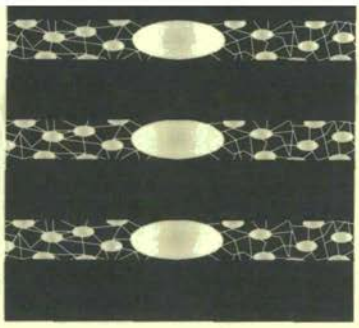
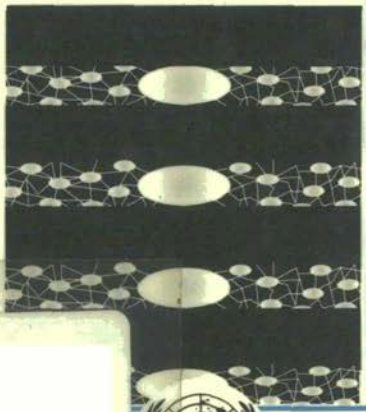


GENETICALLY MODIFIED ORGANISMS

A GUIDE TO BIOSAFETY



Editor:
George T. Tzotzos

UNIDO
United Nations Development Organization

UNEP
United Nations Environment Programme

CAB INTERNATIONAL

Genetically Modified Organisms

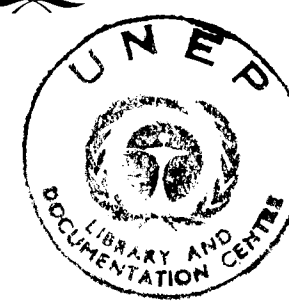
A Guide to Biosafety

Genetically Modified Organisms

A Guide to Biosafety

Prepared by the Secretariat of the
United Nations Industrial Development Organization (UNIDO)
in cooperation with the
International Centre for Genetic Engineering and Biotechnology (ICGEB)
for the UNIDO/UNEP/WHO/FAO Informal Working Group
on Biosafety

Editor: George T. Tzotzos



CAB INTERNATIONAL

CAB INTERNATIONAL
Wallingford
Oxon OX10 8DE
UK

Tel: +44 (0)1491 832111
Fax: +44 (0)1491 833508
E-mail: cabi@cabi.org
Telex: 847964 (COMAGG G)

A catalogue record for this book is available from the British Library.

ISBN 0 85198 972 1

Printed and bound in the UK by Biddles Ltd, Guildford

Contents

Introduction

Understanding the molecular basis of living systems provides phenomenal opportunities in combating disease, increasing agricultural productivity, improving the environment and utilizing hitherto untapped biological resources. The advent of recombinant biology has been hailed as the last significant scientific revolution of this century and has generated in the developing world immense hope for improving human welfare and securing the food supply.

Molecular genetics not only unleashed tremendous power to manipulate live forms, but also increased our insight into gene exchange processes and fate of transgenic organisms. This in turn resulted in some of the concerns about the inherent safety of biotechnology applications and products. It was soon to be realised that a balanced approach was needed to ensure the fruition of the promise of biotechnology without compromizing human health and environmental safety.

The issue of the sustainable management of biotechnology has, therefore, become a key one particularly in the developing world where the necessary standards for the development, handling, application and commercialisation of biotechnology products are often absent and are a major consideration in the work of the United Nations entities.

Perceivable risks to human health and the environment may arise either as a 'direct' result of the properties of transgenic organisms or alternatively as an 'indirect' result of changing socioeconomic conditions brought about by the application of recombinant technologies. The emphasis of this volume is mainly on such 'direct' effects. We believe that our current understanding of biological and environmental processes allows considerable objectivity in identifying and assessing potential 'direct' risks, at least qualitatively. A conscious effort has been made to separate risk assessment from risk management issues as the latter are subject to - among others - cultural perceptions and are at times motivated by socioeconomic interests.

This volume is intended to provide an unbiased technical guide to those dealing with genetically modified organisms at the research or regulatory level. Reference to existing regulations is meant to bring into perspective the different oversight approaches at the national level and thus help the reader derive his/her own conclusions as to what may be optimal in a given situation.

We wish to express our sincere thanks to Dr Y. Pervikov of WHO and Dr M. Malusynski of the Joint IAEA/FAO Division for their support in preparing this volume.

Hamdallah Zedan
United Nations Environment Programme (UNEP)

George T. Tzotzos
International Centre for Genetic Engineering and Biotechnology (ICGEB/UNIDO)

List of Contributors

Philip J. Dale,
Cambridge Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UJ, UK.

John Grinsted,
University of Bristol, Department of Pathology & Microbiology, School of Medical Sciences, University Walk, Bristol BS8 1TD, UK.

Geoffrey Hall,
International Mycological Institute, Bakeham Lane, Egham, Surrey TW20 9TY, UK.

Julian Kinderlerer,
Department of Molecular Biology & Biotechnology, The University of Sheffield, PO Box 594, Sheffield S10 2UH, UK.

Marion Leopold,
Université du Québec a Montréal, C.P. 8888, Succ. "A", Montreal, Quebec, Canada.

Morris Levin,
Maryland Biotechnology Institute, Center for Public Issues in Biotechnology, University of Maryland, College Park, Maryland 20742, USA.

Don Powell,
Cell Determination Laboratory, Department of Development and Signalling, The Babraham Institute, Babraham, Cambridge CB2 4AT, UK.

George T. Tzotzos,
ICGEB Vienna Office, UNIDO, Vienna International Centre, P.O. Box 300, A-1400 Vienna, Austria.

Raymond A. Zilinskas,
Center for Public Issues in Biotechnology, University of Maryland, Biotechnology Institute, 4321 Hartwick Road, Suite 500, College Park, Maryland 20740, USA.

Chapter one

Biological Risk Assessment: An Editorial Overview of Some Key Policy and Implementation Issues

George T. Tzotzos

ICGEB Vienna Office, UNIDO, Vienna International Centre, Vienna, Austria.

For a number of years the promise of biotechnology to alleviate disease and contribute to sustainable forms of development has been dimmed by concerns over the intrinsic safety of transgenic organisms. In the early days of recombinant DNA technology, the complexity of genetic interactions coupled with the lack of practical experience were awesome enough to justify a cautious approach towards the handling and releasing of transgenic organisms. Although, we now have considerable knowledge of the properties of recombinant systems and a vast volume of data gathered from different applications of biotechnology these concerns have not yet diminished. In the developing world, sensitization that countries be turned into testing grounds for new recombinant products has sometimes turned caution into fear. Considerations of this kind have often overshadowed the benefits developing countries are to derive from the application of genetic engineering.

In response to these conjectural or real concerns, the United Nations Industrial Development Organization (UNIDO), the United Nations Environment Programme (UNEP) and the World Health Organization (WHO) formed in 1985 the Informal Working Group on Biosafety. The Group was enlarged in 1991 with the addition of the Food and Agriculture Organization of the United Nations (FAO). The present volume was commissioned by the Group and is meant to help scientists and regulators in conceptualizing the major issues underlying biological safety as well as improve their understanding of how these affect the policy to regulate biotechnology.

The wealth of scientific data and knowledge mentioned earlier has not reduced the controversy of the international debate on biosafety. Efforts to harmonize biotechnology regulations have met with little success. This is because international regulation has not moved in phase with the rapidly expanding frontier of scientific knowledge. To a large extent, it is also due to the manifest differences among countries in:

1. Public perception of biotechnology.
2. Industrial policy.
3. Regulatory capability.

For as long as these differences persist, it may be difficult to achieve a global consensus on a unique set of regulations.

The factors that shape public perception and therefore determine the acceptability or not of biotechnology are dealt with in Chapter 2 of this volume and do not need to be further discussed here.

Industrial Policy

In the industrialized world, the commercial utilization of gene technology is of strategic importance in maintaining and/or increasing global market share. This is quite clearly not the case with most developing countries, where, on the one hand, there is little capacity to engage in commercial biotechnology and on the other conventional genetic technologies can still contribute greatly to national wealth generation.

In the top tier biotechnology countries private investment is directly linked with innovation and timely product development and commercialization are essential for early returns on investment. Delays arising from administrative obstacles, in particular, are thought to increase the risk of investment and be major disincentives for product development.

Regulations have become a prime consideration in forming a product developing strategy, at the corporate level, and part of the overall industrial policy at the national level. The drive to relax regulatory oversight has to be seen as part of the effort to maintain the technological advantage that has been achieved with massive inputs of capital resources. The rationale for regulatory relaxation is based on the severe limitations of the present regulatory regimes in responding to the demands imposed by the rapidly increasing number of recombinant products in a time frame and at a cost that would be acceptable to industry. Their structural shortcomings are becoming manifest through the burdensome bureaucratic procedures and superfluous testing protocols that are inordinately costly and lengthy (De Greef, 1991). The erosion of the basis of the regulatory philosophy by scientific advances and the accumulated experience in dealing with transgenic organisms and products is thought to be justification enough for regulatory review.

The main thesis put forward in calling for the review of procedures is that "engineered" genetic recombination permits much greater predictability of gene expression than is the case with "conventional" methods and that furthermore, transgenes do not represent risks that are conceptually different in nature from those associated with the use of native organisms, or organisms modified by "conventional" technologies (NAS, 1987). This, it is argued, invalidates the very ra-

tionale of legislation, which in many countries is based on the method by which a genetic modification has been brought about. Consequent to this argument, the focus of regulation should be the safety, quality and efficacy of a product (product- or risk-based regulation) (Wyngaarden, 1990). To continue with a process-based regulation would be to stigmatize a technology with a safety record which is, so far, immaculate.

On current trends, it is reasonable to expect a certain convergence in policy from technology-based regulations to product- or risk-based ones and a concomitant simplification of review procedures. This may be easier in those cases where the characterization of the end points of biological risk is relatively easy as in the case of food (e.g. toxicity, allergenicity, lack of nutritional value). To give an example, the concept of substantial equivalence in food safety is currently being debated as a direct attempt to simplify regulatory review. According to this "the need for and extent of any safety evaluation should be based on a comparison between the new food and the analogous conventional food, if one exists" (Miller & Flamm, 1993). Further attempts at simplification, in other areas of biotechnology application, may eventually lead to the exclusion from regulatory oversight of groups of GMOs, products and methods of genetic manipulation.

In environmental applications, however, where the potential hazards cannot often be easily identified, looking alone at the end product of transgenesis is not sufficient as what needs also to be understood is the interaction of the transgene with its ecosystem. The power of recombinant technologies to allow a much greater range of combination of genetic traits than is possible in nature or by using "conventional" methods has a bearing on environmental safety (Tiedje *et al.*, 1989). Consequently, the method of genetic manipulation may constitute a useful trigger for regulatory oversight, while risk could still be assessed with respect to the properties of the GMO alone. It has been suggested that a type of hybrid approach would be preferable to product-based regulations also in terms of administrative simplicity. The latter, despite the soundness of the intellectual premise on which they are based, may lead to regulatory confusion arising from the difficulty to categorize organisms on the basis of distinct risk levels (Lesser & Maloney, 1993).

Regulatory Capability

Biotechnology regulation in industrialized countries has been pro-active. That is, it has been enacted long before the products of the technology came to the market place. The picture in developing countries is one of striking contrast. With the exception of a handful of countries no legislation has been so far enacted to cover the products of recombinant biotechnology. The situation is due to change imminently under pressure from the demand to test and commercialize large numbers of transgenic products, if this lack of regulation is not to constitute yet another barrier to technology transfer.

The adaptation of existing legislation to cover the use and products of recombinant technologies has been proposed as an effective way of moving away from the obsolescent notions of process-based regulation while at the same time avoiding the bureaucratic delays of formulation and enactment of new legislation. The appropriateness of this approach is, however, questioned on the grounds of practicability and - in the case of environmental regulation - on grounds of conceptual soundness (see Chapter 7, pp. 137-139 of this volume).

An alternative approach that has been proposed is that of adopting guidelines instead of regulations as the latter are inflexible and unable to keep up with advances in science and the changing social consensus. Guidelines on the other hand have the necessary flexibility to adapt timely and with a minimum of administrative intervention to such changes (Persley *et al.*, 1992). Although this is, in principle, true, it fails to recognize the fact that most developing countries have no previous history of voluntary compliance to guidelines and codes of conduct. Legislation may, thus, be the only avenue of regulatory implementation. The introduction of broad stipulations permitting interpretation, and of clauses for periodic reviews and amendments may provide the necessary flexibility to respond to the changing demands of the industry and society.

What will eventually determine the appropriateness of any approach is the ability to monitor and overview regulatory compliance. Phrased differently, the capacity to identify, evaluate and manage biological risk. This capacity is generally lacking in the vast majority of developing countries. The reasons for this have been adequately analysed elsewhere (Cohen and Chambers, 1991). The role of international institutions in strengthening national capability for biological risk assessment is the subject of the next section.

International Support Mechanisms

The identification of biological hazards and the evaluation of the effects thereof focuses on the intrinsic properties of the transgenic organism itself, the interaction of the transgene with the receiving environment and its potential effects on target and non-target organisms. A number of biological methodologies have been proposed and these are reviewed elsewhere (Strauss, 1991). They are also dealt with as the central theme of the individual chapters of this volume. Suffice to say here that all of them rely on judgemental reasoning for the prediction of risk. Some, despite their methodological rigour (Royal Commission on Environmental Pollution, 1991, GENHAZ), have been rejected as putting too much strain on resources. They, nevertheless, merit treatment for they provide a good "road map" to direct most of the questions to be asked in ascertaining the safety of transgenic releases.

The "qualitative/judgemental" nature of risk assessment relies on expertise covering a wide range of scientific disciplines. In industrialized countries, regulatory formulation and implementation is being accomplished by national and institu-

tional committees and expert panels. The replication of this model in the developing world would require institutions, human and financial resources that are far beyond the means of the great majority of countries. International development agencies are being called upon to provide assistance in the formulation of regulatory policy and, increasingly, also in the evaluation of specific field trials.

The establishment of international expert panels for risk assessment of field releases of transgenic products in developing countries is useful in the short term, but the limitations of such panels in the long run have to be recognized. Their operation as surrogate to national institutions may prove difficult to sustain under the pressure of the geometrically increasing number of applications for field trials and commercialization of transgenic products and the costs associated with them.

Assistance in defining the terms of reference and the operational framework of national and institutional biosafety committees is likely to have a long term positive impact on strengthening national capacity in biotechnology. International agencies have an important role to play in increasing the awareness of the socioeconomic merits of the technology and in motivating governments to establish such committees as an essential step enabling technology transfer. International assistance in the training of scientists and administrators in the art of risk assessment is the most cost effective measure. Experience has shown that lack of education in the scientific concepts underlying biosafety has resulted in gross misinformation of the general public and in unproductive administrative measures. Human resource development should, therefore, be an integral part of the assistance package offered by international agencies involved in the promotion of biotechnology.

Finally, if demand on human and financial resources is to be reduced without compromising safety to human and animal health and the environment, there has to be a serious effort to move away from qualitative judgment of risk to quantitative risk evaluation. Until now this has been a near impossible task. This is because the range of the scientific knowledge base from which risk assessors have to distil data covers a vast array of disciplines (see Indicative List of Data Requirements for Biological Risk Assessment, below) and at the same time data are being generated at a phenomenal rate.

Data Requirements for Biological Risk Assessment (Indicative List)

1. Parent organism (taxonomy, molecular biology, physiology, reproduction).
2. Transgenic organism (molecular biology, reproduction).
3. Method of transgenesis.
4. Method, amount and frequency of introduction.
5. Fate of transgene (transport, reproduction, transfer, establishment).
6. Toxicity of transgene products and intermediary metabolites.
7. Effective dose for toxicity.
8. Susceptible non-target organisms.
9. Effect on non-target organisms.
10. Site characteristics.
11. Ecological effects.

Navigation and data capture from existing databases is confounded by the heterogeneity of their design. Furthermore, the scarcity of validated models and the fact that very often the outputs of one need to be used as inputs for another makes integration and quantification of the various subsets of the total knowledge base extremely difficult. The management of biological information and availability of tools that permit data interpretation and modelling in risk assessment will, thus, determine the effectiveness and reliability of methodology.

Knowledge system technology, albeit at an early stage of development in biological risk assessment, can be utilized to facilitate regulatory procedures and reduce the burden on human and financial resources. It should eventually become a valuable tool in providing decision support to expert panels.

Investment, therefore, in an integrated information support environment for biological risk evaluation merits serious attention. Such an environment needs to be transparent, requires advanced information management systems and standardized communications and database search protocols. Whereas technically feasible in varying degrees of sophistication, the magnitude of the task and the need for cooperation with the private sector make such proposition possible only as the result of international cooperation.

