recombinant human growth hormone gene driven by a mouse metallothionein-1 promoter (MthGH) (Zhu *et al.*, 1985). In this study, fertilized eggs of goldfish were obtained by artificial spawning and insemination and the chorion of the eggs was removed by digestion in a trypsin solution. A DNA solution containing approximately 10^{5}-10^{6} copies of the MThGH gene was delivered with a micromanipulator into the germinal disc just underneath the second polar body. Fifty percent of the founders were confirmed as transgenic by Southern hybridization. Since 1985, many other aquatic species that have been used in transgenic research, as listed in Table 5. A variety of genes have been used to develop transgenic aquatic and marine organisms, including: marker genes; growth hormone; antifreeze polypeptide; ceciopin; interferon; phytase; human clotting factor VII; reporter genes for contaminants; and GnRH antisense (Hallerman 2003).

5.2. METHODS FOR GENE TRANSFER

There are several methods used to facilitate transfer of genetic material into the germlines of aquatic species as well as techniques used to confer transient expression of transgenes. These are summarized below.

5.2.1. MICROINJECTION

Foreign genes have been successfully introduced into the cytoplasm of fish eggs through microinjection of an egg typically at the 1, 2 or 4-cell stage. Injected embryos have survival rates ranging from 16–85 percent, depending on the species, and the number of transgenic founder fish also varies by species from 5 percent in zebrafish to greater than 70 percent with trout (Sin, 1997). Microinjection of fish eggs with foreign DNA has been

successfully performed in several species, such as rainbow trout, Atlantic salmon, tilapia, medaka, common carp, zebrafish, loach and catfish.

The introduction of foreign genes into pronuclei of fertilized fish eggs is more difficult than for fertilized mammalian eggs because the pronuclei are not readily visible, the egg has a tough chorion, and the perivitelline space is relatively large. In general, three different approaches have been applied to deal with the chorion barrier: removing the chorion by manual dissection or enzymatic digestion (Stuart *et al.*, 1988, Ozato *et al.*, 1986); penetrating the chorion via the micropyle (Brem *et al.*, 1988; Lu *et al.*, 1992); and cutting a hole in the chorion (Chourrout *et al.*, 1986, Penman *et al.*, 1990). Although integration rates of transgenes are generally low and microinjection is a time consuming method of gene transfer, it remains the most commonly used technique to produce transgenic fish

5.2.2. ELECTROPORATION

Electroporation has been applied to the development of transgenic aquatic species to overcome some of the challenges associated with microinjection of fish eggs. With electroporation, cell membranes are reversibly perturbed by sudden changes of electrical fields, facilitating the uptake of foreign DNA through transient pores in the membrane. This method has been exploited successfully for the introduction of cloned DNA molecules into different kinds of cells for permanent transformation or for transient expression of gene products (Lu, unknown). Gene transfer via electroporation has been applied to medaka, goldfish, carp, channel catfish, loach, zebrafish, black porgy, red abalone and surfclams.

There is significant species variability in terms of embryo survival after electroporation. Inoue *et al*. (1990) showed that this technique is effective in producing transgenic fish by directly exposing medaka embryos to brief high voltage electric fields. However, only 25 percent of the embryos that survived the electric shock hatched and of these only 4 percent were transgenic. Copy number varied from 1 to >100. Survival of electroporated eggs and fertilized embryos and the success of transgene integration have subsequently been found to be dependent on the stage of development when eggs and embryos were shocked and the duration and voltage of the electrical pulse (Sin, 1997).

Gene transfer through the electroporation of sperm has been reported in marine molluscs (Hu *et al*., 2000).

5.2.3. TRANSPOSON MEDIATED GENE TRANSFER

A transposon is a segment of DNA having a repeat of an insertion sequence element at each end as well as genes specific to some other activity such as resistance to antibiotics. It is capable of migrating to a new position within the same or another chromosome, plasmid, or cell and thereby transferring genetic properties. Raz *et al.* (1998) demonstrated the utility of transposable elements of the *Tc1/mariner* family for transgenesis in zebrafish. Modified transposons, based on the *Caenorhabditis elegans* Tc3 transposon, were found to transpose from a plasmid donor *in vivo* and integrate into the germline in a transposase-mediated fashion. Tc3 transposase can also mediate excision of zebrafish chromosomal copies of modified Tc3-based Transposons. Expression of genes from the chromosomal copy of the modified transposon was reliable and stable after several generations of germ line transmission. One additional transposon of the *Tc1/Mariner* family, *Sleeping Beauty* (SB) (Ivics *et al.*, 1997) has been demonstrated to be mobilized in zebrafish as well as human cell lines upon overexpression of the reconstituted SB transposase.

