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REPORT OF THE OECD WORKSHOP ON THE MONITORING OF ORGANISMS INTRODUCED INTO THE ENVIRONMENT

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 1992

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ENVIRONMENT MONOGRAPH NO. 52

REPORT OF THE OECD WORKSHOP ON THE MONITORING OF ORGANISMS INTRODUCED INTO THE ENVIRONMENT

A Record of Discussion

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 1992

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Preface

In December 1990 the Danish National Agency for Environmental Protection hosted an OECD Workshop on the Monitoring of Organisms Introduced into the Environment. This report is a record of the discussion of the Workshop, which focused on monitoring associated with small-scale field trials. It sets out major findings and suggestions of the Workshop.

Biotechnology was recognised in 1982 by the OECD Committee for Scientific and Technological Policy as an emerging major field of technology, from which various policy issues would arise with wide scientific, economic and societal impacts. A group of national experts was therefore convened to consider recommendations regarding future OECD work in this area. Their report on *Biotechnology: International Trends and Perspectives* (OECD, 1982) remains an excellent, forward-looking study of many of the issues, problems and opportunities associated with the use of biotechnology. The authors of the report recognised that this area requires a unique multidisciplinary approach, integrating the development and implementation of policy and technical instruments in the areas of science and technology research, industry, agriculture, education, environment, health, and worker safety.

As a follow-up to this activity, a study on "Safety and Regulations in Biotechnology" was undertaken. That study led to a Recommendation of the OECD Council concerning Safety Considerations for Applications of Recombinant DNA Organisms in Industry, Agriculture and the Environment [C(86)82 (Final)] which called, *inter alia*, for further research to improve the prediction, evaluation and monitoring of the outcome of applications of recombinant DNA organisms. This work also led to the publication of *Recombinant DNA Safety Considerations* (OECD, 1986).

In 1988 the Group of National Experts on Safety in Biotechnology (GNE) decided that part of their programme should be the development of general principles which would identify a generic approach and guidelines for the design of small-scale field research with plants and microorganisms. The OECD subsequently sponsored the Workshop in Denmark on approaches and methods for monitoring.

Although it has been reviewed by the GNE, the report of the Workshop does not necessarily represent the views either of the Group of National Experts or of OECD Member countries. Following review by the GNE, the Environment Committee recommended that the Secretary-General make this report available to the public under his own responsibility, which he agreed to do.

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I. Objectives and organisation of the Workshop

This was the first OECD Workshop convened to examine the scientific principles and methodologies available for monitoring organisms within the environment. In his welcoming remarks Mr. Ole Plougman, Deputy Director General of the Danish Environmental Protection Agency, drew attention to the timeliness of the meeting. Mr. Plougman highlighted the need for harmonized international approaches, both in OECD Member and non-Member countries, as biotechnology has moved from the laboratory and greenhouse into the field.

The need for forward-looking and innovative work for the development of an international approach to monitoring was highlighted by the Chairman of the Workshop, Dr. Roger Nourish. Scientific principles and appropriate methodologies for following the environmental fate and impact can enable us to design, evaluate and monitor field tests in the environment. The purpose of the Workshop was to provide a first step in international exchange and co-operation. In this regard, as background, a number of representatives gave oral reports on monitoring and on regulatory and legislative activities concerning field testing in their countries. In addition, more formal presentations (case studies) were given in the following areas: bacteria, viruses, plants and animals.

The Workshop provided a unique opportunity for experts from different countries, as well as diverse scientific and regulatory backgrounds, to exchange views and experiences openly and freely. This document provides a preliminary outline of considerations in approaches and methods for monitoring the fate and impact of organisms introduced into the environment. The utilization of appropriate approaches and methods may contribute to the safe development of the technology and to its beneficial applications. The focus for the discussion of monitoring in this Workshop report is the small-scale field introduction. The term monitoring, as discussed in the context of the meeting, was very broadly interpreted.

The objectives of the Workshop, as agreed by the Steering Group of Experts from Member countries, were:

i) to develop a snapshot of the "state-of-the-art" concerning the monitoring of organisms, genetic material, and environmental processes with particular regard to the introduction of GMOs into the environment;

ii) to explore to what extent principles and guidance can be developed at present;

iii) to identify further data/research, and/or work, needed to develop harmonization of principles, approaches or procedures within the OECD; and

iv) to develop recommendations with regard to the need for, and priorities attached to, further work.

The Workshop was organised around plenary sessions and three working parties on:

i) approaches to the development and use of monitoring;

ii) design and methods for monitoring related to plants; and

iii) design and methods for monitoring related to microorganisms.

Particular emphasis was placed on: acquiring information on the experience gained from the past history of monitoring of various kinds of environmental introductions; approaches, principles and key issues addressed by Member countries; practical methods used; and useful information and data. The Workshop focused, in particular, on basic field research and initial applied field research (see **Figure 1**).

Figure 1. Good Development Principles (GDP) in the context of field research (OECD, 1990a)

Figure 2. Important components for monitoring programme inputs

II. Principal findings of the Workshop

A. General approaches to monitoring

Monitoring of organisms can be used for many purposes, including:

i) to gather information on the performance of a specific organism;

ii) to increase knowledge which might be applicable in many different circumstances; and

iii) to contribute to an assessment of environmental impact.

For instance, the conduct of monitoring for performance could include data about survival, competitive ability, and yield.

Several scientific reasons for monitoring plants and microorganisms were identified during the Workshop:

i) to test assumptions about pre-release evaluation of hazard;

ii) to provide basic information on the biology of organisms and the ecosystem in which introductions take place;

iii) to allow better predictions to be made about probable environmental impacts and performance; and

iv) to provide the data that enable better experimental designs to be developed.

Since there are divergent purposes and reasons for monitoring, deciding on an appropriate protocol for a given field test will depend on those pertinent to the particular field test. In certain cases, monitoring may not be warranted or useful. The decision to monitor and the development of an appropriate monitoring protocol should be a flexible process, involving an assessment by the experimenter and/or other suitable entities of all pertinent available information. This would include information regarding the organism to be introduced (the organism profile), the environment into which the organism is introduced (the environment profile), the procedures employed for the introduction (the introduction profile) and, as appropriate, previous monitoring results. When the term *profile* is used, it is intended to serve as a description of the relevant component which is as complete as possible, based on the information available. The integrated evaluation of the information contained in these profiles for a given introduction provides the basis for the development of a specific monitoring programme (see **Figure 2**).

The organism profile includes knowledge of the organism that contains the trait(s) of interest; the donor organism(s), if any, that contributed a trait(s); the gene(s) coding for the trait and its products; the mechanism used to transfer or confer the trait into the test organism; and any other information about the organism. The environmental profile consists of the site-specific and surrounding area factors involved

in the evaluation of the environment into which the organism is to be introduced. For example, the environmental profile may include data from observations of the site

prior to testing, or from other information systems, e.g. laboratory studies/literature or monitoring of

the make-up and behaviour of the organisms and ecosystems indigenous to the release site. Finally, the introduction profile describes the physical attributes and procedures to be employed at the site during the test.