This volume, it is hoped, will contribute towards the understanding of the scientific principles of risk assessment and of the less tangible issues involved in risk management. Readers from the developing world may derive some guidance on how to set up oversight mechanisms in step with developments in science and international regulatory policy.

References

Cohen, J.I. & Chambers, J.A. (1991) Biotechnology and biosafety: Perspective of an international donor agency. In: *Risk Assessment in Genetic Engineering*, Levin, M. & Strauss, H.S. (eds), pp. 378-392. McGraw-Hill Inc., New York.

De Greef, W. (1991) Regulations and the future of agricultural biotechnology. *Agrofood Ind. hi-tech*, 2, No. 4, 3-7.

Lesser, W. & Maloney, A.P. (1993) *Biosafety: A Report On Regulatory Approaches For The Deliberate Release Of Genetically Engineered Organisms - Issues And Options For Developing Countries*, p.19. Cornell International Institute for Food, Agriculture and Development (CIIFAD), Ithaca, NY.

Miller, H.I. & Flamm, E.L. (1993) Biotechnology and food regulation. *Curr. Opin. Biotechnol.*, 4, 265-268.

NAS (1987) *Introduction of Recombinant DNA-Engineered Organisms into the Environment: Key Issues*, p. 5. National Academy Press, Washington, DC.

OECD (1986) *Recombinant DNA Safety Considerations*, p. 41. OECD, Paris.

Persley, G.J., Giddings, L.V. & Juma, C. (1992) *Biosafety: The Safe Application of Biotechnology in Agriculture and the Environment*, p. 10. International Service for National Agricultural Research, The Hague.

Royal Commission on Environmental Pollution (1991) 14th report: GENHAZ. HMSO, London.

Strauss, H.S. (1991) Lessons from chemical risk assessment. In: *Risk Assessment in Genetic Engineering*, Levin, M. & Strauss, H.S. (eds), pp. 297-318. McGraw-Hill Inc., New York.

Tiedje, J.M., Colwell, R.K., Grossman, Y.L., Hodson, R.E., Lenski, R.E., Mack, R.N. & Regal, P.J. (1989) The planned introduction of genetically engineered organisms: ecological considerations and recommendations. *Ecology*, 70, 298-315.

Wyngaarden, J.B. (1990) The future role of biotechnology in society. In: *Advances in Biotechnology*, p. 260. Swedish Council for Forestry and Agricultural Research, Stockholm.

Chapter two
Public Perception of Biotechnology

Marion Leopold
Université du Québec à Montréal, Canada.

Introduction

How the public perceives biotechnology is of critical importance to countries and firms seeking to compete in this new technological frontier. Public perception can affect the timing and direction of innovation in biotechnology, as well as the rates of diffusion of the technology, its products and services. In fact, significant adverse public reaction to a given product or application may keep the latter from ever reaching the marketplace.

Public perception of biotechnology is not only an important question, but also a complex one. It is shaped by a wide spectrum of factors, including country-specific levels of GNP, income distribution and education; national traditions and history; the role of government, industry, the media and advocacy groups. Public perception also covers a broad range of issues, including human and environmental safety, ethics, legal questions, economic and socioeconomic impact. Furthermore, public attitudes can undergo rapid shifts in response to unforeseen events, such as when a breakthrough biopharmaceutical is brought to market only to be challenged on cost/benefit or risk/benefit grounds. Finally, the impact of public perception on commercial biotechnology cannot be gauged on purely scientific grounds, as that impact may be great even if the perception is founded on scientifically unsound or non-scientific considerations.

In addition, the public itself is not a singular entity, and as such does not represent a homogeneous set of interests, attitudes and values. Accordingly, various public responses must be properly weighted.

This chapter examines public perception as it has evolved since the mid-1970s, and assesses its actual and potential impact upon the development of commercial biotechnology. To that effect due account is taken of the overt expression of that perception, particularly as it has shaped and been shaped by the regulatory process (An Overview of Public Debate on Biotechnology), and the opinions of a larger

spectrum of voices as revealed through surveys (Public Perception of Biotechnology). Finally, the question of public perception is raised in the context of less-developed countries, particularly those that have entered or are attempting to enter the field of biotechnology (Developing Countries).

The following pages deal primarily with biosafety and, to a lesser extent, the socioeconomic impact. Given the objectives of this volume, critical issues such as ethical concerns over the practice of human gene therapy, animal transgenesis, and the patenting of life forms are not addressed.

An Overview of Public Debate on Biotechnology

When the techniques of genetic engineering were still at the embryonic stage in the early 1970s, a group of prominent scientists involved in rDNA research voiced concern about potential biological hazards resulting from certain rDNA experiments. In a letter published in *Science* on July 26, 1974,[1] they called for a voluntary worldwide moratorium on the relevant experiments and for an international conference aimed at discussing biosafety with respect to rDNA experimentation. This conference, which took place in 1975 at the Asilomar Conference Centre in Pacific Grove, California, culminated in the drafting of a statement of principle that presented a proposed set of standards for rDNA research[2]. Immediately following the conference, the newly formed Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH)[3] set to work on transforming the statement into formal guidelines[4]. Many countries would follow the United States lead.

This series of events was unusual in more than one way: the initial debate on biosafety, including the move to establish guidelines, came from within the scientific community itself; public policy issues were introduced and acted upon at a time when advanced biotechnology was still nascent; the considerable, often dramatized national and international press coverage given to these events and to the disagreements they generated among scientists served as an early eye-opener for the public.

Based on strict rules of physical and biological containment of rDNA, the NIH guidelines addressed only federally-funded laboratory experiments. In practice, however, both non-governmental institutions and private firms would comply with the guidelines, presumably to give maximum credibility to their research practices. Scientists and firms also deemed it in their best interest to avoid inflexible statutory regulations. This position proved to serve the research community well; the safety record of laboratory experiments in genetic engineering rapidly demonstrated that concerns related to such experiments had been exaggerated, and containment restrictions were progressively relaxed. A similar pattern emerged in the EC, although individual member states would position themselves differently in this respect.

Shortly after the NIH guidelines were issued in 1976, the first incident of overt public opposition to rDNA research (and to NIH oversight procedures) took place

in Cambridge, Massachusetts. Harvard University plans to set up a genetic engineering research lab drew an angry "not in my back yard" response from local community groups. This in turn caught the attention of the national and international media, as well as that of Massachusetts Senator Edward Kennedy, who was quick to initiate Senate subcommittee hearings on the appropriateness of existing regulations governing research in biotechnology[5].

In the ensuing years, more and more local organizations entered the debate on genetic engineering, particularly in communities where university research was active; following Cambridge's lead, several states and communities introduced their own legislation governing rDNA research. In response to these moves and to what appeared to be a general public concern, more than a dozen bills aimed at regulating genetic engineering were filed in Congress. For lack of adequate support, none of these would be enacted into law, and the position of the NIH as sole regulator was reinforced.

By the mid-1980s, the context in which biotechnology evolved had undergone several important changes. The main thrust of the technology had shifted from the university laboratory to the commercial arena. A significant number of initially sceptical, large corporations had begun to enter the game, primarily through strategic alliances with start-up firms[6]. Government had come to see biotechnology as a critical technology, with an important role to play in international competition. Finally, new public policy and public perception issues were raised, as biotechnology moved into non-medical applications such as agriculture and bioremediation; concerns shifted from potential risks related to the accidental discharge of engineered organisms to health and safety risks associated with the intentional release of such organisms.

Because biotechnology had become an increasingly high-stake economic game and because new elements of potential risk had been introduced, public debate over biosafety and over the need to find a more adequate regulatory mechanism took on a new sense of urgency. On the question of risk assessment related to environmental release, intense controversy developed within the scientific community, pitting molecular geneticists against ecologists, and confronting two competing scientific paradigms[7]. The debate also drew in new advocacy groups including, prominently, environmentalists and, in the EC, their parliamentary representatives. Proponents of biotechnology, such as industry trade associations, individual corporations and related research interests, also began to take a more active stance.

Much of the policy debate would play itself out in the regulatory arena, where the shift in focus from the laboratory to commercial applications had generated the need for additional, sector-specific safety and health rules. In the United States of America, this role would be taken up by the Food and Drug Administration (FDA), the Department of Agriculture (USDA) and the Environmental Protection Agency (EPA) in their respective areas of jurisdiction. For a host of reasons, the regulatory environment became extremely complicated and confusing. Attempts to overcome these drawbacks, including the issuing, in 1986, of a "Coordinated Framework for the Regulation of Biotechnology"[8], were not particularly successful. However, the

fact that no biotechnology-specific legislation was passed left open the possibility of progressive regulatory adjustments. In the EC, stringent, biotechnology-specific directives, covering both products and processes, created other types of externalities.

For industry stockholders, biosafety regulation was a mixed blessing. On the one hand, government approval was a necessary means of "legitimizing" products (and, in the EC, processes); but at the same time, major regulatory delays in most application sectors were creating serious barriers to competitive market entry[9].

Active opponents of biotechnology or of specific applications would continue to work with broad-based coalitions, including, now, environmental groups. Government lobbying, representation at Congressional hearings, litigation, communicating through the media, as well as organized boycotts and the sabotaging of field tests were some of the tactics that would put pressure on the regulatory agencies and interfere with the timely market introduction and diffusion of specific products.

At the present time, an important confrontation between the United States bioindustry and its detractors concerns genetically engineered foods. Having failed to stop innovation in this area, opponents are now fighting for the labelling of such foods. Wary of the impact of organized opposition on public opinion, the FDA, under whose aegis foods are regulated, is presently reviewing its position on the question of identifying the processes used in producing foods.

As the above attempts demonstrate, most opposition to biotechnology has been the work of activists who have put together broad-based alliances of special interest groups. Through skilful use of institutional mechanisms and the media, these constituencies have had considerable success in lengthening innovation time-frames in several biotechnology application sectors. This being said, it would be incorrect to assume that organized advocacy groups and their *ad hoc* allies represent public opinion in general.

Public Perception of Biotechnology

A critical and judicious use of public opinion surveys can be helpful in attempting to establish: (i) how a wide cross-section of the public perceives biotechnology; (ii) what factors shape their perceptions; and (iii) how the latter are likely to affect the market entry and diffusion of the various products and services of biotechnology. Most such surveys conducted to date do not take into account economic factors, such as the question of product pricing. If for no other reason than this, survey results can give only a first approximation of biotechnology's success in the marketplace.

Relevant results of three recent surveys are presented and assessed here[10]. With respect to the Eurobarometer survey, on the whole, the findings were quite similar to those of the two United States studies; for that reason, they are not accounted for separately, except in one instance. It should, however, be noted that differences in

public perception *among* EC member states are often considerable. Cross-country variations are dealt with elsewhere in this chapter.

Survey Results

How the Public Perceives Biotechnology

1. Most people agree that the risks of genetic engineering are largely exaggerated (Survey A).
2. This does not entail an unqualified endorsement of genetic engineering, as a large majority of the public feels that strict regulation is needed (A).
3. In a majority of cases there is a split in public perception between support for (closely scrutinized) small-scale, experimental testing and lack of support for large-scale commercial applications of the technology (A).
4. Generally speaking, the public is disposed to believe that biotechnology may have a favourable impact on food quality and nutrition (B).

Factors Shaping Perception

1. The level of education and religious allegiances are key explanatory variables of the negative perceptions of a minority of the population (Survey A).
2. The lack of majority support for large-scale commercial applications of biotechnology is largely due to the influence exerted by environmental groups. The latter are often given more credibility than federal agencies (A).
3. This may change over the long run if allegations by such groups are successfully counteracted (A).
4. Scientific literacy diminishes the impact of fears induced by environmental groups (A).
5. Available research on public awareness and attitudes does not provide enough elements for the design of effective educational programmes (B).
6. Informal means of diffusing information, particularly the media, face a public that is increasingly avid for information on biotechnology (B).
7. The setting of clear standards is also called for. For example, food labelling information may become a powerful tool for meeting public concerns on biotechnology applications. The usefulness of such a tool depends, however, on levels of education and awareness (B).
8. Trust in government agencies is positively correlated to the level of education and awareness (B).
9. Rhetoric and exaggerated expectations fed by "science fiction" approaches increase uncertainties (B).
10. Media representatives, educators and health professionals are important agents for enhancing public awareness (B).
11. Making the public feel part of the decision-making process plays an important role in public acceptance (B).

12. The reliability of information sources on biotechnology are rated as follows: consumer organizations, 27%; environmental organizations, 23%; schools, universities, 17%; public authorities, 7%; animal-welfare organizations, 5%; religious organizations, 3%; political organizations, 1%; trade unions, 1%; industry, 1%; don't know/no answer, 15% (C).

Effects of Public Perception on Commercial Opportunities

1. The majority believes that fears resulting from risk exaggeration seriously damage product development (Survey A).
2. *Ex-ante*, some degree of private (and public) uncertainty is unavoidable: the full extent of consumer response cannot be realized until products are on the market (B).
3. An increasing awareness regarding food safety coupled with a gap between public acceptance and technical progress may lead to a slowdown in the development of applications and market entry (B).
4. Given an acceptable safety threshold, public acceptance is likely to be more influenced by product price than by product quality considerations (B).

Assessment of Surveys

The Learning Curve in Public Perception

Public perception is a function of five main country-specific variables: economic affluence; education; social and institutional means of participation; cultural and religious values and traditions; and random variables (e.g., particularly traumatic historical events).

The maturity of public opinion and the means of expressing it cannot be taken as a constant over time. A learning process is involved whereby, with social development, public opinion can be expected to play an ever increasing role through ever more sophisticated means of expression and participation. It may be legitimate to wonder whether, even in today's most advanced countries, society has achieved an historical summit in this learning process. This circumstance relativizes the degree of assertiveness of any conclusion arrived at by means of opinion polls.

Target Populations of Advocacy Groups

As already noted, advocacy groups tend to address specific audiences according to the specific impact of different applications. As far as the public at large is concerned, a relationship can be postulated between the level of education and the influence of advocacy groups over different segments of the public. At one ex-

treme, the segment of the public with no scientific literacy or no literacy at all is unlikely to be reached. At the other extreme, the segment of the population that is as highly scientifically literate as to be able to form an opinion of its own is also unlikely to fall under the influence of advocacy groups, which may address themselves to religious, moral or other kinds of prejudices. Finally, there is the intermediate segment of the public, which is literate enough to understand what the issues under debate are about, but not enough to make their own judgement. This is the target segment that is most likely to fall under the influence of advocacy groups as far as the population at large is concerned.

Public Choice as Indicators of "Revealed" Public Opinion

Rational market choice involves perfect information and minimum thresholds of economic affluence. Insufficient market choice leads to a sparsity of evidence on revealed public opinion. This latter situation arises because, in the relative absence of choice, people tend to accept risks, even if they are considerable. Insufficient choice may also lead to the need for public intervention. This situation is found in underdeveloped countries and among the least affluent segments of society in industrial countries.

Relative Competitiveness

If product/process innovation leads to an equivalent product, the public will react to competitive pricing.

Developing Countries

1. In developing countries there is a need for an environment that is institutionally, politically, socially, educationally, and culturally enabling.

2. Commonalities and differences with respect to industrial countries include the following.

(a) Widespread relative market failure makes it difficult to determine "revealed" public perception.

(b) Developing countries are behind on the learning curve.

(c) The same variables as in industrial countries shape public perception; their relative weights change across countries.

(d) Those developing countries that have entered or are attempting entry into biotechnology may also be those where a greater percentage of the population is capable of making rational decisions; however, as in the most industrialized countries, that percentage remains relatively small. In this context, the question of income distribution must be taken into account; countries like Brazil or India have a biotechnology industry but particularly high rates of poverty and

illiteracy. Broad-based public opinion cannot develop when large segments of the population are illiterate and/or uneducated.

(e) Target populations for advocacy groups are, on the whole, much smaller in developing countries. In the least developed countries, these populations may be absent (as may be the advocacy groups).

(f) The role of traditional values and religion may, in many instances, exert more influence on public perception than in industrial countries.

(g) In developing countries, non-governmental organizations are presently the advocates of public opinion.

(h) Because of their neutrality, international organizations, particularly the specialized agencies of the United Nations system, can also be expected to play an important role in enhancing public awareness in developing countries.

Notes

1. Berg, P., Baltimore, D., Boyer, H.W., Cohen, S.N., Davis, R.W., Hogness, D.S., Nathans, D., Roblin, R., Watson, J.D., Weissman, S. & Zinder, N.D. (1974) Potential biohazards of recombinant DNA molecules. *Science*, 185, 303.

