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OTTAWA '92: THE OECD WORKSHOP ON METHODS FOR MONITORING ORGANISMS IN THE ENVIRONMENT

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

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Foreword

This Environment Monograph is the report of the OECD Workshop on Methods for Monitoring Organisms in the Environment, which was hosted by the Canadian authorities in Ottawa on 14-17 September 1992. It has been prepared by OECD's Environment Directorate in collaboration with the Directorate for Science, Technology and Industry.

The background material for the Ottawa Workshop included descriptions of 67 monitoring methods. These methods are presented in a companion document, *Compendium of Methods for Monitoring Organisms in the Environment* (Environment Monograph No. 91).

The Workshop report was reviewed the Group of National Experts on Safety in Biotechnology. Subsequently, the Joint Meeting of the Chemicals Group and Management Committee of the Special Programme on the Control of Chemicals recommended that it be derestricted. It has been made public under the responsibility of the Secretary-General.

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Résumé

Sur invitation des autorités canadiennes, un atelier de l'OCDE sur les méthodes de surveillance d'organismes dans l'environnement a été tenu à Ottawa du 14 au 17 septembre 1992. Il y avait 99 participants provenenant de 13 pays Membres de l'OCDE et de la Commission de l'Union Européenne à cet atelier.

Ce rapport est composé de quatre sections dont la première contient les présentations faites lors de l'ouverture de l'atelier ainsi que la description des objectifs de l'atelier. Les objectifs étaient les suivants :

1) d'identifier les conditions dans lesquelles une surveillance est pratiquement réalisable et efficace d'un point de vue scientifique et de cerner les situations ou tel n'est pas le cas ;

2) de recenser les différentes méthodes de surveillance disponibles et de présenter les options à prendre en compte ; et

3) d'examiner dans quelle mesure les différentes méthodes peuvent être appliquées.

La deuxième section contient les conférences qui ont été faites, entre autres la conférence d'ouverture dans laquelle la nature pluridisciplinaire de la surveillance ainsi que les problèmes de communication qui en decoulent ont été mis en valeur. Les autres conférences traitent de divers aspects importants de la surveillance, des objectifs des différentes methodes et de leurs champs d'application : micro-organismes, plantes et animaux. Certains thèmes communs reviennent dans la plupart de ces conférences. Il en est ainsi de la nécessité de choisir une méthode appropriée au problème, de la bonne définition du problème et du choix de la durée de la surveillance.

Les rapports des trois groupes de travail, micro-organismes, plantes et animaux, sont rassemblés dans la Section III. Les groupes de travail ont examiné les méthodes de surveillance par rapport à un grand nombre de critères tels que : sensibilité, reproductibilité, fiabilité, facilité d'application, exigences en matière d'équipement spécialisé, temps nécessaire à l'identification des marquers, coût, et aspect qualitatif ou quantitatif des résultats.

La Section IV comprend 23 présentations qui étaient affichées et également plusieurs présentations brèves faites au cours de l'atelier.

En réponse à un questionnaire diffusé avant l'atelier, 67 méthodes, décrites en détail, avaient été soumises. Ces méthodes sont disponibles dans un document servant de complément à ce rapport. Il s'agit de la Monographie sur l'Environnement N^{o} 91, **Compendium of Methods for Monitoring Organisms in the Environment**.

Des projets antérieurs du présent rapport ont été soumis pour commentaires au Groupe de l'OCDE d'experts nationaux pour la sécurité en matière de biotechnologie (GNE) en novembre 1992 et mai 1993. Par la suite, la Réunion conjointe du Groupe des produits chimiques et du Comité de gestion du Programme spécial sur le contrôle des produits chimiques a recommandé la mise en diffusion générale du rapport. Le présent document est mis en diffusion générale par le Secrétaire général sous sa propre responsabilité.

I. Introduction

Welcoming remarks

This was the second OECD Workshop to consider methods for monitoring organisms in the environment. The first Workshop, held in Copenhagen in 1991, developed a "snapshot" of the state of the art of monitoring organisms in the environment.[1] This second Workshop was aimed at developing an overview and critique of monitoring methods, from a scientific perspective, as well as of the circumstances in which they could be applied.

In his welcoming address, **Mr David Hendrick, Director-General of MSPD, Agriculture Canada,** welcomed the delegates on behalf of the Government of Canada. He pointed out that biotechnology was an important strategy for the economic growth of Canada. Since 1983, the National Biotechnology Strategy had provided a federal impetus to encourage the development, application, research and commercialisation of the emerging Canadian biotech industry. Over 300 companies were now actively involved in research to develop new products and processes based on biotechnology. Approximately 10,000 products were under development in the medical, agricultural, forestry, mining and waste management sectors.

Mr Hendrick went on to point out that, under the umbrella of this strategy research, networks comprising academics, government researchers and industry collaborators had been established, and that these networks promoted alliances and cooperative ventures in various key sectors.

At the same time, a strong national regulatory framework was under development to ensure that field releases and commercial products would not have unacceptable effects on the environment. This framework would also reaffirm a common regulatory approach for the federal departments involved. Canada had chosen to regulate on a product basis. This allowed the use of existing legislation as the basis for regulating biotech products. For example, novel food guidelines had just been issued for public comment and debate by Health and Welfare Canada. The first commercial foods were expected to be submitted for regulation within the next twelve months.

In the agriculture sector over 200 small-scale field trials of genetically engineered crops, mostly canola, flax, potatoes and soybeans, were approved during the field season of 1992. This was likely to be followed the next year by pre-commercial trials on a large scale. Environment Canada was now entering its last public consultations on biotech products released to the environment. All departments were developing risk-based strategies to regulate the new products.

Mr Hendrick emphasised that monitoring was an essential component of both research and regulatory activities. The opportunity to discuss methods for monitoring organisms released to the environment would allow us to draw from existing methodologies for traditional organisms and to adapt or change them as necessary.

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[1] See OECD Environment Monograph No. 52, *Report of the OECD Workshop on the Monitoring of Organisms Introduced into the Environment: A Record of Discussion* (Paris, 1992).

Finally, he pointed out the importance of compiling reference material which would provide researchers and others with a range of options, as well as scientific considerations, to be taken into account when developing monitoring programmes. This would be a most useful addition to the broad science base on this subject.

In his welcoming address **Dr Terry Walker, Industry, Science and Technology Canada,** picked up the theme of the National Biotechnology Strategy of Canada, stressing the relevance of the OECD for discussing safety matters in biotechnology. He pointed out that the development of guidelines and principles for the regulation of biotechnology products in harmony with those of Canada's major trading partners was a major aspect of the Strategy.

The objectives of the Workshop

In outlining the objectives of the Workshop, **Dr Jean Hollebone, Chair of the Workshop**, began by referring to the previous OECD Workshop on the Monitoring of Organisms Introduced into the Environment, which was held in Copenhagen 1990. She pointed out that this had been the first OECD forum which examined general approaches and methods for monitoring organisms, particularly genetically modified organisms. The emphasis had been on information exchange between experts from different Member countries with diverse scientific and regulatory backgrounds.

The Ottawa meeting was organised as a follow-up Workshop in which the scientific and technical details could be updated and elaborated upon. Dr Hollebone stressed its timeliness in view of significant changes which had occurred in the intervening two years. First of all, the collective experience in the field testing of genetically modified plants and microorganisms was much broader. In Canada alone, for example, in 1992 testing proceeded at over 200 sites across the country as opposed to 26 in 1990. The number of different traits, and the range of organisms, had also increased significantly.

Secondly, many researchers were now moving from small-scale field tests to larger and multi-site testing. This was a prelude to varietal registration and pre-commercial performance testing. Hence, there was a movement from small contained trials, where experimental parameters could be carefully monitored and controlled, to larger trials. The need for appropriate methodologies to determine environmental fate and impact and to design, evaluate and monitor field tests in the environment was now a practical reality.

Dr Hollebone pointed out that the first objective of this meeting was therefore to encourage open discussion and dialogue in order to share approaches and experiences which ultimately could lead to harmonized international approaches. In order to achieve this, the Workshop was designed around the experts to ensure that they had comprehensive input into the working groups and poster sessions. This should enable practical details to be fully discussed, possible solutions proposed, and the most up-to-date information exchanged.

Dr Hollebone stressed that in addition to many of the methods that had been recently applied to the monitoring of genetically modified organisms, there were numerous methods that had been employed in traditional agricultural practices, either in routine environmental monitoring for unmodified organisms or in pure research. Some of these methods may not have been applied as yet to products of biotechnology with environmental implications, but they were an important source of information.

In highlighting the major objectives of this Workshop, Dr Hollebone began by stressing that it was not a regulatory meeting; that is to say, it was not designed to provide regulatory protocols for determining the fate and impact of genetically modified organisms in the environment. However, useful information might come out of the discussions, which could be taken into consideration in the development of regulatory criteria. In addition, Dr Hollebone stressed that the monitoring methods discussed were not meant to be prescriptive in any way.

Decisions about the use of monitoring would depend on three major considerations:

1) our knowledge of the organism, the trait transferred, whether the organism is genetically modified, the host donor and vector, and our familiarity with the final product;

2) the characteristics of the introduction site, that is, the environmental profile, climate, soil, ecology of the site, etc.; and

3) the introduction profile, that is, the specific experimental design appropriate to the experiment, i.e. size, frequency of application, number of sample plots and so forth.

The purpose of this Workshop was to provide scientists and regulators with a set of options from which they might choose or design the most appropriate monitoring method, should monitoring be required. The major objectives of the Workshop were threefold:

1) to identify the various situations under which monitoring is practical and effective from a scientific point of view, and those situations under which it is not;

2) to identify the various monitoring methods available, that is, the range of options available for monitoring; and

3) to determine the applicability of the various methods in order to make this determination.

Many of the key components that should be evaluated before determining the methods' applicability will relate to factors such as the sensitivity of the method, the ease of use or practicality, the requirement for specialized facilities or equipment, the time required to identify markers, the specificity and reliability of the methods, the costs, and any limitations imposed by the method. In addition, the experimental design and purpose of the monitoring are important, because purpose will determine the level of precision that will be required in many cases.

Finally, Dr Hollebone suggested that two key products could come from the Workshop: firstly, a set of proceedings; and secondly, a compendium of methods where the various methodologies would be collated.[2]

[2] See OECD Environment Monograph No. 91, *Compendium of Methods for Monitoring Organisms in the Environment* (Paris, 1994).

II. Formal Presentations

The views expressed in these papers represent the opinions of individual authors. They do not necessarily represent the views of the OECD or of Member countries.

The Keynote Presentation

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The purpose of this meeting is to analyse the available methods and methodologies for monitoring transgenic organisms in the environment. I was asked to set the scientific background, particularly the broad scope of the problem. Dr Hollebone reviewed the objectives of this meeting in the light of the previous Workshop as well as the results of the monitoring questionnaire; and you yourselves have extensive monitoring expertise.

I'll use this opportunity to remind you of some of the problems with effective monitoring and the context in which monitoring will be conducted, which were well described in the previous OECD Workshop and the major important issues raised there. At that time a "broad" definition of monitoring was invoked. For this meeting, we must consider what is meant by monitoring and know where we are using different definitions.

Different goals of monitoring are not always going to be possible from the same protocol. Thus, choices must be made and the primary goal of a particular study must be identified. Moreover, I think monitoring can mean different things to different people.

My own reaction is to think of it beyond field tests, to when the organism is in commercial production. Perhaps that is because I see monitoring before that stage as requiring simply that we apply our expertise to the problem, while meaningful monitoring of commercial transgenics seems impossible. Although it is comforting to view commercial production as the end to regulatory responsibility, the public will hold many portions of government responsible if the organism misbehaves after it is approved for production. Responsibility for public safety does not stop with commercialisation even though many monitoring problems get harder. Thus, we need to keep in mind whether a particular approach is appropriate at some or all stages of the process, as well as to the goals of the monitoring study.

Let me bring to your attention at the outset that there are multiple players in this game. That they have different agendas is obvious, but they also have different "spontaneous reactions". Each group has different stereotypic reactions to the problem, of which all should beware. The precise set of players involved in each monitoring scenario will differ, but I assure you that academics will want to study it forever, industry will be concerned about the financial bottom line, legislators will want laws to govern monitoring, regulators will claim jurisdiction, etc. Working across professional boundaries means learning to get the result you want from another group: to get applied answers from research academics requires finding a way to ask the question correctly. Certainly, never ask "Is more study required?". Perhaps "Which needs more study, maize (*Zea mays*) or sorghum?" or "Should we study reproduction or dispersal first?" might work.

Secondly, placing transgenic organisms in the environment is, by definition, a subject that cuts across disciplines. When talking across disciplines, one finds that each field has amazing levels of jargon and shared beliefs. Everyone should be as explicit and jargon-free as possible. A plant pathologist found dozens of ecological and evolutionary terms in a paper I thought was "public access"; I could not believe that a microbiologist did not recognize the word "introgression". Phil Regal from the University of Minnesota has a number of excellent papers pointing out that there has been a continuous change in how ecologists and evolutionary biologists view the world. You would no more use your 1970 understanding of ecology than use your 1970 understanding of genetics.

Everyone should be careful to be explicit, look for assumptions, and make sure real communication is going on. That sounds like platitudes, but it is critically important for writing monitoring schemes that will be used by academics or industry, and interpreted by regulators, that the strengths and weaknesses of molecular biology and genetics, agriculture or environmental science, and evolutionary ecology are integrated simultaneously.

Monitoring must of course be set into a context. Why are we monitoring? Every one of the diverse group of participants I alluded to above has a different answer to that question. The reactions to new genetic technologies range from:

- It is totally safe technology;
- It will transform life as we know it, greatly improving it;
- It is unnecessary and frivolous; and
- It is risky (highly dangerous) to the quality of life.

How can the same thing be seen so diversely?

Let me remind you of some of the facts underlying these views:

- Plants, animals and microbes are biodegradable;
- These are familiar organisms, with truly minor changes;
- Plants, animals and microbes can increase in number, disperse and evolve; and
- Every technology has had serious downsides (the automobile, a technology we'd never give up, causes many problems: why should this technology be different?).

Probably all these views are true, which is why we are here! This Workshop is to come up with effective monitoring designs. But let me remind you that it is a no-win situation. If the monitoring is effective, there will be no (major) problems and we'll never know what the unregulated biotechnology would have been like. So your efforts will be unappreciated because it will seem there was no risk. If inadequate monitoring leads to discomfort or suffering, then of course the technology and monitoring will be strongly criticized and the criticism received will be warranted! So the rewards are blame or no credit: not a promising role.

Next, I would like to make a few comments about where monitoring fits into the whole process. We are caught between "why be so careful" and "have you checked for the impact on trout, pine trees, maples, deer flies, etc...". Monitoring requires clarity about what is being

sought, such as: to detect the escape of genes to related species; to determine if microbes spread outside the field; or to determine if viruses have alternate hosts. For clear goals it is easier to design sound protocols because the statistical tests are more satisfactory. If the goal is clearly stated, anyone doing the monitoring will recognize a problem and there should be no need to argue about what the result means.

However, since it is new technology, it has never been exposed to "the environment" before. Herein lies the paradox. On one hand, new problems cannot be predicted and on the other hand if we can predict problems, they are not new. Wide-ranging, open-ended monitoring is probably the way to detect new or unique effects of genetic engineering. Yet such monitoring is expensive in time and money. It is also inefficient: surely most studies will find nothing at great expense even if a previously unknown problem eventually turns up. I raise this as a problem for monitoring because it expresses the core of our responsibility to the environment.

Success or failure of biotechnology in the environment will be a complex result of a process of which monitoring is just part. You may have noted, from the nuances of the use of the term monitoring, that I associate monitoring with looking for problems.

Molecular biology and agriculture are used to sort through many mutants, throwing out many new varieties looking for a good one. They focus on clear major and immediate effects and are understandably impatient of checking for obscure effects at great expense. However, environmental and public interest groups are worried about protecting the public and small, unique ecosystems that may never be restored if they are seriously perturbed. So they will err on the side of caution and evaluate minimal differences between, for example, an original and transgenic organism. It will be difficult to develop monitoring schemes that will please both extremes. Economic development also needs to be reconciled with environmental safety when developing monitoring protocols satisfactory to both extremes.

Recognition of the resistance to biotechnology and the need for caution about its consequences is clearly stated by the mere fact of this meeting. This thought should be with us all as we consider studies that repeatedly show this technology to be benign. However, the need to provide cost-effective monitoring to prevent a "logjam" of excessive or overcautious regulation must be recognized.

The monitoring challenge is also going to scale-up with time as the rate at which organisms reach field testing increases, the diversity of organisms and the environments for which they are intended increase, and the uniqueness of the organisms reaching field testing increases. Currently, transgenics are only one or two genes away from naturally occurring mutants. Some do, in fact, occur naturally. But if the technology is half of what it is described as offering, the future will bring quite novel organisms to monitor in multiple environments.

These monitoring pitfalls should be avoided:

- Too much or too little effort is put into monitoring;
- It is unclear what you are looking for;
- Monitoring doesn’t go on "long enough"; and
- There is no appropriate mitigation available.

Let me illustrate these by considering the issue of weeds:

1) Too much or too little effort: Transgenic maize is going to be an important product. Maize itself is not a weed or weedy by almost any definition (a few will come up in a fallow field in the year following a maize crop, but they do not persist) so energy spent monitoring maize as a weed is time and money wasted. Conversely, studies of pollination by bees show that they fly distances that reflect the distance between flowers. So a standardized monitoring design that only samples within a 100 m radius when there are numerous compatible weedy relatives within 300 m is going to be insufficient energy in monitoring, even though adequate sampling of the larger area will require significantly more effort.

2) Unclear goals: The term *weed* is variously defined, so to make the problem more manageable, we would like to prevent "weed problems". But weed problems range from those that employ dozens of people in every agricultural research group to plants that annoy a few homeowners. Governments have ways of deciding when a weed is important enough to warrant research time: can monitoring reflect that? Is it simply pressure from the public that moves researchers to new plants, and if so, how does the public decide that there is a "weed problem"? How many trials are necessary until a crop is deemed safe? What of monitoring after commercial release?

3) Monitoring does not go on "long enough": Populations of all species are capable of exponential growth if unchecked. The problem is how long it takes to get to "problem" densities. What if the time frame is decades? Purple loosestrife (*Lythrum salicaria*) has contributed to considerable environmental problems and to changing aquatic ecosystems in the northern US in a variety of undesirable ways. It took decades to build up to those numbers.

4) What response will be given to what outcome: While problems with purple loosestrife were seen in local areas long ago, as recently as the summer of 1991 letters to *Horticulture* magazine objected to removal of purple loosestrife from US nurseries. At least some of the public still want it. My point is that agreement that it's "out-of-hand" is not going to be easy or uniform. Monitoring protocols should come with definitive statements of what is to be done if specific outcomes are detected. A wide range of "it depends" is reasonable, but intolerable changes and planned responses should be defined.

In conclusion, the Workshop organisers have laid out specific questions and criteria for evaluation of methods. You can see I am concerned about subtle and long-term effects. I would argue for long-term monitoring, and yet, despite laying expensive ecological worries before you, I think that most of the problems to be monitored for are, and will be, less difficult or dangerous than current problems from chemical technology. Therefore, if it is possible to allocate monitoring effort in relation to perceived hazard (absolute and in comparison to other alternatives), both advancement of the technology and public safety would be fostered.

Genetic engineering offers us solutions to important human and environmental problems. As in all technologies, undesirable consequences are likely, thereby presenting a monitoring problem of immense complexity. Yet, despite this difficulty, the simultaneous pressures of public safety and the promise of this technology present us with this challenging problem (which I am, as an academic, delighted to discuss endlessly just for the fun of it). It is with the hope of realistic solutions that this international Workshop has been convened, and so I will end with my title, "Toward a Safe Genetically Engineered Future", expecting this Workshop to move us forward significantly.

Monitoring Rhizobium in the Environment Using PCR

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Rhizobia fix nitrogen in a symbiotic association which takes place in nodules on the roots of legumes. The interaction is very beneficial to farmers since nitrogen is supplied to the crop without the cost of chemical fertilizers. This type of bacterium can be easily isolated from nodules, but cannot be directly cultured from the soil. It is also difficult to identify particular strains once they are isolated from nature. Since rhizobia are excellent candidates for genetic modification to increase their benefits to agriculture, it is important to derive sensitive and specific methods for their detection in the environment. This talk will describe the use of polymerase chain reaction (PCR) as a monitoring tool for this bacterium. The techniques are relevant to field studies of rhizobia, monitoring genetically engineered bacteria, and, in general, to tracking microorganisms in the environment.