There is an interactive process which is dynamic and flexible between the stages of development of a new variety of plant or strain of organism (**Figure 1**). Stage 1 in the development of an organism may involve contained laboratory and/or greenhouse experimentation or microcosm/mesocosm experimentation to provide data that may be used to design subsequent monitoring procedures. Evaluation of data from this stage can provide an understanding of the organism's behaviour in a defined environment. Stage 2, the focus for the discussion of monitoring in this report, involves the small-scale field introduction.

Data obtained at Stage 1 can be included in the evaluation of all the components of the three profiles and can be used to design a field-scale monitoring programme in Stage 2. Thus, for example, when the profiles are analysed the known behaviour of the organism, particularly if it is indigenous to the environment, will provide significant information about the potential behaviour of the organism during the field test.

Stage 3 is the developmental stage. It may encompass possibly longer term testing at multiple sites, and occurs prior to commercialisation of the introduced organism. There may not be a clear distinction between the different stages of testing, depending on the purpose of the particular test, and it may be appropriate to bypass one or more stages of testing. Emphasis needs to be placed on a dynamic and flexible interaction between these stages.

Monitoring can detect environmental impacts which may be beneficial, neutral and/or adverse. Whether monitoring will be useful in providing data about environmental impacts or for assessment of risk will depend upon the information that already exists on parameters including the organism, the purpose of the test, and the characteristics of the site.

Environmental impacts are a consequence of intended and unintended effects. The intended effects are determined by the intended use of the organism. Intended effects are reasonably foreseeable from the information developed for Stage 1. Other effects may also be foreseeable. However, unforeseeable environmental impacts may occur but because these impacts would not be predicted, they could be not be systematically monitored until recognized.

Environmental impacts may result from the presence of the organism in the environment, or the transfer of genetic material from the organism into others, including indigenous members of the same or related compatible species. Detection or monitoring of the transfer of genetic material is easiest when the transfer results in a gene being stably maintained and functionally expressed by the resulting hybrid organism in the environment. However, some experts consider that gene flow in the absence of selection pressure or functional expression may also occur. The relevance of this to the environment has not been determined.

The effectiveness of a monitoring programme can be judged on the usefulness of the data

generated, as these relate to results of previous trials or the design of subsequent ones. The analysis of the data can be used in determining whether field testing of the organism is to continue as planned, to continue with modifications of the type of monitoring done, or to be terminated. The development of a monitoring programme is a dynamic cyclical process in which modifications to monitoring practices can be made during current or subsequent field trials as data from earlier monitoring programmes accumulate and are evaluated.

Much of the methodology required for monitoring of organisms in the environment already exists in the biological and agricultural sciences. Further research to enhance understanding of the normal behaviour of organisms and ecosystems in a variety of environments reflecting those encountered in field trials can contribute to the improvement of approaches to, and methods for, monitoring. Essential support of the continued development of scientific research, and associated education programmes, will be necessary to assure keeping pace with anticipated technological development.

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B. Plants: monitoring approaches and methods

Methods and experience from ecological genetics, plant ecology and experimental agriculture will be of value in plant monitoring. The biology and behaviour of most crop plants is well understood. Assuming that a new plant line is like its agronomic progenitor is reasonable in the absence of any other data.

In this section methods are described by which sampling can be achieved, plants identified and results analysed. Methods are also described which are designed solely to provide information that will allow more precise detection of environmental impacts. The precision of some methods, depending on their purpose, will be enhanced by the use of easily identifiable markers. These methods need not necessarily be used in field trials. All the methods described can be used with plants in general, including those that are genetically engineered, transgenic or indigenous.

Sampling:

All sampling methods can be divided into three broad categories: direct observation, trap plants, and physical methods.

1) Direct observation

In its simplest form this category involves regular and methodical inspection of the trial site both during and after the field test to see if the plant has moved out of the experimental area, or has survived on the site after termination of the experiment. To provide a baseline for this type of monitoring, a pre-release survey for the presence of the plant in the area of the site would normally

be carried out.

2) Trap plants

Trap plants acting as recipients provide a simple way to monitor for potential pollen escape. For pollen trapping to be successful, the flowering characteristics, especially flowering time, of the trap plants should be as similar as possible to those of the test plants, especially in the case of insect-pollinated plants. Trap plants with enhanced nectar production will increase the efficiency of pollen trapping.

3) Physical methods

In cases where pollen is readily identifiable, direct sampling of the atmosphere can provide a suitable form of monitoring.

Identification:

1) Direct observation

For test plants to be identified quickly, easily and unambiguously, they must display a characteristic that is stably inherited and expressed, and different from that displayed by local crops and feral populations of the same species.

2) Trap plants

In cases where there is no cultivation of the same species in the vicinity of the release site, male sterile trap plants provide a simple and efficient means of monitoring as all seed produced by the trap plants must have been produced by pollination from the test plant.

Where suitable male sterile trap plants are not available, the lines used for trapping pollen must differ from the test plant in some characteristic that is stably inherited and expressed. Such characteristics may exist at the following levels:

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

ii) physiological (e.g. resistance to disease or sensitivity to herbicide);

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

iv) molecular (RFLPs).

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) Physical methods

Physical sampling methods are most useful for trapping pollen in which there is very little intraspecific variation. Identification in such cases is only possible to the species level. Similarly, physical sampling for dispersed seed may only allow for the identification of species unless a distinctive marker is present in the plant line.

Experimental design:

Although the exact design of an experiment will vary between 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 experimental design needs to be in a form that any data generated from monitoring can be statistically analysed, and this may require the advice of a statistician;

ii) The objectives for monitoring need to be clearly and unambiguously defined. For example, the level of detection required should be established;

iii) The layout of the experiment needs to be capable of providing the required level of sensitivity;

iv) The best experiment would be designed so that the threshold limits of detection by monitoring are clear and unambiguous;

v) Although in theory genetic markers expressed at any stage of plant development can be used, the choice of trait will affect the efficiency of detection. Particularly in the case of trapping pollen or other propagules, large quantities of pollen may have to be observed and processed. Because of the large numbers involved, cheap, easily identified and readily available markers should be sought in the first instance (a rough assessment of the utility of different markers in monitoring is given in **Table 1**); and

vi) Specialist botanical expertise may be required to identify potential hybrids between a test plant and locally growing wild relatives.

Background monitoring

At some point during the evaluation period, an indication of the performance of a plant in the non-cultivated components of the environment may be useful. This kind of information can

conveniently be grouped under two headings:

i) the ability of the plant to hybridise with phylogenetically close relatives with which it may come into proximity; and

ii) the ability of the plant to survive and persist in non-agricultural situations.

Hybridisation

Although there is a wealth of information about the ability of crop species to hybridise with relatives, much of it is not in a form directly useful for monitoring of plants. The major drawback is that much of this information merely tells us that hybridisation can occur, but not the frequency with which it occurs. Experiments using both natural and artificial cross-pollination can be used to provide

Figure 3. 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.

probable upper and lower limits of the frequency of hybridisation. In such experiments the principles of good experimental design outlined above should be followed. Information on the viability of hybrids and their fertility may also be very useful. An additional problem in inter-species hybridisation is that the ability to hybridise may vary between genotypes. Cross-pollination studies will be most informative when the test plant itself and wild relatives, from a range of different sources, are used.