2. Accounts of the Asilomar conference and of the events that ensued can be found in much of the literature dealing with the history of biotechnology. Among other sources: (a) Gore, Jr., Sen. A. & Owens, S. (1985) The Challenge of Biotechnology. In: *Yale Law and Policy Review,* Vol. 3, No. 2, Spring; (b) Krimsky, S. (1991) *Biotechnics and Society: The Rise of Industrial Genetics.* Praeger Publisher, New York; (c) Olson, S. (1986) *Biotechnology: An Industry Comes of Age.* National Academy Press, Washington, DC; (d) Watson, J. & Tooze, J. (1981) *The DNA Story.* W.H. Freeman & Co., San Francisco; and (e) Yanchinski, S. (1989) *Biotechnology: A Brave New World?* Lutterworth, Cambridge.

3. The RAC was established in October 1974 at the request of the initial group of scientists.

4. The *Guidelines for Research Involving Recombinant DNA.* National Institutes of Health, June 1976.

5. *Oversight Hearings on the Implementation of NIH Guidelines Governing Recombinant DNA Research: Joint Oversight Hearing of the Subcommittee on Health and Subcommittee on Administrative Practices. 94th Congress.* United States Congress. Senate. 22 September, 1976. As referred to by Plein, L.C. (1990) Biotechnology: Issue development and evolution. In: *Biotechnology: Assessing Social Impacts and Policy Implications,* Webber, D.J. (ed.). Greenwood Press, Connecticut.

6. The increased interest of industry is attributable to several factors: rapid progress and promising results on the scientific front; the 1980 United States Supreme Court ruling making genetically engineered organisms eligible for patenting (Diamond v. Chakrabarty); and, the generally pro-technology and deregulatory environment that characterized the Reagan Administration.

7. For more on the scientific debate, see Krimsky, S. (1991) *Biotechnics and Society: The Rise of Industrial Genetics.* Praeger Publisher, New York.

8. Office of Science and Technology Policy, *Federal Register*, 51, June 1986.

9. Time lags due to regulatory externalities stemmed from a complex interplay of factors including, in the United States of America, bottlenecks related to risk assessment, overlaps in regulatory jurisdiction, the fact that some of the agencies were both regulators and promoters of biotechnology, a lack of qualified regulators and adequate infrastructure, and effective lobbying by opponents of biotechnology. Cf. Leopold, M. (1993) The commercialization of biotechnology, the shifting frontier. In: *Biotechnology R&D Trends. Science Policy for Development*, Tzotzos, G.T. (ed.), *Ann. New York Acad. Sci.*, 700, pp. 214-231. The New York Academy of Sciences, New York.

10. (A) United States Congress, Office of Technology Assessment, *New Developments in Biotechnology - Background Paper: Public Perceptions of Biotechnology*, OTA-BP-45, United States Government Printing Office, Washington, DC., May 1987. (Survey conducted in October-November 1986, among a national probability sample of 1273 American adults.). (B) Hoban, T.J. & Kendall, P.A. (1992) *Consumer Attitudes about the Use of Biotechnology in Agriculture and Food Production* (summary of a report to the United States Department of Agriculture, Extension Service), Raleigh, North Carolina. (Survey conducted in February-March 1992, by telephone [random digital dialling] with 1228 adults across the United States of America.). (C) Marlier, E. (1992) Eurobarometer 35.1: Opinions of Europeans on Biotechnology in 1991. In: *Biotechnology in Public: a Review of Recent Research*, Durant, J. (ed.). Science Museum for the European Federation of Biotechnology, London. (Survey conducted in March-April 1991, simultaneously in the 12 countries of the European Community, among a representative sample of 12 800 people 15 years and over.)

Chapter three
Risk Assessment and Contained Use of Genetically Modified Microorganisms (GMMs)

John Grinsted
University of Bristol, United Kingdom.

Introduction

Since genetic engineering first became a practical proposition in the early 1970s, it has become subject to governmental or quasi-governmental control in many countries. The reason for such measures is the perception that there are potential risks inherently associated with this technique that are qualitatively different from and intrinsically more hazardous than those posed by "natural" organisms.

Experience has not supported these conjectures. As regards the properties of an isolated recombinant organism, they are defined by its known components; and as regards the initial construction of libraries for the isolation of the clones, there is no evidence that any unexpected hazards occur.

It is the case, however, that dedicated regulation of genetic modification is in place in many countries, so that the detailed assessment of even the most innocuous experiment involving this technique is a legal requirement. It is also the case that the genetic modification is an additional factor that must be taken into account in any overall risk assessment of genetically modified organisms (GMOs). In this chapter, hazards that are normally associated with microorganisms and how they are handled in the laboratory are described. Hazards peculiar to GMOs, how these hazards can be assessed and the resulting alignment with containment are then discussed. Procedures in the United Kingdom are used as the basis for this chapter. In particular, *Categorization of Pathogens According to Hazard and Categories of Containment*, 2nd edition, 1990 (HMSO ISBN 0 11885564 6) and ACGM/HSE Notes of Guidance for work involving genetic modification have been consulted extensively. The principles underpinning these procedures are universal and inform any sensible assessment of the risks involved.

Categorization of Microbiological Hazards

With respect to assessing risk in the contained use of GMOs, it will generally be microbiological hazards that will be significant: there should be no problem in containing higher organisms. (With flowering plants, proper precautions to contain pollen would have to be taken.) With microorganisms in general, the overriding potential hazard is pathogenicity, the capacity to infect and cause disease within a particular host. Other factors that must be taken into account are any toxic, allergenic, or other biological effect of the non-viable organism, or of its components, or of any product expressed by the organism. The concept of toxicity should not be limited to lethality, but should include such things as mutagenicity, carcinogenicity, neurotoxicity, and environmental effects.

Infection followed by the development of disease will depend on the microorganism's ability to multiply in the host, balanced against the host's capacity to control or limit this proliferation. Thus, a fundamental attribute of bacterial pathogenicity is the ability to enter the host and find a suitable niche. Entry is not simply a casual contact between host and infectious agent, and the microbe's surroundings may change profoundly between the initial site of entry and the final resting place. Thus, many pathogens must possess the genetic machinery that enables growth in, or toleration of, several different environments. Moreover, a specialized pathogenic trait may not be expressed until the infecting organism encounters a particular environment within its host. Generally, then, pathogenicity is not a simple characteristic, and virulence determinants like anti-phagocytic capsules, bacterial toxins, and bacterial pili, as well as more complicated factors associated with entry and persistence within hosts may be involved.

The pathogenic potential of microorganisms varies, and they are usefully categorized according to pathogenicity. This classification can then be used to determine an appropriate level of containment. For example, in the United Kingdom, microorganisms are allocated to one of four levels:

1. Hazard Group 1: Organisms that are most unlikely to cause human disease.
2. Hazard Group 2: Organisms that may cause human disease and which might be a hazard to laboratory workers, but are unlikely to spread to the community. Laboratory exposure rarely produces infection and effective prophylaxis or effective treatment is usually available.
3. Hazard Group 3: Organisms that may cause severe human disease and present a serious hazard to laboratory workers. They may present a risk of spread to the community, but there is usually effective prophylaxis or treatment available.
4. Hazard Group 4: Organisms that cause severe human disease and are a serious hazard to laboratory workers. They may present a high risk of spread to the community, and there is usually no effective prophylaxis or treatment.

Organisms in Hazard Groups 2, 3 and 4 are "pathogens". Organisms allocated to these levels are shown in Table 3.1.

The intention of the categorization is to fit appropriate containment to pathogenic organisms, bearing in mind that containment must be designed to protect both the worker and the public at large. Thus, increasing Hazard Group levels correspond to increasing levels of containment. In matching hazard group with containment level, the pathogenic potential, route of transmission, epidemiological consequences, and host susceptibility are considered. The possibility and consequences of any failure of containment must also be considered, and, where appropriate, emergency plans must be prepared to secure the safety of people and of the environment in the event of an accident.

An important element of containment is strict adherence to standard operating practices and techniques. Good laboratory practice must be the basis of all levels of containment. This consists of the following practices and procedures.

1. Local codes of practice for the safety of personnel must be formulated and implemented.
2. Laboratory personnel must receive instruction in the procedures conducted in the laboratory.
3. The laboratory should be easy to clean. Bench surfaces should be impervious to water and resistant to acids, alkalis, solvents, and disinfectants.
4. If the laboratory is mechanically ventilated, an inward air flow should be maintained by extracting room air, and the system must be interlocked to prevent positive pressurization of the room in the event of failure of the extraction fan. The ventilation system should also incorporate a means of preventing reverse air flow.
5. The laboratory should contain a washbasin or sink that is used only for hand washing.
6. The laboratory door should be closed when work is in progress.
7. Laboratory coats or gowns should be worn in the laboratory and removed when leaving the laboratory suite.
8. Eating, chewing, drinking, smoking, storing of food, and applying cosmetics must not take place in the laboratory.
9. Mouth pipetting must not take place.
10. Hands must be disinfected or washed immediately when contamination is suspected, after handling viable materials, and also before leaving the laboratory.
11. All procedures must be performed so as to minimize the production of aerosols.
12. Effective disinfectants must be available for routine disinfection and for immediate use in the event of spillage.
13. Bench tops should be cleaned after use.
14. Used laboratory glassware and other materials awaiting disinfection must be stored in a safe manner. Pipettes, if placed in disinfectant, must be totally immersed.
15. All waste materials must be made safe before disposal either by autoclaving or by incineration.
16. Materials for autoclaving or incineration must be transported without spillage in robust leak-proof containers.

Table 3.1. Categorization of microorganisms into hazard groups. (This list is based on information given in Categorization of Pathogens According to Hazard and Categories of Containment - see text)

HAZARD GROUP 1

Organisms not in Groups 2, 3 or 4

HAZARD GROUP 2

BACTERIA

Acinetobacter calcoaceticus
Acinetobacter lwoffi
Actinobacillus spp
Actinomadura spp
Anctinomyces bovis
Actinomyces israeli
Aeromonas hydrophila
Alcaligenes spp
Arizona spp
Bacillus cereus
Bacteroides spp
Bacterionemia matruchottii
Bartonella bacilliformis
Bordetella parapertussis
Bordetella pertussis
Borrelia spp
Campylobacter spp
Cardiobacterium hominis
Chlamydia spp (other than avian strains)
Clostridium botulinum
Clostridium tetani
Clostridium spp (other spp known to be pathogenic to man)
Corynebacterium diphtheriae
Corynebacterium spp (other spp known to be pathogenic to man)
Edwardsiella tarda
Eikenella corrodens
Enterobacter spp
Erysipelothrix rhusiopathiae
Escherichia coli (except those known to be non-pathogenic)
Flavobacterium meningosepticum
Francisella tularensis (Type B)
Fusobacterium spp
Gardnerella vaginalis
Haemophilus spp
Hafnia alvei
Kingella kingae
Klebsiella spp
Legionella spp
Leptospira spp
Listeria monocytogenes
Moraxella spp
Morganella morganii
Mycobacterium bovis (BCG strain)
Mycobacterium chelonei
Mycobacterium fortuitum
Mycobacterium marinum
Mycobacterium microti
Mycobacterium ulcerans
Mycoplasma pneumoniae
Neisseria spp (spp known to be pathogenic for man)
Nocardia asteroides
Nocardia brasiliensis
Pasteurella spp
Peptostreptococcus spp
Plesiomonas shigelloides
Proteus spp
Providencia spp
Pseudomonas spp (spp, other than those in Group 3, known to be pathogenic for man)

Table 3.1. cont.

HAZARD GROUP 2 (cont.)

Salmonella spp (other than those in Hazard Group 3)
Serratia liquefaciens
Serratia marcescens
Shigella spp (other than that in Hazard Group 3)

FUNGI

Absidia corymbifera
Acremonium falciforme
Acremonium kiliense
Acremonium recifei
Aspergillus flavus
Aspergillus fumigatus
Aspergillus nidulans
Aspergillus niger
Aspergillus terreus
Basidiobolus haptosporus
Candida glabrata
Candida guilliermondii
Candida drusei
Candida parapsilosis
Candida kefyr
Candida tropicalis
Cladosporium carrionii
Staphylococcus aureus
Streptobacillus moniliformis
Streptococcus spp (except those known to be non-pathogenic for man)
Treponema pertenue
Veillonella spp

Conidiobolus coronatus
Cryptococcus neoformans
Cunninghamella elegans
Curvularia lunata
Emmonsia parva
Epidermophyton floccosum
Exophialia dermitidis
Exophialia jeanselmei
Exophialia richardsiae
Exophialia spinifera
Exophialia werneckii
Fonsecaea compacta
Fonsecaea pedrosoi
Fusarium solani
Fusarium oxysporum
Geotrichum candidum
Hendersonula toruloidea
Leptosphaeria senegalensis
Vibrio cholerae (incl El Tor)
Vibrio parahaemolyticus
Vibrio spp (other species known to be pathogenic for man)
Yersinia enterocolitica
Yersinia pseudotuberculosis subsp *pseudotuberculosis*

Madurella mycetomatis
Madurella grisea
Malassezia furfur
Microsporum spp
Neotestudina rosatii
Phialophora verrucosa
Piedraia hortae
Pneumocystis carinii
Pseudallescheria boydii
Pyrenochaeta romeroi
Rhizomucor pusillus
Rhizopus microsporus
Rhizopus oryzae
Sporothrix schenckii
Trichophyton spp
Trichosporon beigelii
Xylohypha bantiana

Table 3.1. cont.

HAZARD GROUP 2 (cont.)

PARASITES (infective stages)

Acanthamoeba spp
Ancylostoma duodenale
Angiostrongylus spp
Ascaris lumbricoides
Babesia divergens
Babesia microti
Balantidium coli
Brugia spp
Capillaria spp
Clonorchis sinensis
Cryptosporidium spp
Dipetalonema perstans
Dipetalonema streptocerca
Diphyllobothrium latum
Drancunculus medinensisa
Entamoeba histolytica
Fasciola gigantea
Fasciola hepatica
Fasciolopsis buski
Giardia lamblia
Hymenolepis nana (human origin)
Hymenolepis diminuta
Loa loa
Mansonella ozzardi
Necator americanus
Onchocerca volvulus
Opisthorchis spp
Paragonimus westermanni
Plasmodium spp (human and simian)
Pneumocystis carinii
Schistosoma haematobium
Schistosoma intercactum
Schistosoma japonicum
Schistosoma mansoni
Strongyloides spp
Taenia saginata
Taenia solium
Toxocara canis
Teichinella spp
Trichomonas vaginalis
Trichostrongylus spp
Trichuris trichiura
Trypanosoma brucei subsp
Wuchereria bancroftii

VIRUSES

Adenoviridae
Arenaviridae
(other than those in Hazard Groups 3 and 4)
Bunyaviridae
Hazara virus
Caliciviridae
Coronaviridae
Herpesviridae
Cytomegalovirus
Epstein-Barr virus
Herpes simplex viruses types 1 and 2
Herpesvirus varicella-zoster
Human B-lymphotropic virus
Orthomyxoviridae
Influenza viruses types A, B and C

Table 3.1. cont.

HAZARD GROUP 2 (cont.)

Paramyxoviridae
- Measles virus
- Mumps virus
- Newcastle disease virus
- Parainfluenza virus types 1-4
- Respiratory syncytial virus

Papovaviridae
- BK and JC viruses
- Human papillomaviruses

Parvoviridae
- Human parvovirus (B19)

Picornaviridae
- Acute haemorrhagic conjunctivitis virus (AHC)Coxsackieviruses
- Echoviruses
- Hepatitis A virus (human enterovirus type 72)
- Polioviruses
- Rhinoviruses

Poxviridae
- Cowpox virus
- Molluscum contagiosum virus
- Orf virus
- Vaccinia virus

Reoviridae
- Human rotaviruses
- Orbiviruses
- Reoviruses

Rhabdoviridae
- Vesicular stomatitis virus

Togaviridae
- Alphaviruses
- Flaviruses
- Rubivirus (rubella)

Unclassified viruses
- Hepatitis non-A non-B viruses
- Norwalk-like group of small round structured viruses
- Small round viruses associated with gastroenteritis

Unconventional agents associated with:
- Creutzfeldt-Jakob disease
- Gerstmann-Sträussler-Scheinker syndrome
- Kuru

HAZARD GROUP 3

BACTERIA

Bacillus anthracis
Brucella spp
Chlamydia psittaci (avian strains only)
Coxiella burnetii
Francisella tularensis (Type A)
Mycobacterium africanum
Mycobacterium avium
Mycobacterium bovis (excluding BCG)
Mycobacterium intracellulare
Mycobacterium kansasii
Mycobacterium leprae
Mycobacterium malmoense
Mycobacterium paratuberculosis
Mycobacterium scrofulaceum
Mycobacterium simiae

Table 3.1. cont.