The method

PCR is a method by which a defined segment of DNA present on a small amount of template can be specifically amplified. The procedure requires template DNA, short oligonucleotide primers which are complementary to the ends (5'-3') of a short (approx. 500 bp) segment of the template, a thermostable polymerase such as Taq DNA polymerase, and deoxynucleotides and buffers appropriate for DNA synthesis by extension of the primers when annealed to their target template. The reaction is done in a temperature cycler in which the temperature is changed in each cycle to successively melt the DNA at high temperature, and then to permit primer annealing and extension at relatively lower temperatures. With each such cycle the number of short target DNA molecules is doubled, such that after about 25 cycles the target sequence will predominate to the extent that it can be readily detected. The procedure is very sensitive since only a few template molecules are required to initiate the synthesis reactions. It is highly specific because the primers will only anneal to specific locations, complementary to their sequences, on the template.

To apply PCR to detection of a bacterium in the environment, a sample is taken from the field, such as soil or a nodule, and processed to generate a sample of template DNA for the PCR reaction. If the bacterium being monitored is present in the sample, and if the PCR primers are specific for a small segment of its genome, than a positive PCR reaction will occur. The processing method to prepare the template is critical to the success of the method and will vary to optimise its recovery. For *R. meliloti* in soil we have used a microfuge tube procedure in which the bacteria are extracted from the soil, then broken by sonication or glass beads. The crude preparation containing soil microorganism DNA is then used as a PCR template. For nodules, the bacteria are extracted from crushed nodules, but sufficient free DNA is present to serve as a template without mechanical breakage. To obtain a semiquantitative estimate of the number of cells in the sample, a series of ten-fold dilutions of the template are made prior to the PCR reaction. The production of an amplified DNA fragment is detected by agarose gel electrophoresis.

The PCR primers determine the specificity of detection of the target bacteria. For unmodified bacteria, primers are readily derived which will detect bacteria at the species level. For example, we have used primers complementary to two 20 bp sequences in the *nod* C gene of *R. meliloti* in order to nonspecifically detect *R. meliloti* strains in field samples. To detect a specific strain of unmodified bacteria it is necessary first to identify and characterise a portion of its genome having a unique sequence, in order to design appropriate specific primers. A more direct approach is deliberately to mark a strain of interest by modifying it to contain a unique sequence for PCR. When properly chosen, the inserted DNA will enable the detection technique to be absolutely strain specific. The DNA introduced may be genetically active, such as when used for a genetic modification, or inactive, if its sole purpose is to serve as a sequence for detection by PCR. However, another consideration in strain marking is that generally we are unable to prepare modified microorganisms without leaving selective markers in the genome, such as antibiotic resistance genes, as a necessary consequence of the manipulations.

Field testing of a marked *R. meliloti*

This monitoring technique has been field tested by using it to detect a marked *R. meliloti* strain in soil and nodules from alfalfa plots. Since the marking procedure is a genetic modification, the field release was subject to Canadian regulations concerning the release of genetically engineered microorganisms in the environment. This proposal to use a modified *R. meliloti* in field experiments was used as a test case for the Canadian regulatory system, and involved joint consideration of the application by all Canadian government agencies concerned with the use of microorganisms in the environment. Official approval for the release was obtained from the F. P. and I. Branch of Agriculture Canada.

A *R. meliloti* strain, R692, was derived by insertion of a 1.7 kb DNA fragment from transposon Tn903 into the *nod* megaplasmid between the *nif*HDK and *fix*ABC operons. The *nod* megaplasmid is a very large plasmid (about 1500 kb) which is nonconjugative and stable. The DNA fragment was inserted at a location between genes such that there was no loss of any genetic function. It was introduced by techniques that select for an homologous recombination event into the genome, such that there were no cloning vehicles left in the final construct. The only other genetic change was the introduction of a kanamycin resistance gene because of the need to select the insert during the strain construction. This resistance was not otherwise used, since it was too low for selection of the bacteria from field samples. For monitoring, only the PCR detection technique was used to detect the insert.

R. meliloti R692 was used for field experiments on the Central Experimental Farm in Ottawa in the summer of 1991. The bacteria were sprayed onto freshly seeded alfalfa plots to obtain soil titres ranging from 10^0 to 10^7 bacteria per gram of soil. The plot was sampled at weekly intervals to test for bacteria in the soil by the PCR technique. Nodules were sampled from the plants when established (four weeks) and tested using the same detection procedure. The experiments have revealed that the titre of the bacteria in the soil decreased rapidly after inoculation, dropping by about a factor of ten per week. Horizontal movement of the bacteria was undetectable by analysis of soil samples in and adjacent to the plot. Very limited movement was detected by analysis of nodules from plants adjacent to the sites where the bacteria were applied. Tests of vertical location of the bacteria in soil cores showed that the bacteria were initially dispersed to a depth of about 18 cm, and subsequently retained viability in a region 2-8 cm from the soil surface. Nodule formation and occupation by the marked *R. meliloti* was found to increase with the amount of the inoculum applied to the soil. It was found that 10^4-10^5 bacteria per gram of soil was sufficient to give maximum number of nodules per plant and to result in 70-90 per cent occupancy by the marked strain. As few as ten bacteria per gram of soil resulted in the detection of the marked strain in nodules.

This field test demonstrated that PCR is practical for use in monitoring microorganisms in the environment. Application of the technique did not require culturing of the microorganism, and the presence of a short marker DNA segment in the genome of the target bacteria was sufficient to render it detectable amidst a large, heterogenous population of soil microorganisms.

Present research to improve the marking and monitoring techniques

Our laboratory and field experience with marking and monitoring *R. meliloti* has demonstrated three areas of research that can be expected to improve the utility and sensitivity of the technique. These areas are: a) development of methods and vehicles for marking strains rapidly and without leaving unwanted genetically active DNA; b) deriving methods to synthesise and clone synthetic DNA which can be introduced into bacteria to mark them; c) improvement in the sensitivity of detection of template DNA in soil by identifying and removing a potent inhibitor of the PCR reaction. The following is a description of our present research in these areas.

In order to make changes to bacteria without leaving markers we have tested the use of plasmid vehicles which, when introduced into bacteria, cause a defined change in the genome by homologous recombination and then leave the cell together with all markers. For marking, the change to be effected is the insertion of a unique, genetically inactive segment of DNA for detection by PCR. The introduction is effected by incorporating the desired segment into the middle of DNA which is homologous to the desired site of insertion using recombinant DNA or DNA synthesis techniques. The insert, bracketed by homologous DNA, is introduced into *R. meliloti* within a plasmid carrying a selectable marker, tetracycline-resistance (Tc^r). By using a ColE1-based plasmid, such as pBR322, which cannot replicate in *R. meliloti,* selection for Tc^r resistant bacteria results in colonies in which the plasmid has recombined into the genome by a single crossover between the homologous DNA segments on the plasmid and in the genome. The result is an integrated plasmid bracketed by the native and introduced homologs, the latter containing the insert sequence to be introduced. The plasmid also contains a *sac*B gene which encodes the enzyme levansucrase. In the presence of sucrose the enzyme catalyses the synthesis of branched-chain fructose polymers (levans) which are lethal to the cell. By plating on media containing sucrose, and with Tc^r selection removed, a second recombination event which reverses the integration is selected for. Depending upon the location of the second crossover, the insert can be left in the genome, while the plasmid is lost. The net result is the recombination of the insert into the selected genomic location without leaving the plasmid or its markers. We have used this technique to insert inactive synthetic DNA into the genome that has been subsequently used for detection by PCR. Our initial testing of this approach used a plasmid construction in which the *sac*B gene was added after the homologous DNA segment. We are presently building a vehicle of this type which is ready to use except for cloning in the desired homologous segment.

To mark a strain it is necessary to insert a unique DNA fragment. In most cases, as for the Tn903 fragment described above, a DNA fragment derived from an unrelated source can be used to serve as a unique marker. Another source is to use synthetic DNA, since as little as a 20 bp insert to serve as the site for one primer could serve to mark a strain. Automated DNA synthesisers permit the synthesis of larger DNA fragments so that the homologous DNA segments to bracket the insert can also be synthesised. In our experiments we have synthesised overlapping single stranded DNA fragments and used PCR to generate double stranded DNA from them for cloning. In this way we produced a 500 bp fragment containing cloning sites in the ends of the fragment, and two 140 bp segments homologous to a continuous 280 bp internal segment of the *R. meliloti* chromosome. The fragment also contained a central 145 bp segment of inactive DNA for which we produced several primers in different orientations. This fragment has been cloned into the *sac*B-Tc^r plasmid described above, and subsequently the system was

used to introduce the 145 bp insert into the genome. We have demonstrated that the resultant strain can be specifically detected by various combinations of internal and external primers. Further work must be done on the use of synthetic DNA for marking and strain modification to determine parameters such as the minimal sizes of homologous DNA that are required to effect the two recombinations in this system.

A third area of research involves the preparation of template DNA samples from soil. Despite the potential of PCR to detect one to ten cells in a sample, we have not achieved sensitivities of detection of less than about 10^3 bacteria per gram of soil. The difficulty is that there is a potent inhibitor of the PCR reaction present in soil, requiring that template DNA samples be diluted 10^2 to 10^3-fold to dilute out the interfering substance. To identify the inhibitor, we used PCR reactions spiked with template to give positive reactions, and assayed soil fractions to identify those which would cause inhibition. In this way we have purified a high molecular weight, brown substance that is the PCR inhibitor in our soil extracts. This inhibitor is probably the same as has been reported by other laboratories using DNA from soil, since we have isolated it from diverse soils, and its characteristics appear to correspond with published information. The substance sticks to microorganisms during their removal from soil, and subsequently binds to DNA after lysis of the cells. We are presently characterizing the inhibitor to determine methods to remove it during the preparation of the template DNA.

In summary, I have described the application of methods using PCR for the sensitive and specific detection of microorganisms. The techniques include methods for preparing template DNA from field samples, and also for marking strains for their detection. It is hoped that these experiments and approaches will contribute to the goal of safe and effective use of microorganisms in the environment.

European Botanical Files in Relation to the Safety of Genetically Modified Plants: A Case Study in the Netherlands

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1. Summary

A numerical code on the exposure of indigenous wild plants to gene flow from crops has been developed, indicating the level of intensity which is necessary for monitoring transgenetic crops.[3] Observations on introductions (escapes and casuals) from 40 cultivated species found in the wild flora have been presented from the archives and herbarium collections present in the State Herbarium.

2. Introduction

Since 1902 the State Herbarium of the Netherlands (Rijksherbarium, Leiden) supported and managed the monitoring of the wild flora of the Netherlands. The results are the three volumes of the *Atlas of the Netherlands Flora* (Mennema et al. 1980, Mennema et al. 1985, Van der Meijden et al. 1989), depicting the monitoring results for the period 1902-1949 and 1950-1979. It is also available as a databank on a grid scale of 5 x 5 km^2. For the period 1980-1989 we now have ready a databank on a grid scale of 1 x 1 km^2, containing 3.5 million observations. All observations in both databanks can be tracked down, either by annotated field notes or by herbarium specimens.

As a by-product of these activities an enormous amount of observations became available of records from non-indigenous species found in the wild, like casuals and escapes. When it became apparent that the Ministry of Housing, Physical Planning and Environment (VROM) wanted to have facts on gene flow from cultivated plants to the wild flora, we suggested that we would study the existing archives and herbarium specimens. Should this succeed, then the accumulated results from a period of *one century* would become available. So we started our job to make Botanical Files of 40 cultivated species, most of them crops (De Vries et al., 1992). This selection does not mean that there are actually plans to produce GMOs in all species; the files merely give background information for the government commission called VCOGEM, which has to deal with problems of risk assessment for GMOs.

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[3] See also Summary XXII, "Numerical codes for considering biological properties of host organisms in particular environments," on page 102 of this Workshop report.

3. Starting points and definitions

3.1. General

The first questions are: what is monitoring, and how can it be done? The subject of our study is the host plant, not the GMO itself nor the insert. We understand monitoring plants as: checking the occurrence of changes in the behaviour of (host) plants.

The necessary knowledge is:

- taxonomy of the host plant; it is for instance not advisable to define "canola" as a monitoring subject, but specifically either *Brassica rapa* (= *campestris*) or *Brassica napus*, because both species behave differently in escapability characters;
- ecology of the host plant;
- distribution area and density;
- dispersal/gene flow properties.

Apart from this, you need a series of observations in time.

Important questions to assess the value of monitoring host plants are the following:

1) Are there enough records of introduction in literature and herbaria?
2) What is meant by nature?
3) What are the criteria for effective gene flow from culture to nature?
4) What are the pros and cons of this study?
5) Can this study be done for larger areas than the Netherlands?

3.2. Herbaria as sources of information

It is important to give some information about herbaria. Our Dutch State Herbarium in Leiden contains about 3.5 million specimens from all over the world. From these we have ± 300,000 "wild" specimens from the Netherlands, belonging to ± 2,500-3,000 species (of which 1448 indigenous). The specimens are selected in a rather peculiar way. First they are selected by a plant collector, who is in most cases an expert. Plant collectors mostly collect only rare plants, like rare indigenous plants, spontaneous hybrids (which have to be checked), rare escapes, casuals. Also the curator of an herbarium is selective in including specimens in the large herbarium collection. As a result, in most large herbaria rare plants are strongly over-represented. This makes herbaria useful as sources of information on gene flow from culture to nature. The species concept used is the so-called modern morphological species concept.

3.3. Gene flow

Gene flow is possible by:

- diaspores (propagules; mostly seeds);
- pollen; gene flow by pollen can be divided into two categories:
    - intraspecific: by crossing events;
    - interspecific: by hybridisation.

3.4. Effective gene flow: the viability criterion

A very important criterion for what effectively may happen in nature is the criterion for a plant to belong to the Netherlands' flora. We followed the criterion developed for our definition of "indigenous" plants from the Check-list of the Netherlands' flora (Van der Meijden et al. 1990, that is: the viability to survive in the Dutch climate. How can we ascertain this? In general, we need to have observations of three successive generations, in three distinct localities.

What are the consequences of this criterion for monitoring gene flow from culture to nature?

1) Experimental hybrids are of no significance: only those hybrids that are actually observed in nature are of importance;
2) Wild relatives that cross or hybridise with the cultivated species are under scrutiny; no other wild relatives.

3.5. Circumscription of "nature"

Nature is part is the environment. Which habitats do we include for our purpose? There are the following possibilities:

1) all ecosystems (including cultivated fields);
2) all ecosystems except artificial habitats (cultivated fields, gardens, plantations);
3) all ecosystems except artificial and unnatural habitats (urban habitats, waysides).

If you choose the latter, 85 per cent of the surface of the Netherlands falls out of the scope. The first circumscription is obviously too wide. So we chose the second one.

4. Results

4.1. Hybrids in the flora of the Netherlands

Concerning events of gene flow by pollen: what do we know about the distribution of spontaneous hybrids in the wild flora of the Netherlands? The number of Check-list species amounts to 1448. During the last two centuries, 228 interspecific hybrids have been observed (once or more), none of them viable. The number of viable hybrids (i.e. hybrids that have established) is ten, of which five hybrids have only vegetative dispersal, and five also have a partial generative dispersal. It is interesting to note that of the thousands of ornamental hybrids cultivated, no escapes are recorded at all (the one established ornamental hybrid, *Helianthus rigidus* x *tuberosus*, is probably an introduction from North America).

4.2. Introductions into the wild flora

Concerning events of gene flow by propagules, we present the following numbers of diaspores introduced into the Netherlands:

Sources:

- by trade: each year billions of non-indigenous seeds;
- by cultivation: seeds of 2000 ornamental herbaceous species;
- by cultivation: seeds of 1500 woody species.

From our archives and the herbarium (over 200 years):

- not viable introductions: 1000 species of casuals and 1000 species of escapes;
- viable: 220 species (= 16 per cent of the total number of indigenous species).

4.3. Summary of results

Thus, the use of the viability criterion means that:

- of all hybrids observed in nature, 228 are not viable and only ten match the criterion;
- of all seeds of originally non-indigenous species, only 220 species have succeeded in becoming part of the flora of the Netherlands in the last 200 years.

One of the conclusions may be: Holland is extremely "chilly" to immigrants.

5. Code for gene flow

To bring a kind of standardization in the data on gene flow, we developed a numerical code, the Dpdf-code. *Dp* concerns pollen dispersal, *Dd* dispersal of propagules, and *Df* gives the possible frequency of gene flow to nature.

5.1. Dispersal of pollen

The summarized code for Dp (dispersal of pollen) is:

0 = no crossing or hybridisation possible in the absence of wild relatives
1 = no crossing or hybridisation observed with any wild relative
2 = crossing or hybridisation observed with a wild relative only on experimental sites
3 = ± sterile hybrids observed in nature
4 = crossing with conspecific wild populations must be expected (cannot be detected by normal morphological methods)
5 = both crossing and hybridisation will occur in nature
9 = crossing or hybridisation events uncertain

5.2. Dispersal of propagules

The summarized code for Dd (dispersal of propagules) is:

0 = no escapes observed in nature
1 = no viable off-spring observed
2 = temporary establishment (mostly vegetatively)
3 = viable introductions observed
4 = escapes untraceable morphologically because conspecific relatives grow in the neighbourhood
9 = escapes doubtful

5.3. Frequency

The summarized code for Df (frequency) is:

0 = no wild conspecific populations in nature
1 = wild populations rare
2 = wild populations not common
3 = wild populations very common
9 = distribution wild populations uncertain

This code means for instance that if the wild populations are rare, the possibility for crossing or hybridisation is low.

6. Examples

We'll give three summarized examples from the Botanical Files.

6.1. Tomato

Tomato (*Lycopersicon esculentum*): Dpdf: 0.2.1

Dp = 0: No wild Lycopersicon species. (Note: If tomato is regarded as belonging to the genus *Solanum*, Dp would be 1, as no crossing or hybridisation has been observed with any of the five wild relatives in the Netherlands.)

Dd = 2: Frequent casual, sometimes shortly establishing.

Df = 1: Lasting wild populations rare.

Conclusion: Very low exposure of gene flow from culture to nature must be expected.

6.2. Beet

Beet (*Beta vulgaris*): Dpdf: 4.1.1

Dp = 4: Crossing with wild subsp. *maritima*.

Dd = 1: Sometimes escaping, not establishing.

Df = 1: Wild subspecies rare.

Figure 1 gives the distribution of wild and cultivated beet in the Netherlands: it shows that beet is cultivated throughout the country, but that the wild populations are only coastal, and rare.

Conclusion: Local exposure of gene flow from culture to nature must be expected.