Survival and persistence

The characteristics of plants that can affect their survival and persistence occur at all growth stages **(Figure 3)**. Experimental methods to measure such parameters have been developed in the disciplines of ecology and experimental agriculture. There will be considerable variation between species in the types of experiments required to measure survival and persistence, but again the principles of good experimental design outlined above will help optimise the data-gathering exercise. The parameters measured in such experiments will vary under different conditions, so tests under different experimental conditions will help provide a more comprehensive picture.

General findings for plants

1) As much relevant information as possible should be gathered from available sources (e.g. laboratory, greenhouse trials, literature, etc.) to aid in the assessment and prediction of the performance and behaviour of the test plant in field testing. Information on the genetic composition of the plant and the parent plant, and related crop plants, may be useful in predicting behaviour, performance and potential impact on the environment. Assessments based on these predictions may be confirmed through monitoring.

An understanding of the genetics of a trait in a plant may prove useful in anticipating the behaviour and/or the characterisation of certain plants in the field. Polymerase chain reaction (PCR) has been used to characterise a gene introduced into rapeseed and its interspecific hybrids.

Monitoring methods which utilise marker genes inserted using molecular biology may be useful in estimating the ability of a plant to establish in both cultivated and uncultivated situations. Kanamycin resistance has been used to estimate outcrossing rates between cultivated potatoes and their wild relatives. In cases where screening for a particular trait is laborious, a more easily screened "reporter" gene that is under control of the same promoter, or is closely linked genetically to the trait, could be considered.

2) There are many areas where information on the basic biology of the plant species being field tested may not be currently available. Monitoring may be an important tool in filling such data gaps. When the longevity of seeds in soil is not known, for example, the duration of post-harvest monitoring cannot be determined. In this case, the monitoring programme actually provides information on the basic biology of the species and data on which further monitoring can be based.

3) Pre-release assessments may consider the possibility of movement and establishment of the test plant from the field test site, or transmission of genetic material to other varieties or related species. However, the behaviour of the plant, or expression of the introduced genetic material, may

differ between contained glasshouse and field conditions. Even the most comprehensive set of contained experiments cannot cover all possible combinations of environmental conditions. Pre-release assessment may suggest a high probability of plant movement, establishment or outcrossing. This may be tested by monitoring of small-scale field experiments.

4) Experience from traditional plant breeding and laboratory testing is useful in designing confinement conditions in small-scale field trials. However, confinement cannot always be assumed until tested and verified, at some point, under field conditions. For example, stability of a male sterility trait may need to be verified through actual monitoring in the field.

Competitiveness

5) Experiments to monitor the competitiveness of a test plant with other organisms (plants, animals) in the environment may neither be necessary nor the purpose of a small-scale field test. However, it may be desirable to assess the competitive ability of the plant before or during developmental testing. Traditional ecology protocols and methods may be adequate for this assessment.

6) Following germination, seedlings still have to establish themselves to produce a mature plant. But the rate of establishment of germinated seeds is dependent on the environment in which the seed has landed. In small-scale field trials, establishment rates may be easily monitored since the test plant may be easy to recognise. Different monitoring strategies for establishment rates may be required depending on the scale of the test.

General approach to testing

7) Both ecological and genetic methods are needed in efficient monitoring systems.

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

9) In general, experimental design may need to be customised (i.e. on a case-by-case basis) in order to meet the specific concerns related 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.

10) It cannot be assumed that:

i) the structural integrity and control of a genetic construct will be retained under field conditions; or that

ii) genetic constructs will behave in a Mendelian manner.

Gene insertions by rDNA techniques may display higher or lower rates of deletion, excision, amplification or transposition than genes introduced by other means. 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 reliable.

11) Principles of good experimental design to provide data capable of statistical analysis should always be followed.

C. Microorganisms: monitoring approaches and methods

The diversity of microorganisms, and their potential for use in unconfined areas, can present a significant challenge to the design and success of any kind of monitoring. Thousands of microbial varieties have been used as pesticides, as vaccines, for bioremediation, and for many other purposes. Increasingly, they will be tested over progressively larger areas of the environment during the coming years as important new environmental applications are discovered. Consequently, monitoring may provide useful information about the behaviour of microorganisms, including those that have been modified using molecular techniques.

Monitoring issues

1) Designing a monitoring programme

Designing a monitoring programme starts with a clear definition of the programme's objectives based on available knowledge of the microorganism to be released, the release environment and the release protocol. It is balanced by the degree of uncertainty and the predictability of predicted 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-characterised genera or species may suggest specific testing needs (e.g. pathogenicity or toxicity), although this may not replace completely laboratory/small field trials. The only way to take the information base beyond that of laboratory trials and modelling is to test the fate of the microorganism, or one similar, in a controlled and monitored field trial.

2) Extraction procedures

Extraction, i.e. the removal of a quantifiable and representative number of microorganisms from the existing population, is fundamental to analysis of microbial propagules in the environment.

Most procedures for monitoring microorganisms involve the extraction of cells; dilution plate counts (see below) involve the use of partial extractions. The most efficient extraction involves initial concentration of biomass, followed by extraction of cellular contents. The benefit of organism extraction is that a wide range of analyses may then be done with the extracted biomass. The cells, when concentrated and purified, can be used for DNA and RNA analyses without complication from contaminating humic acids and clays. Purified, concentrated cells can be analysed by target-directed probes involving antibody-specific and DNA/RNA-specific probes. It is therefore possible, by cell extraction, to retrieve cells selectively by antibody capture, selective plating and nucleic acid probe analysis of samples.

3) Detection methods

The techniques discussed below have been used or are expected to be used to monitor microorganisms in the environment.

i) Plate count techniques

Plate count techniques have been used in most environmental introductions of microorganisms to date. These techniques are inexpensive, well established, and generally, although not universally, applicable. Plate counts rely on the ability of microorganisms to multiply on agar media. It should be recognised that microorganisms may have dormant stages, especially if subjected to environmental stresses. In this state they may remain viable though not culturable by conventional plating techniques. High background levels of microorganisms can interfere with quantification, although this may be overcome by the use of specific media or selective agents added to the medium. Combining plate culturing with other techniques such as colony blot, ELISA or DNA (RNA) hybridisation may increase the sensitivity of the monitoring programme.