HAZARD GROUP 3 cont.

Mycobacterium szulgai
Mycobacterium tuberculosis
Mycobacterium xenopi
Pseudomonas mallei
Pseudomonas pseudomallei
Rickettsia-like organisms
Rickettsia spp
Salmonella paratyphi A, B, C
Salmonella typhi
Shigella typhi
Shigella dysenteriae (Type 1)
Yersinia pseudotuberculosis subsp *pestis*

FUNGI

Blastomyces dermatitidis
Coccidioides immitis
Histoplasma capsulatum var *capsulatum*
Histoplasma capsulatum var *duboisii*
Histoplasma capsulatum var *farciminosum*
Paracoccidioides brasiliensis
Penicillium marneffei

PARASITES (infective stages)

Echinococcus spp
Leishmania spp (mammalian)
Naegleria spp
Toxoplasma gondii
Trypanosoma cruzi

VIRUSES

Arenaviridae
- Lymphocytic choriomeningitis virus

Bunyaviridae
- Bunyamwera supergroup
  - Oropouche virus
- Phleboviruses
  - Rift Valley fever
- Hantaviruses
  - Hantaan (Korean haemorrhagic fever)
  - other hantaviruses

Hepadnaviridae
- Hepatitis B virus
- Hepatitis B virus + Delta

Herpesviridae
- *Herpesvirus simiae*

Poxviridae
- Monkeypox virus

Retroviridae
- Human immunodeficiency viruses (HIV)
- Human T-cell lymphotropic viruses

Rhabdoviridae
- Rabies virus

Table 3.1. cont.

HAZARD GROUP 3 (cont.)

Togaviridae
- Alphaviruses
  - Eastern equine encephalomyelitis
  - Venezuelan equine encephalomyelitis
  - Western equine encephalomyelitis

Flaviviruses
- Japanese B encephalitis
- Kumlinge
- Louping ill
- Murray Valley encephalitis

Flaviviruses (cont.)
- Powassan
- Rocio
- St Louis encephalitis
- Tick-borne encephalitis
- Yellow fever

HAZARD GROUP 4

BACTERIA, FUNGI, PARASITES
None

VIRUSES

Arenaviridae
- Junin virus
- Lassa fever virus
- Machupo virus
- Mopeia virus

Bunyaviridae
- Nairoviruses
  - Congo/Crimean haemorrhagic fever

Filoviridae
- Ebola virus
- Marburg virus

Poxviridae
- Variola (major & minor) virus

Togaviridae
- Flaviviruses
- Tick-borne viruses
  - Absettarov
  - Hanzalova
  - Hypr
  - Kyasanur Forest
  - Omsk
  - Russian spring-summer encephalitis

This level of containment is appropriate for work with microorganisms of Hazard Group 1 (i.e., non-pathogens). A summary of the UK requirements for work at higher levels of containment is shown in Table 3.2. Levels 3 and 4 are high containment, in which all work is carried out in a microbiological safety cabinet; they are particularly relevant where there is potential danger from aerosols.

It must also be remembered that the environment as a whole must be considered. Thus, while the considerations above refer specifically to hazard to human health, there are microorganisms that are pathogenic for other animals or for plants. Use of such pathogens may be restricted for this reason, and, in any case, such possible effects in case of accidental release must be considered in a risk assessment, and suitable containment must be used.

Should GMMs Be Treated Differently from Other Microorganisms?

The possibility that genetic modification might affect the host range of the host organism, or its capacity to utilize different substrates, or might convert the host into a pathogen, or alter its balance with ecologically interrelated populations must all be considered. But such considerations of possible hazards would be called for by any newly-isolated organism. Are there hazards unique to GMMs, which demand a different set of criteria for the risk assessment? It is certainly the case that recombinant organisms will often contain genes and produce compounds not normally found in microorganisms, and these factors must be taken into account. But is this so different from assessing the hazards associated with a newly-isolated natural microorganism? A different sort of judgement must be made in assessing possible hazards associated with gene libraries, in which there may be millions of different clones. The assessment here is not that different from deciding on the hazard posed by a clinical specimen, in which there may be pathogens.

With a fully-characterized recombinant organism (a clone of *E. coli* K12, for instance), there is no reason to think that the hazard will be any more than indicated by the insert and its product. The risk assessment will then simply be a consideration of the extent of expression of the insert and the chance of damage caused by the product of such expression. With a clone using an uncharacterized host (a primary cell line, for instance), more care would be taken (as would be the case with non-GMMs) due to the possibility of activation of unknown agents or pathways (e.g., retroviruses or oncogenes). With a recombinant virus, the possibility of infectious spread must of course be seriously considered, but then this would be done anyway when working with any virus.

The major unknown is not with isolated clones, which will have been characterized to some extent, but with gene libraries (i.e., during the original isolation of the clones). Here there is the potential for completely unknown combinations of sequences with unknown effects. It was this that was the major original worry that

precipitated the current regulation of genetic modification. This point can be approached in a number of ways; for instance, one might consider the chances of unexpectedly deriving a pathogenic strain of the host bacterium, or of unexpectedly changing a eukaryotic cell line; and there is also the experience of twenty years of work in this field.

As discussed briefly above, pathogenicity is not a simple characteristic. Many genes must interact appropriately for a microbe to cause disease: the pathogen must possess and appropriately express characteristics such as recognition factors, adhesion ability, toxigenicity, and resistance to host defence systems. Single-gene modifications of organisms with no pathogenic potential or history, or the introduction of several genes not contributing to pathogenicity do not appear likely to result in unanticipated pathogenicity. Moreover, with respect to pathogenicity, considerable experience and extensive data can be used to define parameters of concern. For instance, with one of the favourite hosts, *E. coli* K12, many of the characteristics associated with wild-type *E. coli* (which is a Group 2 pathogen - Table 3.1) have been lost, including the cell-surface K antigen, part of the LPS side chain, the adherence factor (fimbriae) that enables adherence to epithelial cells of human gut, resistance to lysis by complement, and some resistance to phagocytosis. The genetic information for four of these five characteristics is widely separated on the chromosome, and it is reasonable to conclude that there is practically no chance of accidental transfer of these genes during a genetic modification experiment. In addition, *E. coli* K12 does not survive very well outside specific growth media in the laboratory. Without the capability to survive in the environment, and to infect hosts, very few conceivable hazards that might be inadvertently incorporated would pose much risk. So, with such a well-characterized and enfeebled host cell, it seems extremely unlikely that anything untoward could happen by accident. And all the evidence of the last twenty years supports this contention.

With a less well-characterized host, the unexpected is, perhaps, more likely, simply because less is known about the organism, and there is no experience of use. This could, for instance, easily be imagined with primary mammalian-cell lines. It is inconceivable that the cell culture in itself could pose any threat (assuming that it were properly handled), but one could imagine activation of viruses - although general experience with such cell lines suggests that this is unlikely.

The construction of gene libraries from all types of organisms in many types of host cells has a long and safe record. Although this does not, of course, prove that a disaster could not happen (the hypothetical event may only occur at very low frequency), it does, in conjunction with the theoretical considerations, lead to a feeling of quiet optimism that the primary isolation of clones is basically a safe procedure (unless, of course, the intention is to isolate some harmful characteristic).

Risk Assessment of GMMs and Assignment to Containment Level

The GMM is simply a potential pathogen, which might also have an effect on the environment if it were to escape. The three components of the GMM are the host organism, the vector, and the insert. (Where the vector is a virus, the recombinant virus will be a GMM in its own right, and the "host" and the "vector" will be the same.) These components can be separately assessed, and the amalgamation of the assessments gives a measure of the risk associated with the recombinant organism as a whole and indicates an appropriate containment level. The characteristics of the host and of the vector often provide a degree of "biological containment", where the enfeebled state of the host, and/or the inability of the vector to transfer to other organisms impede spread of the recombinant outside the laboratory.

The **host** itself, if a microorganism, will probably already have been assessed as to its pathogenicity: for instance, it might be in a list such as that shown in Table 3.1. This assessment will be the basis for further considerations. (*Prima facie*, the level of risk for the GMM cannot be less than that of the host itself.) It is, of course, usually the case in genetic engineering experiments that, at least with bacteria, the host is non-pathogenic (Hazard Group 1 in Table 3.1). Another factor that must be considered is the capacity of the host to survive outside the laboratory. This would be of relevance in the event of an accidental release from containment; if the host could become established in the environment, then so would the recombinant DNA ("vertical transfer" of the recombinant molecule). *E. coli* K12 hardly survives in the environment: it is "disabled". There are also especially disabled strains of *E. coli* K12 (such as MRC1 and 1776), which could only survive in the most specialized growth media. *Aspergillus oryzae*, *Bacillus subtilis* and *Saccharomyces cerevisiae* can also be considered to be especially disabled. Eukaryotic cell lines also cannot, of course, persist independently outside the laboratory, and, as long as they are unable to colonize the worker and contain no known adventitious agent, they also can be considered to be especially disabled. An essential requirement in deciding whether a cell line fulfils these criteria is that it should have had a long history of safe, uneventful use in the laboratory.

The **vector** has to be considered both for its own potential for pathogenicity and for its ability to transfer to other organisms ("horizontal transfer" of the DNA). This latter is important in consideration of the consequences of accidental release since it could result in transfer of the recombinant DNA to organisms that have access to various different niches in the environment.

Vectors for bacteria. Most vectors used in *E. coli* contain no sequences encoding pathogenic characteristics. They do, however, usually contain selective markers such as resistance to antibiotics. These characteristics must be

Table 3.2. Summary of requirements for the four levels of laboratory containment in the United Kingdom

Requirements	Containment levels			
	1[a]	2	3	4
Laboratory suite isolated	No	No	Partial	Yes
Laboratory sealable for fumigation	No	No	Yes	Yes
Ventilation:				
inward airflow/negative pressure	Optional	Optional	Yes	Yes
through safety cabinet	No	Optional	Optional	No
direct mechanical	No	No	Optional	Yes
mechanical through independent ducting	No	No	Optional	Yes
Airlock	No	No	Optional	Yes
Airlock with shower	No	No	No	Yes
Wash handbasin	Yes	Yes	Yes	Yes
Effluent treatment	No	No	No	Yes
Autoclave on site	No	No	No	No
Autoclave in suite	No	Yes	Yes	No
Autoclave in lab:				
free standing	No	No	Optional	No
double ended	No	No	No	Yes
Microbiological safety cabinet / enclosure	No	Optional	Yes	Yes
Class of cabinet / enclosure		Class I	Class I/III	Class III

[a] Containment level 1 is equivalent of 'good laboratory practice' (see text).
Source: Adapted from ACGM/HSE Note 8.

considered in the context of current medical practice, in particular whether the genes involved could be transferred to strains where their presence would prejudice treatment. The particular markers commonly used (resistance to penicillin, or to tetracycline, or to chloramphenicol, for example) already occur widely in the environment, and their escape would not cause a problem. As regards the potential transferability of the vector, most commonly-used *E. coli* vectors are transfer-deficient. There are two factors that must be considered here: some plasmids encode functions that enable the plasmid to be transferred to other bacteria (they are, thus, Tra^+); others are Tra^-, but can be mobilized by Tra^+ plasmids - they are Mob^+. So, it is possible to have plasmid vectors that are Tra^+ (self-mobilizable), or Tra^-, Mob^+ (mobilization-defective) or Tra^-, Mob^- (non-mobilizable); vectors in these groups show decreasing capabilities of transfer to other microorganisms. The host range of plasmid vectors is also relevant; for instance, some plasmids can transfer to and be maintained in many different genera (e.g., RSF1010 and derivatives, RP4 and derivatives). Such plasmid vectors obviously increase the chances of transfer to and establishment in other organisms of recombinant DNA.

Vectors from other bacteria must be considered in a similar way: many genera contain plasmids that have the capacity for transfer. Table 3.3 shows the mobilization potential of some commonly-used bacterial vectors.

Vectors for higher eukaryotes. Some of the vectors shown in Table 3.3 are shuttle vectors designed for construction of recombinants in *E. coli* and then for studies of transient or stable expression in animal or plant cell lines. Some of these vectors contain sequences from eukaryotic viruses, such as mouse mammary-tumour virus or SV40. Such vectors can be considered not to be harmful as long as no infective particles are involved (see discussion of infective vectors below). Vectors which integrate into the chromosome may also be considered to be non-mobilizable.

Eukaryotic viral vectors. Certain vectors for eukaryotic cells are based on explicit pathogens (viruses), which may indeed retain some of their pathogenic characteristics. Some such vectors are infective but replication-defective, so that they can deliver the DNA to the target cell, but cannot replicate as viruses there (examples are retrovirus-based vectors). Here, one must consider what type of cells are susceptible (if human cells can be infected, the worker may be at risk), and what chance there is of correction of the replication deficiency by complementation by or recombination with latent viruses in the host. If the vector is a replication-proficient virus, consideration must be given to the chances of the insert affecting the pathogenicity or the tropism of the virus.

Access is a measure of the probability that a recombinant DNA could enter the environment (including the human body) and survive there. It is a function of the host and of the vector. For instance, a non-mobilizable vector (see

Table 3.3. Mobilization properties of various vectors[a]

Non-mobilizable vectors	
E. coli	pAT153, pACYC184, pBR327, pBR328, pUC series, pBluescript II, pMTL20, pBS, pGEM, pBEMEX, pUR222, pUCBM, pSP series, pEX series, pCAT series, pT3/T7, pEUK, pMAM, pMSG, pEMBL, pSELECT Criteria for non-mobilizable vectors are considered to be met by bacteriophage λ vectors with restricted host range, such as λCharon 3A, λgt10, λGEM, λEMBL, λgt11, λZAP Criteria for non-mobilizable vectors are considered to be met by bacteriophage M13 vectors used in a host containing a Tra⁻ F plasmid
B. subtilis	pUB110, pC194, pS194, pSA2100, pE194, pT127, pUB112, pC221, pC223, pAB124, pBD series
Mobilization-defective vectors	
E. coli	pBR322, pBR325, pACYC177, pKK233-2, pKK338-1, pBTac1, pBTrp2, pKC30, pKT279, pFB series, pNO1523, pSVL, pKSV10, pGA482, pNOS, pHSV106

[a] See text for definitions.
Source: This list is based on information in ACGM/HSE/DOE Note 7.

Table 3.3) used in a strain of *E. coli* K12 would have a very low access, as would any vector in a cell line, provided that no infective virus is involved. In the case of recombinant infective viruses, access to sensitive hosts will be very high and the result may be a productive infection resulting in more recombinant particles.

The **insert** is, of course, the component that requires the most careful assessment (the host and vector will usually already be well characterized and properly assessed). The major point with the insert is the possibility of gene expression and the hazards posed by the gene product. These considerations give rise to two more risk factors, damage and expression.

Damage is associated with the known or suspected biological activity of the DNA or of the gene product it encodes, and the levels and nature of the product required to elicit this activity. Factors such as the activity of the expressed protein and any toxic, allergenic, or pathogenic effects that it might have are relevant.

It may be that the biological activity of a protein is dependent on the host cell system in which it is expressed. For example, some proteins expressed at high levels in *E. coli* are incorrectly folded and are present in insoluble, biologically inactive inclusion bodies. Another example of dramatically different effects depending on the location of the insert would be an oncogene: in a bacterium, even if expressed, it would be harmless; the DNA has to get into a mammalian cell to exert the effect. The full biological activity of other molecules is dependent on post-translational modification, which will only occur in certain host cells (usually eukaryotic). A further consideration should be whether the protein is synthesized as a fusion product. Thus, careful consideration of the details of the recombinants often indicate that *prima facie* damage factors can be mitigated. Guideline examples given in the United Kingdom for levels of damage are:

(a) A toxic substance or pathogenic determinant that is likely to have a significant biological effect.

(b) A biologically active substance which might have a deleterious effect if delivered to a target tissue, or a biologically inactive form of a toxic substance which, if active, might have a significant biological effect.

(c) A biologically active substance which is very unlikely to have a deleterious effect, or could not approach the normal body level (e.g., less than 10% of the normal body level).

(d) A gene sequence where any biological effect is considered highly unlikely either because of the known properties of the protein or because of the high levels encountered in nature.