5.2.4. RETROVIRUS MEDIATED GENE TRANSFER

Methods have been developed utilizing retroviruses as vehicles for gene transfer. When the retrovirus penetrates a cell, the resultant viral RNA is converted to double strand DNA which enters the nucleus and integrates into the host genome through a site-specific recombination event. The provirus becomes part of the host genome, and the stability and maintenance of the transgene is far superior to that achieved using other gene transfer systems (Sin, 1997).

Broad host range (pantropic) replicative-defective retroviral vectors have been used to infect fish cell lines and newly fertilized finfish and shellfish eggs such as medaka, zebrafish and surf clam (Lin *et al.*, 1994; Lu *et al.*, 1996, 1997). Stable transgenic medaka and surf clams have been produced by electroporating these pantropic vectors into newly fertilized embryos (Lu *et al.*, 1996, 1997). Sarmasik *et al.* (2001) used retroviral vectors to

infect immature gonads of live-bearing fish and crayfish *in situ* and result in the production of transgenic individuals by crossing transformed animals with their untransformed counterparts. Retroviral infection and microinjection were compared in zebrafish; the two methods were equally efficient in passing the transgene into eggs, but there was wider variability in the extent of reporter gene expression among those founders that were microinjected (Linney *et al.*, 1999).

Retroviruses can be employed in gene transfer with technical ease and are effective with an absence of any apparent effect on the viability of the infected cells. Retroviral expression can be modulated via the enhancer sequences in the viral long terminal repeat, which determines tissue tropism and the type of disease caused by a particular virus. Typically, organisms transformed with a retrovirus have one transgene copy with a single site of insertion and no transgene rearrangements.

5.2.5. SPERM MEDIATED GENE TRANSFER

Sperm soaked in DNA solution have been used to successfully transfer foreign genes to eggs during fertilization. This technique has been successfully applied to sea urchins (Arezzo, 1989), zebrafish (Khoo *et al.*, 1992) and goldfish (Yu *et al.*, 1994). The efficiency of sperm-mediated gene transfer has been improved through electroporation of the sperm, however, chromosomal integration of the DNA of interest remains variable (Sin, 1997, Levy *et al.*, 2000).

5.2.6. LIPOSOME MEDIATED GENE TRANSFER

As reviewed by Sin (1997), liposome mediated gene transfer (or lipofection) makes use of the interaction between positively charged lipid molecules and the negatively charged DNA molecules. Because the surfaces of biological membranes are also negatively charged, liposomes can readily associate with the negatively charged plasma membrane and transfer nucleic acids into cells. The mechanism involved in the internalization of the DNA is not yet understood. It has been shown that the rate at which the liposome binds to the plasma membrane determines the overall uptake rate, and lipid specificity for specific cell types also plays an important role for uptake.

Two plasmids have been successfully transferred by lipofection into the embryos of African catfish, however transgene expression decreased as the larvae aged, suggesting that the plasmid DNA was lost through degradation (Szelei *et al.*, 1994).

5.2.7. PARTICLE BOMBARDMENT

Microparticle bombardment or biolistic transformation, a method commonly used in plant biotechnology to produce transgenic monocot species, has also been applied to develop transgenic fish. Linearized or plasmid DNA is coated onto tungsten or gold particles which are forcibly discharged, from a gun-like apparatus, into eggs. Particle bombardment has been used to transfer genes into loach, rainbow trout, zebrafish, brine shrimp and sea urchin. Most biolistic research with aquatic species to date has used marker genes, with variable success; integration and germline transmission of transgenes has yet to be consistently demonstrated.