Selective plating procedures often rely on genetic markers, such as resistance to antibiotics. Concern has been expressed about the release of genetically engineered microorganisms that carry antibiotic resistance genes. Marker systems such as the *lacZY* and *lux* genes can be used to avoid these concerns.

ii) Most probable number techniques

Most probable number (MPN) techniques have been used to monitor microorganisms in the environment. The use of these techniques may be limited when there is a requirement in the monitoring programme for specific identification and quantification of a particular strain. Like plate counts, MPN methods rely on the ability of the microorganisms to grow in artificial media, and also on their ability to produce a measurable change, e.g. colour or turbidity. Microbial concentrations may therefore be underestimated using this technique.

iii) Direct microscopy

Direct microscopy techniques are commonly used to monitor environmental microorganisms. A range of methods are available, including light microscopy with or without specific staining, epifluorescence microscopy, immunofluorescence microscopy and electron microscopy. These methods

count directly the collected microorganisms and therefore may give a value independent of the recovery losses inherent in cultivation techniques. The main disadvantage is the difficulty that an inexperienced observer has in identifying specific microorganisms from their cell shape, which probably limits the use of these techniques to research purposes. Additionally, there is difficulty with some of these methods in distinguishing between live and dead or dormant cells. Vital fluorescence staining, e.g. with acridine orange, can overcome this problem while immunofluorescence staining, coupled with direct microscopy, may be a way to differentiate between a specific microorganism and background microorganisms.

iv) Immunological techniques

Immunological techniques have not been used for primary detection of microorganisms in the environment. However, some ELISA and fluorescent antibody techniques are becoming routine for other applications. In principle, they are strain-specific. Detection is dependent on surface antigens and consequently may be compromised by polysaccharide layers that mask them. Direct methods may have relatively low detection limits, e.g. approximately 10^4 cells/g soil. Immunological techniques do not depend on culturing the microbes. While these techniques will positively identify viable non-culturable microorganisms, they do not distinguish between living and dead ones.

v) Nucleic acid techniques

Nucleic acid techniques using, for example, probes or the polymerase chain reaction have been used in several field tests, including those of genetically engineered organisms for identification purposes and to detect gene transfer. These techniques offer high specificity and sensitivity. Results are currently qualitative, but may be at least semi-quantitative in the future. The methods rely on the extraction of nucleic acids from microorganisms or environmental samples, and consequently they may be used to monitor viable non-culturable or non-viable microorganisms. Extractions must be optimised for sample type and type of microorganism. Often it is necessary to culture the microorganisms before extraction. *In situ* hybridisation using oligo probes can be used to detect specific organisms.

4) Gene transfer

If there is a possible concern about the consequences of gene transfer, the likelihood that it will occur should be considered. While the predicted frequency of gene transfer to or from microorganisms will usually be very low, the large number of microorganisms released, even in a small-scale experiment, significantly increases the likelihood of gene transfer occurring. Therefore, monitoring may consider:

i) the function and origin of the gene;

ii) an analysis of the factors which may affect gene transfer;

iii) gene transferability; and

iv) the probability of the maintenance and expression of the genetic information.

5) Effects monitoring

Effects upon the environment which result from release of microorganisms are predominantly influenced by time. Consequently, a programme for monitoring the effects of a release into the environment may need to be divided into those effects occurring in the short term, arbitrarily set at one year, and those occurring in the long term.

i) Short-term effects

A first consideration for any released microorganism is quantification, relative to a specific environmental effect. For example, the number of antibody-producing animals could be determined relative to the number of animals ingesting an engineered vaccine strain.

A second consideration could be to what extent a release has augmented effects occurring naturally in the environment. For example, the population level of an introduced nitrogen-fixing bacterial strain could be determined relative to the total number of indigenous nitrogen-fixing bacteria in the soil.

Certain characteristics of the environment may serve as indicators of environmental condition and can be monitored to determine the impact of a release. Physico-chemical properties of soil (e.g. water potential, pH, nutrient levels, redox potential) may be measured over time. Sensitive steps in biogeochemical cycles may be determined at regular intervals, before and after application of test strains. It may be appropriate to monitor the biological diversity of microbial communities and the fate of a functional gene of interest, especially when horizontal transfer is of concern. Monitoring environmental parameters is useful, not only to evaluate the intended result of the application but also to provide an early indication of unintended, though not necessarily unpredicted, changes.

ii) Long-term effects

Where there is an opportunity for extended interactions of a microorganism with any particular ecosystem, there may be a greater opportunity for expression of some environmental effect. Such extended interaction may be influenced by prolonged exposure of the ecosystem to the introduced microorganism, repeated applications of microorganisms to a particular site, or dissemination of an established population to adjoining habitats. The effect may be through a cumulative or synergistic action with other microorganisms within the particular ecosystem.

It may be difficult (if not impossible) to predict all of these effects before field testing. Consequently, preparing to monitor them quantitatively will not be possible. While the prediction of long-term effects with any certainty is clearly limited, certain approaches to defining the potential for an effect may be possible. For example, if the released microorganism has insecticidal properties, a programme of regular visual inspection may form the basis of routine monitoring. Careful consideration of microbial interactions monitored in the short term could provide clues to a potential for other effects to occur in the long term. It may be possible to predict these effects from characterisation of the microorganisms in different environmental media, or with mixed populations of microorganisms, or such clues could result from pre-release monitoring.

To manage successfully a programme of experimental releases progressing from smaller to larger scale, testing and evaluation of the observations made at one stage will exert an influence upon

testing at a succeeding stage. Monitoring, if necessary, could be redefined to be made more or less stringent, alternative methods could be employed, or other changes could be made to evaluate better the potential for long-term effects. A key consideration is the practicability of the monitoring programme. It will be less feasible, in terms of sample and data handling, to use an intensive programme that was designed to monitor small-scale releases when assessing large-scale releases.

6) Sampling programmes

Design of specific sampling programmes to monitor introduced microorganisms needs to be a flexible process which fully takes into account the organism profile, the environmental profile and the introduction profile **(Figure 2)**. Superimposed on these considerations is the additional need to ensure:

i) that site-specific variability is minimised;

ii) good sampling practice, including attention to the storage and transport of samples;

iii) the use of existing information on environmental sampling for microorganisms; and

iv) continued optimisation of sampling procedures based on monitoring results.

III. Suggestions concerning future work and priorities

A. Future research on plants

1) Background information

More attention needs to be given to the development of background information, including information on:

-- use of parental plants as predictive models for new plant lines;

-- pollination biology, e.g. dispersal gradients;

-- seed production and persistence of seed banks in soil;

-- factors affecting establishment ability and competitive ability; and

-- stability of genetic markers for the identification of modified plants.

2) Trap plants

"Designer" trap plants could be tailored with readily identifiable markers for use with major transformed crop species.

3) Diagnostics

Immunological methods need to be developed for quick and easy diagnostics. Attention also needs to be directed towards the development of easy-to-use diagnostic kits capable of screening samples quickly under conditions other than those of specialised laboratories.

B. Future research on microorganisms

Microbial ecology in general is an area that may provide information on the following points:

1) Culturability of strains

One of the drawbacks of monitoring effects on microorganism populations present in the environment is that only a small percentage (estimated by some to be approximately one per cent) of the total microorganisms present is culturable. The reasons for this are not well understood. Meaningful monitoring will require improved recovery of microorganisms that are representative of the total, in number and diversity.