(e) No foreseeable biological effect (e.g., non-coding DNA sequence).

Expression is a measure of the anticipated or known level of expression of the inserted DNA. The identity of the promoter must be considered: the insert itself might contain one, but generally the promoter will be part of the vector, particularly where a high level of expression is sought. The level of expression from the most active promoters can result in the insert directing synthesis of greater than 10% of soluble protein in the host cell. Activity will decrease through increasingly less efficient promoters, to no promoters at all. And, of course, the insert may only contain non-expressible DNA.

In the United Kingdom, numbers are put on Access, Expression and Damage, and the product of the three numbers then determines the containment level appropriate for the GMM. (This final numerical procedure is not appropriate with viral vectors because they are infectious agents.) Here are some examples of containment levels assigned to the use of various GMMs after assessment following the above guidelines: over-expression of harmless proteins using standard pBR322-based vectors in *E. coli* K12, containment level 1; over-expression of harmless proteins in eukaryotic cell lines (assuming no infective virus is involved), contain-

ment level 1; over-expression of a biologically active protein such as interleukin-2 using a mobilization-defective vector in *E. coli* K12, containment level 2; a replication-defective retroviral particle carrying the interleukin-2 gene and capable of infecting human cells, containment level 2; a replication-defective retroviral particle carrying an oncogene and capable only of infecting murine cells, containment level 2; a replication-defective retroviral particle carrying an oncogene and capable of infecting human cells, containment level 3.

In addition to these assessments of isolated clones, gene libraries have to be assessed. Here there is the problem of the unknown; in particular, how should "damage" be assessed. If a eukaryote is the source of the library, it is likely that a genomic library will be less hazardous than a cDNA library, since it is likely that only in the latter will most genes be expressible. But even with cloning from the chromosome there is the possibility of picking up some adventitious agent such as a latent virus. As stated above, all the evidence suggests that such unexpected outcomes are unlikely. Thus, if the origin of the library is a harmless, well-defined organism, it is reasonable to assume that there is very little chance of hazard, and containment level 1 is appropriate. If the library comes from a pathogen, however, the overall level of containment should be appropriate for that pathogen (Table 3.1); when defined clones have been isolated from the library, realistic measures of damage can be assigned, and it may be that a lower level of containment will be appropriate.

Work Under Containment (Large Scale)

Industrial scale growth of GMMs is not intrinsically more hazardous than the use of the organism in the laboratory; it is mainly the scale of operation, and hence the possible escape volume, concentration, and duration of exposure, that have increased. Balanced against this is that most of the uncertainties related to the organism at the laboratory stage have been eliminated: the organism will be strictly defined, and the chances of the production strain becoming pathogenic are negligible. In most cases, traditionally safe microorganisms will have been modified by inserting segments of DNA to facilitate manufacture of new products, and no safety considerations will be raised beyond those that might be called for by the products themselves.

The crossover point between "lab scale" and "large scale" has often been taken as 10 litres. This is clearly arbitrary, and, in the United Kingdom, the definition with respect to work involving GMMs is "the use of a cell or organism constructed by genetic manipulation, for example, in a laboratory-scale reaction vessel, for pilot-plant work or commercial manufacture". Recommendations for containment on a small scale are not wholly appropriate for large-scale production, but act as a starting point for assessment: the underlying philosophy in the assessment for the laboratory-scale experiments will be relevant in determining the nature and extent of the control measures required. Standards of work protection should not fall be-

low the small-scale guidelines, but additional expertise may be required to establish specific containment criteria. But there is, of course, a huge resource of existing experience in fermentation techniques in which the strict application of containment has been necessary.

There are steps involved in downstream processing, each of them requiring individual assessment. For example, any consideration of risk must take into account factors such as whether the organism is killed in the fermenter before downstream processing. Methods of processing microorganisms on a large scale have the potential for aerosol generation and widespread contamination. In some cases, the risks presented by other aspects of the process and by the product may dictate the level of physical containment.

The vast majority of large-scale applications of GMMs will use organisms of intrinsically low risk, which warrant only minimal containment. This level of minimal containment is known as Good Large-Scale Practice (GLSP). GLSP will involve no containment measures beyond those required for process needs. The following fundamental principles of occupational safety and hygiene should be applied for GLSP as well as for all levels of containment:

1. To keep workplace and environment exposure to any physical, chemical, or biological agent at the lowest practicable level.
2. To exercise engineering control measures at source and to supplement these with appropriate personal protective clothing and equipment when necessary.
3. To test adequately and maintain control measures and equipment.
4. To test when necessary for the presence of viable process organisms outside the primary physical containment.
5. To provide training of personnel.
6. To formulate and implement local codes of practice for the safety of personnel.

To allow designation of GLSP, the overriding consideration is that the GMM must not be a pathogen, should be as safe in the bioreactor as the host organism, and should have no adverse consequences in the environment. In arriving at the designation, environmental aspects should be taken into account. It is an inherent feature of large-scale work, and especially of GLSP, that microorganisms will be released incidentally at various stages of the fermentation process and at the early stages of downstream processing. These organisms impinge on the environment. The following factors should be considered in an assessment of the potential risk to the environment:

1. The volume/biomass likely to be released.
2. The known or predicted behaviour of the organism, including factors affecting its survival, multiplication, and dissemination.
3. The description of the ecosystems into which organisms could be disseminated, and the known or predicted impact in such ecosystems, including effects on

plants, animals and microorganisms (e.g., pathogenicity, toxicity, virulence, allergenicity, colonization).

4. The availability of techniques for the detection, identification, and monitoring of the organism, and for detecting transfer of new genetic material to other organisms.

Conclusions

It is obvious that there are characteristics unique to GMMs that must be properly assessed so that a realistic estimate of the risk involved in using such organisms can be made, and appropriate containment can be prescribed. The principles and examples discussed above provide a basis for this assessment.

It should also be obvious from the preceding discussion that there is no real scientific justification for special regulations for genetic engineering; the potential risks are just another factor that has to be considered in risk assessment. Although it is probable that the public is not so sanguine, and that, politically, dedicated rules are required, the regulation of safety must command respect of operators as well as the public. This requires that rules be soundly based. While the vast majority of experiments involving genetic modification are manifestly harmless and present negligible risk, in many countries, all such experiments are subject to over-rigorous, officious regulation.

Chapter four
Safety in the Contained Use and the Environmental Release of Transgenic Crop Plants

Philip J. Dale[1] & Julian Kinderlerer[2]

[1]*Cambridge Laboratory, John Innes Centre, Norwich, United Kingdom.*
[2]*Department of Molecular Biology & Biotechnology, The University of Sheffield, Sheffield, United Kingdom.*

Introduction

During the last ten years, significant advances have been made in methods of handling DNA in the laboratory and in ways of introducing genes into crop plants. These have provided new opportunities to modify world crops in novel ways. Biotechnology will have a profound effect on the ability to provide plants with many new properties. It will be possible to produce plants that have enhanced resistance to pests and diseases, have modified protein and oil content, and have improved nutritional properties. There will eventually be opportunities to protect crop plants against environmental stress, such as drought, high salinity, or cold. Biotechnology is also capable of providing new pharmaceutical and other chemical substances synthesized *in situ* within crop plants in environmentally acceptable ways. As a consequence, the consumer is likely to gain from a wider choice of plant-derived products. However, the ability to insert into crops genes derived from unrelated organisms, and even to synthesize genes in the laboratory, causes concern among some people. The insertion into plants of a *Bacillus thuringiensis* gene responsible for the production of an insecticidal protein, for example, may give rise to the evolution of insect-resistant strains (Williamson, 1993).

The new technology is regulated by statute or convention in almost all countries in the industrialized world. Traditional methods of modifying plants[1] tend not to be regulated, although in many countries there are some controls on the introduction of novel (imported) species. Historically, crop plants have not been subjected to formalized risk/safety analysis or risk management as there is a mass of knowledge, understanding and experience regarding the procedures for managing the introductions of crop plants developed by a wide range of breeding methods

(OECD, 1992). In traditional breeding, crops are improved by cross-pollination between plants with desirable characteristics, followed by selection of progeny with new gene combinations. Improvement by plant breeding methods is possible when the genes controlling the characteristics of interest are found within the crop species itself or within species that are sexually compatible with it. Various techniques have been used over the history of traditional plant breeding to increase the choice of genes available through traditional breeding, and they include now embryo culture, ovary culture, and protoplast fusion. Even when a novel hybrid plant can be obtained, there may be failure of chromosome pairing, or the genetic recombination necessary to introduce the foreign genes into the crop species.

Transgenic Plants

It was only about 20 years ago that enzymes were discovered that were able to cut DNA molecules at specified sites (restriction enzymes), and others that were able to join DNA fragments together again (ligases). These enzymes have made it possible to develop recombinant DNA techniques to create, in the laboratory, new combinations of genes and gene control sequences.

In the early 1980s it became possible to introduce laboratory-modified DNA sequences into whole tobacco plants, and to ensure that the introduced genes (transgenes) were inherited in a simple Mendelian manner through pollen and egg cells. A transgenic plant is one which has received a segment of DNA that has been manipulated by recombinant DNA techniques, and the foreign segment of DNA has been integrated into the plant's genome.

Since the early developments in recombinant DNA technology, a wide range of restriction enzymes have been discovered which are able to cut DNA at a variety of specific positions along the molecule. There has also been development and refinement of DNA hybridization techniques, which make it possible to detect specific DNA sequences in plants, to determine the numbers of copies and the structural integrity of the transgenes inserted. Recent advances in genetic mapping techniques have also made it possible to determine the chromosomal location of a transgene within the total plant genome.

Methods of Producing Transgenic Plants

One of the principal hurdles that have prevented the routine modification of crop plants is the difficulty of introducing foreign DNA into a plant cell. The process of DNA introduction into plants (called transformation) was first achieved in tobacco, and has been relatively easy to achieve in other solanaceous species (potato, petunia, various *Nicotiana* species). With certain other crop species, especially the cereals, it has been less easy. Species that have been transformed to date are shown in Table 4.1. Many approaches to transformation have been attempted, and the successful methods used at present fall into four groups (Potrykus, 1990, 1991).

Table 4.1. Principal plant transformation methods in current use and examples of the crop species in which they have been successful

Species (crop)	Method			
	Agrobacterium	DNA uptake into isolated protoplasts	Micro-projectile bombardment	DNA uptakeinto partially digested, immature embryos
Actinidia deliciosa (kiwifruit)	+			
Avena sativa (oats)			+	
Beta vulgaris (sugarbeet)	+			
Brassica carinata	+			
Brassica juncea	+			
Brassica napus (rapeseed)	+	+		
Brassica oleracea (various)	+	+		
Carica papaya (papaya)	+		+	
Cucumis melo (melon)	+			
Cucumis sativis (cucumber)	+			
Dactylis glomerata (cocksfoot)		+		
Dendranthema indicum (chrysanthemum)	+			
Dianthus caryophyllus (carnation)	+			
Festuca arundinacea (tall fescue)		+		
Fragaria ananassa (strawberry)	+	+		
Glycine max (soybean)	+	+	+	

Table 4.1. cont.

Gossypium hirsutum (cotton)	+		+	
Helianthus annuus (sunflower)	+			
Juglans regia (walnut)	+			
Lactuca sativa (lettuce)		+		
Linum usitatissumum (flax)	+			
Lycopersicon esculentum (tomato)	+			
Medicago sativa (alfalfa)	+			
Nicotiana tabacum (tobacco)	+			
Oryza sativa (rice)		+	+	
Picea glauca (white spruce)			+	
Pisum sativum (pea)	+			
Populus (poplar)	+		+	
Prunus armeniaca	+			
Prunus domestica (plum)	+			
Solanum muricatum (pepino)	+			
Solanum tuberosum (potato)	+			
Triticum aestivum (wheat)			+	
Vitis vinifera (grape vine)	+			
Zea mays (maize, corn)		+	+	+

For references see Dale *et al.* (1993).

Agrobacterium

The *Agrobacterium* method of transformation is used widely to transform dicotyledonous species. There are two principal species, *Agrobacterium tumefaciens* and *A. rhizogenes*, which in their wild-type form are pathogens causing crown gall disease and hairy root disease, respectively. Many dicotyledonous species are susceptible to infection by *Agrobacterium* species (De Cleene & De Ley, 1976), brought about by the incorporation of genes from an independently replicating plasmid within the *Agrobacterium* cell, which then become incorporated into the host plant. The introduced DNA modifies the phytohormonal levels within infected cells, and either causes a disorganized proliferation of cells and the formation of a gall (*A. tumefaciens*), or the production of a mass of roots covered with hair (*A. rhizogenes*) (Bevan, 1984).

The disease-causing genes are carried between specialized T-DNA (transforming DNA) border sequences on the independently replicating circular plasmid DNA molecule. By recombinant DNA methods, it has been possible to remove the disease-causing genes, so that the *Agrobacterium* organism is no longer pathogenic. New vector plasmids have also been constructed which enable foreign genes to be inserted between the T-DNA borders. *Agrobacterium* cells carrying the gene(s) of interest are then incubated with cultured cells of the recipient crop plant, and transgenic plants are regenerated from them. Only a small proportion of the treated plant cells eventually become transformed, so it is usually necessary to incorporate selectable marker genes (usually conferring resistance to a particular antibiotic) between the T-DNA border sequences. To select the transgenic plants, the corresponding antibiotic is added to a plant regeneration medium where only transgenic plants are able to grow normally.

The major restriction to the use of this technique is that many plants, particularly cereals, are extremely difficult to transform because of the lack of a wound response (Potrykus, 1991).

DNA Uptake into Plant Protoplasts

Protoplasts are plant cells that have had their cell walls removed by enzymatic treatment. They can be produced from various parts of the plant (often from leaves or hypocotyls) and are bounded by the plasma membrane. This membrane is delicate and its integrity can be affected by polyethylene glycol treatment or by passing an electrical current through a protoplast suspension. The DNA to be introduced is added to the medium surrounding the suspended protoplasts, and the chemical or electrical treatment allows the DNA to enter. In a small proportion of protoplasts, the foreign DNA becomes incorporated into the cell genome.

As with the *Agrobacterium* method, it is usual to insert a selectable antibiotic-resistance marker gene in order to select the transformed protoplasts and the cell colonies that develop from them. Plant-tissue culture procedures are subsequently used to regenerate whole transgenic plants.

Particle Bombardment

The *Agrobacterium* and protoplast methods have often proved to be inadequate for the transformation of recalcitrant species. Cereals are not normally hosts to *Agrobacterium*, and routine and reliable regeneration of plants from cereal protoplasts is difficult and often dependent on the genotype. The technique of particle bombardment was developed in an attempt to overcome some of these problems; it involves coating metal particles (usually tungsten or gold particles, 1mm in diameter) with DNA and shooting them into plant cells capable of subsequent plant regeneration. Particles can be propelled by various means, including 0.22in. blank cartridges, compressed gasses, or the instantaneous evaporation of a water droplet caused by an electrical discharge. The small metal particles, with their DNA coating, enter the plant cells and become lodged there. In a tiny proportion of the recipient cells, the DNA becomes incorporated into the genome, and transgenic plants can be regenerated from them. As with the other methods, it is usual to incorporate a selectable marker gene.

Partial Digestion of Cells in Multicellular Structures

A method which now looks promising for the transformation of cereals is the partial digestion of immature embryos with enzymes, followed by the stimulation of DNA uptake by exposure to an electrical current (electroporation). Eventually, plants are regenerated from the transformed cells of the embryo. This method has the potential advantage of using immature embryos which often have a high capacity for plant regeneration. More experience with this approach will be required before it can be established how widely applicable it may be for the transformation of a range of gramineous species.

Number and Position of Transgenes Inserted

The number of copies of transgenes that become incorporated into the nuclear genome varies among independently transformed plants and with the method of transformation used. The integration of one T-DNA copy with the *Agrobacterium* method is common, but higher numbers are observed. Multiple T-DNA inserts do occur, and occasionally the presence of 20–50 copies is reported. The position of insertion of T-DNA within the nuclear genome is believed to be random. In several studies, the position of T-DNA has been mapped using the Restriction Fragment Length Polymorphism (RFLP) method, and there is yet no evidence that there are preferred sites of insertion (Chyi *et al.*, 1986; Ambros *et al.*, 1986). Most genes are incorporated into the nuclear chromosomes, but there are reports of transformation of the plastid component of the cytoplasmic genome (O'Neill *et al.*, 1993).