6.3. Ryegrass

Ryegrass (*Lolium perenne*): Dpdf: 5.4.3

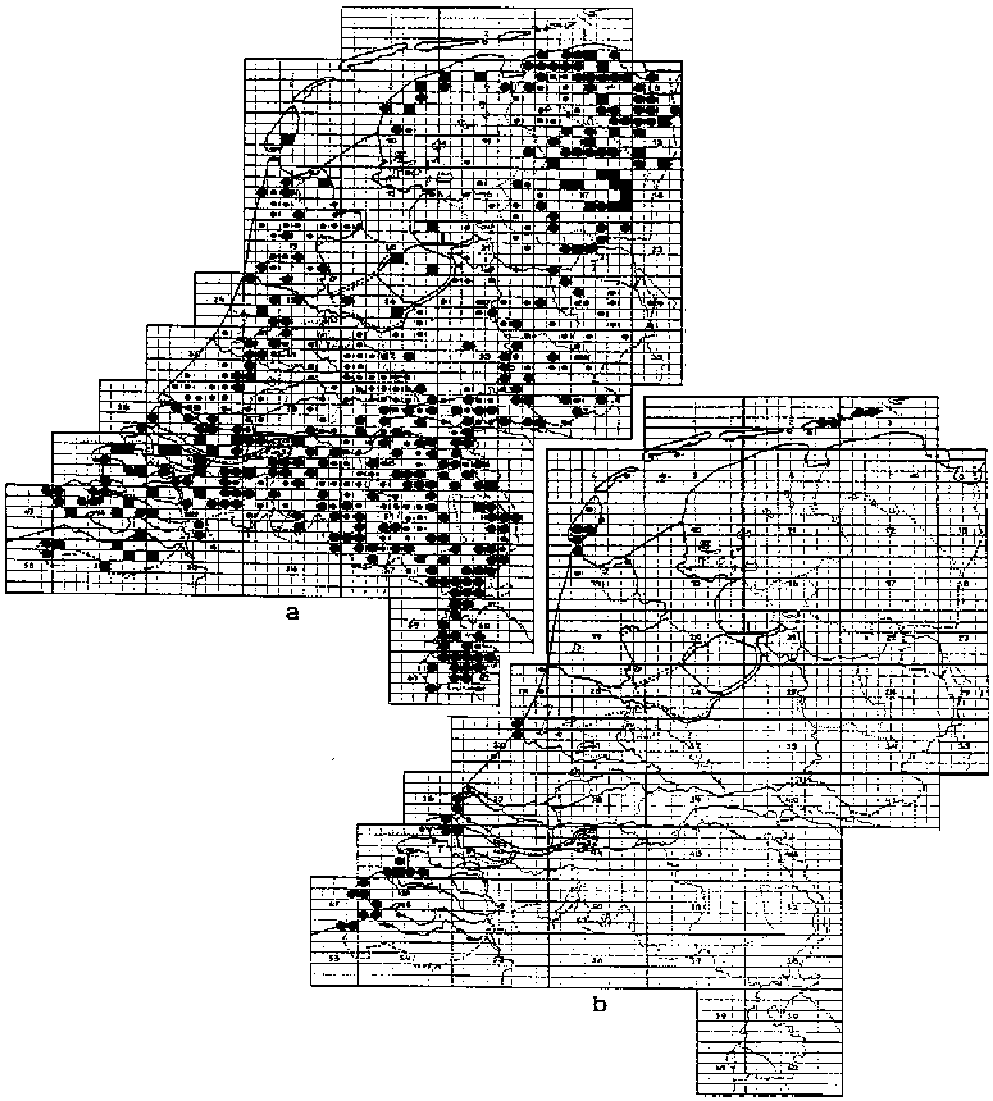
Dp = 5: Crossing with wild *Lolium perenne*, hybridisation with wild *Lolium multiflorum* and with wild *Festuca pratensis.*

Dd = 4: Escapes from cultivation are untraceable.

Df = 3: The wild species is very common.

Conclusion: High exposure of gene flow from culture to nature must be expected.

Figure 1 Distribution of cultivated (a) and wild (b) *Beta vulgaris*



distribution cultivated (a) and wild (b)
Beta vulgaris

7. Results

What are the results of this study of 40 species of cultivated plants?

Low exposure rates are expected in 50 per cent of the cases.

Local: 15 per cent
High: 25 per cent
Further study necessary: 10 per cent

8. Discussion

What are the pros of this study?

1) It is a quick method because underlying facts are already available; our study of 40 cultivated species took 18 months (including publication).

2) It gives a tool to classify "weediness" into a standardized numerical code.

What are its limitations?

1) The viability criterion poses problems with ornamental plants, because the parentage of most ornamentals is not sufficiently known. It also poses problems with woody plants, because they live too long for normal monitoring.

2) It gives no information on GMOs themselves.

3) It gives only information on a part of the questions on risks: only the expected *exposure* of nature to gene flow from crops is presented. In fact, it indicates the *level of intensity* which is necessary for monitoring transgenetic crops.

The most important question is: Can we produce European or North American botanical files? Or: Is the amount of already available information sufficient to make reliable botanical files? We hope the OECD can coordinate future studies on this subject.

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Measuring Gene Flow from Farmed to Wild Fish Populations

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Introduction

Species of animals and plants are composed of populations that are more or less different in genetic characters and life history, and we often refer to the genetic population structure of a species, meaning the distribution pattern of genetic variation within and among the populations. In some species the populations are very similar, and alleles appear to be exchanged rather freely over large geographical areas. Many marine fish species may illustrate this situation. In other species, the populations differ in genetic composition, and the gene flow is restricted (Ferguson 1989).

A battery of biological mechanisms that serve to restrict gene flow among species and populations have been identified (Mayr 1977). Some of these isolation mechanisms operate on the distribution of organisms in space and time, some operate on behaviour, or colour and sound signals, while others are based on morphology, reduced offspring viability and fertility, etc. Many of these isolation mechanisms are associated with developmental costs, and therefore it is suggested that they also represent evolutionary benefits. Accordingly, the organisation of genetic variability into species and populations, and the evolution of isolation mechanisms, are regarded as being of adaptive significance, allowing for development and preservation of adaptive gene combinations.

Following the increased interest in release of hatchery fish in enhancement programmes and ocean ranching, in fish farming, and now in transgenic fish, attention has been focused on the potential genetic and ecological effects of this activity on wild populations, possibly leading to breakdown of isolation mechanisms, increased gene flow among populations, and hybridisation and introgression between species.

The development of Norwegian salmon farming was a great success in many respects, creating thousands of jobs and optimism in the small coastal communities along the Norwegian coast during a period of general economic recession. However, following rapid development, it soon became evident that salmon farming, like most other industries, had impact on the environment. Reference is made to some key figures to illustrate the problems. Norwegian salmon production increased very fast from about 4000 tonnes in 1980 to more than 160,000 tonnes in 1990, while the total annual world catch of wild Atlantic salmon is less than 10,000 tonnes, according to official statistics. The number of wild spawners in Norwegian rivers has been estimated at about 100,000 individuals, while the number of salmon escaping from farms reached about 2 million in 1989. Escaped salmon were recorded in significant numbers in many salmon rivers as well as in coastal areas. As it is known from numerous studies that genetic changes are common in reared fish populations, and that genetic variability is easily lost, there has been a lot of concern about the future of the wild salmon stocks, and the problem has been put on the agenda of both national and international meetings (NASCO/ICES).

Two areas of concern were soon recognized:

1) How can escaped salmon be identified and distinguished from wild salmon?

2) What are the genetic and ecological consequenses of escapement?

With respect to the first question, it turned out to be possible to distinguish escapes from wild salmon by external features, such as fin erosion and scale loss, if the individuals had escaped as adult fish. Fish that escape as juveniles are difficult or impossible to identify. Further, it was found that isomeric forms of components in the fish feed, reflected in the flesh, were useful in distinguishing between farmed and wild salmon.

The genetic and ecological questions associated with the escapement were more complicated. First of all, no diagnostic genetic character was found that could be used to identify farmed salmon and gene flow to wild populations. It was therefore concluded that to evaluate the genetic and ecological effects of escapement, a series of field experiments using genetically marked farmed fish would have to be conducted.

Three studies at the Institute of Marine Research

Genetic marking methods are very useful in fundamental research on gene flow among wild populations and introgression between species, but they also offer good opportunities to study the migration of alleles from cultured to wild populations (Skaala et al. 1990). The method depends on the existence of adequate isozymes or DNA fragments that can be used as markers. The number of isozyme markers will vary from species to species. At the Institute of Marine Research we have identified genetic markers in three fish species, i.e. brown trout (*Salmo trutta* L.), Atlantic salmon (*Salmo salar* L.) and cod (*Gadus morhua*). The purpose for all of the experiments is to estimate the reproductive success of reared fish that escape or are being released, and to estimate gene flow from reared to wild populations. However, the experimental set-ups differ somewhat. Here, I will concentrate on the experiments where brown trout is used as a model species to study genetic impact on wild stocks from farmed fish. We have been using two types of genetic markers: 1) biochemical-genetic markers or isozymes coded by spesific loci, and 2) morphological-genetic markers, i.e. a genetic variation in spotting pattern (Jørstad et al. 1991).

The brown trout is an excellent model species in this context for several reasons. First of all, the brown trout is an opportunistic species. It occupies a great range of different habitats and forms populations with different life histories. This implies that populations tend to diverge, and genetic distance is built up among populations. Further, the brown trout is regarded as one of the most polymorphic vertebrae species, and biochemical genetic markers are abundant. Also, it has been hypothesised that adverse effects of population mixing should be most pronounced and easy to detect in species where a large portion of the total genetic variability is distributed among populations. There are also other practical advantages with the brown trout compared to Atlantic salmon, as the individuals are smaller, robust and also abundant in smaller streams, well suited for sampling and field experiments.

Gene flow and genetic changes can easily be detected by using genetically marked populations. However, we also wanted to know if effects on more ecological parameters of the wild trout population could be detected. This was more difficult, as we did not know what effects we could expect and what to look for. Finally, we decided to have some baseline information on abundance of juveniles, individual growth rates, mortality of juveniles, size and age of smolts, size of eggs, etc.

All three experiments on salmonids, two including brown trout (T1 and T2) and one including Atlantic salmon (S1), are carried out in the River Øyreselv, draining into the Hardangerfjord on the west coast of Norway (**Figure 1**). In section A, above a waterfall impassable for ascending anadromous fish, freshwater resident trout is the only fish species found, while section B, located near the sea, is inhabited by anadromous trout and salmon. The lower part of the river is divided in two parallell branches, each about 400 m long. In the first experiment, T-1, reared and genetically marked mature individuals were released in both sections of the river. In section A, video observations on the spawning behaviour of reared and wild trout were carried out, and ten reared individuals were kept in an enclosure for this purpose. In locality B, a mark-recapture study on juveniles was carried out to estimate the number and survival of different genotypes in offspring resulting from wild and reared trout, and from a combination of the two, and to assess changes in population parameters. The major effort has therefore been concentrated to locality B.

The experimental set-up in experiment T1 gives an opportunity to concentrate on the activity and success of mature individuals. One of the advantages here is that we know the exact number of introduced spawners, and therefore it is possible to quantify the magnitude of the manipulation at the time of reproduction. The disadvantage of this set-up is that if the spawning success of introduced fish is low, there will be a correspondingly low number of offspring and a risk of a sampling problem. In the second experiment, T-2, we introduced 2200 juvenile trout in the river, all of which were homozygous for the visible marker. In this experiment the number of genetically marked fish is larger, and we will first concentrate on interactions in the juvenile phase. We expect this group to reach maturation in 1994/1995. If the survival rate is good, it represents a new gene pulse.

The aim of experiment S-1 is to quantify reproductive success of ocean ranched Atlantic salmon, and the gene flow from ocean ranched to wild salmon stocks. The study is running parallel to a larger experiment on ocean ranching. Genetically marked smolt were released in June 1992, and the first spawners are expected to return in 1993. By 1994, then, we will have the first genetic data from the experiment with Atlantic salmon.

Genetic markers and electrophoresis

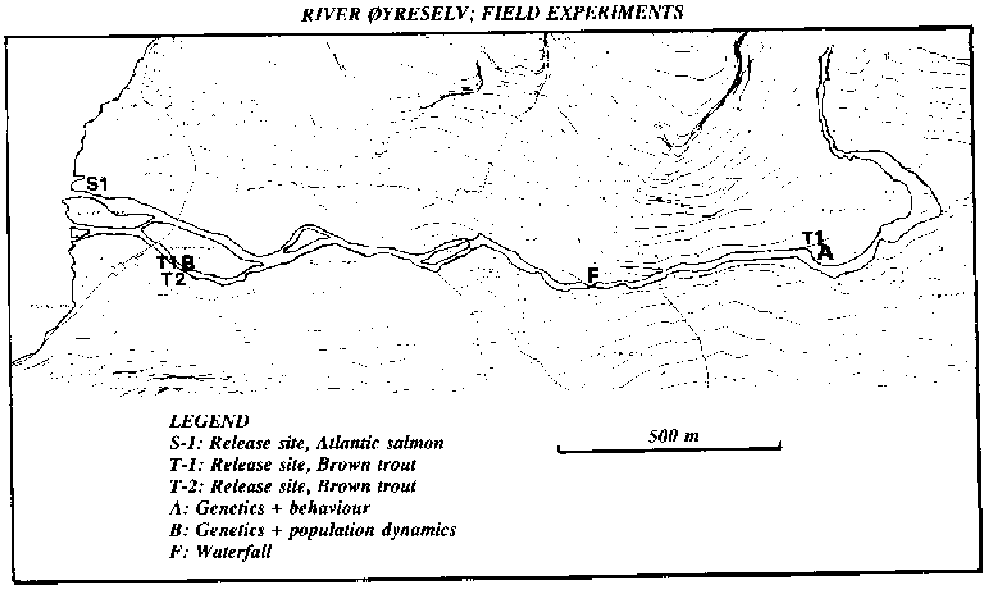
In 1986 production of a genetically marked trout strain was initiated. Data on the performance of the different genotypes were recorded. During 1988, genetic information on wild trout populations was sampled to find an adequate locality for the experiment, and electrophoretic studies were conducted. These included the following twelve enzyme systems: aspartate aminotransferase (AAT, 2.6.1.1), alcohol dehydrogenase (ADH, 1.1.1.1), adenylate kinase (AK, 2.7.4.3), creatine kinase (CK, 2.7.3.2), esterase (EST, 3.1.1.-) glycerol-3-phosphate dehydrogenase (G3PDH, 1.1.1.8), isocitrate dehydrogenase (IDHP, 1.1.1.42), lactate dehydrogenase (LDH, 1.1.1.27), malate dehydrogenase (MDH, 1.1.1.37), malic enzyme (MEP, 1.1.1.40), glucose-6-phosphate isomerase (GPI, 5.3.1.9), and phospho glucomutase (PGM 5.4.2.2), encoded putatively by 32 loci.

In the first study (T1) we were using two independent types of genetic markers, biochemical-genetic markers, i.e. isoenzymes, and a visible, morphological-genetic marker, i.e. a natural variation in spotting pattern, to quantify the genetic impact from the reared fish. The visible marker is a variant in spotting pattern on the body that can easily be distinguished from the spotting pattern commonly found in brown trout. Individuals homozygous for this genetic variant have a spotting pattern consisting of tiny black spots, and at the juvenile stage they lack the common parr marks. Heterozygous individuals are intermediate between the fine-spotted and common genotypes in appearence, as they have irregular and broken-up parr marks (Skaala and Jørstad 1987, 1988).

The number of wild spawners in section A was estimated by diving surveys and fish counting, and a sample of adult fish was collected by gillnetting to record the length distribution and weight of spawners. In section B, the number of spawners is estimated by electrofishing and diving surveys. Based on population estimates and the genetic marking, the number in each year-class and the survival of offspring resulting from wild and reared fish are assessed. 0+ parr were sampled by divers and by electrofishing. In section B, the number of 0+, 1+ and older was estimated by the mark-recapture method (Peterson).

In autumn 1989, genetically marked spawners were released in spawning areas for wild trout in sections A and B. The number of released reared fish was 43 in section A and in 104 in section B, which in both sections exceeded the number of wild spawners. Each released spawner was also tagged individually with Floy anchor tags. Diving surveys and video recordings were conducted during the late autumn and winter to estimate the number of released trout still present at the spawning grounds. Samples of the 1990 year-class have been collected in 1990, 1991 and 1992 for genotyping.

Figure 1 Map of River Øyreselv: release sites for trout (T) and salmon (S)



Gene flow from reared to wild populations

The visible marker is rare, and has only been found in three populations in Norway, none of these near the location of the present experiment. Enzyme electrophoresis revealed that the MDH-2*152 allele common in the reared strain was rare in the population in section A (0.02). We also found that the LDH-5*100 allele, present in high frequency in the marked strain, was present in very low frequency (0.03) in the anadromous trout in section B in River Øyreselv and the neighbouring rivers. Therefore one visible marker and one isozyme marker were available in each section. All samples of wild trout from year-classes hatched before the release experiment conformed to the expected Hardy-Weinberg distribution. Out of 43 0+ parr sampled in section A in October 1990, two individuals homozygous for the visible marker, and seven heterozygous individuals were found, giving a frequency of 0.128 of the visible marker allele. Statistically significant differences were found in genotypic distributions between the 1990 year-class and previous year-classes in both the visible marker ($p<0.0001$) and the MDH-2* marker ($p<0.01$). In contrast with the earlier year-classes, the 1990 year-class deviated significantly from expected distribution in the spot controlling locus as well as in MDH-2*. The genetic contribution from the farmed trout in section A, by September 1990, was calculated as 19.2 per cent based on the isozyme marker MDH-2*, and 12.8 per cent based on the visible marker. The population was also sampled in August 1992, and the frequency was then 0.108. No homozygotes for the spotting pattern nor isozyme marker were found.

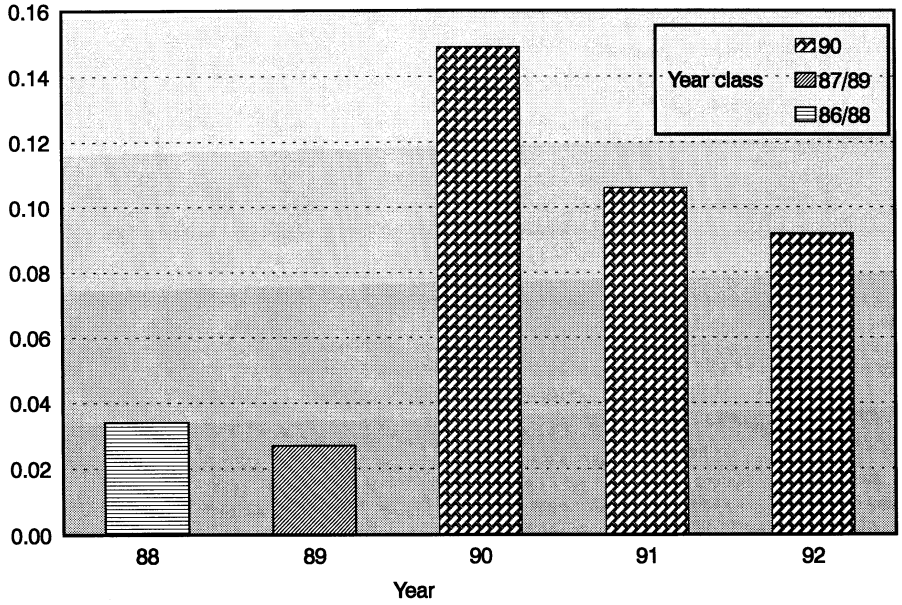
In section B, the visible marker was only observed in heterozygous form, as no fine-spotted individuals were found. In October 1990, 20 heterozygotes were recorded in this year-class, giving a frequency of the visible marker of 0.075. The frequency dropped to 0.03 between October 1990 and February 1991. By June 1991 the frequency was found to be 0.027. The frequency of the isozyme marker LDH-5* varied from 0.027 to 0.034 in year-classes between 1986 and 1989, while in the 1990 year-class this allele was found in 26 out of 134 individuals, giving a frequency of 0.149, significantly higher than in previous year-classes. By June 1991, the frequency of the LDH-5* marker had decreased to 0.106. Observed deviations from expected Hardy-Weinberg equilibrium were significant in both visible marker and in LDH-5*. By June 1992, the frequency of the isozyme marker had dropped further to 0.092 (**Figure 2**).

The genetic contribution from farmed trout was calculated as 16.3 per cent based on the allozyme marker (LDH-5*), and 7.5 per cent based on the visible marker, by September 1990. By June 1991 the genetic contribution from farmed trout in section B had dropped to 10.9 per cent based on the isozyme marker and 2.7 per cent based on the visible marker. Statistically significant differences were found in genotypic distributions between the 1990 year-class and the previous year-classes in both the visible marker and in the isozyme marker (LDH-5*).

In section A there is a fairly good agreement between the change in allelic frequencies estimated by the visible marker and the isozyme marker, LDH-5*, while in section B the observed change in allelic frequency is greater in the isozyme marker. The change observed in the isozyme marker in section B is comparable to the observed changes in section A, while the observed change in the visible marker is somewhat lower.

The relative reproductive success (RRS) of farmed trout compared to wild trout, calculated by 1990, from the frequencies isozyme markers, was 31 per cent in section A and 25 per cent in section B. The data from section B also indicate that the mortality rate of hybrids was higher than that of offspring from wild spawners, possibly due to differences in size (Skaala 1992).

Figure 2 Frequency of marker allele in year classes 1986/88 and 1987/89 before introduction and in the 1990 year class with the introduced genetic material from the hatchery trout



Conclusions

Genetic changes were recorded in both of the wild trout populations. In general, the visible marker is less effective than the isozyme markers in discriminating between alleles originating from farmed and wild stocks. The advantage of the visible marker is, however, the possibility to sample and type without killing a part of the population.