2) Host range

Our knowledge of the factors determining the host range of many microorganisms is at best limited; and at worst these factors are unknown. When considering monitoring priorities, it would be very helpful to have basic information regarding fundamental pathogen-host interactions. This might also suggest approaches to reduce effectively or to limit host range where appropriate.

3) Microbial interactions

Research on basic population dynamics and functional relationships of microorganisms could improve our ability to predict the fate and effects of microorganisms.

4) Influence of fauna

Information regarding interactions of microorganisms with higher organisms in an ecosystem is often non-existent; yet resident fauna, e.g. earthworms, may act as microbial reservoirs. This may be a special niche where genetic transfer occurs.

5) Gene transfer

While genetic exchange between microorganisms has been extremely well characterised in the laboratory, very little is known about transfer in environmentally relevant situations or the important environmental parameters that influence them. Estimation of gene flow into indigenous species can only be made by informed predictions. The organism characteristics and interactions that allow gene transfer need to be considered in the context of environmental complexity. Procedures need to be developed to test for gene transfer, from introduced microorganisms to the natural population and from natural populations to introduced microorganisms. Additional work might be specifically directed towards the minimisation or prohibition of gene transfer.

6) Selection and evolution

Organisms and virions recently isolated from natural habitats are commonly used as hosts and vectors of foreign genes. Microorganisms modified in this way commonly express lower fitness when released back into their natural habitats. If this is the case, positive selection of mutants and genetic rearrangements may cause a drift in chromosomal genes towards a new genotype with better fitness.

For monitoring purposes, methods for genetic profile surveillance of the microorganism are needed, as well as methods to monitor fitness with time.

7) Nutrient cycling and depletion

While most microorganisms are adaptable in function, allowing elemental cycling to continue, some may tend towards functional redundancy while others, e.g. those in parasitic and commensal relationships, can be very restricted in their habitat. Competition by an introduced microorganism may lead to displacement of key species within a functional organisation and this in turn may influence

major element cycling or result in nutrient depletion. At present, the interaction between microorganisms in the ecosystem is not well understood.

8) Biodiversity/community structure

Methods for defining biodiversity and community structure analysis are available for monitoring purposes, but they need to be tested and documented. This includes genetic, physiological and immunological approaches.

9) Sample extraction

Extraction techniques include isolation of whole viable cultivable or non-viable cultivable cells, or DNA or RNA, from complex media such as soil, plant material, or run-off water.

Many of the advanced molecular techniques for monitoring require intensive treatment or handling of samples, as well as extraction. Much more work is needed to validate extraction procedures. Ways to improve extraction sensitivity are needed.

10) Markers

The development of efficient, environmentally neutral and readily identifiable markers for microorganisms needs to be encouraged, to provide alternatives for use in field testing. The use of particular markers in field situations, with information on their relative success, could profitably be recorded in a central data bank.

11) Biological containment

Research on the development of constructs and host genotypes which limit the survival and dispersal of microorganisms and introduced genes is needed. Constructs should be tested under ecologically relevant conditions.

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Annex I National reports qnd selected case study presentations

A. National Reports

The following information on national considerations concerning the monitoring of organisms introduced into the environment has been summarised by the rapporteurs and the OECD Secretariat from reports submitted to the Workshop.

Austria does not currently have any legislation covering the release of GMOs. Scientists working in the field follow international voluntary guidelines, but no releases of GMOs to the environment have yet taken place in Austria. A Governmental committee was recently established to prepare a comprehensive law for the entire field of biotechnology. It is intended that legislation will be based on two recent EEC Directives.

Although Belgium has no specific legislation covering the release of GMO's, all the notified field experiments have been covered by the framework of existing product laws (e.g. on seeds and drugs).

Moreover, with a view to a "vertical" implementation of the "horizontal" European Directive on the Deliberate Release of GMO's (90/220/EEC), the different competent authorities operating at various levels in Belgium are adapting these product laws.

Aspects related to monitoring of GMO's have received utmost attention by the competent authorities during the review procedures. The submitted proposals have been treated, and this along the lines of the OECD recommendations and the quoted EEC Directive, on a case-by-case basis.

The monitoring requirements have been adapted as well to the eventual hazards associated with a particular release and the containment measures involved (e.g. deflowering, use of male sterile plants).

In Canada, all products of biotechnology are regulated, at virtually all stages of product development, under a number of different legislative acts. Monitoring at each stage of development in the life-cycle of a biotechnology product is an integral part of the risk assessment process.

In reviewing monitoring activities in Canada, several common themes or principles can be determined under the various administrations and their legislation:

i) Monitoring is required as a basic component of any field trial or experimental permit granted to date;

ii) Monitoring programmes are used to ensure trials are conducted properly;

iii) Monitoring is used to provide information on the validity of the risk assessment and decision-making criteria used in establishing the experimental conditions and trial restrictions/limitations in the first instance, e.g. on the stability of the construct, its expression, the fate and safety in the environment, etc;

iv) Monitoring provides information on the actual environmental impact and behaviour of an environmental release, and safety information with regard to workers and non-target species;

v) Information from monitoring is useful in deciding whether the product may move to the next stage of development, e.g. larger scale, less restricted conditions;

vi) Monitoring provides information that will assist in the knowledgeable prediction of environmental impact/behaviour for other (related) organisms.

Monitoring requirements in Canada are in general decided on a case-by-case basis. To date, current releases have been restricted to thirty-one trials of genetically modified plants, and the commercial licensing of a genetically modified *E. coli* bacteria-toxoid for vaccination of swine. Releases of genetically modified microorganisms have not occurred to date, but draft guidelines have been or are being developed under both the Pest Control Products Act and the Canadian Environmental Protection Act.

In Japan, a framework for risk assessment of rDNA organisms is being developed. Under this system, the risk is to be evaluated for each individual case, based on current knowledge. The procedure to be followed includes, *inter alia*, the examination of monitoring and controlling procedures (including emergency response plans) of the field tests. Only when these are considered to be adequate can the field test proceed. There have been no releases to date.

A release scheduled for 1991/1992 of a *Pseudomonas paucimobilis* for bioremediation of xenobiotics would be subject to monitoring for plasmid transfer, survival, persistence and transport in soil, interaction with microbial communities and influence on N-, P-, and C-cycles. Specific detection methods must be available, as well as methods for mitigation of adverse effects.

The importance of environmental monitoring is emphasized in a report of February 1990 to the environmental agency. The recommendations include methods and schemes for sampling as well as specifying items such as survival, reproductive ability, mobility, genetic stability and mobility of genes, changes in host range and influence on target and non-target organisms. Further focus was put on evaluation of the monitoring methods and results.

In the Netherlands, a new law was passed in 1990 implementing the EEC Directive on releases of GMOs. Monitoring has been an important element in the review and permitting process, and permit renewal is given after the examination of monitoring results. Six field tests of genetically modified potatoes and two releases of microbes have taken place so far. The need for intensive monitoring of the potatoes was avoided through strict confinement, by deflowering and by using male sterile plants. The rDNA Committee has been examining in some depth the issue of genetic transfer associated with the release of genetically modified microorganisms and is commissioning further studies in this area.