5.2.8. STEM CELL TRANSFECTION

More recently the use of embryonic stem cells (ESC) as a method for inducing transgenesis has been advocated. These cells are undifferentiated and remain totipotent, so they can be manipulated *in vitro* and subsequently reintroduced into early embryos where they can contribute to the germ line of the host. In this way genes could be stably introduced or deleted (Melamed *et al.*, 2002). Despite the early success of ESC technology in mice, the uptake of the technology for fish has been slow, although early precursor cells (Mes 1) have been cultivated from medaka and show many of the same features as mouse ESC. Studies by Hong *et al.* (1996, 1998, 2000) showed that 90 percent of host cell blastulae transplanted with Mes 1 cells developed into mosaic fry, and these cells became integrated into organs derived from all three germ layers, and differentiated into various types of functional cells.

5.2.9. TRANSIENT GENE EXPRESSION

Transient gene expression in fish, such as DNA vaccination, has important implications for aquaculture. Ramos *et al.* (2005) reported the oral delivery of a construct expressing the beta-galactosidase reporter gene into fish by encapsulating the DNA in chitosan and incorporating it into fish feeds. Reporter gene expression was observed in the stomachs, spleens and gills of fish fed with flakes containing the chitosan-DNA complex.

5.3. POTENTIAL RISKS ASSOCIATED WITH GENE TRANSFER TECHNIQUES

Given the variety of techniques that may be applied to transfer foreign genes into aquatic species, the question arises as to whether any of these techniques has the potential to increase any capacity for horizontal gene transfer from the transgenic aquatic organism to other organisms in the environment. In their review of applications of biotechnology techniques for animal biotechnology, the Committee on Defining Science-based Concerns Associated with Products of Animal Biotechnology specifically identified transposons and viral vectors as being of particular concern as regards the potential for HGT of the novel gene (NRC 2002).

The Committee's report states "*When viral vectors are used for the introduction of genes into the germline of animals, there exists a potential for inadvertent transmission of the gene to other individuals (not necessarily of the same species). This undesirable effect could occur if such an animal were to be infected with a virus sufficiently similar to the vector to package the vector into virions*". The use of pantropic retroviruses to transform aquatic species should therefore consider the natural occurrence of viruses related to the vector in the receiving organism, and the existence of native homologues of the novel gene in the receiving environment.

The Committee also raised a concern about the use of *mariner* and related transposons such as *Sleeping Beauty* to introduce germline DNA given that related elements have been found in large numbers in the human genome as well as planaria, nematodes, centipedes, mites and insects (NRC 2002). The Committee members speculated that HGT via transposition among highly diverse hosts could be possible if these sequences were mobilized by the constructs used to transfer *mariner*-like elements into the germline, where their insertion into genes could result in unexpected genetic damage. It was also suggested that this potential avenue to HGT could be minimized or eliminated by expressing the transposase in the trans configuration and deleting the gene for these enzymes from the transgene construct, so that once inserted into the host genome the element is immobilized.

6. CONSIDERATIONS FOR RISK ASSESSMENT AND RISK MANAGEMENT OF CONTAINED NOVEL AQUATIC ORGANISMS

6.1. ASSESSING RISK IN A REGULATORY CONTEXT

Scientific risk assessment is the cornerstone of biotechnology regulatory systems and public policy decisions related to the safety and acceptability of genetically modified organisms (GMOs). Even in countries that have incorporated structures and mechanisms for including non-safety (*i.e.*, socioeconomic) issues in the decision-making process, a strong scientific capacity and knowledge base is viewed as key to identifying hazards, and assessing their impacts and likelihood.

The classic paradigm of risk assessment is that it is a science-driven process that quantitatively evaluates the probability of risk, largely removed from the emotive factors and other biases that influence risk perception (NRC, 1983). In this context, risk is the chance that some harm will result from some postulated hazard, and risk assessment is "the process of obtaining quantitative or qualitative measures of risk levels, including estimates of possible health effects and other consequences as well as the degree of uncertainties in those estimates" (Fiksel and Covello, 1986). The objective of risk assessment is to produce neutral and transparent risk information, including the identification of possible risk management and mitigation measures, to inform the decision-making (risk management) function.