The experiment T1 reflects a situation where a massive swarm of farmed spawners enter a natural spawning habitat about one month before the initiation of the spawning activity of the wild population. Still, the presence of a high number of reared spawners seems to have had little influence on the reproductive success of the wild anadromous brown trout, as the numbers of 0+ parr originating from wild parents actually were higher in August 1990 than in the two preceding years. The number of genetically marked 0+ parr present in August 1990 was much lower than expected from the number of genetically marked spawners, and demonstrates a lower reproductive success of the released fish. Furthermore, the frequency of the introduced genetic material is declining.

It has been demonstrated that genetic material originating from a farmed strain, and introduced in the two populations in the River Øyreselv, has been incorporated in the gene pools of the wild trout populations. The results suggest, however, that farmed spawners may have a reduced reproductive success compared to wild fish. From the present experiments, we conclude that the relative reproductive success of the hatchery fish compared to the native populations is about 25-30 per cent. Based on population estimates and genetic markers, we recorded a strong decline in the frequency and absolute number of individuals carrying hatchery alleles during an observation period of about one year.

It is important to underline that the adaptability and reproductive success of reared or farmed fish in natural environments is dependent on several circumstances, such as the life stage at which the fish escape. For example, fish that escape at an early stage in life may have more time to acclimatize before spawning, and may therefore do a lot better than fish that escape as ripe spawners just before the spawning season. Further, there will be differences between species. Therefore, no single experiment will give a complete picture of the actual genetic impact on wild populations from hatchery stocks. It is quite possible to imagine a scenario where farmed individuals compete successfully at the spawning grounds, and mate among themselves as well as with wild individuals. If their offspring then compete successfully and survive, the outcome may be a change in the genetic characteristics of the wild populations. On the other hand, if their offspring compete less successfully, with a correspondingly high mortality a whole year-class may be strongly reduced.

From the present data we conclude that a single introduction of hatchery fish is not necessarily critical to the native population, as the reproductive success of farmed fish may be low and the survival rate of their offspring may be reduced. However, when there is a more frequent input of farmed fish, as may often be the case in the real world, the genetic characteristics of the wild stocks will be altered, even when the farmed fish have a reduced redproductive success. There is still little information on the ecological consequenses of such gene flow. We believe that a series of different experiments will have to be conducted to get a proper understanding of the genetic and biological effects of escaped and released farmed fish.

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Methods for Monitoring Microorganisms in the Phyllosphere

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Introduction

In this short article we would like to take the opportunity to present an overview of the methodologies available for the monitoring and identification of bacteria in the leaf and root environment. The criteria used in the selection of a candidate bacteria, the merits and limitations of the identification techniques, and the marker genes available for the construction of genetically modified microorganisms (GMMOs) suitable for field release will be discussed.

We have been involved in the development and application of monitoring methodologies for the isolation, detection and characterisation of bacteria associated with the leaves (phyllosphere) of sugar beet and wheat. Particular emphasis has been placed on the development of a genetically modified microorganism suitable for environmental release which will allow the impact, the potential for gene transfer and the risk associated with such a release to be assessed. Through the availability of the GMMO and the application of monitoring methodologies, it was considered that a better understanding of the microbial ecology of the plant habitat could be developed. Below we outline the experimental strategy for the generation of a GMMO suitable for environmental release into the phyllosphere of crop plants.

Selection of candidate bacteria

The candidate organism should be drawn from the natural microflora of the target habitat and be able to survive and colonize once released. An understanding of the microbial ecology and potential for and frequency of gene transfer in the chosen environment is therefore essential. Such data will be imperative for making valid predictions of the likely risk and environmental impact associated with the release of any GMMO. To ensure the stable inheritance of the markers, they should be inserted into the chromosome and not be associated with any mobile genetic elements. For the effective study of released bacteria, sensitive and specific detection methodologies are required which allow for the monitoring of both the GMMO and the novel genes.

The target habitat

The phyllosphere is perhaps the most important agrochemical target for the application of biological agents for crop protection or yield enhancement. The phylloplane probably comprises the largest surface area of any terrestrial habitat, yet relatively little is known about its microbiology. It experiences extremes of chemical and physical environments and it is a site to which microorganisms disperse, borne on rain, dust, wind and by insects, birds and animals. It is actively colonized by a variety of bacteria, yeasts and filamentous fungi which are well adapted to this harsh habitat (Andrews and Hirano 1992). The necessity of colonizing bacteria to be highly adapted to this habitat may be reflected in their ability to respond very rapidly to adverse as well as optimal conditions to ensure survival. Such responses may include high affinity for natural substrates, short generation times, improved survival mechanisms and enhanced rates of gene transfer or genetic reassortment. The phylloplane is also considered to be a site for the support of bacteria, fungi or yeast populations which may function as indicators of environmental change and atmospheric pollution (Fenn et al. 1989).

Monitoring the microflora of the phylloplane

The techniques adopted should allow the accurate detection and identification of the organisms under study. Tracking released organisms may provide insight into their temporal and spatial distribution in the target habitat, important for the development of a better understanding of population ecology, diversity, succession, survival and persistence of the inoculum and indigenous populations. By addressing these aspects, it will be possible to correlate data to better describe genetic diversity of the community and the adaptations that organisms go through to survive in a given habitat, so that more effective and persistent vectors for biological control can be developed.

Isolating and identifying bacteria

There are no universal or perfect methods for the detection or enumeration of bacteria in the environment (Drahos 1991). Each method has its strengths and limitations and should be chosen to suit the environment in which the study population occurs. The most widely applied methods are still those based on the use of general or selective media for the isolation of microorganisms from suspensions of sample. This may well be adequate if, for example, pseudomonads are under study, as they are considered to be fully culturable *in vitro*, whereas the detection of other bacteria that produce viable but non-culturable stages may require detection by molecular methods.

Automated systems for identifying clinical specimens have been described, but few of them are useful for the identification of environmental isolates. Traditional biochemical assays and commercial kits are often laborious to perform and are difficult to interpret when applied to environmental samples. Therefore, standard methods have to be adapted to describe the microbial components isolated from the study habitat. The limitations of such an approach are that relatively few isolates can be investigated and fewer can be precisely identified. Commonly described phenotypic methods for comparing isolates include the analysis of constitutive macromolecules, electrophoresis of polypeptides by SDS-PAGE (Lambert et al. 1987), gas chromatography of extracted fatty acids (MIDI-MIS, Newark, USA) (Thompson et al. 1993a) or novel substrate utilisation, API (Marvingui et al. 1992) or BIOLOG (Hayward, USA) (Garland and Mills 1991). Genotypic methods rely on the identification and comparison of particular nucleic acid sequences unique to the host organism and depend on DNA fingerprinting by RAPD analysis, pulse field electrophoresis of macrorestriction fragments, and the use of hybridisation methods for the detection of restriction length polymorphism (RFLP) and to target specific

messenger RNA or ribosomal RNA (Pickup 1991). Phenotypic methods are normally dependant on the isolation of bacteria able to grow on laboratory media and although genotypic analysis can be applied to isolated organisms, it is not essential. Alternative methods based on the application of target directed amplification (PCR) (Hadrys et al. 1992) or fluor-labelled oligonucleotides specific to naturally amplified target rRNA sequences (Amman et al. 1990) permit the study of populations *in situ* (Hahn et al. 1992) or unable to grow *in vitro*. In addition, the use of epifluorescent immunomicroscopy with specific antibodies to the target bacteria has been shown to be suitable for the direct detection and enumeration of bacteria in environmental samples (Morgan et. al 1991). At present, however, it is only possible to genetically modify or produce antibody against those bacteria that can be cultured. Therefore, monitoring methods should be optimised for the simple detection of viable organisms *in vitro. In situ* determinations remain the ultimate objective of most microbial ecologists, as they require minimal sample disruption and allow real time detection.

Although many of the molecular methods for detection are highly specific, they are none the less time-consuming, costly and complex techniques that limit the size of sample that can be studied. What is important for monitoring purposes, especially for the tracking of a released GMMO, is that the methodology adopted be reproducible and provide data of statistical significance (Donegan et al. 1991). To this end, a method should be both practical and simple to perform. It should be sensitive, reliable and permit the quantifiable detection of the target.

When investigating bacteria in the natural environment, novel isolates are common. This can and does lead to confusion over their taxonomy. Therefore, reliable methods have to be developed for identification and comparison of isolates. An approach we have successfully adopted is that of constructing habitat-specific libraries from the fatty acid profiles of isolated bacteria analysed by MIDI-MIS apparatus (Thompson et al. 1993a). These libraries allow the positive identification of strains repeatedly sampled over consecutive seasons. Although the availability of such analytical methods permits the detailed study of populations, they may not be sensitive enough for monitoring a released organism. This is especially relevant to the situation where the recipient is a natural component of the microflora of the study habitat. For monitoring purposes, it is therefore necessary to be able to rapidly identify, and preferably select for, the organism under evaluation. One practical approach to meet these criteria is to introduce, by genetic manipulation, novel phenotypes.

Commonly used marker phenotypes:

i) Selectable phenotypes

Marker genes that express products that modify or exclude biocides permit the isolation of the bacteria from the indigenous sensitive background. There are a limited number of such genes which include: antibiotic resistance (kanamycin, erythromycin, streptomycin, spectinomycin, ampicillin, chloramphenicol, tetracycline, gentamycin), resistance to heavy metal toxicity (mercury, copper, zinc, cadmium), arsenite resistance, Bialaphos resistance, utilisation of novel substrates (lactose) or xenobiotics. The inclusion of one or more of these selectable phenotypes is at present unavoidable in the genetic modification of the bacteria. Cloning methodologies are still for the most part dependent on the introduction of one or more of these genes into recipients for the selection of the modified bacteria. However, the retention of selectable traits in the GMMO greatly facilitates monitoring and increases the sensitivity of detection assays based on isolation and cultivation.

ii) Screenable phenotype (enzymatic and colormetric markers)

These include the introduction of traits including bioluminescence (*lux*), colorimetric enzyme activity *xyl*E, *lac*Z, and *gus*A which respectively code for catechol 2,3 dioxygenase (catechol to a soluble yellow dye), ß-galactosidase (X-gal to blue product) and ß-glucuronidase (X-gluc to a soluble blue-indigo dye).

Each of the genes can be modified or adapted to optimise expression or have expression controlled by an inducible promoter. Cassettes on disarmed transposons carried by suicide integration vectors have been described to facilitate the marking of isolates (Barry 1986, Shaw and Kado 1986, Herrero et al. 1990). Obviously the introduced gene(s) must be expressed in detectable quantities by the candidate (recipient) organism. To date, some of the genes described above have been successfully introduced into a variety of bacteria, plants, yeast, fungi and nematodes. However, there are only a limited number available. To optimise their use for the identification of the recombinant, more than one phenotype is typically included in the GMMO. In preliminary investigations and to satisfy the requirements of the regulatory authorities which oversee the release of GMMOs into the environment, GMMOs have been marked with genes to facilitate their detection. However, viable GMMOs which function as vectors for the delivery of biologically active gene products, e.g. insecticides or fungicides, are under development and will be important for assessing biological impact in the future.

The limitations in the use of these genes depend on the habitat under investigation and the level of natural activity. In the United States, a number of viable bacteria have been released into the phytosphere; a fluorescent pseudomonad carrying the *lac*ZY genes in its chromosome (Drahos et al. 1992), ice nucleation-deficient P. syringae (Lindow and Panopoulos 1988), pytopathogenic *Xanthomonas campestris* constituatively expressing bioluminescence (Shaw et al. 1992), and a commercial product, InCide™, that contains the endophyte *Clavibacter xyli* expressing the delta endotoxin of *Bacillus thuringiensis.*

The combination of natural or introduced resistance markers, for example the ability of the recombinant pseudomonad containing *lac*ZY to utilise lactose as a sole carbon source, allows the simple detection of the bacteria by plating methods (Drahos et al. 1986). In practice, all of the available marker genes require the extraction and plating of samples before the recombinant can be identified.

Of the marker genes available, those for the expression of bioluminescence (light emission) have been considered the most appropriate for the direct detection of bacteria. Such sensitivity, where practicable, may permit assessments of cellular activity and the location of individual cells or clonal populations. The *lux* operon when constituatively expressed is energy-demanding and usually deleterious to the recombinant's competitive survival in the environment. However, the complete operon (*lux* CDABE) has been successfully incorporated into the chromosome of the phytopathogen *X. campestris*, which was able to survive in the phytosphere after injection into target cabbage plants. As the recombinant retained its pathogenicity, it infected the plant and survived in detectable amounts in growing tissue. The level of light emitted by infected leaves was shown to correlate with the number of cells that were extracted (Shaw et al. 1992). However, in naturally competitive habitats bacteria have only been marked with the *lux*AB luciferase genes to reduce the metabolic load on the cell, which then has to be provided exogenously with the aldehyde substrate prior to evaluating light emission. Luminometers can detect low levels of light emission from very small quantities of cells in suspensions of environmental samples, and although it is theoretically possible to detect a single cell, complex charge coupled devices are required to provide the necessary sensitivity (Rattray et al. 1990). Bioluminescence has been estimated to allow the direct detection of a 1000-fold fewer cells than *lac*Z-based systems on the rhizosphere of wheat (deWeger et al. 1991). Although autophotography permits the spatial distribution of the inocula to be determined on roots

extracted from the soil, it offers no benefit over simple agar overlay and incubation methods which can be applied to marked bacteria (McCormack and Bailey, unpublished observations). Where light emission is only employed as an additional phenotypic marker for the identification of recombinant bacteria isolated onto agar, it is considered less versatile than the other more reliable colorimetric markers currently available (i.e. *lac*Z, *xyl*E).

Extraction of samples

Many methods have been published which describe protocols for the extraction of microorganisms from environmental samples (Fry 1993, Macdonald 1986). All methods vary in complexity and can only be chosen after a number of factors have been considered: is the method reproducible? can a sufficient number of samples be extracted in the time available? can a number of detection methods be applied to the extract?

Extraction for analysis

Homogenisation may release antimicrobial agents, contaminants or nutrients from tissue which can affect the organism's ability to grow on minimal or selective media and limit detection with nucleic acid probes. Abrasion with 10 per cent (w/v) sand or direct washing can be successful but variable. In the phytosphere abrasion has been useful for isolating bacteria which colonize the leaf or root surface. Direct leaf press onto agar, and the overlay of roots with agar, though not quantitative, are useful methods for determining the spatial distribution of bacteria. Homogenisation of replicates is generally considered to be the most reproducible and appropriate mechanism for extracting and enumerating populations. Any extractive method that requires dilution and isolation of samples on agar introduces bias towards the abundant fast-growing heterotrophic organisms. However, by using selective agars, specific populations can be studied. What is important is that the method chosen should suit the type of analysis to be performed and not be varied between samples.

Our own investigations have been based on the description of community diversity following the identification of numerically abundant isolates collected from the root and three different leaf types (emerging, fully expanded and senescing) over entire growing seasons for sugar beet and wheat (Thompson et al. 1993b, Legard et al., manuscript in preparation). An important aspect of any approach taken to understand the ecology of the phyllosphere is that the sampled microbial community is well characterized or identified. To do this, we have used a semi-automated system to identify the fatty acid profile extracted from isolated colonies and compared them with data bases to ensure accurate identification of bacteria (Miller 1982). By applying the method to the identification of isolates collected over three successive seasons from an experimental plot of sugar beet, we have been able to generate a habitat-specific data base which provides accurate and reliable identification of isolates. From these studies, the temporal and spatial distribution of bacterial populations in the phyllosphere of sugar beet was determined, thus allowing predictions of the seasonal occurrence of specified groups to be made.

Pseudomonad populations persist and survive well in the phyllosphere and rhizosphere. *Erwinia* spp. are an important part of the bacterial community of leaves. Arthrobacter and other typical Gram-positive soil organisms decline in numbers on healthy leaves as the season progresses. However, as leaves age and begin to senesce, the relative numbers of Gram-positive component (best described as that which contaminates the leaf surface on emergence through the soil) is maintained at low numbers by rain splash and dust from the soil until the leaf begins to decay, releasing sufficient nutrients to negate the competitive pressures encountered on healthy leaves. Other population successions have also been observed. For example, the fluorescent pseudomonads, which are the most frequently isolated group of organisms (from

sugar beet and wheat phytosphere), change in their relative species composition as the plant tissues mature.

Using FAME-MIDI analysis, the fluorescent pseudomonads, represented by *Pseudomonas aureofaciens* isolates, predominated and were present on sugar beet plants throughout the growing seasons studied in 1990 1991 and 1992. The *P. aureofaciens* group is a major component of the community, which peaked around 125 days post planting (<40 per cent of the sample) and was maintained thereafter at low levels (>0.01 per cent) on the plant. Its preferential habitat was the surface of emerging and expanding leaves (10^7 cfu/g) as well as the rhizoplane of developing roots (10^8 cfu/g). By concentrating specifically on the detailed comparison of isolates taken from soil, immature and mature leaves, and to identify two distinct populations, population A was sampled throughout the season whereas population B was only isolated from plants on a single occasion during an unusually hot and arid period in the summer of 1990. These observations illustrate that changes in plant tissue physiology and extreme changes in environmental conditions are able to select or enrich for a subset of populations on the phyllosphere.

The niche preference of the fluorescent pseudomonads for healthy growing tissue has been exploited in the development of the recombinant strain. The candidate organism was selected from this well described fluorescent pseudomonad group, for which a basic understanding of its natural ecology had been determined. As the organism's preferred habitat appeared to be young tissue, it was considered suitable, as following release the recombinant may become self-limiting as the plant matures. In addition, because the wild type persists from season to season, detailed analysis and monitoring of a released bacterium would allow trends in its relative abundance and distribution in the phytosphere to be investigated to determine dispersal mechanisms and whether the soil acts as a natural reservoir for the bacteria which colonize plants germinating in successive seasons. To make a valid assessment of risk and impact, and to better understand microbial ecology, it was considered essential that an indigenous organism should be selected as a candidate for genetic modification and release. The choice of markers should facilitate their unequivocal detection and be simple to use. Therefore from the study of the microbial ecology of the phyllosphere of sugar beet and wheat an indigenous, non-pathogenic, plasmid-free bacteria was selected as a candidate for genetic modification.

On the basis of these criteria, an indigenous, non-pathogenic, plasmid-free, fluorescent pseudomonad was isolated from the phytosphere of mature sugar beet (*Beta vulgaris*) grown at the study site in Oxford. The candidate, SBW25, was a member of the most abundant (temporally and spatially) fluorescent pseudomonad population, *P. aureofaciens* (Bailey and Thompson 1992, Thompson et al. 1993a,b) or on wheat (unpublished observations). It is also able to colonize both sugar beet and wheat leaves and roots following seed inoculation in non-sterile soil.

Introduction of marker genes into the chromosome of SBW25

The recombinant organism SBW25EZY-6KX was marked by the introduction of two unique sets of marker genes into two well separated chromosomal loci. Markers were introduced into the chromosome by site-directed homologous recombination. No vector sequences or remnants of mobilisable genetic elements remained in the recombinant. The strategy was to generate a GMMO in which the introduced exogenous DNA was as genetically stable and heritable as any other chromosomal gene or region. Two chromosomal sites were chosen, which had no apparent effect on the ability of the recombinant to colonize plants or compete with the wild type when compared *in vitro*. The first chromosomal site, Ee (5kb) is unique to a sub-group of very closely related bacteria. The second site, 6 (7.2 kb) differentiates closely related

fluorescent pseudomonads isolated from the phytosphere by RFLP patterns. The two separate chromosomal sites and the particular combination of marker genes facilitate the detection of gene transfer. It was considered unlikely that both sets of markers would be transferred simultaneously in the event of chromosomal mobilisation. The combination of markers provides highly sensitive monitoring methodologies for both the introduced genes and the recombinant bacteria.