In New Zealand, the risk assessment and recommendation for monitoring are made by an advisory expert body, the Interim Assessment Group (IAG), which refers to the applications for deliberate releases and greenhouse tests submitted to the Ministry for the Environment. IAG reviews the results of the monitoring. Monitoring of the releases is performed, but the IAG recommends that an outside institution or person be involved in this.

The IAG presently functions on the basis of voluntary compliance by the applicants, but legislation is being prepared to establish a Hazard Control Commission for assessing and licensing hazardous substances and new organisms. Six GMOs have been field tested in New Zealand.

In Sweden, until 1990, there was no regulation governing releases of GMOs. However, the only previous small-scale field test, carried out in 1989 (oilseed rape modified for higher protein content), was reviewed under a voluntary scheme. An amendment to an existing law in 1990 gave the possibility of granting permits for modified plants, and a further oilseed rape field test in 1990 was reviewed under the new law. A monitoring programme was undertaken for detecting volunteer plants and for monitoring gene transfer by pollination. Currently, the Swedish Environment Protection Agency is initiating a programme for developing monitoring requirements for deliberate releases of GMOs into the environment.

In the United Kingdom, until July 1990 proposals for GMO releases were reviewed by the Government's Advisory Committee on Genetic Manipulation and the Interim Advisory Committee on Introductions. These Committees have now been replaced by the Advisory Committee on Releases to the Environment (ACRE).

The regulatory authorities have treated proposals on a case-by-case basis and have made monitoring recommendations when it was thought that they would enhance the safety of a release proposal. The monitoring arrangements associated with most release proposals are therefore a combination of the proposers' experimental design modified in the light of advice from regulatory authorities. The Advisory Committee on Releases to the Environment (ACRE) has set up a working group on monitoring. Part of the terms of reference for the group includes the review of past and existing monitoring requirements, with a view to preparing future guidance.

Twenty-nine releases have so far taken place in the United Kingdom, of which 22 are of crop plants. Monitoring has involved at least the following:

-- pre-release "monitoring" to describe, for example, the indigenous fauna and flora at a proposed release site;

-- "monitoring" during a release to measure, for example, the persistence and/or dispersal of the organism or genetic material from the original release site;

-- post-release "monitoring" which would include, for example, arrangements to ensure that the site has been cleared of the GMO.

In the United States, monitoring guidelines specifically for microorganisms are being developed by the Environmental Protection Agency. Figure 4 shows an outline of the scheme envisaged in these guidelines. The intensity of monitoring to be recommended for a particular release will depend on both the degree of uncertainty and the level of possible adverse effects associated with that release (Figure 5).

In the European Community, the Directive on the Deliberate Release of Genetically Modified Organisms to the Environment (90/220/EEC) requires that case-by-case environmental risk assessment should be carried out before any release of GMOs is made, whether in a field test or by marketing a product, as the basis for receiving the consent of the authorities for the

release. Information on the monitoring to be undertaken for a release is part of the assessment of the release. More specifically, information may be required on:

-- methods for tracing the GMOs, and for monitoring their effects;

-- specificity (to identify the GMOs, and to distinguish them from the donor, recipient or, where appropriate, the parental organisms), sensitivity and reliability of the monitoring techniques;

-- techniques for detecting transfer of the donated genetic material to other organisms;

-- duration and frequency of the monitoring.

Monitoring provides the data to verify the assumptions made in the risk assessment, and to enable better assessments for future phases of a release. It also provides an indication of whether the released organism has spread beyond the designated area, and hence triggers the emergency response which may be necessary if the GMO spread needs to be controlled.

Figure 4. **Scheme for monitoring guidelines being developed by US EPA**

Figure 5. **Determination of monitoring intensity (US EPA)**

B. Case study presentations

The following case studies were presented to the Workshop by individual experts and the OECD Secretariat. The summaries have been prepared by the rapporteurs and the Secretariat.

Release of genetically modified *Clavibacter xyli*

The modified *Clavibacter xyli* carries the gene for endotoxin production from *Bacillus thuringiensis*. The host organism is a natural pathogen inhabiting the xylem of Bermuda grass.

The modified bacterium was released with the Bt-toxin in various modified versions in 1988, 1989 and 1990 in multiple trials. Monitoring experience from former trials, as well as from laboratory and greenhouse testing, was used to revise and improve the monitoring programme as the releases progressed.

The modified bacterium was stabbed into the stems of corn plants or applied to seeds. The test site was surrounded by corn trap plants and buffer zones. A fallow zone separated the site from surrounding corn fields.

The purpose of the release was to evaluate the activity of the toxin against the European corn borer, to determine the level of protection achieved, to examine the persistence of the modified bacteria in plant vegetation, litter and debris, and to measure gene transfer and gene segregation under field conditions. The dissemination of the modified bacterium by mechanical or insect vectors was also monitored. Transfer by insects and transfer to non-target plants were monitored, as well.

Samples were taken from the sap of leaves and other plant parts, soil and run-off water. Sampling was also performed to determine presence of the bacterium in insects. Weedy species of plants, and trap and sentinel plants, were also assayed for its presence. Crop residues were monitored for survival and decline of bacterium. Monitoring for survivors over winter was done using sentinel Bermuda grass grown on-site after termination of the release.

Low-sensitivity methods were used for detection of the modified organism. The use of more sensitive methods had shown that those susceptible young plants infected will always grow to a point where detection is possible with less sensitive methods.

Monitoring showed that the modified bacterium does not spread to corn plants under natural conditions. Mechanical transmission through tools injuring the plants was seen as an effective route both in Bermuda grass and corn. There was no transmission of the bacterium via seeds. Survival in debris and soil declines rapidly under warm conditions, more slowly under colder ones. No colonisation was detected on volunteer plants, indicating low or no survival.

No modified bacterium was detected in run-off water. The bacterium was detected in some insects. Insects were not seen as vectors for transmission from plant to plant, although only a few insects were tested.

It was emphasized that the monitoring programme, and the samples and tests made, were determined by the careful study of the ecological behaviour of the parent organism, and by laboratory and former field testing comparisons with the modified *Clavibacter*. Extensive data had been provided to establish a background on the biology of the organism. Further, the conditions specific to the release site were evaluated and taken into consideration.

Release of genetically modified *Pseudomonas syringae* and *Pseudomonas fluorescens*

Genetically modified *Pseudomonas syringae* and *Pseudomonas fluorescens* bacteria deficient in production of ice nucleating surface proteins were released by spraying onto strawberry and potato plants at two release sites. The purpose was to test the ability of the bacteria to prevent post injuries to plants.

The mutant bacteria had retained their normal ecological characteristics of growth and colonisation ability for leaf surfaces. They were genetically stable.

The test sites were 891 m^2 and 1776 m^2 respectively, with buffer zones of 15 m and 30 m. The site and the application by spraying were prepared so as to give the data good statistical quality with replicas and controls.

The applications were performed under calm conditions, with wind speeds of 0.7-0.2 m/second at 1 m above ground.