Structure and Expression Stability of Transgenes

Structural rearrangement of inserted DNA can occur, and it is often desirable to use molecular analysis to determine whether the introduced DNA is intact. The expression of introduced genes can vary considerably among different independently transformed plants. In some instances there is a positive association between the level of expression of a transgene product and the number of transgene copies present, but this is not always the case, and a negative association has also been reported (Hobbs *et al.*, 1990; Jefferson *et al.*, 1990; Blundy *et al.*, 1991). It is known that transgene expression can be down-regulated or switched off. This gene suppression is often associated with methylation of cytosine nucleotide residues at particular locations within the introduced DNA (Selker, 1990; Scheid *et al.*, 1991).

Another problem arises from the tissue-culture process used to regenerate transgenic plants, which is known to introduce variation (somaclonal variation). This is not influenced directly by the transgene(s) inserted, but it is a change originating from the process of growing plant cells in culture. Somaclonal variation can be epigenetic or genetic. If epigenetic, the variation is not inherited in subsequent sexual generations. Genetic variation originates from gene or chromosomal mutation, and is heritable. To overcome the practical problems associated with variation in transgene expression and somaclonal variation, in practice, it is necessary to produce a range of independently transformed plants (frequently more than 100) and to select individual genotypes which have the desired phenotype (Larkin & Scowcroft, 1981; Karp, 1991; Dale & McPartlan, 1992).

The consequences of somaclonal variation in transgenic plants are not likely to be any more significant than those of its presence in their non-transgenic counterparts. In the production of a transgenic plant variety it will be necessary to eliminate any undesirable variation of this kind during the selection programme. The consequences of instability in transgene expression will lead in most cases to reduced transgene expression and to a change in phenotype such that the transgenic plant becomes more like its non-transgenic original. However, if the function of a transgene is to down-regulate the expression of an undesirable plant product, for example, the consequences of instability may potentially present a hazard that would need to be considered as part of a risk assessment (see below).

Review of Existing Regulations

According to the estimates of the Organisation for Economic Co-operation and Development (OECD, 1993), there had been 864 releases of modified plants up to 1992. Of these, 316 had occurred in the United States of America, 302 in Canada, and 217 in the European Community. Releases have occurred in at least 22 countries (Dale *et al.*, 1993).

The regulations governing the use of transgenic plants in containment and following release into the environment vary considerably between countries. There has been, and continues to be, a gradual move from voluntary schemes to statutory

ones, particularly for intentional releases into the environment. Where possible, existing legislation has been applied to the production, maintenance and release of genetically modified plants, but frequently these have not been adequate to cover the potential environmental effects from the release of transgenic plants.

In the United Kingdom, for example, contained work has been covered by the Health and Safety at Work Act of 1974 (Royal Commission on Environmental Pollution, 1989). Since physical and biological containment implied little risk to the environment, the presumption had been that those at risk were only those working with modified organisms, or working in the same facility where such organisms were contained. The United Kingdom regulations covering the manufacture or use of genetically modified organisms in containment have recently been changed to bring them into line with the European Directives. The term "containment" is used in describing safe methods for managing infectious agents in the laboratory environment where they are being handled or maintained. The purpose of containment is to reduce or eliminate the exposure of laboratory workers, other persons, and the outside environment to potentially hazardous agents (US HHS, 1993).

In 1990, the Commission of the European Communities issued Directives on the contained use of genetically modified microorganisms (CEC, 1990a) and the deliberate release into the environment of genetically modified organisms (CEC, 1990b). These Directives are binding on all EC countries. It is the responsibility of each of the member countries to bring into operation national legislation enforcing the Directives. By the end of 1992, national legislation had been adopted in Denmark, France, Germany, the Netherlands and the United Kingdom. Ireland and Italy had adopted enabling legislation which was to allow regulations to be introduced. Belgium, Greece, Luxembourg, Portugal and Spain and were in the process of introducing the necessary legislation.

In the United Kingdom, new sets of regulations for both contained use (of microorganisms as required by the Directives, and separately of other organisms, such as plants) and deliberate release came into force in February 1993. These are intended to implement the requirements of the European Directives, the Environmental Protection Act, 1990, and the Health and Safety at Work Act, 1974.

In the United States of America, three agencies share the responsibility for regulating the use of transgenic plants, whether in containment or in environmental applications. They are the United States Environmental Protection Agency (EPA), which is responsible for care of the environment, the United States Department of Agriculture (USDA), which is concerned with the safety of crop plants and the wholesomeness of food products, and the Food and Drug Administration (FDA), which has a mandate to ensure the efficacy and safety of food and pharmaceutical products (Levin & Strauss, 1993). These agencies have laws which charge them with the responsibility for humans, crops and the environment. They are also responsible for achieving a balance between risks and benefits. The laws relevant to products of gene technology are systematically presented in a coordinated framework published in the Federal Register in June 1986. The principal United States statutes regulating biotechnology are the Federal Insecticide, Fungicide and Ro-

denticide Act, which regulates pesticides, including modified organisms where they are used as pesticides (FIFRA: 7 USC, sec 136-136y), the Toxic Substances Control Act, which authorizes regulation of new and existing chemical substances, including microorganisms (TSCA: 15 USC, secs 2601-2654), and the Federal Food, Drug and Cosmetic Act, which regulates food derived from microorganisms, plants, and animals, food additives, pharmaceuticals, cosmetics, and medical devices (FFDCA: 21 USC, secs 301-392). In addition, the Federal Plant Pest Act and the Plant Quarantine Act (7 USC, sec 150aa-jj) cover a number of transgenic plants and microorganisms. In the United States of America, the deliberate release of a modified organism, whether at an experimental or "marketing" stage, may be subject to a number of federal laws, and more than one agency may have to be consulted. In addition, a number of States (particularly Wisconsin, Minnesota and North Carolina) have adopted their own legislation, or review any intention to release modified organisms under existing Federal and State legislation (Royal Commission on Environmental Pollution, 1989; Levin & Strauss, 1993). In general, a plant is regulated if it is being imported, moved interstate or released, *and* if the donor or the vector are plant pests. Plant pests may be so designated by the Director of Biotechnology, Biologics and Environmental Protection (BBEP), or are listed in statute (7 CFR), or are unclassified. If a plant has pesticidal properties it would also be regulated under the FIFRA rules. A plant may be removed from the list in §340.2 by the Director of BBEP in response to a petition, and therefore be exempt from regulation. It is also possible for any person to petition the Director to add a plant to the list, bringing it within the regulatory system. The USDA is obliged to respond to any given petition within 180 days.

In the European Union, authorization for experimental releases of modified plants is given in the individual countries after a risk assessment has been made. This risk assessment is based on data supplied by the applicant and is carried out by the "competent authority"[2] within the relevant country. The decision must be given within 90 days of application. In the United States of America, authorization depends on the USDA making an assessment of the impact of the release on agriculture and the environment, again on the basis of data submitted by the notifier. The USDA has to deliver its opinion within 120 days. If the modified plants contain pesticides, the EPA also has to carry out a review.

The use of existing legislation in the United States of America means that regulation is *product-specific*. New legislation in the European Community has made the regulatory regime *process-specific*: it is triggered by the process by which the transgenic plant is made. These are examples of the different approaches to legislation which have been adopted. In practice, they converge. Countries all round the world are adopting models related to these, depending mainly on the type of legislation already in place. Latin American countries, for example, are assumed to have plant quarantine systems and regulations to deal with plant introductions. It is assumed that these will have to be altered to deal with genetically modified organisms (Inter-American Institute for Cooperation on Agriculture, 1991).

In the next few years, transgenic plants will move into widespread use as crop varieties, which will imply the possibility of both intentional and unintentional movement across national borders. Therefore, international harmonization of standards and procedures is essential. The OECD has been active in developing guidelines for the contained use of transgenic organisms and for their release. These activities have led to the production of reports (OECD, 1986, 1990) and of a discussion document (OECD, 1992). Although the OECD has no statutory authority, it has stimulated international discussion and has defined procedures which member countries may agree to adopt. The main impact of the work of the OECD is in influencing policies in its member countries and thereby aiding the move towards similar approaches and standards. It has significantly influenced the formulation of regulations in industrialized countries.

Various international initiatives are now being mounted with developing countries through several organizations, including the United Nations Industrial Development Organization (UNIDO) and the United Nations Environment Programme (UNEP). The aim of these initiatives is to facilitate the development of common standards of biosafety. As part of these activities, UNIDO, on behalf of a Joint Working Group on Biosafety made up of representatives from UNIDO, UNEP, WHO and FAO, has published a "Voluntary Code of Conduct for the Release of Organisms into the Environment" (UNIDO, 1991). In addition, UNIDO is promoting an international Biosafety Information Network and Advisory Service (BINAS), whose role is to provide access to data and advice from experts in the setting-up of national authorities - where they do not already exist - that are qualified to handle releases of genetically modified organisms.

In many countries, regulatory oversight of the introduction of modified organisms into the environment is triggered by the process by which a product is made, rather than by the actual properties of the product. Although the eventual risk assessment regards the modified plant, taking into account parental and vector properties, the reason for regulation is that the plant has been made using the new techniques. It is argued that some products may be dangerous because of their novelty, and that their novelty arises from the process by which they are produced (Williamson, 1993). It is the likelihood of damage to the environment (including human health and safety) which provides the rationale for regulation. The novelty of the product within the receiving environment implies a risk which needs assessing. The method used in making the plant is not necessarily important, although gene technology allows modifications which could not have been achieved using other methods. If process is the basis for regulation, plants made using traditional techniques may not be regulated, yet may pose a greater risk to the environment than those made using the new methods. The precision obtainable through current gene technology may result in a lower risk because the effects are more predictable. This argument implies the imposition of regulation on all introductions, regulation for particular properties (e.g., plants carrying pesticidal properties), or complete deregulation of introductions. The European Community has chosen to use the process of gene modification to trigger a risk assessment and regulatory

system. The United States of America has chosen to regulate only those introductions which include a known and regulated phenotype. Although the most important criterion for regulation is the protection of the environment, many countries have assumed that public acceptance of the products of the new technologies is a legitimate reason for regulation. A stringent risk assessment, open to the public in both the European Community and the United States of America, is likely to do more to reassure those concerned about the use of the new technology than any other approach to the introduction of modified species of crop plants. The Inter-American Institute for Cooperation on Agriculture considers that one of the reasons for regulation is "addressing the legitimate concerns of the general public regarding the safety of biotechnology" (1991).

Review of Risk Assessment Methodologies

There are various approaches to risk assessment. Those addressing the contained use of genetically modified plants are concerned with establishing good standards of laboratory practice, the efficiency and security of containment facilities, and the effects of the modified organisms on human health (OECD, 1986; Royal Commission on Environmental Pollution, 1989; CEC 1990a; Levin & Strauss, 1993). Most schemes assessing the risks do so under the headings "Access", "Expression" or "Damage". "Access" is a measure of the probability that a modified "microorganism"[3] (or the DNA within it) will be able to enter the human body and survive there. "Expression" is a measure of the anticipated or known level of expression of the inserted DNA. "Damage" is a measure of the likelihood of harm being caused to a person by exposure to a modified organism (Advisory Committee on Genetic Manipulation, ACGM/HSE, 1986, 1993). Until recently, in most countries, assessment of risk to the environment from the contained use of modified organisms was not required. In Europe, CEC Directive 90/219 (CEC, 1990a) requires an assessment of risk to the environment even though the containment (which may be physical, chemical, or biological) is intended to stop escape or to minimize the probability of escaped organisms surviving in the environment.

What are the hazards posed by modified plants? Unwanted attributes of crop plants may include the tendency of a self-pollinated line to outcross because of self-sterility or other factors. There may also be a tendency of the plant to become a weed. The modified plants may yield toxic substances in the product, or the target range of the toxin deliberately inserted into the modified plant may differ from that of the donor organism. The response of the modified organism to other organisms in the environment, and the reaction of other organisms to the modified plant may change (OECD, 1992). Any of these may pose a risk to humans working with or consuming the products, or to the environment. Where pesticides are introduced into the modified crops, the actual response may be much wider than expected, with consequences for the ecosystem. In addition, the modified plant may display unwanted changes in appearance, susceptibility to environmental stress, or end-use characteristics. "In many cases, the effects have been scale-dependent and, there-

fore, become apparent during the scale-up process" (OECD, 1992).

It has been proved that it is virtually impossible to formulate a "quantitative", structured method for the assessment of risk to the environment resulting from the deliberate release of a modified plant. For example, it is not thought possible to estimate the probability of a plant becoming a weed when released, as the characteristics of "weediness" are not easily defined (Fitter *et al.*, 1990; Williamson *et al.*, 1990). "The complexities of the natural environment and ecological interactions mean that risk estimation is, in most cases, more a question of qualitative evaluation rather than of quantitative analysis" (UK DOE, 1993). Therefore, risk assessment procedures for the release of transgenic plants require a detailed comparison of the transgenic plant with the plant genotype it was derived from. The procedures also require consideration of the interaction of the modified organism with the particular environment into which it is to be introduced. Although the methodologies in various countries differ, most ask similar, detailed questions about the modified organisms and the environment in attempting to perform a risk assessment. The system used by most countries lists a very large number of questions relating to the organism, release site, and the wider environment (Table 4.2). The information required is essentially the same in all countries. It involves a number of steps which identify the hazards associated with an introduction into the environment (UK DOE, 1993).

Containment

Physical Containment in the Laboratory

It is generally assumed that there is a progression from laboratory cell culture, through growth room, glasshouse and experimental field trial, to the possible commercial release of modified plants. Information from each stage of this "step-up structure", as well as from equivalent structures for releases of similarly modified organisms, or about the use and release of modified organisms in other countries should be available in the formulation of a risk assessment.

Cultures of plant cells fall within the scope of the CEC Directive on the contained use of modified microorganisms (CEC, 1990a). Once they are planted out, however, they are no longer thought of as microorganisms, and different controls apply within the member countries of the European Community. The physical containment of transgenic plants within the laboratory, tissue-culture rooms, and growth cabinets is maintained by good laboratory practice. Unlike microorganisms, plants can be monitored relatively easily. Care has to be taken to ensure that pollen and seeds produced under laboratory conditions are prevented from escaping. Care should also be taken to label plants correctly and to prevent mislabelling or the inadvertent mixing of different transgenic plants. As part of good laboratory practice it is also essential to maintain a high level of quality control of DNA sequences, constructs, transgenic plants, especially those received from other laboratories, and verify the reproducibility of experimental results.

Table 4.2. A summary of the information required to submit a proposal for the field release of transgenic plants in the European Community[a]. (This provides the basic information for risk assessment[b])

---

1. General information
   Name and address of the organization wishing to release transgenic plants, including the names and qualifications of the personnel responsible

2. Information about the DNA donor organism, the recipient plant species and the transgenic plant
   2.1 Characteristics of the transgene donor organism(s) and of the recipient plant species, including:
   - scientific name and taxonomic details
   - geographic distribution
   - potential for genetic exchange with other organisms
   - genetic stability
   - pathogenicity
   - toxicity
   - allergenicity

   2.2 Characteristics of the gene vector used to introduce the transgene(s) into the recipient plant species, principally:
   - the nature and source of the vector
   - properties of the DNA sequences present in the vector

   2.3 Characteristics of the transgenic plant including:
   - a description of the DNA sequences and the methods used to prepare and insert the DNA introduced
   - the extent to which the introduced sequences are limited to the DNA required to perform the intended function(s) in the transgenic plant
   - a description of the transgenic plant
   - a description of how the genotype and phenotype of the transgenic plant differ from those of the plant from which it was derived
   - stability and level of expression of the transgene(s)
   - allergenicity or toxicity of the transgenic plant products

3. Information about the conditions of the release and the receiving environment
   3.1 A description of the proposed release, including:
   - purpose of the release
   - proposed planting date
   - plot size
   - number of transgenic plants
   - agronomic methods
   - methods of eliminating the transgenic plant material if found to be necessary

Table 4.2. cont.

3.2 A description of the release site and the wider environment, including:
- geographical location
- proximity to humans
- local flora and fauna
- target and non-target ecosystems

4. Information about the interaction between the transgenic plants and the environment

4.1 Characteristics of the transgenic plant which may affect its survival, multiplication and dissemination

4.2 A description of the interaction of the transgenic plant with its environment, including:
- relevant information obtained from earlier release studies on the likely environmental impact
- the possibility for gene transfer to other plants or to micro-organisms
- the possibility for dispersal of the transgenic plants themselves or their propagules
- methods used to verify genetic stability of the transgenic plants

4.3 An assessment of the potential environmental impact, including:
- the likelihood of excessive plant population increase
- the influence on non-target organisms

5. Information on monitoring, control and emergency response plans

5.1 A description of monitoring techniques, including:
- methods for identifying the transgenic plants
- methods for identifying the transgenes if transferred to other plants or organisms

5.2 A description of methods for controlling the site, including:
- minimizing spread of transgenic plants
- methods to protect the site from intrusion

5.3 A description of methods of discarding waste plant material

5.4 A description of emergency plans to removed or destroy the transgenic plant material and to terminate the experiment if it is considered necessary

[a] From EC Directive 90/220.
[b] Risk to human health and the environment.