Marker gene cassettes

A kanamycin resistance-catechol 2,3 dioxygenase gene cassette (KX) was constructed and inserted into one site on the chromosome. The *lac*ZY operon (Barry 1988) was removed from the flanking Tn7 IS regions, and inserted into a second site at least 1 mb away (approximately 20 per cent of the chromosome) (Bailey et al., manuscript in preparation). Isolated chromosomal fragments from SBW25 genomic banks were physically mapped and the appropriate cassette inserted. After isolation, the marked chromosomal fragments were transformed into the recipient strain and recombinants (which arise by homologous recombination between the marked fragment and the chromosome) were selected on lactose minimal medium (*lac*ZY) and media containing kanamycin (100 μg/ml). Phenotypic detection was confirmed by treatment with X-gal (blue colonies) or catechol (yellow colonies). All three marker genes are constitutively expressed. When used in combination with *Pseudomonas*-selection agar, containing kanamycin and X-gal, KX recombinants can be selected from a background of 10^{12} cells and detected at amounts in the order of 10 cfu/g environmental sample. Gene transfer events can be assayed by the treatment, with catechol, of kanamycin-resistant white colonies isolated on media containing kanamycin and X-gal. Those that turn yellow are genotypically characterized to determine whether *lac*Z expression has been lost or whether the KX cassette has been transferred to a novel host.

In vitro and in planta growth

In vitro growth studies in non-selective broths and co-cultivation of the recombinant with the wild type demonstrate that they have the same mean generation time and that they compete equally well even after daily passage (1/1000 dilution) for 20 days. Following seed inoculation and planting in non-sterile soil collected from the field site, the introduced bacteria successfully colonized the developing plants. SBW25EeZY-6KX colonized the plant similarly to the wild type and behaved as predicted from the ecological studies of fluorescent pseudomonads in the field. The recombinant colonized emerging immature leaves, expanding mature leaves and growing roots. Maximal levels were observed on developing tissue, where the inoculum represented almost 100 per cent of the sampled bacteria, $>10^7$ cfu/g. As the plants matured, recombinant populations declined allowing normal community succession.

In glasshouse and microcosm studies, the application of recombinant is assumed to result in the saturation of the available niches normally occupied by related indigenous fluorescent pseudomonads. Once the environment changes, as the plant matures, they are no longer suited to this group of bacteria and their populations are succeeded by other community members.

Conclusions

We have attempted to briefly describe the monitoring of bacteria in the phytosphere and the development of a GMMO suitable for release into the phytosphere of sugar beet and wheat.

At the time of completing this article, consent under the Environmental Protection Act 1992, of the United Kingdom to release the bacteria in the spring of 1993 had been granted.

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Monitoring the Flow of Pollen in Crop Plants

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Introduction

Most cultivated crop species are the products of millions of years of evolution under natural selection and thousands of years of conscious or unconscious selection by man. During this latter period, many of the mechanisms which ensure survival and dispersal in natural conditions have been substantially modified in order to increase productivity under the somewhat unnatural conditions of agriculture. Many examples can be cited, but perhaps one rather typical example is the loss of the brittle rachis characteristic of wild barley species such as *Hordeum spontaneum* in its human-aided evolution to the cultivated form *H. vulgare*. In some cases, the cultivated form of a species has so diverged from its original wild ancestors that only the cultivated form is known, e.g. maize (*Zea mays*). In others, usually where cultivated origins are more recent, both wild and cultivated forms continue to inhabit the same broad geographic or ecological zones and their division into different species seems more a taxonomic convenience than a true biological distinction, as both wild and cultivated forms remain fully cross-compatible and capable of producing fertile hybrids, e.g. cultivated beet, *Beta vulgare*, and wild beet, *Beta maritima*.

Bearing these points in mind, we thought it would be useful to consider some of the potential hazards of genetically modified plants and why gene flow or pollen movement between and within them may be relevant to such perceived hazards.

Aspects of hazards

i) New toxins

The products of novel genes, engineered into crop plants, may benefit those plants by enhancing disease and pest resistance, for example, but those same products may be hazardous to the consumer – human or animal – if included in their diet. This is a situation with which conventional plant breeders have been obliged to contend for many years. Examples include:

- glycoalkaloids in potato tubers, whose levels are routinely assayed in breeding programmes and controlled by statutory or voluntary guidelines (Morris and Lee 1984);

- SMCO in forage brassicas which cause haemolytic anaemia in ruminants, but may actually be beneficial in human diets (Whittle et al. 1976);

- covicine and vicine in faba beans, which cause favism in some humans and limit use in poultry rations, etc. (Marquard 1989).

Assessment of these "risks" may more properly be addressed by toxicologists than a plant breeder, whose main problem may be to determine whether a novel "toxin" only affects a proportion of the population, as, for example, in the case of gluten in wheat and wheat products. However, if the "toxin(s)" can be transmitted via pollen into the seed, as can erucic acid levels in oilseed rape, some knowledge of pollen movement between cross-compatible crops will be necessary to ensure that crops grown for, say, an industrial purpose are sufficiently isolated from others of the same species grown for consumption.

ii) New pathogens

Perhaps the best example of this is afforded by the introduction of virus coat protein genes into plants in order to enhance their resistance to those viruses (Barker et al. 1991). Could this result in a novel strain or pathotype of the pathogen capable of attacking a wider host range or utilising a novel vector? Any possibility of this, one would hope, will be explored before the release of such engineered crops into the agricultural environment, and pollen movement from field experiments into adjacent crops prevented.

iii) Enhanced weediness

Weeds can best be described as "plants out of place" – volunteer potatoes in a pea crop are weeds. Should a novel gene confer such a selective advantage to an otherwise agricultural crop species that it becomes more likely to survive and reproduce outside the normal crop rotation system, the degree of "risk" could range from becoming a minor nuisance to becoming a major agricultural or ecological problem. Parallels may be drawn here, perhaps, with the escape of alien species into environments which lack the natural predators and pests that control them in the environment from whence they came. Examples such as prickly pear in Australia, and Japanese knotweed (*Reynoutria japonica*) in the UK, amongst others, are well documented (Elton 1958). Gene flow and pollen movement will clearly be factors in assessing this "risk".

iv) Outcrossing to other plants

The possibility of gene transfer to weed species with consequences similar to iii) above are clearly problems to be addressed. The likelihood and the distances (genetic and geographic) of such spread require to be assessed, particularly if the novel genes are likely to enhance the aggressiveness of existing weeds or upset the ecological balance by affecting, for example, the insects which predate them.

v) Unforeseen effects of alien DNA

Almost by definition, one cannot entirely anticipate unforeseen consequences of the insertion of alien DNA. But, hopefully, more accurate estimates of gene flow and effective isolation distances will go part way to "putting the genie back in the bottle" if it escapes.

SCRI research is currently directed to many aspects of risk assessment (see poster), but we will address specifically the question of gene flow via pollen. We are actively looking at gene flow/pollen movement in several crop species, for various reasons:

- They are crops with which we are familiar and have expertise and experience;
- They are crops which are capable of being genetically engineered (indeed they include crops where engineered varieties are close to commercialisation);
- They are crops which exhibit a range of reproductive biology and diverse origin – endemic to UK, Europe or introduced. These crops are raspberry, blackcurrant, potatoes, oilseed rape and barley.
- We wish to study pollen movement on an agricultural scale rather than on the somewhat artificial, experimental scale imposed on GMO release experiments

With respect to this latter point, all five crops are important and widely grown in the vicinity of the institute. Angus is the most important seed potato growing county in Scotland. It is the major centre for the soft fruit industry in the UK and the most concentrated area of production in Europe. In recent years, 25 per cent of the Scottish oilseed rape crop has also been grown in Tayside.

Involvement by the Institute in gene flow experiments with GMOs has been somewhat inconclusive and, even had it not been, it is difficult to conceive how the results might have been extrapolated to similar GMOs grown in the real life agricultural context. Similar research on potatoes in New Zealand (Tynan et al. 1990), tobacco in the UK (Paul et al. 1991) and oilseed rape in France (Chevre et al. 1992) has demonstrated that some pollen movement/gene flow is to be anticipated between different genotypes of these species and, in the latter case, between species under field conditions. However, the experiments were on a small scale relative to agricultural practice when agricultural crops of these species will be grown on many hectares of land. Moreover, current regulatory controls regarding the release of genetically modified organisms into the environment preclude the practicability of scaling up such experiments to an agricultural scale (EC Directive 90/220).

We are therefore predominantly using "natural" genes to monitor gene flow. The main problem is that we are attempting to quantify low frequency events. Thus it is necessary to identify genes whose phenotypic expression is easy to recognise, which will enable the rapid and efficient screening of very large numbers of plants. This research is at an early stage, but I would like to refer to some of the genes we have identified and are using to monitor pollen movement in the three crops to which I have referred, and some preliminary results:

Current research at SCRI

1) *Rubus (raspberry)*

The Tayside region contains a high concentration of raspberry crops. There have been several surveys of the wild populations, and the SCRI breeding programme has been well documented since its inception in 1957. *Rubus* is a perennial crop capable of reproduction vegetatively and by true botanic seed which has a long viability; its pollen can remain viable under some conditions for up to one month and it is normally insect-pollinated. Two spontaneous mutant genes have been released into the environment within the last 30 years. Both have been extensively used in the breeding programme at SCRI, and one has been widely distributed in

commerce in named cultivars. A recessive mutant for spinelessness is present in a heterozygous state in the cultivar Glen Clova (released in 1969), which occupied 90 per cent of the acreage by 1980, and the cultivars Glen Moy and Glen Prosen (released in 1984), which jointly occupied 30 per cent of the acreage by 1991 in a homozygous state. The L_1 gene – a dominant mutant which is expressed in the phenotype by several "gigas" symptoms, i.e. larger than normal stipules and sepals, found in the cultivar Delight, confined to the SCRI site – was widely used in the breeding programme since 1958. A retrospective comparison comparing the incidence of these two genes in seed collected in 1960 and 1990 from "wild" and feral populations of *Rubus*, from 84 sites in Scotland, has failed to find either gene in the 1960 samples, but a low frequency of the "spine-free" phenotypes were identified in the 1990 samples. Only 39 spine-free seedlings were observed in more than 10,000 raised; of 120 genotypes within 2 km of SCRI, none had the L_1 phenotype and only six were spine-free – at four sites only and all within 50 metres of raspberry crops. The tentative conclusions to date are that gene flow from cultivated to wild *Rubus* does occur but that, over a 30-year period, it has been minimal and possibly limited to vegetative escape rather than via pollen or seed. The work is ongoing (McNicol 1992).

2) Potatoes

Solanum tuberosum is not native to Europe and there are no known endemic species of the solanaceae with which it is capable of hybridisation. Cultivars are clones and are normally reproduced by seed tuber. Many (older) cultivars tend to be shy flowering and produce few berries, if any. Several modern cultivars, however, are freely flowering and produce very large numbers of berries – up to three tonnes per acre have been recorded. Since any one berry can contain more than 100 true seeds, the quantity of true seed that can be deposited in the soil after harvest is clearly very substantial. Research at SCRI has shown that this true seed can retain its viability in the soil for many years and is a cause of some concern to growers as the volunteer potatoes, owing their origin to this seed bank (Lawson 1983). This can provide an unwanted "bridge" between potato crops, exacerbating problems of disease, particularly viruses and soil-borne pests, as well as causing difficulties for vining peas if potatoes feature in the same rotation, where the immature berries are difficult to distinguish from peas. The potato flower possesses no nectary and is not attractive to honeybees, but its anthers provide an abundant source of pollen for bumblebees and hoverflies, which are known to frequent them. The tetraploid potato is self-compatible, and it has usually been assumed that open pollinated berries are primarily the consequence of selfing. However, recent data from field experiments with genetically modified potatoes have confirmed unpublished SCRI data that some cross pollination does occur (Tynan et al. 1990). How far the pollen can move is not known. Fortunately, a number of widely cultivated varieties possess major dominant genes which other equally widely cultivated varieties lack. SCRI research is directed towards exploring these genes as natural markers to trace pollen movement between varieties in agriculture and in large scale experimental plots. Two characters of particular value to this exercise are field immunity to PVX in cultivars such as Maris Piper and Cara, absent in cultivars such as Desiree and Pentland Crown, or resistance to the golden cyst nematode (*Globodera rostochiensis*) governed by the dominant gene H_1 also present in the former two but neither of the others (Anon. 1993). This being our first full season, no data are yet available.

3) Oilseed rape

Brassica napus is a natural amphidiploid of the two diploid species, *B. oleracea* and *B. campestris*. Whilst truly wild, weed species of the latter are recognised, *B. napus* is thought to have arisen spontaneously in agriculture and truly wild populations are unknown. The cytogenetic relationships of the brassicae and the ease with which members of this diverse family

can be intercrossed in breeding programmes is well documented (McNaughton 1976). However, on a note of caution, crosses that appear to have been very difficult to secure, or required intervention with techniques such as embryo-rescue in controlled breeding programmes, may be not so infrequent as these experiences suggest in nature. Recent work in France has shown that even intergeneric crosses between *B. napus* and *Raphanus raphanistum* can occur under some field conditions (Chevre 1992).

Although both its constituent diploid species are obligate outbreeders with well defined sporophytic self-incompatibility systems, *B. napus* is (usually) self-compatible. Historically, breeders of *B. napus* crops have tended to treat them as inbreeders and most cultivars have been highly inbred and homogenous. Some have indeed been produced by the doubling of haploids produced from anther culture and so are completely homozygous. However, pollination is normally effected by insects – the flowers are particularly attractive to honeybees – and so the species may be regarded as a facultative inbreeder capable of varying degrees of outcrossing. The reproductive biology is, however, itself a target of both conventional breeders and genetic engineers who, with varying degrees of success, are converting some *B. napus* crops into outbreeders, utilising self-incompatibility or male sterility systems, in order to produce hybrid varieties. This approach will, itself, dramatically affect pollen movement or gene flow within and between cultivars and related (weed) species if successful.

Oilseed rape pollen is heavy and "sticky"; its free movement in air, unaided by bees, is generally considered rather limited. As a general rule, isolation distances of c. 20 metres between high and low erucic acid varieties is deemed sufficient to prevent unacceptably high contamination of the one by the other. Studies by SCRI and collaborators investigating putative links between human allergy problems and oilseed rape appear to confirm that the levels of pollen in the atmosphere, proximal to an oilseed rape crop, decline fairly rapidly with distance. Breeders have tended to err on the safe side perhaps, by isolating elite stocks of cross-compatible brassica seed crops by approximately 1000 metres from each other.

We have had some difficulty identifying potentially useful marker genes in existing oilseed rape cultivars, as we have done in raspberry and potatoes. Isozyme markers, RFLPs and RAPDs, will prove useful in positive identification of parentages of hybrid seedlings once they are identified, but are impractical for the routine rapid screening of the large numbers necessary to detect low frequency events. Nevertheless, the use of pollen traps and bait plants has already produced data in our first season that suggest oilseed rape pollen may be capable of more extensive movement and effective fertilisation of compatible genotypes than considered hitherto.

In one experiment emasculated plants of a rapid cycling *Brassica napus* genotype have set seed, coincident with the trapping of oilseed rape pollen in an adjacent trap, at a distance of approximately 2.5 kilometres from the nearest oilseed rape crop in flower. These seeds have yet to be germinated and their genotypes confirmed, but it does appear to confirm similar findings in the USA in the case of wild radish (Klinger et al. 1991).

Tentative conclusions

Pollen movement between and within crop species and their wild relatives is likely, resulting in gene flow between them. The genetic and geographic distances likely to be important are unclear and will vary considerably between species. The consequences of such gene flow will also vary considerably, dependent on the particular gene or genes involved, the crop species and the environment. However, in some instances it may already be deduced that escape of novel genes into the environment is a certainty. Whether they stay there or not will depend on whether they convey any advantage to their recipients. What the consequences will be is a matter of conjecture.

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Problems Associated with the Release of Rabies Vaccines into the Environment

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The discovery by Baer and co-workers (1971) that foxes could be immunized by oral administration of an attenuated rabies virus strain, triggered research projects aimed at the application of oral immunization of carnivores. This led to a successful field experiment in Switzerland in 1978. Since then several other countries have undertaken vaccination field trials. The most important conclusions to be drawn from the field applications of vaccine baits in Europe and in Canada are that it is possible to immunize sufficient numbers of animals by bait in order to stop the spread of the disease into rabies-free areas and to eliminate the disease from enzootic areas. In European areas where rabies was eliminated, a minimum of 50 per cent and often 80 per cent of the fox populations had been immunized. In areas freed of fox rabies, the disease also disappeared from all other species. The disease did not reappear spontaneously from undetected reservoirs after fox vaccination campaigns were discontinued. However, rabies was occasionally able to re-invade a fox population from a contiguous infected area (Wandeler 1991).

The technologies and attenuated rabies vaccines presently being used for field application are effective in foxes, but generally are not adequate for the control of the disease in other carnivores. Great efforts are being put into developing vaccines for other species. A vaccinia rabies-glycoprotein recombinant virus has been extensively tested in the laboratory, and field trials for its applicability to control raccoon rabies in the Mid-Atlantic States of the USA are in progress. The same vaccine has already been in use (for foxes) in field experiments in Belgium and France since 1988 (Brochier et al. 1991). Adenovirus rabies-glycoprotein recombinants are presently being developed and tested in Canada for vaccinating skunks and other carnivora by the oral route.

The release of genetically altered infectious agents (attenuated viruses and recombinants) always has ecological impact, and many concerns have been expressed (e.g. Regal 1986). Possible environmental impacts of the release of genetically modified viral vaccines can be classified as follows:

1) The genetically modified virus vaccine causes mortality: The vaccine virus itself (e.g. SAD and ERA in rodents) or a derivative (mutant, recombinant) of it may cause mortality in target or in non-target populations. Differences of vaccine pathogenicity for different species (e.g. SAD; carnivores – rodents; HAV5: Mus – Sigmodon) are not always predictable. Deletions in the viral genome created for accommodating the insert may alter the species specificity and pathogenicity. Horizontal transmission leads to adaptive evolutionary changes of vaccine virus properties. Thus far the laboratory studies with SAD and V-RG, and subsequent field investigations, have not produced any evidence that vaccine virus spreads among target or non-target species or that they change host specificity and pathogenicity.

2) Altered selection pressure on rabies causes the spread of mutants: Street rabies virus may change antigenic properties under the unusual selective pressure of an immune host population. Some of the new recombinant vaccines give only partial protection and set the scenario for the selection of escape mutants. Such mutants do not necessarily have properties leading to horizontal transmission. No such events have been observed in monitored oral vaccination zones of Central Europe and Ontario.

3) Altered host population dynamics have ecological effects: The mass vaccination of wild carnivores against an important mortality factor affects their population dynamics and may also alter their population density. This again will bring changes for the species they prey on and for their competitors.

Careful evaluation and surveillance are indicated when live vaccines are to be released into wildlife populations. A valuable catalogue of considerations has been published by the Ecological Society of America (Tiedje et al. 1989). There is agreement that each case needs to be assessed individually. This conclusion was also reached by the majority of national biosafety committees.

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III. The Discussion in the Working Groups

Introduction

A number of points raised during the early part of the Workshop were relevant to the subsequent discussions within the Working Groups. As pointed out by the Chair of the Workshop, Dr Jean Hollebone, the purpose of the Workshop was not to examine the premises which might lead to the initiation of a monitoring programme, but to examine critically the techniques that could be adopted to follow the fate of a novel organism, or gene, introduced into the environment, once the decision to monitor has been taken.

The keynote speaker, Dr Kathleen Keeler, also provided an excellent background to the discussions of the Working Groups, with a provocative and wide-ranging presentation which emphasised the multidisciplinary nature of monitoring and the communication problems to which this could give rise. In turn, these difficulties of communication place an onus on the members of the scientific community to clarify their shared assumptions and language.

Dr Keeler continued by pointing out the paradox of monitoring – an effective monitoring programme which identifies safety problems and allows their correction is doing no more than it ought, but a programme which fails to detect an unanticipated problem is criticised. She also pointed out that although the conduct of a monitoring programme is fully within the domain of science, the results will often be intended for use in a more public arena in which interested constituencies may place different interpretations on the results.

In addition, the six invited speakers, from wide and varied backgrounds, provided stimulating background with presentations which encapsulated many of the different objectives that monitoring methods might fulfil. Several themes kept recurring throughout the presentations:

1) The methods have to be appropriate for the problem;

2) The problem has to be well defined; and

3) It is important to choose the appropriate time scale over which to conduct the monitoring activity.