Risk = Exposure X Hazard

As it is commonly expressed, risk is a product of both hazard (*i.e.*, the degree of severity of the adverse consequence) and the likelihood of exposure to the hazard. Minimal risk situations occur when either the hazard is judged to be insignificant, or the probability of exposure to be very small, or both. In considering the estimation of risk, it is useful to distinguish different types of hazard:

- Probabilistic: The hazard exists AND has occurred at least once. For example, airplane crashes. Based on prior crash data, a frequency of occurrence can be calculated and the risk can be quantified.
- Hypothetical: The hazard has not occurred BUT there is a scientific line of reasoning to support its existence. For example, development of an antibiotic resistant pathogen as a result of horizontal gene transfer (HGT) from plants to microbes. The transfer of plant genes and their stable integration within the genome of bacteria present in either the gut or soil has not yet been observed in nature. Yet, based on our knowledge of molecular biology and observations of HGT between other organisms, we can hypothesize that it may occur between plants and microbes and can estimate an approximate likelihood based on the step-wise probability of individual preconditions. A property of hypothetical hazards is that specific hypothesis-testing models or scenarios can be developed.
- Speculative: The hazard has not occurred AND there is NO reasonable scientific line of reasoning to suggest that it will. For example, an adverse long-term health effect as consequence of chronic consumption of a currently approved genetically modified food (*e.g.*, insect-resistant maize) specifically because it was genetically modified. Speculative hazards are not generally amenable to rigorous hypothesis testing. With respect to genetically engineered plants, many of the concerns voiced by overly precautious parties are based on speculative hazards.

The focus of risk assessment should be on asking empirical questions about probabilistic or hypothetical (possible) risks, not speculative (scientifically indefensible) risks. That is, risk should be something that is testable by empirical means, rather than based on unsubstantiated or illogical possibilities. For example, the possibility of altering a plant's potential for weediness as a consequence of genetic engineering is something that can be evaluated by assessing specific characteristics of the modified plant (*e.g.*, seed dormancy and germination rates, seed dissemination, time to maturity and competitiveness) in relation to species of known weediness, such as the conventionally bred crop. Placing the emphasis on empirical questions and testable risks implies that disputes or uncertainties can be resolved through further study and analysis, something which is not possible for speculative risks. Focusing on testable risks does not imply that "no evidence" means "no risk," or that new analytical methods cannot or should not be developed and applied. It is precisely the role of basic research to discover new knowledge that could be elaborated into testable hypotheses or analytical methods once the knowledge has been revealed. As such, risk assessment is complemented by basic research, but should not become a surrogate for it. In the face of scientific uncertainty, or when risk assessment results are inconclusive, it is essential that improved analytical tools be developed and that provisional risk management decisions be taken on a precautionary basis.

When approached in this manner, the risk assessment process is reserved for experts only and is not open to considering normative questions, such as ethics or socioeconomic impacts. Risk assessments are not the appropriate vehicles for assuaging public fears (that is, perceptions of risk) or proving social benefit. In practice, it is rarely that clear cut. It is difficult to dissociate the perceptions of risk from risk assessment, and impossible to ignore the uncertainty in science that limits objective quantification of risk.

6.2. GUIDANCE FOR CONTAINMENT AND DISPOSITION OF NOVEL AQUATIC ORGANISMS AND WASTE DERIVED FROM THESE

The Office of Laboratory Security (OLS) of the Public Health Agency of Canada, provides guidance to researchers about the use, including decontamination and disposition, of biohazardous material in laboratory settings through its Laboratory Safety Guidelines. The Guidelines, updated in 2004, were initially developed to guide government, industry, university, hospital, and other public health and microbiological laboratories in their development of biosafety policies and programs. The Guidelines also serve as a technical document providing information and recommendations on the design, construction and commissioning of containment facilities. While the Guidelines emphasize biosafety as related to human pathogens, they are used as a source of guidance for public and private sector laboratories undertaking any research that requires biological containment.

The mandate of the OLS is to ensure effective, evidence based biosafety interventions on a national basis by means of regulatory control, surveillance, applied research and the provision of information for laboratory operations throughout Canada. The Guidelines provide guidance as to the classification of biohazardous materials according to risk and assist in the development of containment protocols and facilities for biohazardous materials and is the basis for institutional biosafety programs at research institutes in Canada.

The Health Canada Office of Laboratory Security (OLS) Guidelines provide a classification structure for determining the level of risk associated with biological agents. Risk is assigned according to the potential for harm to human, environmental and animal health with the emphasis placed on human health protection.

Laboratory animals and recombinant DNA and genetic manipulation are addressed generally in the OLS guidelines (see Appendices 1 and 2, respectively).