Treatments of the sites included 1) controls with water only, 2) *P. syringae* ice minus, 3) *P. fluorescens* ice minus, 4) mixtures of wild type *P. syringae*, and 5) spraying with fungicides and bacteriocides.

The data developed during the release confirmed laboratory results: the bacteria were able to colonize plant surfaces when sprayed onto plants with insignificant numbers of wild type ice plus bacteria already present.

The main objective of the monitoring performed was to test a number of sampling devices for monitoring of bacteria in the air with the potential to spread outside the site by aerial drifting.

Of the five types of sampling devices tested, glass impingers and gravity plates (open petri dishes) were most useful in terms of costs, ease of use and reliability of data produced. Apart from these two devices, six-stage Andersen samplers, Regnier samplers and sentinel potted plants were used.

Extensive coverage of the potential air drift made it possible to establish a detailed pattern for the movement of the applied bacteria.

Generally, the manner of application combined with wind conditions ensured that spread beyond the sites was minimal. Extrapolation of data suggested that more than 99.99% of the sprayed bacteria settled directly onto plants and soil at the test site. Though only a minimal number of bacteria drifted beyond the site and buffer zones, it was suggested that aerosol drift was significantly influenced both by the size of the area and by the concentration and amount of bacteria applied. It was also

concluded that once bacteria were in an aerosol, a relatively large percentage were able to drift from the original site of application. The drift is further dependent on the size of the nozzle, which determines droplet size and the potential number of bacteria in the droplets.

A recommendation was made to minimise the number of bacteria contained in aerosols, as this seemed to be the key factor in potential dissemination beyond the site of release.

Release of genetically modified baculovirus insecticides

Baculoviruses are used as "natural" insecticides to control certain invertebrate pest species. It is envisaged that the efficacy of these insecticides might be improved by incorporating genes which kill the host more quickly. A number of field trials of modified baculovirus have been made. Each release took place in animal- and insect-proof enclosures.

These field trials require effective methods to monitor the outcome of virus infection in insect larvae, and the persistence of virus on leaf surfaces and in soil. The virus infection may be diagnosed using simple observation of the pathology, followed by more critical examination using DNA probes in hybridisation experiments. The presence of residual virus in soil and on leaf surfaces is most readily monitored by using a biological indicator species in the laboratory to amplify low levels of virus in field-collected samples.

Release of genetically modified Vaccinia virus carrying rabies virus glycoprotein

The genetically modified Vaccinia Copenhagen strain was released on an island. The rabies virus glycoprotein was inserted into the thymidine kinase gene of the Vaccinia virus, significantly reducing pathogenicity. The virus was released in baits targeted towards raccoons, for the purpose of vaccination against rabies.

The monitoring protocols of the released virus were designed based on knowledge of the biology of the target and non-target organisms that might be in contact with the virus. Establishing a background picture of the area through pre-release monitoring had been an important part of preparatory work. In the case of predominant carnivores, including raccoons, up to two years of such work had taken place.

Monitoring was for effect, as this was more practical than monitoring for the actual virus/organism itself. This approach also gave information on whether there really was an effect of the organism rather than focusing solely on whether the organism dispersed. Monitoring covered the fate of 3 120 baits hand-distributed over 312 hectares for a period of 30 days. Most baits were contacted by raccoons before the end of 10 days. Fewer than 12 untouched baits were removed from the site after this period. Surveillance included keeping of photographic and written records of animal tracks, inspection of randomly chosen bait stations, and determination of the contacting animals. Foxes and, for the most part, raccoons contacted the bait. There was no avian contact.

Live trapping of raccoons was used to assess adverse clinical signs and pathological reactions, with comparison of trapped animals from the release site and control areas. A total of 209 raccoons, including recaptures, were evaluated during a 30-day period. No signs of gross lesion, weight loss or abnormal behaviour were observed. No Vaccinia virus has been isolated from the blood samples or from the oral and fecal swabs taken. Further analysis is ongoing.

Monitoring of raccoons included the use of radio collars for determining activity and mortality. A number of rodents were trapped on the site. Some foxes and rodents were trapped in control areas. Blood samples and oral and fecal swabs will be analysed for the presence of virus and antibodies. Researchers and other personnel involved in the release were also tested for virus, pre- and post-release, with no serum antibody rise detected even in samples taken from a researcher bitten by a vaccinated animal. Survivability of the virus in baits was monitored using baits specifically distributed for this purpose. Additional monitoring concerned climatic conditions, such as temperature and precipitation, and the influence of insects on bait degradation.

Gene dispersal from oilseed rape produced with DNA technology

Genetic transformation is now routinely applied to improve agronomical characteristics of rapeseed (*Brassica napus*). Normally, however, a risk assessment is required before cultivation of transgenic plants by farmers. The first step in this case was to assess gene dispersal by pollen transport and by crossing to wild species, using a phosphinotricin-resistant spring rape (Westar R.).

Outcrossing of transgenic rapeseed to related weed species

Five wild species related to rapeseed were selected: *Brassica oleracea, B. nigra, B. adpressa, Sinapis arvensis,* and *Raphanus raphanistrum*.

Ovary culture was applied to reciprocal crosses to get hybrids between these weeds and transgenic lines. One hundred nine hybrids were obtained. No seed was produced without embryo rescue. Crosses with *B. oleracea*, which is one parent of rapeseed, were the most successful. A maternal effect was revealed: better results were obtained with rapeseed as female parent except in the case of *Raphanus raphanistrum*, for which no significant effect was revealed. Hybridisations with *B. nigra* or *Sinapis arvensis* were unsuccessful when they were used as female.

After crosses between F1 hybrids and *B. oleracea, B. adpressa, S. arvensis*, and *R. raphanistrum*, the first BC1 plants were obtained by using ovary culture; this study is still in progress.

Characterisation of the F1 hybrids

Ten isozyme systems were studied in the different species and in the derived hybrids. They allowed confirmation of the hybrid structure of the plantlets at the two-leaf stage.

Chromosome counting in dividing cells or in pollen mother cells was done to characterise the different hybrids. Most of them had the expected triploid structure (ACX): haploid chromosome complement rapeseed (AC) and one genome of the wild species (X). Mature pollen was stained with acetocarmine, and pollen fertility was assessed by the percentage of stained grains. Fertility ranged from 0 to 59 per cent in triploid hybrids.

The bar gene was characterised in the hybrids by studying its expression (Basta spray) and its presence (PCR). Up to this point, a good correlation was found between bar gene presence and Basta resistance except in the case of two plants where the bar gene was not expressed.

Trials in insect-free cages

Experiments were carried out in 1989 and 1990 in 36 cages of $9m^2$, each containing a beehive to provide a high pollination rate. Seeds were sown in a growth cabinet (10^oC, short day regime), then transplanted in the cages late in the season to prevent accidental crosses with other cultivated or wild Brassicae. Different proportions of transgenic rapeseed and wild plants (wild mustard and wild radish: 32/4, 24/12 and 18/18) were used to allow different pollination conditions. In addition, there was male sterile rapeseed in the cages to test interspecific hybridisations without competition from rapeseed pollen.