As pointed out by Dr Hollebone, a key aspect of the discussion in the Working Groups was that monitoring techniques have to be reviewed with respect to a number of general criteria: sensitivity; repeatability; reliability; ease of use and practicability; requirements for specialized equipment or facilities; time to identify markers; throughput; cost; and whether the results are qualitative or quantitative. These themes were taken up within the individual groups.

The Microorganisms Working Group

Background

Part of the remit of the group was to define the applicability and value of techniques available for the monitoring of microbes. This included techniques available for use in model systems, following the release of microbes to the open environment, or in the detection of indigenous species.

Objectives:

1) identify the various monitoring methods available (i.e. the range of options);

2) determine the applicability of various methods; and

3) identify the various situations under which monitoring is practical and effective and those situations under which is not.

For the purposes of evaluating current methodologies, the following considerations have been made:

- sensitivity; threshold/limit of detection in both lab and field samples
- reliability; accuracy of applied technique
- value; cost, effort and time involved
- application; diversity of techniques and ease of use
- reproducibility; accuracy of repeat samples
- specificity; precision of assay
- practicality; ease of use/need for complex facilities, apparatus
- extraction; sample preparation
- limitations; pitfalls/drawbacks

Note: The choice of method is dependent on the need for, or desirability of, quantitative versus qualitative information. It also depends on whether detection is to be of a cell protein, phenotypic marker or specific nucleic acid sequence.

The choice of method must be relevant to the size of the study plot and the handling of samples collected. All quantitative methodologies MUST generate statistically valid data.

Monitoring issues

Designing a monitoring programme

Designing a monitoring programme for microorganisms introduced to the environment starts with a clear definition of the programme's objectives based on available knowledge of the microorganism to be detected, the release environment and the release protocol. It is balanced by the degree of uncertainty or predictability of effects associated with the release. Satisfying all objectives may be restricted by limitations of available methodology. In these instances, there may be the need to select complementary procedures or to consider the development of new techniques.

Developing a microorganism profile begins with a complete description of the particular strain. This can be based upon available information on the biology of the organism, highlighting characteristics relevant to its behaviour in the environment, and supplemented by tests done with samples taken from the release site. For many microorganisms, previously published information may provide, at most, sufficient information for satisfactory pre-field release evaluations and, at the least, a useful adjunct to pre-field release testing. The relatedness of the test microorganism to well characterized genera or species may suggest specific testing needs (e.g. pathogenicity or toxicity), although this may not replace complete laboratory/small field trials. The only way to supplement the information base beyond that obtained from literature, laboratory trials and modelling is to test the fate of the microorganism, or one similar, in a controlled and monitored field trial.

Techniques

Plate count techniques

Plate count techniques have been used in most environmental introductions of microorganisms and for the detection of microorganisms. These techniques are inexpensive, well established and generally, although not universally, applicable. These methods rely on the ability of microorganisms to multiply and form colonies on agar media. It should, however, be recognized that microbes may have dormant stages, especially when subjected to environmental stresses and when other organisms indigenous to the study habitat are viable but non-culturable. In addition, high background levels of microorganisms can interfere with quantification. This, of course, may be overcome by the use of specific media or by the addition of selective agents or novel substrates added to the medium. By combining plate culturing with techniques described below, increased sensitivity and specificity of detection can be introduced into the monitoring techniques. Selective plating procedures often rely on genetic markers either naturally present or introduced by recombinant DNA methodologies. Examples of these markers include genes or operons which encode resistance to antibiotics and heavy metals, or provide the ability to utilise novel substrates and enzymes detectable by the conversion of substrates to coloured compounds or the emission of light. Secondary detection methods based on colony blotting include the application of antibody for ELISA and nucleic acid hybridisation.

Most probable number (MPN) techniques

MPN methods rely on the ability of microorganisms to grow in artificial media and on their ability to produce a measurable change. By enrichment in selective or non-selective media, the number of propagules is increased to allow specific detection. Typically, as few as 1 cfu/g may be detected. Identity is confirmed by plate methods, assessment of novel activity, reaction with specific antibody, or the use of nucleic acid probes.

Evaluation of culture techniques

Sensitivity: Using selectable markers for detecting microorganisms introduced into soil, aquatic or terrestrial environments can be as low as 1 to 20 CFU/g tissue.

Reliability: Providing samples can be sufficiently well extracted (see below) and are culturable (see below) this is an extremely reliable, accurate and easy-to-use method.

Value: Inexpensive. Cost, however, will be significantly increased by the use of expensive substrates or by the adoption of secondary screening protocols and colony blotting.

Application: Widely applicable to all/most environments.

Reproducibility: Typical variation within samples. Variation between samples dependant upon source and characteristics of study organisms.

Specificity: Determined by the use of selective media (antibiotic and/or heavy metals), unusual carbon sources, or the use of differential media. For example, inclusion of *lac*ZY gene that permits pseudomonads to grow on lactose as a sole carbon source. The use of King's B media and derivatives for pseudomonads and media which select for particular groups of organisms.

Practicality: No specialized skills or facilities required beyond the typical laboratory.

Extraction: Limitations in quantitative evaluation dependant upon the efficiency of extraction. Homogenous suspension of single cells necessary.

Extraction is the key to all methodologies based either on the culture or other detection methods described.

Limitations: Environmental samples may be contaminated with tissue, organic compounds, etc. which affect the selectivity of the media employed. This is especially relevant when employing minimal media. At high concentrations of contaminating material, selection may be prevented. Poor dispersal and clumping of cells can lead to inaccurate quantification. Microbial contaminants may interfere with growth of the target organism. Not all bacteria can be cultured.

Microscopy

Microscopy remains a traditional microbiological technique. A range of techniques can be applied for the enumeration and specific detection of microorganisms using both light and epifluorescence microscopy. Bacteria can be enumerated directly, and vital staining can distinguish viable cells (e.g. acridine orange, DAPI, media and nalidixic acid). Immunofluorescent staining or the use of fluorochrome-labelled oligonucleotides directed against unique ribosomal RNA sequences are effective methods for the differentiation of specific microorganisms within a mixed culture.

Sensitivity: Few organisms, e.g. *Vibrio*, can be distinguished on the basis of morphology alone. The application of specific probes, however, allows differentiation. Limits of detection relate to field of view (1 in 10^5). Visualisation is matrix-dependant. Most techniques have been developed and are applicable to the detection of aquatic microorganisms.

Reliability: Dependant upon the experience of trained personnel and the type of probe applied. Under these conditions, the method is highly reliable.

Value: Expensive initial investment. Time-consuming, but one of the few available methods for direct observation for organisms such as VAMs.

Application: Limited for monitoring. But valuable when used in conjunction with other techniques, i.e. antibody oligonucleotide probes, vital staining and for bioluminescence.

Reproducibility: Operator dependant.

Specificity: Improved when used in conjunction with other methods.

Extraction: Limited by contaminating materials. However, the embedding and sectioning of tissues or soils, etc. can allow the undisturbed observation, *in situ*, of organisms to provide a sense of habitat and distribution.

Practicality: Not practicable for the handling of large numbers of samples. It is also limited to organisms found in sufficiently high concentrations. Has been found to be useful for observing microbes collected in animal and air samples.

Limitations: The main problems associated with microscopy techniques are mainly ones of sample preparation and the contamination of the sample with environmental materials. These contaminants often result in false positives either in direct counting or in the absorbance of fluorescent probes. In addition, plant material and soil can often autofluoresce, requiring the use of complex filters.

Marker genes

A variety of functional genes are available for insertion directly into the genome of isolated bacteria. These genes can be placed, with a variety of methods, onto either the chromosome or plasmid DNAs. Such marker systems permit the sensitive detection of recombinant microorganisms by either plating methods or the targeting of these exogenous sequences by the use of nucleic acid probes. Used alone or in conjunction with other markers, they can provide exquisite sensitivity for the isolation and identification of bacteria. The choice of markers is very much dependant upon the environment under investigation, i.e. background activity, and the type of monitoring protocols required for the selection of bacteria amongst the background of indigenous strains. Efforts should be made to avoid the use of resistance markers for clinically important antibiotics and the use of selective substrates which may be hazardous to the operator. An example of this would be the widespread use of heavy metals for selection. Genetic modification and the selection of appropriate promoters may be necessary to permit sufficient expression of gene products in different bacterial genera (**Table 1**).

Table 1 Evaluation of Marker System for Detection of Genetically Engineered Microorganisms

MARKER SYSTEM	SEN	REL	VAL	APP	REP	SPE	EXT	PRA
1. Bioluminescence (*lux*)	+	+	+	+	+	+	+/-	+
2. Lactose (*lac*ZY)	+	+	+/-	+/-	+	-	-	+
3. *xyl*E	+	+/-	+	+/-	+	-	-	+
4. Antibiotics	+	-	+	+/-	+/-	+/-	-	+
5. Heavy metals	+	+/-	+/-	+	+/-	+/-	-	+/-

SEN = sensitivity
REL = reliability
VAL = value
APP = application
REP = reproductivity
SPE = specificity
EXT = extraction
PRA = practicality

Application: The sequences for all these genes are known. Therefore, secondary or complementary detection methods based on nucleic acid techniques can be applied.

Limitations: For detection the organism must remain viable and culturable to express the phenotype. This limitation is avoided with the use of bioluminescence as a marker where the application of luminometry permits detection of cellular activity in solution. Other limitations relate to the presence of similar phenotypes in the study habitat. For example, typical background counts of bacteria in soil samples that convert X-gal (*lac*Z) can be in the region of 10^5 per gram. It has also been observed that target sequences for nucleic acid probes against *xyl*E are common in soils contaminated with hydrocarbons. Antibiotic-resistant natural microorganisms may also be common in environmental samples. Luciferase activity has not been reported in terrestrial habitats, but can be a problem in aquatic environments. For *in situ* detection of luminescence, the use of CCD has demonstrated that single cells potentially may be detected. However, its application to the localisation and detection of *lux*-containing bacteria on leaf surfaces may be problematical. Extended incubation in the dark of plant tissue is often required to reduce or remove background fluorescence. It is therefore essential that these parameters in the study habitat are fully understood when enumerating any microbes.

Nucleic acid techniques

Nucleic acid techniques using probes or the polymerase chain reaction have been applied for the identification of microorganisms from environmental samples. These techniques are applicable not only for monitoring the organism itself, but also for the detection of gene transfer. These methods can offer both high specificity and sensitivity. Although these methods have traditionally been used in conjunction with cultured samples, they can also be used for the direct evaluation of target sequences from environmental samples. Three methods are currently available:

1) target specific amplification by the polymerase chain reaction;

2) direct hybridisation; and

3) use of oligo-nucleotide probes for the detection of cellular ribosomal RNA.

Table 2 Evaluation of Nucleic Acid Techniques

	Specific amplification	DNA probing
Sensitivity	High	Medium
Reliability	Yes	Yes
Value	High cost High benefit	High cost
Application	Universal	Universal
Reproducibility	Yes, highly	Yes, can be
Specificity	Absolutely, to degree required	Absolutely, to degree required
Extraction	Complex	Complex
Practicality	Lab technique	Lab technique
Limitations	Non-quantitative patented	Quantitative

Limitations: Generally costly and specialized equipment and facilities are necessary. However, colony hybridisation can be an effective and highly sensitive method for monitoring microorganisms which contain identified unique sequences. Other potential limitations include the use of radioactive chemicals, and availability of specific probes or oligo-nucleotides. Purification of nucleic acid is problematic from environmental samples; for example, contamination of samples with inhibitors of PCR is common. Methodologies for each sampled habitat and for the sampling of different groups of microorganisms need to be established. PCR methodologies are highly sensitive. Theoretically, a single copy of the target nucleic acid sequence can be detected. Normally, at least 10^3 copies of target are required for PCR methods and 10^5 copies are necessary for detection using radio-labelled probes in membrane supported hybridisations (e.g. Northerns, Southerns, etc.) in nucleic acids isolated from environmental samples. The use of oligo-nucleotide probes directed against rRNA offers highly specific and sensitive detection of microorganisms. Limitations include the need for epifluorescent microscopy and methodologies for the permeabilisation of fixed cells, especially in the case of Gram-positive microbes. Recent developments utilising enzyme-conjugated oligonucleotide probes will help to overcome some of these difficulties.

Immunological Techniques

Fluorescent antibody (FA) and ELISA techniques have been used successfully in environmental microbiology for the detection and enumeration of microorganisms at the genus, species and strain level. The application of microscopy of flow cytometry allows the direct enumeration and localisation for target cells.

Sensitivity: Potentially high. Direct enumeration in the order of 10^3 cells/ml in laboratory samples and 10^4-10^5/per gram in a typical soil extract. With cell enrichment or amplified ELISA methodologies, as few as one cell per sample well may be detected. Antibody techniques can also be applied to identify the products of microorganisms, i.e. the expression of a particular introduced antigen from a recombinant organism or the detection of a toxin to allow the assessment of biological activity *in situ*. This approach has potential to distinguish between quiescent and metabolically active cells.

Reliability: Each assay must be validated. Care must be taken to ensure the inclusion of appropriate controls. The degree of reliability can only be determined once specificity has been unequivocally assessed.

Value: Potentially, a very valuable technique. Equipment costs for ELISA readers are in the order of $US 10,000. Thousands of samples a day can be routinely and inexpensively screened.

Application: Extremely versatile. Little to moderate training required.

Reproducibility: 95 per cent reproducible.

Specificity: Can be very high and tailored to meet the needs of the target assay.

Practicality: Methods are available for on-site detection of antigen. Limited technical training is required and can be completely automated. Speed of assay can be from ten minutes to four hours. Also applicable to colony lift ELISAs.

Extraction: Lends itself to simple protocols.

Limitations: The preparation of monoclonal or polyclonal sera can be expensive and requires the use of animals. The identification and selection of target antigens and epitopes is often difficult. For example, in natural environments, surface antigens may be masked by capsular components. Further, the expression of phenotypes in laboratory cultures may not be the same as organisms isolated from the environment. The production of antibody to certain microorganisms such as fungi is often difficult. The limited application and use of antibody techniques in environmental microbiology in comparison to those methods available for the detection of nucleic acids underlines these difficulties. In addition, soils may contain inhibitory compounds.

Conclusions

A diverse collection of monitoring methodologies are currently available which meet the needs of most monitoring strategies. At present, plate count methods remain the simplest and most valid techniques for the detection of marked organisms. By the application of antibody or nucleic acid methodologies, the sensitivities of plate count methods can be enhanced.

Suggestions for further work in the scientific community:

1) Improve extraction methods from environmental samples.

2) Improve range of available culture media.

3) Improve sensitivity of PCR by eliminating inhibitory environmental compounds.

4) Develop *in situ* methods for identifying and localising microorganisms, evaluating their *in situ* activity and diversity.

5) Develop methodologies for monitoring the impact of introduced microorganisms on indigenous populations of microorganisms.

6) Improve methods for detecting gene transfer and evaluating gene transfer frequency.

7) A comprehensive literature review of all available marker genes should be generated.

8) The key to evaluating environmental impact depends on a clearer understanding of microbial ecology.

9) Develop automated and rapid methods for identification and classification – probably at the nucleic acid level. A unified and freely accessible data base for rRNA sequence would facilitate such an objective.

10) Incorporate and utilise available data bases when considering a release.

11) Workshops should be organised that are specifically designed to address the above considerations.

The Plants Working Group

Monitoring approaches and methods

In considering monitoring approaches and methods, it is first important to consider whether monitoring is appropriate for a given transgenic plant in a given set of circumstances.

Methods and experience from ecological genetics, plant ecology, taxonomy and experimental agriculture will be of value in the monitoring of test plants. This report concentrates on those methods by which sampling can be achieved, plants, propagules or genes identified, and results analysed. Also included are methods designed solely to provide further information for the organism, environment and introduction profiles for future releases. The precision of most methods will be enhanced by the use of easily identifiable markers, and all of the methods described can be used with plants in general, including transgenic or indigenous plants.

Sampling and identification

This section outlines the major approaches to the sampling and identification of test plants and the detection of pollen movement from test plants. These can be divided into two broad categories: direct observation, based on visible morphological characters, and interception of pollen by biological or physical methods.

1) *Direct observation:* In the case of pre- and post-release surveys, this takes the form of regular and methodical observation of the trial site to identify, record and/or remove plants of the species or its relatives. The area for, and duration of, direct observation should take into account the reproductive biology of the species (see **Figure 1**).

For plants where seed predation is known to occur (e.g. small fruit, tree fruit, sunflowers, etc.) it may be difficult, or impractical, to monitor seed movement. In confined experiments, prevention of seed dispersal (e.g. by netting against birds) is likely to be more practical than monitoring of seed dispersal, although difficulties could arise with wind-dispersed seeds. Similarly, movement of vegetative propagules, e.g. tubers, corms and bulbs, by small animals may be difficult to monitor.

In large-scale post-test monitoring, direct observation may be practical in conjunction with crop rotation. Monitoring is likely to consist of sampling based on good experimental design (e.g. quadrat analysis) rather than an exhaustive search of the release site and its surroundings.

The use of direct observation in the monitoring of released test plants depends upon the display of a conspicuous morphological character which is stably inherited and expressed, and different from that of local crops and feral populations of the same species.

2) *Interception of pollen (biological)*: There are two reasons for wanting "trap" plants in the vicinity of, or as a surround to, a transgenic crop plant field trial. Although in some circumstances the same "trap" plant may serve both purposes, this may not necessarily be so. It is therefore useful to distinguish between the two reasons for using "trap" plants and to highlight the desirable characteristics independently for both.

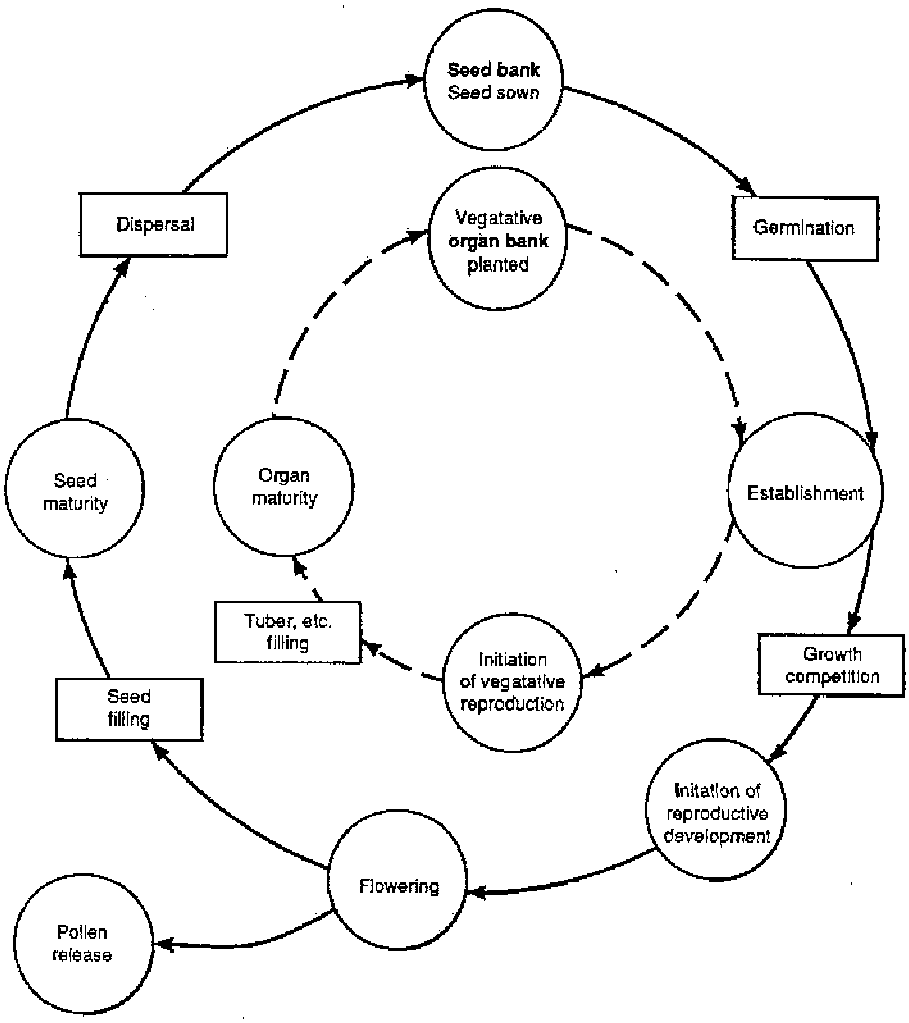
   i) *"Trap" plants as a barrier to pollen escape/gene flow*: In this case the trial plot will be closely surrounded by "trap" or guard plants whose function it is to "capture" pollen from the test plants and minimize its escaping from the trial area. In this instance, the "trap" plants should be as similar to the test plants as possible, i.e. fully cross-compatible, morphologically indistinct, and with a flowering period coincident with or, preferably, in advance of and extending beyond that of the test plants. In the case of insect-pollinated species it may be useful if the "trap" plant possesses features which make it more attractive to insect pollinators, e.g. ample nectar supply for bees, abundance of pollen for hover flies, etc. If the "trap" plants bear fruit at the termination of the experiment, the area occupied by them requires the same post-release monitoring as the test plants and the area they occupied.

   ii) *"Trap" plants as detectors of pollen movement*: In this instance the trap plants are intended to capture pollen from the test plants in order to provide information on geographic and taxonomic distance over which gene flow may occur. The "trap" plants may be of several forms – determined by the purpose of the experiment.

      a) *Geographic distance*: In this case the objective is to determine how far pollen can move from source and affect fertilization. The "trap" plants should be fully cross-compatible with the source plants and be as similar as possible to the source plants in their flowering characteristics, especially flowering time and duration. Since the objective may be to determine very low frequency events, trap plants should be prevented as far as possible, biologically (e.g. by use of genetic male-sterility) or mechanically (e.g. by emasculation), from self-pollinating or cross-pollinating with each other (or with other cross-compatible species not involved in the experiment). The source plants should possess some readily identifiable marker which is absent in the trap plants, preferably dominant in expression and phenotypically easily identified in seed, or plants grown from seed produced on the trap plants. It should also be possible to confirm/refute the source of pollen which gives rise to putative hybrids using molecular or biochemical markers, which are usually co-dominant.

      b) *Taxonomic distance*: In this case the trap plants may be of closely related species of the same genera or family in order to determine the likelihood/ possibility of gene flow between the test species and its near relatives, particularly weedy forms. In this instance cytological examination may also be a means to identify putative hybrids.