In addition to the OLS Guidelines, Fisheries and Oceans Canada and the Canadian Food Inspection Agency are developing guidelines for the containment of aquatic animal pathogens in laboratories and live animal holding facilities.

6.3. CONSIDERATIONS FOR THE EXPERT PANEL

The members of the Expert Panel are requested to evaluate, comment on and supplement the information provided in this document, and to provide insight into the possible nature, magnitude and source of risk, if any, to the environment or human health that may be presented by the HGT of free DNA or DNA associated with waste from novel aquatic organisms including somatic cells in waste effluent. In addition, the Panellists are asked to consider possible frameworks for categorizing hazards associated with transgenes and to address the circumstances under which such hazards may result in significant risk to the environment or human health. The Panellists are also requested to provide science-based regulatory options to contain and mitigate risks related to HGT of DNA from waste biomass from novel aquatic organisms and effluent.

In order to stimulate dialogue, the Expert Panel members are asked to respond to each of the following:

1. Based on the literature reviewed herein, it would appear that there is significant evidence that the potential for HGT of free, recombinant DNA released from the decomposition of carcasses, somatic cells in effluent water or other waste associated with novel aquatic organisms to prokaryotes or eukaryotes is extremely remote. **Are there any conditions or circumstances which could conceivably alter this conclusion?**

2. There are many transformation techniques used to produce novel aquatic organisms. **Which of these, if any, could enhance HGT from carcasses, somatic cells or other waste associated with novel aquatic organisms after material disposition (*e.g.*, in a landfill)? Are there specific types of vectors or regulatory elements that should be taken into account when assessing the potential for, and risk associated with, HGT? And how do such parameters influence HGT frequencies or risks associated with HGT?**

3. **Can categories of hazard be applied to different classes of transgenes? If so, what criteria or parameters should be used to define these categories and can the Panel provide a framework for these?**

   For example, the following framework is offered as a starting point for the Panel's consideration:

   Possible categories of hazard for transgenes introduced into novel aquatic organisms:

   a. High hazard transgene:

      i. Gene that could confer enhanced pathogenicity to potential recipient bacteria (potential positive fitness change);

      ii. Gene that could alter the ecology of potential recipient bacteria or increase environmental fitness (positive fitness change);

      iii. Gene encoding a protein with known mammalian toxicity (neutral to positive fitness change);

      iv. Others?

   b. Medium hazard transgene:

      i. Gene encoding a protein allergen (bacteria are generally regarded as NOT being a source of allergenic proteins) (negative to neutral fitness change);

ii. Others?

c. Low hazard transgene:

i. Gene derived from the same environment as potential recipient bacteria;

ii. Gene encoding an enzyme of known function and not associated with bacterial pathogenicity nor mammalian toxicity;

iii. Others?

4. Risk assessment also needs to consider the ecology of the transgene donor organism. For example, if the native homologue of the transgene can be found in the same environment as the transgenic organism (or its waste) or if the transgene is functionally redundant then it can be expected that potential recipient bacteria have already been exposed to the gene or its functional homologue, hence there should be no significant additional risk to the environment or human health resulting from HGT. **Is this consideration a valid conclusion in the context of the disposition of carcasses, somatic cells in effluent or other waste associated with novel aquatic organisms? Should such materials or effluent be treated prior to their disposition (*e.g.*, in a landfill) and, if so, what treatment methods should be considered?**

5. The considerations for the Expert Panel, as posed above, focus primarily on potential risks that may be associated with HGT and the disposition of carcasses, somatic cells in effluent or other waste associated with novel aquatic animals. **The Panel is asked to comment on any additional or different considerations that may be specific to the disposition of novel aquatic plants.**

6. The Panellists are asked to contribute any other ideas, opinions or guidance that they feel may be useful to Fisheries and Oceans Canada as regards assessing potential risks associated with HGT from novel aquatic organisms.