These experiments provided 540 000 seeds of rapeseed in 1989 (rapeseed was burnt before maturity in 1990), 130 000 seeds of wild mustard and 36 000 seeds of wild radish. None of the seeds of the wild species were tested for their hybridity up to that date since their germination rate was very low (13 per cent in greenhouse).

Since different procedures intended to increase the germination rate failed, a first approach of hybrid screening was begun using DNA probes on seed extracts. In addition, seeds (from more than a million flowers) were obtained for male sterile rapeseeds, of which only 1 480 were sown and shown to be hybrid with the transgenic rapeseed. This indicated that no crosses occurred with wild mustard or radish, but that short distance (5m) pollination may occur by wind through the cage.

Trial in the field

As a low hybridisation rate may be expected, experimental design was carried out in a larger area in 1990 (225 m^2) to obtain more flowers and more opportunities for hybridisation. In another experiment, *B. adpressa* was grown with a male sterile rapeseed (1:1) in 640 m^2 isolated plots. Sixty-three gr hybrid seeds (100 seeds/m^2) were harvested on male sterile line.

Dispersal of the gene through pollen in rapeseed

In 1990, pollen dispersal from a transgenic spring rape resistant to phosphinotricin was studied at three locations using the following circular design:

-- a central plot of non-transgenic rapeseed (diameter: 1m);
-- a circular plot of transgenic rapeseed (diameter: 9m);
-- a surrounding circular field of non-transgenic rapeseed (diameter: 105m). Male sterile rapeseed plots were transplanted during one field trial at various distances from the transgenic rapeseed (0,1,2,3,6,12,24 and 48 meters).

Transgenic and non-transgenic lines flowered at the same time. Nectar production was about the same. Insect pollinators were mainly honeybees (86%). The honeybee population was homogeneous on the field.

To estimate cross pollination according to the distance from the central transgenic plot, seed samples were harvested at various distances on:

-- transgenic plants (control);
-- non-transgenic plants (central plot, surrounding plot at 0,1,2,3,6,12,24 and 48 meters);
-- male sterile plants at the same distances.

Herbicide resistance tests were performed at one site on rapeseed volunteers, to establish pollen and seed dispersal in the field.

Release of genetically modified sugar beets

This deliberate release concerned two types of genetically modified sugar beets: one made resistant to a herbicide, Roundup, and the other to the viral disease Rhizomania. The sugar beets were released both in a vegetative and, for a few, in a flowering stage. Most of the monitoring conditions and most of the monitoring programme were based on normal plant breeding practices. The release was performed in reproductive isolation. It was prepared according to a step-by-step procedure, with monitoring prior to the release performed under laboratory, growth chamber, and greenhouse conditions.

From pre-release monitoring it was established that the modified beets behaved in the same manner as the non-modified host beets with respect to izozime patterns, morphology, phenology and physiological parameters. DNA probes were used to establish the identity of the modified plants. Reproductive isolation was achieved through removal of plants prior to bolting, or covering of flowering plants with bags.

The efficiency of bagging in preventing pollen escape was monitored prior to the release using model plants (beetroots) in bags and sterile male sugar beets as pollen recipients. A small flow of pollen was detected, supposedly during watering and application of insecticides. Consequently, the final release conditions were modified so that the test plot was surrounded by a 2 m row of hemp. This was done partly to protect against wind dissemination and partly to allow the sticky hemp plants to catch escaping pollen.

It was recommended that the assumptions for the condition (in this case the reproductive isolation) should be carefully evaluated before final conditions were laid down.

Monitoring during actual release of the modified plants consisted mainly of the same investigations as those carried out in the greenhouse, including monitoring for pollen flow and efficiency of the bags. Herbicide resistance was also monitored. At this stage no monitoring was carried out of competitiveness or ability to hybridise with other sugar beets or wild relatives. It was suggested that such monitoring might be appropriate for a larger scale release in the future.

The efficiency of the introduced herbicide resistance was very low. This was especially the case for released plants obtained from seed produced by hybrids of genetically modified sugar beets, with full resistance, and non-modified ones. Plants developed from cell culture showed some functional resistance.

Where bagging was used, monitoring showed some escape of pollen even with the more stringent confinement measures. Pollen also succeeded in crossing the hemp barriers.

The monitoring showed that absolute confinement or reproductive isolation is difficult to achieve and that measures applied to this end (for example, bagging) should be carefully evaluated.

Further, it was concluded that care must be taken when extrapolating from greenhouse and other pre-release testing. In this instance there was a great difference in the expression of herbicide

resistance, which was almost non-functioning in the field. Genetic stability seems to have been influenced by field conditions, although the reason is not known.

It was concluded that the monitoring requirement can be met through building upon practices already used in plant breeding, provided that these practices are carefully evaluated and appropriately tested.

Monitoring genetically modified invertebrates

The indications are that there will be many releases of genetically modified insects, and other invertebrates, in the future. Not only are there a number of potential applications in biocontrol, but it is also likely that scientists will wish to carry out environmental releases for pure research purposes.

Two research projects involving insects have therefore been started. The first project involves laboratory microcosms of fruit flies (*Drosophila spp*.). These microcosms mimic natural communities in that they contain several stably co-existing species. The experimental work includes the "invasion" of microcosms with genetically modified Drosophila to measure the impact on established communities. Research will take into account the two distinct issues of monitoring the impact of invading species, and monitoring the impact of invading genes.

The second project is a field-based study. The rationale is to take advantage of "natural" invasion by a previously non-indigenous moth, *Phyllonorycter leucographella*. Invasion by this moth was first noticed in 1989, so that it is a recent invader. Although the moth itself is small, its larvae feed on a common "evergreen" plant (*Pyracantha spp*.) and cause characteristic easily identifiable damage to the leaves. This invasion can be used to test the feasibility of various monitoring methods which might be applied to future releases of genetically modified invertebrates. In particular, the work has been designed to involve the general public in monitoring the spread of this invader.

Genetic impact of aquaculture on natural fish population

Culture of Atlantic salmon (*Salmo salar*) in sea cages (fish farms) is among the fastest growing segments of the aquaculture industry. Due to incomplete containment, however, vast numbers of Atlantic salmon escape annually from fish farms. In Norway, the escapees currently outnumber wild fish in many spawning populations.

In an attempt to foresee the genetic consequences of these releases on native populations, the literature concerning the impact of intentionally and accidentally released salmonids in Atlantic as well as Pacific drainages was reviewed.

A wide variety of outcomes, ranging from no detectable effect to complete introgression or displacement, have been observed following the release of cultured salmonids into natural settings. Where genetic effects on performance traits have been documented, they have always appeared to be negative in comparison with the unaffected native populations. These findings are consistent with the theoretical implications of increasing gene flow from cultured stocks to locally adapted wild populations. They raise concerns over the genetic future of many natural populations of salmonid fishes.

Complete containment of farmed fish is the single most important step that has to be taken in order to develop aquaculture without compromising the genetic and ecological integrity of extant salmonid populations. Effective monitoring of gene flow from cultured to wild populations must also be implemented.

Annex II

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