Figure 1 Generalised life cycle for plants



The persistence of a plant in the environment, beyond the agricultural setting, depends on successful completion of all the stages of its life cycle. Knowledge of the rates at which these stages and the processes linking them are completed in crop plants, relative to indigenous genotypes or species, may indicate the probability of a crop plant establishing and persisting away from the experimental release site in instances where these characters are not already well understood.

Experiments on geographic/taxonomic gene flow using "trap" plants may generally be equally well carried out using appropriately marked non-transgenic plants. The tests may also be performed using a transgenic pollen source, particularly if appropriate markers are unavailable or if there is a reason to think that the flowering and pollen behaviour, and maturity dates, of transgenic plants may be affected. For insect-pollinated species, the use of trap plants with enhanced nectar production could overestimate pollen flow, and conversely male-sterile trap plants could underestimate pollen flow because they are less attractive to the pollinators.

As with direct observation, the genetic lines used for trapping pollen must differ from the test plant in some characteristic that is stably inherited and expressed or otherwise detectable. Such characteristics may exist at the following levels:

i) morphological (e.g. flower colours, seed shape and leaf shape);

ii) physiological (resistance to disease or sensitivity to herbicide);

iii) biochemical (e.g. allozymes); and

iv) molecular (RFLPs, RAPDs, VNTRs).

For markers at the morphological and physiological levels, the efficiency of monitoring is increased if the trap plants carry the recessive phenotype of characters for which the test plant has the dominant trait. Where a series of releases is contemplated, the construction of trap plants with specific markers to assist identification could be considered.

3) *Interception of pollen (physical)*: These methods include the use of a range of physical traps such as adhesive surfaces. The approach is of most use in isolated releases where there is a low probability of intercepting pollen from other sources. Microscopic examination will permit identification at the species or genus level, depending on the variability of pollen morphology, although a distinctive marker could permit identification of pollen originating from test plants.

Experimental design

Although the exact design of an experiment will vary among releases because of differences in the biology of plant species and sites, some general principles apply in all situations. For monitoring results to be useful, the experiment should adhere to the principles of good experimental design:

i) The biology of the recipient plant must indicate that there is a need for field monitoring that is sufficient to justify the costs and effort;

ii) The objectives for monitoring need to be clearly and unambiguously defined;

iii) The experimental design needs to be in such a form that any data generated from monitoring can be analysed statistically, and this may require the advice of a statistician; and

iv) The design of the experiment needs to be capable of providing the required level of sensitivity; achievable levels of detection should be established at the outset.

The relative merits of different marker systems

Table 3 summarises the extent to which different markers which could be used in monitoring fulfil eight criteria governing their efficiency in a monitoring programme. It should be noted that in the case of laboratory-based molecular and biochemical methods, the rate of progress in automating the techniques is such that throughput using these methods could be increased dramatically in the near future.

Extended and indirect monitoring

There are circumstances under which extended monitoring of plants could be considered. Certain gene insertions may result in the release to the environment of proteins and other substances which could have direct, or indirect, effects on subsequent crops. The impact would depend upon the nature of the inserted gene(s), the product released, its persistence in the environment and the crop rotation. Monitoring of this kind charts the impact of the transformed crop upon its environment, rather than the migration of transgenic material.

There are several methods described in the literature by which the dispersal of genes may be estimated indirectly. These methods are all based on the notion that the spatial distribution of genotypes in populations may be used to infer underlying patterns of gene flow. These methods use variations of the F-statistics of Sewall Wright and depend upon the existence of populations which are in equilibrium with respect to patterns of inbreeding and migration (gene flow). Clearly, therefore, these methods cannot be used to analyse gene flow in short-term experiments with crop plants. However, if suitable biological models exist, for example natural populations of close crop relatives, then estimates of the levels of gene flow can be obtained from these and, by inference, for the crop species themselves.

A major shortcoming of even well designed experiments to measure gene flow directly is that very large experiments are needed to estimate rare, long distance dispersal events. Depending on the trait, these rare events may be of significance since, as the size of the release increases, the expected number of rare events which will actually occur increases and may reach a point where they become very likely. In contrast, the indirect measures of gene flow outlined above, by their very design, give estimates of gene flow events which actually have taken place. Providing that the populations used have been in existence for a number of generations, the analysis of genotype distributions will be based upon many years' actual gene flow and these methods will be superior in detecting rare events.

It should be noted, however, that the detection of gene flow *per se* from transgenic plants to other plants does not necessarily imply either that a particular gene will become fixed in the recipient population or that environmental impacts will ensue.

The criticism that the use of biological models introduces a degree of approximation to the estimates for the related crop species is certainly valid. However, it is of considerable importance to describe the fate of an engineered construct should it escape into related weed species. In this respect, the methods of indirect estimation of gene flow become directly relevant to the problem.

Table 3 The relative merits of different marker systems

Scale: 4 (favourable) to 1 (unfavourable)

Marker System	Reliability	Repeatability	Throughput	Sensitivity	Ease of Use	Special Requirements	Time for Identification	Unit Cost
Seed colour and shape	1-4	4	4	1-4	4	none	4	4
Plant and flower morphology	1-4	4	4	1-4	4	none	4	4
Comment: Reliability varies owing to variation in phenotypic expression caused by underlying genetics/development/plasticity. Repeatability depends upon distinctiveness of marker (including whether quantitative or qualitative).								
Disease resistance	1-4	1-4	2-4	2-4	1-2	2-3	1-4	1-2
Resistance to herbivores	1-4	1-4	1-3	2-4	1-2	2-3	1-4	1-4
Herbicide tolerance	2-4	3-4	3-4	3-4	4	1-3	3-4	3-4
Antibiotic resistance	2-4	3-4	3-4	2-4	4	1-2	3-4	3-4
Comment: Reliability varies according to the underlying genetic basis of resistance, and its expression. Repeatability varies similarly. Throughput and time depend upon plant stage at which resistance may be scored, type of herbivore. Cost can be high because of maintenance of disease, herbivore, etc.								

Table 3 (continued)

Marker System	Reliability	Repeatability	Throughput	Sensitivity	Ease of Use	Special Requirements	Time for Identification	Unit Cost
Secondary metabolites	2-4	4	1-4	2-4	1-4	1-4	3-4	1-4
Protein profiles	2-4	2-4	3	2-4	2-3	4	2	2
Allozymes	3-4	4	2-3	3-4	2-3	4	2	2
Antibodies	3-4	4	3	2-3	3-4	1-3	3-4	1-4
Comment: Low scores on reliability because of lack of experience, variation in metabolic states. Repeatability may be a problem between laboratories. Several characteristics depend upon the complexity of the analytical procedures and the availability of diagnostic kits.								
Nuclear DNA	4	4	1-3	4	2-3	4	2-3	1-2
Extra-nuclear DNA	4	4	1-3	4	2-3	4	2-3	1-2
Construct DNA	4	4	1	4	2-3	4	2-3	2-3
Comment: High reliability depends upon a distinctive marker and a well established technique.								

Feedback to the profiles

Even though there must be a clear understanding of background information on the organism, environment and site introduction before monitoring commences, much of the data accumulated during a monitoring programme can be fed back into the biological profile to facilitate future decision-making. In particular, monitoring experiments can provide very useful quantitative information on the life cycles of test plants [e.g. longevity of seeds and vegetative organs in soil, date of first flowering of perennials (Figure 1)] which, in turn, can inform decisions on the timing and duration of monitoring. Similarly, monitoring experiments can provide useful information on unforeseen characteristics of the test plant and its genetic material, where they occur.

The following **General Principles** are also important.

1) Both ecological and genetic methods are needed for efficient monitoring.

2) When genetic markers are needed, as a first choice the least expensive, easiest to use and most readily available markers should be chosen, so that experiments can be run on a sufficiently large scale to ensure good statistical repeatability.

3) In general, experimental design will need to be customised (i.e. on a case-by-case basis) in order to meet specific concerns in relation to the test plant or genetic construct, the type of proposed release, the nature of the test site, and the potential impact on the environment.

4) It cannot be assumed that the control and expression of a genetic construct will be retained under field conditions. Attempts to predict the behaviour of these constructs on the assumption that they will always be present in a single copy per genome, or that they will always be expressed in their original form, may not always be justified.

5) Some perennials and ornamentals, including trees and grasses which have substantial wild populations, pose unique monitoring problems.

The Animals Working Group

Introduction

In the OECD Workshop held in Copenhagen in 1990 to examine the scientific principles and methodologies available for monitoring organisms within the environment, there was no discussion of animals. The animal kingdom is very large and includes a great variety of life forms living in all possible habitats. At the Ottawa Workshop it was agreed to consider the present state of monitoring, and the immediate prospects, for those animals of interest in biotechnology.

It was apparent that there were experts on, and reports concerning, three main groups at the meeting. These were: animals involved in recombinant vaccines, organisms used in aquaculture, and terrestrial arthropods. These three groups are considered in more detail below. But the meeting also reviewed the animal kingdom as a whole to see where monitoring might be needed in the future.

The Animal Kingdom

Vertebrates

Mammals, birds and, in the foreseeable future, fish will be involved with recombinant vaccines, as discussed below. All three groups may also interact with biotechnology by being transgenic. For some time now there have been many transgenic laboratory and agricultural mammals. Up to now, monitoring has not been considered practical, necessary or effective for any of these. This may change, and monitoring may possibly be required for transgenic (laboratory and agricultural) birds, and also for transgenic fish other than those used in aquaculture, which are discussed below. The general principles of monitoring animals, listed below, would apply.

Arthropods and molluscs

Certain terrestrial insects and mites are already the subject of considerable interest and are discussed below. The same principles would likely apply to most other terrestrial arthropods and molluscs. As these groups are so varied, new and special situations can be expected.

Some groups of molluscs and crustacea, such as oysters and crabs, are used in aquaculture. Other marine, and possibly freshwater, benthic organisms would probably be monitored in the same way.

The group did not discuss the problems of monitoring other types of arthropods and molluscs. It was thought unlikely that there would be any OECD interest in them in the near future.

Other metazoan invertebrates

There may be a need to consider methods for monitoring nematodes and leeches in the future. It is less likely that other groups will become of interest. Such methods may well be very different from the ones discussed here.

General principles in monitoring animals

There are two principles that apply particularly to the monitoring of most animals.

The first is that it may be desirable or necessary to mark animals. There are many ways in which this is done, varying from heavy objects such as radio transmitters to molecular markers.

The second is that the animal may have to be trapped or refound. Some of these methods are indicated in the insect section below. Some traps act passively on the animal, others may require the animal to react and come to the trap. Other techniques for recovering marked (or unmarked) animals resemble those used for microbes, for instance those involving extraction.

As both marking and trapping of animals are affected strongly by the biology of the animal, the appropriate methods may have to be considered case-by-case.

Monitoring of live recombinant viral vaccines for wildlife immunisation

I. Identification of monitoring situations:

- characterization of vaccine master seed and testing of vaccine lots for purity, consistency, and *in vitro* genetic stability
- testing of vaccine in laboratory trials in the target species for efficacy, pathogenicity (mortality and morbidity), excretion, latent infections, and genetic stability
- testing of vaccine in laboratory trials in non-target contact species for pathogenicity (mortality and morbidity), excretion, latent infections, and genetic stability
- pre-release collection of background information in target area: possibilities of human exposure, animal species composition, bait consumers, prevalence of antibodies and viruses related to the vector and gene insert, natural occurrence of biomarkers
- monitoring of field applications of vaccine for efficacy, viral persistence in target and exposed non-target populations, vaccine virus dissemination, vaccine mutants and naturally arising recombinants, vaccine-related morbidity and mortality, bait species specificity, bait acceptance by target species, bait uptake by non-target species, changes in the target virus population, changes in ecological equilibria (competitor and prey populations)

II. Monitoring methods

- morphopathology of exposed animals (pathology, histopathology, immunohistochemistry, *in situ* hybridisation)

- vaccine virus detection: classical methods of virus isolation, identification with immunological methods and nucleic acid analysis (PCR, REA, probe hybridisation, sequencing)

- genetic stability: nucleic acid analysis, biological testing of recovered mutants if necessary

- monitoring of targeting efficacy: application of biomarker technology, "scent station" technology, seroconversion rates

- census methods for monitoring target and non-target populations (bait consumers, competitors, prey)

III. Applicability of methods

WHO, OIE, EC, etc. recommend all of the above methods, when applicable, on a case-by-case basis. WHO, OIE, and/or EC guidelines should be followed.

Monitoring transgenic fish in the environment

Requests to release new strains of transgenic fish into natural or semi-natural ecosystems will likely increase in the near future.

The introduction of transgenic fish into aquaculture situations will be, in most cases, into structures that provide physical confinement, such as:

1) research facilities;

2) land-based test facilities;

3) land-based production facilities; and

4) confined facilities in lakes, rivers and oceans.

It is anticipated that introductions into open waters will also be considered in the future.

With every containment facility there is a degree of risk that the physical barriers will break down and the transgenic fish will escape to the natural environment. In many cases, recovery of released transgenic fish from the wild will not be possible due to the difficulty in sampling and capturing individuals from aquatic environments such as the ocean.

Consequently, when the possibility exists for reproductive interaction with wild members of the species (or other species through hybridisation) or for establishing feral populations, a second level of containment such as sterilisation or monosex technologies should be implemented to reduce these risks.

However, because neither the physical nor biological confinement and/or containment measures available today are completely effective, it is essential that all transgenic fish programmes implement systems that will allow effective monitoring of the transgenic organism or the new gene construct in the local fish populations.

Methods

The methods available for monitoring transgenic fish in the natural environment can be broken down into three areas:

1) sampling;

2) detection of transgenic organisms as well as gene constructs; and

3) determination of biological and environmental effects.

A difficult aspect of monitoring fish in the wild is that of developing sampling methods that can obtain sufficient individuals from appropriate locations at the correct time. The number of fish examined must be appropriate to the local population size, and to the estimated numbers of transgenic organisms in the vicinity.

For some species such as salmonids, reproductively competent individuals return to specific streams or hatcheries to spawn and can be more easily accessed than, for example, migratory marine species. In many cases it may be difficult or impossible to sample sufficient individuals cost-effectively or within a short period of time.

Prior to the introduction of a transgenic fish species, information is required to estimate potential genetic or environmental impacts, including:

1) characterization of local species with respect to their habitat ranges, population numbers, biological characteristics, and genetic status (isozyme, morphological, or molecular markers);

2) estimation of fitness parameters of the new transgenic strain relative to wild stocks (survival, fecundity, behavioural characters, competitive ability relative to its own or other species);

3) studies examining the effect of releases of marked, unmodified fish at the proposed location;

4) review of the current status of knowledge of the interaction between wild and cultured individuals of the species under consideration, as well as the impact to date of introductions of exotic organisms to nature; and

5) stability of genetic modification in the genome and in populations.

Sampling methods are specific to the biology of the species and the number of escaped transgenic fish relative to natural population sizes, but include recovery from hatcheries or commercial catches of fish, seining, gill netting and trapping.

Wherever possible, genetically modified fish should be physically marked or tagged to allow simple and immediate identification of escaped individuals. Effective marking methods include:

1) fin clipping;

2) freeze branding;

3) nose tags;

4) passively induced transponders (PIT) tags;

5) morphological markers;

6) biochemical markers (strain-specific allozymes); and

7) molecular genetic polymorphisms.

Morphological markers will be most useful when large numbers of individuals will have to be screened, for example if very few individuals escape into large natural populations.

In subsequent generations, detection of the transgenic status of an individual can be accomplished by the application of the polymerase chain reaction (PCR) to a small piece of tissue, such as fin, blood or scale. Laboratory methods are available that allow processing several hundred samples per person day with high sensitivity, specificity, reliability, and at moderate cost.

An important part of the monitoring efforts will be registries of research being conducted in secure facilities and registries of any proposed introduction into confined facilities and/or the open environment.

An accurate and complete description of the gene construct present in transgenic organisms, and a reliable protocol for identifying the construct, should be required as part of the database in such registries. Reference should also be made to the International Council for the Exploration of the Sea (ICES): Guidelines regarding Introductions and Transfers, and Guidelines regarding the use of Genetically Modified Aquatic Organisms.

Terrestrial arthropods

There are several (perhaps up to 30) million insect species. They live in various habitats and their monitoring could be useful, or necessary, in agriculture, forestry or medicine.

Monitoring methods usually apply to the most mobile life stages, normally the adult insects. In some cases, as with parasitoids, monitoring larvae (that live within the host insect) may be most effective.

There are many reasons to monitor insects. For instance, monitoring agricultural or forestry pests could result in a reduction of pesticide use. In medical entomology, vector species can be monitored to detect pathogens. Monitoring insects is mandatory for quarantine, and possibly for conservation purposes, as well as in any case of deliberate release of species in a new environment (e.g. classical biological control, genetically modified insects).

There is abundant literature on methods of insect monitoring. With respect to arthropods, monitoring can be done to assess population levels or to study insect attributes. **Insect populations** may be monitored with either relative or absolute methods. Because capture levels cannot be converted into absolute units (i.e. number of individuals per thousand), traps are considered frequently as relative methods. In contrast, data from sampling programmes can often be converted into absolute units. Therefore, sampling is useful in studies where a relationship is to be established between population levels and damage (e.g. economic impact of pests).

The applicability of monitoring methods depends on the arthropod targeted. **Table 4** presents the evaluation of several methods to monitor insect pests. The data are applicable to a majority of insect pests, but clearly monitoring methods must be chosen on a case-by-case basis. Several components have been taken into account to assess monitoring methods. For instance, light traps 1) are generally non-sensitive; 2) are easy to use; 3) do not require specialized equipment; 4) require much time to identify markers (species or attributes of species); 5) are not specific; 6) are variable in reliability; 7) are costly to operate; and 8) are useful to capture species of restricted number of taxa, namely lepidoptera and, to a lesser extent, coleoptera, hemiptera and diptera.

For monitoring specific insect groups such as parasitoids (**Table 5**) or coccinellid and carabid predators (**Table 6**), fewer methods can be used. This is a consequence of the life cycle characteristics of these groups and a lack of research data.

Population levels of important groups of arthropods such as phytophagous and predatory mites can only be monitored effectively by visual observation, or with a leaf brushing machine. Consequently, mite monitoring is expensive. This situation is likely to remain as such in the future.