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8. APPENDIX 1: OLS GUIDANCE FOR LABORATORY ANIMALS

Program Specific Guidelines for Laboratory Animals: General Requirements (Health Canada 2004)

Work with animals poses a variety of unique hazards, including exposure to infectious agents (naturally occurring or experimentally produced), animal bites and scratches, kicks and crushing injuries, allergies and physical hazards (noise, temperature). In addition to keeping infectious agents from spreading to laboratory workers there is a need to address, in the equipment and practices of animal facilities, the issues of cross-contamination between animals and of keeping adventitious agents from inadvertently infecting experimental animals (also referred to as "barrier" facilities). Animal facilities for work with small and large animals should be designed and operated in accordance with the Containment Standards for Veterinary Facilities[1], published by the Canadian Food Inspection Agency, the Guide to the Care and Use of Experimental Animals[2], published by the Canadian Council on Animal Care and other CCAC guidelines and policies (as revised from time to time). Institutions using animals for research, teaching and testing should consider obtaining a CCAC Certificate of GAP (Good Animal Practice®). There are other international recommendations which can provide further assistance with the assessment of hazards associated with the care and use of research animals[3-5].

Ideally, animal facilities should be a physically separated unit, but if they adjoin the laboratory the animal rooms should be separated from other activities in the laboratory to allow for isolation and decontamination as required. As general protocols cannot anticipate the specific requirements of each experiment, specific entry and exit protocols for scientific staff, animal handlers, animals, biological samples, equipment, feed and wastes should be developed for each project.

Animal rooms for small animals should be designed for ease of cleaning and disinfection, and have a minimum of built-in equipment. A small preparation area, storage area and handwashing sink are usually all that are required. As well, the design should facilitate the use of containment caging systems and support facilities for animal procedures, cage washing, waste disposal and food/bedding storage. Recent technological improvements have been incorporated into a wide variety of housing systems to provide control of microenvironmental factors such as temperature, air exchange and humidity. Descriptions of currently used caging and bedding disposal systems have been provided elsewhere[6].

At least one-fifth of people who work with laboratory rodents, guinea pigs and rabbits develop allergies[7]. Allergic conditions may result from contact with animal fur or hair, bedding and animal wastes. The allergy may manifest itself immediately or may be acquired over a succession of exposures to the allergen. Symptoms range from mild rashes to severe asthma. Unnecessary exposure to these allergens can be minimized through engineering controls, ventilation, use of isolators and containment caging systems, and appropriate use of respiratory and other personal protection[3,4].

Containment facilities for large animals are unique, in part because of the large quantity of infectious microorganisms that may be present in the animal cubicle. Unlike a laboratory room, where the BSC provides primary containment, the large animal cubicle serves as both the primary and secondary barrier. Particular attention must be given to the use of protective clothing and equipment by staff entering an animal cubicle contaminated with large volumes of infected animal waste. Floor drains connected to an effluent sterilization system are employed at containment levels 3 and 4 to effectively remove and treat infected animal wastes. Special care must also be taken to avoid serious injuries (*e.g.*, crushing) that could occur when handling large animals. Physical barriers, restraints and gating systems should be designed and used to prevent such injuries. The handler must have knowledge of the animal's general characteristics, such as mentality, instincts and physical attributes.

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9. APPENDIX 2: OLS GUIDANCE FOR RECOMBINANT DNA AND GENETIC MANIPULATION

Genetic methods, such as natural selection, cross breeding, conjugation and transformation, have been used for many years to change biological species and organisms. These methods have been supplemented by newer and much more efficient ones, of which the best known are the techniques of recombinant DNA. Some newer techniques include the production of transgenic plants and animals; the cloning of microbial toxin or other virulence genes in an expression vector or in a host background in which it may be expressed; and the production of full-length infectious viral clones, including the reconstruction of infectious virions from recombinant constructs (reverse genetic engineering).

The initial fear of possible risks arising from organisms altered by this technology led Canada, the United States and Great Britain, among other countries, to develop stringent biosafety guidelines. Experience rapidly showed that the initial fears were not justified and that most recombinant DNA research in itself does not pose any specific risks to biological safety[1].

Guidance in how to assess potential risks in recombinant DNA research is available[2,3] but can only be very general. Factors to consider when determining the containment level of a recombinant organism should include:

1. the containment level of the recipient organism;
2. the containment level of the donor organism;
3. the replication competency of the recombinant organism;
4. the property of the donor protein to become incorporated into the recombinant particle; and
5. potential pathogenic factors associated with the donor protein.

Each case needs to have a risk assessment, as it is not realistic to try to define in advance all the possible genetically engineered organisms that might be created or used in the laboratory. Assistance with the risk assessment can be provided by the Office of Laboratory Security, telephone (613) 957-1779.