Physical Containment in Specially Designed Glasshouses

Growing and handling transgenic plants in a containment glasshouse is essentially the same as in the case of those in the laboratory and involves the use of good laboratory type practices. Glasshouses should be designed to withstand freak weather conditions and to keep insects out and pollen in. Decisions need to be made, usually with the guidance of an institutional biosafety committee, on the nature of the containment procedures appropriate for the particular type of transgenic plant. For example, work involving transformation using sequences from non-indigenous pathogens are likely to require very high containment facilities and procedures. These may include controlled and filtered airflow through the glasshouse environment. Control of water outlets and sterilization from the glasshouse may also be appropriate. Autoclaving soil and plant material coming out of the glasshouse will also be necessary. Good labelling practice is important. A review of "Good Practice" that might be adopted is in ACGM, Note 10, which provides guidance on work with modified plants and plant pests (ACGM/HSE, 1989).

Environmental Releases

A comprehensive risk assessment procedure is required once transgenic plants are released into small scale experiments and eventually into commercial production, because once released, recovery of the transgenic plants or pollen and propagules cannot be assured and is often not possible or practical. It is against this background that the scientists responsible for the release, the institutional biosafety committees and the international biosafety groups must assess whether it is acceptable to release specific transgenic plants and if necessary, what restrictions should be imposed.

There are various procedures which can achieve a level of "field containment" which aims to restrict or limit the possible environmental impact of the transgenic plant release experiment. Field containment measures can include isolation from sexually compatible species, prevention of flowering, use of male sterile lines and the use of thorough plant disposal and subsequent monitoring procedures.

Data Required for Risk Assessment

An example of the information required for risk assessment is given in Table 4.2. The method of presenting data varies from one authority to another, but the information required is essentially the same. The categories outlined in Table 4.2 will need some explanation. The process involves the identification of possible hazards associated with the modified organism, the environment into which it is to be released and the interaction of organism and environment. Once the hazard has been identified, it is possible to assess both the risk of that hazard occurring and the

magnitude of harm it may cause. If the hazard is small and the probability of it happening is high, the risk to the environment remains small.

General Information

It is considered important that the staff and the institution responsible for carrying out the release have a sufficiently high level of expertise and experience to carry out the proposed release of transgenic plants, and to be responsible for any field containment and monitoring that is deemed necessary.

The DNA Donor Organism, the Recipient Plant Species, and the Transgenic Plant

Donor Organism. It is essential to have information on the donor organism and the recipient plant species. Information on the recipient species will establish a baseline against which to compare the transgenic plants. Knowledge of the donor species will highlight the kind of information required from the transgenic plant. If the donor is a plant pathogen, for example, this will raise questions in the risk assessment exercise about the possibility of recombination between the integrated DNA from the pathogen and pathogens that may infect the transgenic plant subsequently. The possibility of transcapsidation may also need to be considered where the sequences inserted code for a viral coat protein to confer resistance to certain viral diseases. When the modified cell is infected by another virus, transcapsidation of the latter with the coat protein of the original donor virus might be possible, and this may, in turn, affect viral host range.

Transformation Vector. Information is required on the DNA vector used during the transformation process employed to introduce the transgenes. Antibiotic-resistance genes are generally used to facilitate the screening of transformed cells. Other DNA sequences may act as linking sequences with the vector, or may provide other functions associated with the use of recombinant DNA methods. With certain transformation systems, carrier DNA is sometimes used to aid the transformation process. It is therefore necessary to know the nature of this DNA, so that any consequences can be considered during the assessment process.

Transgenic Plant. It is important to give a description of the transgenic plant, including molecular data on the inserted transgenes, the stability of expression, whether there is any change in allergenicity, toxicity, and the capacity of the transgenic plant to persist in agricultural habitats or to invade natural habitats. It is essential here that the corresponding unmodified plant genotype is used as a control, so that changes in plant phenotype caused by the transgenes can be measured.

The Conditions of the Release and the Receiving Environment

Although the scientific or commercial purpose of the release may not have any risk consequences, it is important that the biosafety groups charged with the assess-

ment of risk have perspectives against which to assess the release. The risk to the environment requires qualitative judgements; therefore, an essential part of the risk assessment philosophy is a case-by-case analysis, and that, based on the accumulated experience, there is a progression towards streamlined and simplified procedures where appropriate (see Conclusions, below). Providing information on the objectives of the release, its size and design, and the agronomic treatments to be used is important both for the risk assessment of the particular release and for the national and international learning process.

Ecological information on the release site is also important. This should include a survey of plant species that might be growing in the vicinity of the release, as well as information on what is known about the nature of pollen dissemination and the distances over which pollen can give successful pollination.

The location and type of the anticipated target organisms must be specified. The target organisms are those which the transgenes are targeted to affect. For transgenes containing the insecticidal Bt protein, it may be a particular class of insect pest; for a viral coat gene, it would be a particular viral pathogen. Non-target organisms are those which are not the primary target of the modification, and they include those that are affected inadvertently. If the transgene has an effect on an insect that is not considered to be a pest, this should be noted. There should also be a consideration of whether the transgenic plant becomes a better or worse host, and/or is harmful to organisms that might be associated with the crop. The risk of harm to the environment includes harming non-target organisms.

The Interaction Between Transgenic Plants and Their Environment

In order to determine the impact of the transgenic plant on its environment, it is important to describe changes in the transgenic plant that may alter its invasiveness in wild habitats, its persistence in agricultural habitats, or its ability to propagate itself sexually or asexually. It is also important to take note of earlier studies with similar transgenic plants. It is necessary to determine the possibility of the transfer of the transgene to the same or a related plant species (wild or cultivated) or to microorganisms, and - if this is the case - what the consequences of that gene transfer might be.

Monitoring, Control, Waste Treatment and Emergency Plans

Once plants are released from containment, and particularly if they are allowed to flower and set seeds, the plants, seeds and pollen carrying the transgenes may move out of the immediate release environment. An important part of the risk assessment is to determine the extent to which it is possible to monitor transgenes after the release, and the efficiency with which it is possible to destroy plant material if it becomes necessary. Efficient methods of identifying transgenic plants, or transgenes present in non-target species may be necessary. This may be done by a visual marker (e.g., β–glucuronidase), a selectable marker (e.g., antibiotic resistance), or molecular analysis (e.g., PCR and Southern hybridization).

There are ways of minimizing genetic exchange which might be considered (see below). It may also be appropriate to describe ways in which plant material can be destroyed at the end of the release experiment or, if necessary, during the course of the experiment.

Carrying Out the Risk Assessment

The first releases of a modified plant will be experimental. Commercial releases may follow once the results of the experimental release have been analysed. It is at this experimental stage that the major risk assessment will have to be made and evaluated. The aim of the risk analysis is to identify either changes in the experimental protocol or methods by which the GMO may be confined in order to minimize risk to the environment or to human health.

The information outlined above is usually assembled by the scientists wishing to carry out the release of transgenic plants. Although the act of assembling the information inevitably involves a consideration of potential risks by the release team, the risk assessment process should be carried out by a multidisciplinary biosafety committee made up of people with expertise in genetics, molecular biology, environmental science, agriculture, plant pathology, and other fields where appropriate. It may even be useful to include representatives of local government and local environmental pressure groups in order to ensure public satisfaction with the care being taken to minimize risk to humans or to the environment.

The first level of assessing a release proposal should be at the institute level - the assessment should be carried out by an institutional biosafety committee. The benefit of carrying out an assessment locally is that the members of the committee will have local knowledge and usually have a good understanding of the scientific principles. It also provides a way of spreading local responsibility for the release to members of the institute that are not directly involved in the release. The institutional biosafety committee would also have responsibility for monitoring the site after the release, informing people at the institute of the release and of the risk assessment, and enhancing accountability. It would monitor the activities of the release team and ensure that any unexpected events are reported.

This risk assessment involves detailed consideration of all parts of the proposal, calling for further information if required, and requesting, where necessary, additional precautions. When the institutional biosafety committee has approved the conditions for the experimental release to its satisfaction, there may still be other levels of risk assessment.

For example, these may be national biosafety committees, which are also multidisciplinary. These bodies similarly go through the release proposal and can make further recommendations or require that the proposal be changed. The oversight process may involve interviews with members of the release team. Finally, international or regional biosafety committees may also be set up (like the one established by the European Union). These may become appropriate as regional trade agreements are ratified.

What Kind of Risks Might Be Identified?

Assessing risk is not an exact science. It is difficult to put a value on the degree of risk. It is never possible to establish that releasing a transgenic plant will involve no risk. All of the activities people are involved in pose a degree of risk, no matter what kind of precautions are taken. The essential feature of risk assessment is to determine how the transgenes might alter risk compared with the non-transgenic crop; therefore, the starting point must be the use of the unmodified crop plant as a baseline against which to compare the effect of the inserted transgenes. There will undoubtedly be questions that cannot be answered because the relevant data are not available. For instance, it is not known for certain whether plant genes can be transferred to microorganisms, and there is much we do not know about the nature and consequences of "gene flow" (see next section) from conventionally bred crops to related weed species. Biosafety committees have to take into account both knowledge and ignorance to arrive at a decision on the acceptability of a release and what additional information or precautions may be necessary.

In cases where detailed scientific knowledge is not available, it is important to use the experience of conventional plant breeding to aid the risk assessment process. Plant breeding has been carried out (first as the result of serendipity and later intentionally) for thousands of years, and many of the genes being inserted by recombinant transformation fall into classes very similar to those manipulated by the conventional plant breeder.

Potential Risks from the Release of Transgenic Plants

One of the major concerns associated with the release of modified organisms is that the inserted information may be transferred to wild populations. The process of introgression is of concern to many authors as a mechanism which may lead to undesirable traits being transferred from modified organisms (Gregorius & Steiner, 1993). Interspecific hybridization is a common process, notwithstanding the barriers that exist to crossbreeding, but most hybrids are rare and the majority are sterile. "Gene flow" is believed to be highly restricted (Levin, 1984), but there is some evidence that this may be misleading (UK DOE, 1993). Modified plants could, in theory, become weeds difficult to control, possibly in contexts other than their normal agricultural environment. A review of risk assessment of genetically modified plants introduced into the environment has appeared recently (Dietz, 1993).

There are several routes of transgene escape, as for example when the plant invades seminatural habitats, or when the transgene is transferred to another crop or to a wild relative (possibly by introgression), and persists on the agricultural land, in verges, ditches or waste-tips. It is likely that the spread of transgenes can be checked with methodology similar to that used to detect any other single gene trait (UK DOE, 1993; p. 30).

What then are the risks associated with the release of modified organisms?[4] Is it possible to confine the modified organism within the release site, and if it were to

"escape", would it pose a problem for the environment? Could it, for example, survive or persist outside the managed "site" within which it has been released? There are differences in the potential of crop plants to transfer from the environment in which they are placed, and in their ability to establish feral populations. If this happened, would it matter? Could the inserted genes be transferred to other plants of the same type or to wild relatives?

The risk assessment must take the host or parental plant as its starting point. Is the host plant capable of surviving outside the normal agricultural environment? Does it have relatives in the external or agricultural environment into which gene transfer is possible? The modification must then be considered both in terms of making any resulting transgenic plant more likely to survive and with respect to the safety of the gene product in the environment.

Some areas that need to be considered are outlined below:

Selectable Marker Genes

Most transgenic plants contain a selectable marker gene that is used during the transformation process. The characteristics of a marker gene are that:

1. It should enable stringent selection with a minimum of non-transformed plants escaping the selection.
2. The selection should result in a large number of independent transformation events and should not interfere significantly with regeneration.
3. The marker should work well in a large number of species.
4. There should be an assay which allows confirmation of the presence of the marker.

Antibiotic-resistance genes are used as they meet all of the above criteria. The *nptII* gene, which confers resistance to the antibiotic kanamycin, is most commonly used. This antibiotic has been superseded for clinical use in the United States of America. The resistance marker also gives resistance to the antibiotic neomycin, which is still prescribed in some countries for clinical and veterinary use. There are also other aminoglycoside antibiotics that are used as markers. Although several extensive studies have concluded that the likelihood of the transfer of this gene from transgenic plants to microorganisms is negligible, and that the transfer would probably be of little consequence if it did happen (Calgene, 1990), there is a continuing debate about whether it is acceptable for this kind of selectable marker gene to be present in commercial transgenic varieties.

Alternatives to antibiotic-resistance markers have been used. These include herbicide-resistance markers (Yadav *et al.*, 1986). There have been a number of suggestions for replacing antibiotic marker genes in plant systems. The Cre-lox system involves the production of two sister lines of modified plants, one containing a gene coding for a bacterial recombination enzyme, the other containing the antibiotic-resistance gene flanked by sites for the action of the enzyme. When the

two lines are crossed, the marker gene should be excised (Dale & Ow, 1991). It is believed that this system would be difficult to apply in vegetatively propagated food crops, such as potatoes.

Gene-fusion markers are used to confirm the expression of proteins within the inserted gene, rather than for the selection of the modified organism. One of the most commonly used markers is the β–glucuronidase gene from *Escherichia coli* (Jefferson *et al.*, 1986).

Herbicide Tolerance

A range of herbicide-tolerance genes have been introduced into various crop plants. Herbicide resistance was one of the first traits subject to genetic modification once the mechanisms of resistance had been characterized. In general, the resistance is a dominant single gene trait (Mazur & Falco, 1989).

One of the principal attractions of this application is that it supplies a means of providing selectivity for herbicides that are quickly degraded in the environment. An environmental risk that needs to be considered is whether the transgenic crop plant that is herbicide-tolerant may become a weed that is then difficult to control. Another factor that needs to be considered is the likelihood of the herbicide-resistance genes becoming established in weed populations by hybridization between crop and weeds. If the hybrids are fertile, they may be difficult to control in an agricultural system which depends for weed control on the same herbicide, or in adjacent crops that are dependent on that herbicide. If there are other adjacent populations of the same crop which have been modified to be tolerant to different herbicides, would a crop plant resistant to multiple herbicides pose additional problems within either the agricultural or the natural environment?

Were the herbicide tolerance to be transferred to non-managed, non-agricultural species within the "wild" environment, would there be cause for concern? Such environments are not normally subject to herbicide treatment, and the presence of wild relatives of crop species displaying resistance may be of little significance. The risk assessment should attempt to identify the possible consequences of such a transfer.

Pest and Disease Resistance

Transgenes conferring pest or disease resistance could bestow a selective advantage on a crop plant and make it more persistent on agricultural land and more invasive in wild habitats. The transgenes could similarly confer a selective advantage if transferred to related wild plant species. Another aspect that needs to be considered is the effect of the resistance genes on pest and pathogen populations. If the transgene provides a very efficient defence, it is possible that the pest or pathogen will rapidly become resistant. This is a phenomenon that is well known in conventional plant breeding, and the concern is arguably more about devising a sound agricultural strategy than assessing risk, but the possibility of using the same

resistance gene in a range of different crops by transformation means that this prospect has to be taken seriously.

One of the most important uses of this technology for the insertion of genes leading to pest-resistance has been the use of Bt toxins. A problem that may be associated with the use of Bt toxins is the evolution of resistance in the target pests. This resistance is due to reduced affinity for the toxin to a mutant membrane receptor (Gill *et al.*, 1992). Transgenic crops containing proteinase inhibitors may pose similar problems, and their use should be carefully planned to avoid the evolution of resistant pests.

Viral coat-protein genes are frequently used to give protection against particular viruses. There is currently a debate about whether this strategy might modify the host range of plant viruses by a process of transcapsidation. Creamer & Falk (1990) have detected transcapsidation events in mixed infections of luteoviruses in the field. This process does not in itself create a new virus, as the coat-protein genes are unaltered, but may temporarily alter the specificity. There are also concerns over the recombination between the viral sequences expressed by the plant and those of the infecting virus. The concerns will relate to the presence of other viruses capable of transcapsidation within the receiving environment.