Monitoring of **insect attributes** refers to activities such as assessing levels of resistance to insecticides, or quality control of wild or reared beneficial insects. Genetically modified insects fall into this category. Standard molecular biology techniques are widely applicable to insect science. For instance, PCR techniques are used to identify insect strains. This approach could be important for commercial applications such as quality control of biocontrol agents.

Monitoring insect attributes will be increasingly important in the future. For instance, many insect pests are now resistant to insecticides. Continuous monitoring of resistance levels is required to design sound management programmes in many agricultural crops.

For monitoring the establishment and spread of introduced arthropods, various mark-recapture techniques are applicable. Physical methods (e.g. clipping, radioactive labelling, colour marking, tagging) have been widely used in research. Genetic markers such as polymorphic traits have also been used in population genetic studies.

Genetic markers will likely be more important in the future in either commercial or research contexts.

Table 4 Monitoring insect pests

	Sensitivity of method	Ease of use (practicability)	Requirement for specialised facilities or equipment	Time required to identify markers	Specificity	Reliability	Cost	Any limitations imposed by method
Traps								
Light	low	high	low	high	low	variable	high	mostly lepidoptera, few coleoptera, hemiptera and diptera
Bait	high	high	low	low	low	low	low	applicable to some species
Pheromone	high	high	low	low	high	high	low	mostly lepidoptera
Sticky	low	high	low	low	low	high	low	manpower, taxonomy
Pitfall	variable	high	low	high	low	low	low	empty traps
Suction	high	high	high	high	low	high	high	manpower, electrical power
Malaise	high	high	low	low	low	high	low	manpower
Emergence	high	high	intermediate	low	intermediate	high	low	phototactic (+)
Sampling								
Visual obs.	high	high	low	low	high	high	high	plant architecture
Vacuum	low	high	intermediate	high	low	high	high	moisture and height of vegetation
Sweep net	low	high	low	high	low	high	intermediate	

Table 5 Monitoring insect parasitoids

	Sensitivity of method	Ease of use (practicability)	Requirement for specialised facilities or equipment	Time required to identify markers	Specificity	Reliability	Cost	Any limitations imposed by method
Traps								
Light	not applicable							
Bait	not applicable							
Pheromone	not applicable							
Sticky	low	high	low	low	low	low	low	identification, weak response
Pitfall	not applicable							
Suction	high	high	high	high	low	high	high	manpower, electrical power
Malaise	high	high	low	high	low	high	high	manpower
Emergence	high	high	intermediate	low	intermediate	high	low	phototactic (+)
Sampling								
Visual obs.	variable	high	low	low	high	low	high	darkness, canopy
Vacuum	variable	high	high	high	low	high	high	moisture and height of vegetation
Sweep net	low	high	low	high	low	high	intermediate	

Table 6 Monitoring coccinellid and carabid predators

	Sensitivity of method	Ease of use (practicability)	Requirement for specialised facilities or equipment	Time required to identify markers	Specificity	Reliability	Cost	Any limitations imposed by method
Traps								
Light	not applicable							
Bait	not applicable							
Pheromone	not applicable							
Sticky	not applicable							
Pitfall	variable	high	low	intermediate	low	intermediate	low	carabids only
Suction	high	high	low	low	low	intermediate	intermediate	related to habitat, electrical power
Malaise	not applicable			high				
Emergence	not applicable							
Sampling								
Visual obs.	high	high	low	low	high	high	intermediate	coccinellids only
Vacuum	high	high	intermediate	low	low	high	intermediate	moisture and height of vegetation
Sweep net	high	high	low	low	low	high	intermediate	

IV. Summaries of the Poster and Short Presentations

I. Modelling gene dispersal from genetically modified organisms: the case of herbicide resistant crops

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Summary

Many genetically modified plants have been transformed to be resistant to herbicides. The risks associated with these transgenic crops need to be quantified before they are released for field trials or commercialisation. Computer simulations were used to compare risks and to estimate what parameters are important to limit escapes of transgenic pollen into weedy relatives.

Hybridisation barriers play an important role in gene flow. However, limited success in crosses between wild and cultivated plants may result in a high frequency of resistant weeds at equilibrium. The models indicate that gene transfer risks decrease when a physical linkage exists between the resistant gene and genes controlling major domestication characteristics.

II. Evaluation of different techniques to monitor bioluminescent microorganisms in soil or non-marine water samples

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Conclusions

It could be shown that bioluminescence may be used successfully as a marker for environmental monitoring purposes. The *major advantages* of the use of bioluminescence as marker for bacteria to be released in non-marine environments are its specificity, the potential to monitor the marker *in situ* (i.e. without extraction and cultivation of the cells), in real time, and the possibility to process and quantify a high number of samples during a working day.

The *major restriction* of all monitoring methods based on bioluminescence is that they can only detect cells where the introduced *lux* genes are actively expressed by the respective bacteria. Dead or quiescent cells will be missed.

The use of bioluminescence as a marker of deliberately released microorganisms depends to a great extent on the knowledge of strain- or sample-specific features and cannot be generalised for every monitoring situation.

Factors that might have impact on the expression of the genes in the particular strain or on the detectability of the light production should therefore be found out for each organism individually before it is released into the environment.

III. Effect of soil structure on the leaching of bacterial inocula through soils

Eric Paterson, J.S. Kemp, E.A. FitzPatrick, C.E. Mullins, M.S. Cresser and S.M. Gammack
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Discussion

The pattern of leaching of the GMM could be interpreted in terms of structural differences between the soils used.

Insch and Craibstone are freely draining soils. Leaching of GMM was characterized by a distinct breakthrough peak during the fourth and fifth rainfall events. This corresponds to displacement of inoculum through water-filled conducting channels by rainwater.

Cruden Bay soil is poorly draining, having fewer water-conducting channels. The GMM was detected in leachate during the first rainfall event, and numbers declined logarithmically thereafter. This is the result of inoculum displacement through a small volume of conducting channels and dispersive mixing of soil water through impeded drainage, particularly because of the more massive subsoil.

IV. Surface antigens of vegetative rhizobium cells may be diminished or absent when in the bacteroid form

Perry Olsen, Mandy Collins and Wendell Rice
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Beaverlodge, Alberta
Canada

Conclusion

Conclusions reached about the reactivity of anti-Rhizobium antibodies based on ELISA evaluations using vegetatively cells may not always be validly extrapolated to nodule typing. The usefulness of such antibodies in nodule typing needs to be empirically verified. Questions arise about the presence or absence of identifying antigen in nodule material in relationship to a variety of such factors as nodule age, nodule origin (field or lab), pH of growth, moisture stress, etc.

V. Effects of inoculative method and size of indigenous population of *Rhizobium meliloti* on nodulation of alfalfa

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Conclusions

Granular inoculant applied to the side of the row after stand establishment had no effect on nodulation or yield of alfalfa grown on moderately acid soil.

Granular inoculants applied with or below alfalfa seed significantly increased biomass yield and nodule weight as compared to seed-applied inoculation when the indigenous population of *R. meliloti* was small.

Granular inoculants applied with or below the seed increased nodule numbers and the proportion of nodules occupied by the inoculant strain in the presence of either small or large indigenous populations of *R. meliloti*.

Increases in nodule numbers and nodule occupancy were not reflected in increases in biomass yield when a large population of indigenous *R. meliloti* was present, indicating that the indigenous strain(s) co-occupied the nodules with the inoculant strain, resulting in smaller, less efficient nodules.

VI. Risk assessment studies at the Scottish Crop Research Institute on the potential hazards of genetically modified organisms

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VII. OECD Directorate for Agriculture Co-operative Research Project on Biological Resource Management 1990-1994

Project Head: Camille Raichon
Secretary: Fay Evans

Organisation for Economic Co-operation and Development
2 rue André-Pascal, 75775 Paris Cedex 16, France

VIII. Detection of low numbers of flavobacterium P25 in soil

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Conclusions

Current immunological techniques are unable to detect low numbers (<1000 per gram) of specific bacteria in soil. Immunofluorescence microscopy, ELISA, immunoblots and flow cytometry are all less effective at detecting P25 in soil than viable plate counts. However, time-limited selective enrichment enables P25 cells to be detected and enumerated when present in soil at densities as low as two culturable cells per gram.

IX. Survival and impact of genetically engineered microorganisms transformed with mercury resistance gene in the water and soil environment

Osami Yagi, Kazuhiro Iwasaki and Hiroo Uchiyama
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Conclusions

The mercury resistance gene was a good marker to detect the GEMs. One CFU of GEMs per mL in water and 10 CFU per gram soil were detectable by this method.

No significant differences of survival were observed in water microcosms between genetically engineered and wild type *P. putida* strains.

The survival rates of *E. coli* and *K. oxytoca* were higher than *Pseudomonas* species in soil microcosms.

The population of heterotrophic bacteria and fungi were not influenced by the introduction of GEMs.

X. Monitoring latent baculovirus infections in insect populations using the polymerase chain reaction method

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2. School of Biological Sciences, Oxford Polytechnic, Oxford, United Kingdom

Summary

A stock of *M. brassicae* insects has been found to harbour a latent MbNPV; another stock of insects of the same species is uninfected.

The latent virus may be activated by closely related or distantly related baculoviruses.

The PCR may be used to detect the latent virus in apparently healthy insects, thus avoiding the need to superinfect insects.

The PCR method developed is amenable to scale-up, thus facilitating analysis of multiple samples, e.g. in 96-well dishes (future work).

XI. Phenotypic and genotypic analysis of bacterial populations isolated from the phylloplane of sugar beet

M.J. Bailey, R.J. Ellis, P.B. Rainey and I.P. Thompson
Institute of Virology and Environmental Microbiology
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XII. PCR and isoenzyme analysis. Methods for identification of gene transfer between species

Rikke Bagger Jorgensen and Bente Andersen
Plant Biology Section
Environmental Science and Technology Department
RISO National Laboratory
Roskilde, Denmark

Conclusions

Natural hybridisation between oilseed rape (*Brassica napus*) and *B. campestris* takes place. The hybrids can backcross with *B. campestris* in the field. Backcrosses can produce *B. campestris*-like plants (AA). The *B. campestris*-like plants have a high fertility and cross readily with wild type *B. campestris*. Therefore transgenes integrated on the A-genome of oilseed rape can be introgressed to *B. campestris* rather easily.

The transfer of specific C-chromosomes to the backcross plants is investigated. This knowledge may enable design of transformation vectors that enhance integration of genes at places where they are least likely to be transferred, i.e. vectors carrying sequences homologous to the chromosome parts that are seldom transferred.

XIII. Measuring the persistence and survival of PGPR on canola and soybean overwinter, using spontaneous antibiotic markers

E.M. Tipping[1], G.L. Brown[1], R.M. Zablotowic[2] and J.W. Kloepper[3]

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3. Department of Plant Pathology, Auburn University, Auburn, Alabama, United States

Conclusions

Although the sample size is small, it appears that pseudomonads survive overwintering better than do strains of the genus *Serratia*.

Recolonization can occur after replanting untreated seeds, although population densities do not reach the levels detected in the previous growing season.

Strains which persisted at high levels throughout the growing season were better able to survive overwintering and colonize subsequent crops after replant.

Most strains present at low levels at the end of the growing season (less than log 2/g root) were not able to re-establish high population levels in the subsequent year, although strains 25-33 and G20-18 were exceptions.

XIV. Oligonucleotide probes to detect bacteria introduced in the environment

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Conclusions

The oligonucleotide defined from the spacer between the 16S and the 23S rDNA gene of strains QP5Rif and R17-FP2 shows excellent specificity for these two bacteria. Tomatoes which had not been inoculated with these bacteria did not yield bacterial colonies hybridising to this probe.

Tomatoes were inoculated with rifampicin-resistant bacteria, and bacterial colonies were recovered. The proportion of colonies hybridising to the probe was consistently lower on a general medium (TSBA) than on a selective medium (TSBARif), again showing that the probe discriminates between inoculated and resident bacteria.

XV. Alternative method for the production of microthreads used in the study of airborne survival of microorganisms outdoors

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Conclusions

Production of microthreads using a modified commercial glue gun proved to be a simple, inexpensive alternative to the use of spider's web. With adequately controlled temperature, 30-40 frames could be wound per hour by a practised operator, a considerable improvement on what could be achieved using spiders.

Despite the difference in diameter of the two fibers, survival of suspended bacteria exposed to toxic factors in the air was similar. Thus the alternative thread can be used with confidence as a replacement for spider's web and is now standard practice in our laboratory.

XVI. GMO release: risk assessment using an insect system

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XVIII. The CORINE Project

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Some results from the 1985-1990 programme

In response to the information needs on the state and evolution of the European environment, the Commission of the European Communities set up the CORINE Programme. Carried out between 1985 and 1990 in collaboration with groups of experts from the Member States, this work improved the availability, comparability, coherence and use of information on the state of the environment within the European Community.

Given the wider role of this task, covering all facets of the state of the environment for a territory greater than 2 million square kilometres, the CORINE Programme concentrated on the collection and collation of information useful for Community environmental policies on topics of priority concern: e.g. biotopes of major importance, acid deposition, the Mediterranean environment.

The Programme has provided a considerable number of results that are equally important at international, EC, national and regional levels. These results can be grouped into three groups:

- the CORINE information system and its databases;
- nomenclatures and definitions;
- co-operation, agreements and know-how within European networks of experts.

The aim of the CORINE Programme was to verify the usefulness of a permanent information system on the state of the environment for Community environmental policy, to check the technical feasibility of creating such a system, and to identify the conditions required for its installation and functioning.

The CORINE results demonstrate that a permanent information system on the state of the environment in the Community is necessary and technically feasible. Moreover, the Programme has made it possible to clarify with precision the conditions required for realizing and running such a system. It was particularly in the light of these results that the Council of Ministers for the Environment decided to transform the CORINE prototype into a permanent information system within the framework of the European Environment Agency.

This poster illustrates the CORINE information system. A detailed description of the work carried out and of the results obtained will be presented in a series of reports, manuals and maps, currently being prepared for publication.

XIX. Genetic monitoring of the invasion of britain by the cynipid gallwasp *Andricus quercuscalicis*

Graham Stone, Imperial College, London; Paul Sunnucks, Institute of Zoology, London; and Karsten Schoenrogge, Imperial College, London, United Kingdom

The cynipid gallwasp *Andricus quercuscalicis* (Burgsdorf 1783) has invaded northern and western Europe from its native range in southeastern Europe and Asia Minor. This has been made possible by human dispersal of one of its host plants – the Turkey oak, *Quercus cerris*. This species has a life cycle involving obligate host alternation between Turkey oak and the English oak, *Quercus robur*. While English oak is common and widespread in much of northwestern Europe, over much of its introduced range Turkey oak is rare and patchily distributed.

We predict the distribution of Turkey oak to have had severe genetic effects on *A. quercuscalicis*. The wide separation and rarity of Turkey oaks are predicted to have caused serial genetic bottlenecks in the invading *A. quercuscalicis*, resulting in considerable loss of genetic variability. The pattern of loss of variability is predicted to be random.

Of 13 scoreable electrophoretic loci, eight showed allelic variation. These loci were scored for 823 individual asexual females of *Andricus quercuscalicis* from 39 populations through the native and invaded range. Allelic diversity and mean heterozygosity both decrease significantly with distance from the native range.

The patterns of change in allele frequencies do not suggest the existence of a selection gradient. Rather they suggest a random walk in gene frequencies associated with repeated westward colony formation involving small numbers of founders from nearby source populations. Colonization by repeated numbers of small steps is also suggested by the high correlation between the geographic position of the sample sites and phylogenetic relationships between them.

Allozyme data suggest that the British population of *A. quercuscalicis* is the result of a small number of founders arriving in the east, with loss of a further two alleles in the extreme southwest.

XX. The genetically modified organisms research programme: science informing policy in the UK

The genetically modified organisms (GMO) research programme, run by the **UK Department of the Environment (DOE)**, aims to determine the hazards, and assess the risks, which the release of GMOs may pose to the environment. The programme, which spends around 1.3 million pounds each year, currently consists of 21 contracts, which range in size from six-month reviews (desk studies) to five-year research projects. Work focuses on issues relevant to the current release situation in the UK, concentrating on crop plants and microorganisms (although some work on animals is included). Where possible, "conventional" organisms are studied rather than GMOs. Advice on the concept of the programme is provided by the UK government's advisory committee on releases to the environment, although new ideas from the wider scientific community are also sought.

XXI. Monitoring the communities of natural enemies associated with the galls of *Andricus quercuscalicis* (Hymenoptera: Cynipidae); an invader in Britain

K. Schonrogge, Imperial College in London, and G.N. Stone, Imperial College in London
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XXII. Numerical codes for considering biological properties of host organisms in particular environments

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A numerical code evaluating the exposure of nature to gene flow from crops has great potential usefulness. Using this kind of code, it is important to be explicit about how the data are evaluated, allowing disagreement while working towards data-based, well defined consensus.

A weakness is that there is probably insufficient data on escapes for certain areas because escapes are usually not included in data bases on the wild vegetation.

In fact, a two-part code describing 1) dispersal of pollen and 2) dispersal of seeds and dispersal units should suffice. Having determined the dispersal code of the crop, this is only a small part of the whole risk assessment. Frequency must be considered, but is so dependent on specific climatic and other abiotic factors that it is difficult to express in one figure.

The code for pollen disposal may be:

0 = no crossing or hybridisation possible in the absence of wild relatives
1 = no crossing or hybridisation observed with any wild relative
2 = crossing or hybridisation observed with a wild relative only on experimental sites
3 = + sterile hybrids observed in nature
4 = crossing with conspecific relatives in the neighbourhood must be expected
5 = both crossing and hybridisation will occur in nature

The code for seed dispersal may be:

0 = no escapes observed in nature
1 = no viable off-spring observed
2 = temporary establishment (mostly vegetatively)
3 = viable introductions observed
4 = escapes untraceable morphologically because conspecific relatives grow in the neighbourhood

Viability = viability in a specific climate, as ascertained by at least three successive spontaneous generations, or at least three localities.

Also see page 27 of this Workshop report (paper by Dr. Van der Meijden.)

XXIII. A monitoring case study: Is heterologous transencapsidation a problem with plants transgenic for potato leafroll virus coat protein gene?

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During the past few years coat protein genes representing most groups of plant viruses have been used to transform plants in attempts to develop coat protein-mediated resistance. There are some concerns with this strategy for developing virus resistance, particularly the possibility of recombination between the transgene and other viruses that may infect the plant. With luteoviruses there is also concern over the possible heterologous encapsidation of other viral RNAs with the expressed coat protein, as this is known to occur in natural systems with several luteoviruses.

Potato plants transgenic for the coat protein gene of potato leafroll virus were infected with either potato virus S, potato virus X, or potato virus Y by mechanical transmission. The plants were determined to be infected with the inoculated viruses by ELISA. Aphids (*Myzus persicae*) were given a three-day acquisition feed on these plants four weeks after inoculation. 1000, 1000 and 1500 aphids from PVS, PVY and PVX infected plants respectively were then transferred to healthy normal potatoes for a one-week inoculation access period.

With PVY and PVS, the aphids were placed in a petri dish with moist paper filters overnight after the acquisition feeding and before the inoculation access feeding, so that the non-persistently transmitted PVY and PVS would not be transmitted unless their RNAs were heterologously encapsidated in PLRV coat protein. After one week the aphids were killed by spraying with Pirimor. Four weeks after the beginning of the inoculation access feeding, the plants were tested for the presence of PVS, PVX or PVY, whichever virus source the aphids had fed on during the acquisition access feeding.

All plants on which the aphids had inoculation access feeds tested negative for PVX, PVS or PVY. These results suggest that PVS, PVX and PVY are not readily encapsidated in PLRV coat protein. PLRV is not known to heterologously encapsidate other viral RNAs in nature. It will be interesting to have similar tests done with luteoviruses that are known to encapsidate other viral RNAs. Infectious transcripts of PLRV inoculated onto plants transgenic for coat protein or other structural genes will be useful in evaluating recombination between viral transgenes and infecting viruses.

Risk assessment of viral transgenes will vary from virus group to virus group. Assessment of risk should take into consideration how the virus is transmitted in nature and what we know or do not know about the mechanism of transmission. It should be possible to build biological containment into constructs, such that in the event of recombination the new virus is not vectored in nature. We also need to consider what controls the host range of a virus so that the ecology and epidemiology of a virus are not altered dramatically.

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