The vast majority of recombinant research involves only the remotest possibility of creating a hazard, because the source of the DNA being transferred, the vector and the host are all innocuous. However, some genetic manipulation does raise significant possibility of risk. In general, if none of the components of the genetic manipulation presents any known hazard and none can be reasonably foreseen to result from their combination, then no biohazard restrictions are needed. If one of the components of the reaction is hazardous, then, in general, discussion of the containment level required should start at the level appropriate to the known hazard. Its containment level might be increased or decreased according to such considerations as the particular gene being transferred; the expression of the gene in the recombinant organism; the biological containment offered by the host vector systems; the envisaged interactions between the gene being transferred and the host vector systems; and the viability of the host vector systems. In any research with genes coding for hazardous products, host vector systems with limited ability to survive outside the laboratory should be used; their use will reduce the level of containment required.

Examples of such considerations follow:

1. A recombinant vesicular stomatitis pseudotype virus expressing a different viral glycoprotein would be at level 2 because the virus is replication-deficient.
2. A recombinant vesicular stomatitis virus expressing a different viral glycoprotein would be at least at the level of vesicular stomatitis virus since the virus is replication-competent and could have an altered tropism.
3. A recombinant vaccinia virus expressing a different viral glycoprotein would be at the containment level of wild type vaccinia virus since the protein does not get incorporated into the virus particle, and it is unlikely that this manipulation will change the biological properties of the recombinant virus.

References:

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2. National Institutes of Health. Guidelines for research involving recombinant DNA molecules. Federal Register (with subsequent amendments) 59 Federal Register 34496, July 5, 1995.

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Appendix 3: Meeting Agenda

Expert Panel Meeting on the Potential Risks Associated With Horizontal Gene Transfer from Novel Aquatic Organisms

January 11, 2006
West Vancouver, British Columbia

Agenda

Time	Topic	Speaker
0830	Welcome and Background	J. Beardall
0840	Canadian Science Advisory Secretariat	J. Rice
0850	Questions from the Discussion Document and Charge to the Panel	M. McLean
0900	Round Table Introduction of Panellists	All
0915	Panel Response: Question 1	All
1030	Refreshment Break	
1100	Panel Response: Question 2	All
1230	Lunch	All
1330	Panel Response: Question 3	All
1500	Panel Response: Question 4	All
1600	Refreshment Break	
1615	Panel Response: Question 5	All
1700	Panel Response: Question 6	All
1730	Summary and Next Steps	J. Rice and J. Beardall
1800	Meeting Closed	

Annexe 3 : Programme de la réunion

Réunion du groupe d'experts sur les risques potentiels liés à la transmission horizontale de gènes d'organismes aquatiques à caractères nouveaux

11 janvier 2006
West Vancouver, Colombie-Britannique

Programme

Heure	Sujet	Orateurs
8 h 30	Mot de bienvenue et contexte	J. Beardall
8 h 40	Secrétariat canadien de consultation scientifique	J. Rice
8 h 50	Questions sur le document de discussions et directives pour le groupe d'experts	M. McLean
9 h	Table ronde – Présentation des membres du groupe d'experts	Tous
9 h 15	Réponse du groupe d'experts : Question 1	Tous
10 h 30	Pause	
11 h	Réponse du groupe d'experts : Question 2	Tous
12 h 30	Dîner	Tous
13 h 30	Réponse du groupe d'experts : Question 3	Tous
15 h	Réponse du groupe d'experts : Question 4	Tous
16 h	Pause	
16 h 15	Réponse du groupe d'experts : Question 5	Tous
17 h	Réponse du groupe d'experts : Question 6	Tous
17 h 30	Sommaire et prochaines étapes	J. Rice et J. Beardall
18 h	Fin de la réunion	

Appendix 4: List of Attendees

Fisheries and Oceans Canada: Expert Panel Meeting on the Potential Risks Associated With Horizontal Gene Transfer from Novel Aquatic Organisms

Vancouver, British Columbia - January 11, 2006

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Annexe 4 : Liste des personnes présentes

Pêches et Océans Canada : Réunion du groupe d'experts sur les risques potentiels liés à la transmission horizontale de gènes d'organismes aquatiques à caractères nouveaux

Vancouver, Colombie-Britannique - 11 janvier 2006

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