Resistance to Stress Conditions

Resistance to stress conditions such as drought, saline soils, heavy metals, cold, high temperature is a complex phenomenon; therefore, the isolation of genes conferring enhanced stress resistance will take time. Plant genes induced by stress (heat, cold, salinity, heavy metals) have been identified (Fraley, 1992), so it is likely that plants with enhanced tolerance will become available in the next few years. Transgenic plants of this type will present a particular challenge for risk assessment because this change may enable plants to grow in habitats where they were unable to grow before, and may confer a selective advantage on plants to which the transgenes may transfer by cross-pollination.

The exotic-species model, where a plant is transferred to a different country and becomes a dominant species, is sometimes used to illustrate what might result from the release of transgenic plants. While this is not a good general model for assessing the consequences of inserting one or a few genes into a crop to modify it in very specific ways, it may be relevant to instances where plants are modified to make them grow in new kinds of habitats. The results of a risk assessment in these cases may be that more information is required on the nature of any competitive advantage conferred by the transgene under the specific conditions of the release.

Toxicity or Allergenicity

The gene product may be toxic in the plant or parts of the plant, or it may modify the allergenic properties of the crop for food or products in the environment (e.g., pollen). Toxicity may apply to organisms other than the target organism, and harm may be inflicted on the ecosystem.

Consequences of the Large-Scale Use of Transgenic Crops

The risk assessment required when considering the widespread use of a transgenic crop variety is essentially the same as for small-scale releases, but there are some important differences. Field containment measures, including those to prevent flower production and to destroy all plant material on the release site (field plot), are no longer possible. The risk assessment must therefore take into account the possibility of cross-pollination between the transgenic crop and adjacent non-transgenic crops and weed species. Transgenic plants will have the opportunity to become established in a range of natural habitats, and there will be the possibility of a recurrent migration of pollen transferring the transgenes to wild populations. In habitats where the crop plants vastly outnumber the sexually compatible wild species, the transgenes may become established in those wild populations even if they confer a selective disadvantage compared with the wild-species counterparts not carrying the transgene.

There will be the opportunity for transgenic plants to be taken intentionally or inadvertently to other countries, including to those geographical areas that have a different spectrum of sexually compatible weed species. A small-scale risk assessment considers the distribution of sexually compatible species in the location of the release site. Large-scale releases raise the question as to what geographical limits should be placed on the environment considered in the assessment scheme.

Rare events which might not be evident in small-scale releases may be significant on a large scale. For example, transgene instability on a small scale may be unacceptable in crop varieties used on a large scale.

There are also questions of agricultural strategy. For example, how far is it prudent to progress towards the introduction of several different herbicide-resistance genes into a crop species? Will this lead to multiply-resistant weeds? Also, what will be the consequences of the same or similar pest- or disease-resistance genes being present in many different crops. The experience of conventional plant breeding points to the increased likelihood for resistant pests and pathogens to emerge.

Other scale-dependent questions regard the toxicity and allergenicity of the crop and the nature of the breakdown products of the transgenes when the plant decays.

Options for Managing Risk

The opportunities for managing risk in small-scale releases are principally concerned with achieving an acceptable level of field containment and of monitoring the site during the release and afterwards. Increased containment can be achieved by the inclusion of buffer crops, or cages, or by bagging flowers in the field. Permission for the first release experiments in the United Kingdom required the deflowering of all plants in the experimental plot. While ensuring that the probability of transferring the introduced gene to other plants was low, this procedure could only be applied to small-scale field trials.

The options for containment following widespread release of a transgenic crop variety are limited, and a risk assessment before the commercial release of a transgenic variety must take this into consideration.

It is important that the risk assessment leading to commercial release is thorough and open to scrutiny and considers all the evidence available from the small-scale releases carried out with the same and similar transgenic plant material. As mentioned in the previous section, there may be difficulties associated with large-scale releases. Some mitigating action might be considered for large-scale releases.

1. There is now a wide range of plant promoters available for giving transgene expression in specific tissues in the plant. It may be desirable to restrict the expression of a particular protein to those parts of the plant where it is required. In the case of a pest that attacks leaves, for instance, it would suffice if insecticidal protein were expressed only in leaves. There may also be opportunities in the future to use a constitutive promoter to give expression in most parts of the plant, but to use a strategy to switch off transgene action in very specific tissues, for example, in pollen, where there may be the possibility of an allergic response.
2. The use of male sterile transgenic plants may be considered desirable to prevent the dispersal of transgenes by pollen.
3. There may also be instances where it is possible or desirable to grow transgenic crops in areas where no sexually compatible wild relatives grow naturally.
4. In order to prevent genetic contamination, it may be occasionally desirable to grow a particular type of transgenic crop (e.g., oilseed crops with a particular fatty acid composition) in areas free from non-modified crops.
5. It may also be necessary to call in a variety if the results are considered unsatisfactory.

Conclusions

From the earliest releases of transgenic plants, the intention was to progress case by case, to build up experience, and on the basis of that experience, to simplify the regulatory requirements and oversight. In the European Union and the United States of America, streamlining or fast-tracking is now developing. In both regulatory systems, particular crop species and transgenes are being identified that have been tested in release experiments many times and have provided acceptable results. In these instances, the time taken for regulatory approval has been reduced substantially. The harmonization of release criteria and the move towards simplified regulatory oversight will no doubt continue to be an area of active international discussion in the coming years (UK DOE/ACRE, 1994a,b).

One of the disadvantages of discussing the process of risk assessment is that it places undue stress on risk and largely neglects benefits. The ability to transfer genes across wide taxonomic boundaries provides a very powerful means in research seeking to understand how genes are regulated and how they influence phe-

notype. It provides a valuable opportunity to extend the advances achieved by conventional plant breeding and to improve crop plants in many novel ways that are relevant to developing countries.

In looking forward to the widespread international use of transgenic crop plants, it will be necessary to go beyond a focused consideration of environmental impact in the immediate release environment, since we all have a responsibility to our communal global environment.

Notes

1. "Traditional breeding means practices which use one or more of a number of methods (e.g., physical and/or chemical means, control of physiological processes) which can lead to successful crosses between plants of the same botanical family" - a definition agreed by the European Community (UK DOE/ACRE, 1993). Throughout history, these methods have allowed the selection of desirable or required traits in plants.
2. A "competent authority" is one or more designated governmental organizations responsible (in each member state) for the regulation of activities involving genetically modified organisms.
3. "Microorganism" as referred to here includes all cells in tissue culture.
4. "Risk" in the context used here is risk to humans and the environment, including other crop plants.

References

ACGM/HSE (1986, 1993) Note 7: Guidelines for the risk assessment of operations involving the contained use of genetically modified microorganisms. Advisory Committee on Genetic Modification, Health & Safety Executive, London.

ACGM/HSE (1989) Note 10: Guidelines for the risk assessment of operations involving the contained use of plants and plant pests. Advisory Committee on Genetic Modification, Health & Safety Executive, London.

Ambros, P.F., Matzke, M.A. & Matzke, A.J.M. (1986) Detection of a 17kb unique sequence (T-DNA) in plant chromosomes by *in situ* hybridization. *Chromosoma*, 94, 11-18.

Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res.*,12, 8711-8721.

Blundy, K.S., Blundy, M.A.C., Carter, D., Wilson, F., Park, W.D. & Burrell, M.M. (1991) The expression of class I patatin gene fusions in transgenic potato varies with both gene and cultivar. *Plant Mol. Biol.*, 16, 153-160.

Calgene (1990) *Kan^r Gene: Safety and Use in the Production of Genetically Engineered Plants, Request for Advisory Opinion*. Calgene Inc., California, USA.

CEC (1990a) Council Directive of 23 April 1990 on the contained use of genetically modified microorganisms. Reference no. 90/219/EEC. *Off. J.* L117, Volume 33, 8 May 1990. Commission of the European Communities, Brussels.

CEC (1990b) Council Directive of 23 April 1990 on the deliberate release into the environ-

ment of genetically modified organisms. Reference no. 90/220/EEC. *Off. J.* L117, Volume 33, 8 May 1990. Commission of the European Communities, Brussels.

Chyi Y.-S., Jorgenson, D., Goldstein, S., Tanksley, D. & Loaiza-Figueroa, F. (1986) Locations and stability of *Agrobacterium*-mediated T-DNA insertions in the *Lycopersicon* genome. *Mol. Gen. Genet.*, 204, 64-69.

Creamer, R. & Falk, B.W. (1990) Direct detection of transcapsidated barley yellow dwarf luteoviruses in doubly infected plants. *J. Gen. Virol.*, 71, 211-217.

Dale, E.C. & Ow, D.W. (1991) Gene transfer with subsequent removal of the selection gene from the host genome. *Proc. Natl. Acad. Sci. USA*, 88, 10558-10562.

Dale, P.J. & McPartlan, H.C. (1992) Field performance of transgenic potato plants compared with controls regenerated from tuber discs and shoot cuttings. *Theor. Appl. Genet.*, 84, 585-591.

Dale, P.J., Irwin, J.A. & Schefler, J.A. (1993) The experimental and commercial release of transgenic crop plants. *Plant Breed.*, 111, 1-22.

De Cleene, M. & De Ley, J. (1976) The host range of crown gall. *Botan. Rev.*, 42, 386-466.

Dietz, A. (1993) Risk assessment of genetically modified plants introduced into the environment. In: *Transgenic Organisms*, Wörmann, K. & Tomiu, J. (eds.), pp. 209-227. Birkhäuser Verlag, Basel, Switzerland.

Fitter, A., Perrins, J. & Williamson, M. (1990) Weed probability challenged. *Bio/Technology*, 8, 473.

Fraley, R. (1992) Field testing genetically engineered plants. In: *Current Combinations in Molecular Biology: Improvement of Agriculturally Important Crops*, Fraley, T., Frey, N.M. & Schell, J. (eds.), pp. 83-86. Cold Spring Harbour Press, Cold Spring Harbour, New York.

Gill, S.S., Cowles, E.A. & Pietrantonio, P.V. (1992) The mode of action of *Bacillus thuringiensis* endotoxins. *Ann. Rev. Entomol.*, 37, 615-636.

Gregorius, H.-R. & Steiner, W. (1993) Gene transfer in plants as a potential agent of introgression. In: *Transgenic Organisms*, Wörmann, K. & Tomiu, J. (eds.), pp. 83-107. Birkhäuser Verlag, Basel, Switzerland.

Hobbs, S.L.A., Kpodar, P.& Delong, C.M.O. (1990) The effect of DNA copy number, position and methylation on reporter gene expression in tobacco transformants. *Plant Mol. Biol.*, 15, 851-864.

House of Lords, Select Committee on Science & Technology: Regulation of the UK biotechnology industry and global competitiveness. Written evidence received up to 30 April 1993. Her Majesty's Stationery Office, London.

Inter-American Institute for Cooperation on Agriculture (1991) Guidelines for the release into the environment of genetically modified organisms. Interamerican Institute for Cooperation on Agriculture, San José, Costa Rica.

Jefferson, R., Burgess, S.M. & Hirsh, D. (1986) β-Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl. Acad. Sci. USA*, 83, 8447-8451.

Jefferson, R., Goldsborough, A. & Bevan, M. (1990) Transcriptional regulation of patatin-1 gene in potato. *Plant Mol. Biol.*, 14, 995-1006.

Karp, A. (1991) On the current understanding of somaclonal variation. In: *Oxford Surveys of Plant Molecular and Cell Biology*, Miflin, B.J. (ed.), pp. 1-58. Oxford University Press, Oxford, UK.

Larkin, P.J. & Scowcroft W.R. (1981) Somaclonal variation - a novel source of variation

from cell cultures for plant improvement. *Theor. Appl. Genet.*, 60, 197-214.

Levin, D.A. (1984) Immigration in plants: an exercise in the subjunctive. In: *Perspectives on Plant Population Ecology*, Dirzo, R. & Sarukhan, J. (eds.), pp. 242-260. Sinauer Associates Inc., Massachusetts, USA.

Levin, M. & Strauss, H.S. (1993) Overview of risk assessment and regulation of environmental biotechnology. In: *Risk Assessment in Genetic Engineering*, Levin, M. & Strauss, H. (eds.), pp. 1-17. McGraw-Hill Inc., New York.

Mazur, B.J. & Falco, S.C. (1989) The development of herbicide resistant crops. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 40, 441-470.

OECD (1986) Recombinant DNA safety considerations. Organisation for Economic Co-operation and Development, Paris.

OECD (1990) Good developmental practices for small scale field research with genetically modified plants and microorganisms: a discussion document. Organisation for Economic Co-operation and Development, Paris.

OECD (1992) Report of the OECD workshop on the monitoring of organisms introduced into the environment. Organisation for Economic Co-operation and Development, Paris.

OECD (1993) Field releases of transgenic plants, 1986-1992: an analysis. ISBN92-64-14046-8. p. 40. Organisation for Economic Co-operation and Development, Paris.

O'Neill, C., Horvath, G.V., Horvath, E., Dix, P.J. & Medgyesy, P. (1993) Chloroplast transformation in plants: polyethylene glycol (PEG): treatment of protoplasts is an alternative to biolistic delivery systems. *Plant J.*, 3, 729-738.

Potrykus, I. (1990) Gene transfer to plants: assessment and perspectives. *Physiol. Planta,* 79, 125-134.

Potrykus, I. (1991) Gene transfer to plants. *Annu. Rev. Plant Physiol.*, 42, 205-225.

Royal Commission on Environmental Pollution (1989) 13th Report: The release of genetically engineered organisms to the environment. HMSO, London.

Royal Commission on Environmental Pollution (1991) 14th Report: GENHAZ. HMSO, London.

Scheid, O.M., Paszkowski, J. & Potrykus, I. (1991) Reversible inactivation of a transgene in *Arabidopsis thaliana*. *Mol. Gen. Genet.*, 228, 104-112.

Selker, E. (1990) Pre-meiotic instability of repeated sequences in *Neurospora crassa*. *Annu. Rev. Genet.*, 24, 579-613.

UK DOE (1993) Research Report No. 1: Genetically modified crops and their wild relatives - a UK perspective. Department of the Environment, London.

UK DOE/ACRE (1993) Guidance Note 1: The regulation and control of the deliberate release of genetically modified organisms. Department of the Environment, London.

UK DOE/ACRE (1994a) Guidance Note 2: Fast track procedures for certain GMO releases. Department of the Environment, London.

UK DOE/ACRE (1994b) Guidance Note 3: Reduced information requirements for releases of plants under the GMO regulations. Department of the Environment, London.

UNIDO (1991) Voluntary Code of Conduct for the Release of Organisms into the Environment. July, 1991. United Nations Industrial Development Organization, Vienna.

US HHS (1993) Biosafety in Microbiological and Biomedical Laboratories. US Department of Health and Human Services, Centres for Disease Control and Prevention & National Institutes of Health, HHS Publication No. (CDC) 93-8395.

Williamson, M. (1993) Regulation of the UK Biotechnology Industry and Global Competitiveness. In: Written evidence submitted to the House of Lords Select Committee on Science and Technology, pp. 206-207. HMSO, London.

Williamson, M., Perrins, J. & Fitter, A. (1990) Releasing genetically engineered plants. Present proposals and possible hazards. *Trends Ecol. Evol.*, 5, 417-419.

Yadav, N., McDevitt, R.E., Bernard, S. & Falco, S.C. (1986) Single amino-acid substitutions in the enzyme acetolactate synthase confer resistance to the herbicide sulfometuron methyl. *Proc. Natl. Acad. Sci. USA*, 83, 4418-4422.

Chapter five
Environmental Release of Genetically Modified Rhizobia and Mycorrhizas

Geoffrey Hall

International Mycological Institute, Egham, Surrey, United Kingdom.

Introduction

Powerful new molecular methods for DNA manipulation provide a means of constructing microorganisms with genotypes which would not occur by natural exchange, and with a degree of precision not previously available. These novel genetic combinations can be produced so that the activities of microorganisms in existing processes are improved or new functions introduced. This chapter concentrates specifically on the genetic manipulation of rhizobia and mycorrhizas, their application in agricultural ecosystems and the hazards, risks and benefits this work may generate. The development of safe work procedures to answer public concern and allay fears commonly expressed about this emergent technology is included.

Why Modify Microorganisms Genetically?

Microorganisms have had many beneficial applications in agriculture and have been used safely for decades, bringing great benefits to humans. In many instances, these applications can be performed more effectively by microorganisms that have been genetically modified, either by classical means (e.g., spontaneous mutation or recombination) or by molecular techniques (e.g., site-directed mutagenesis, DNA cloning and cell fusion). Molecular methods have two advantages over classical methods. First, the precision of many of the molecular methods allows fully characterized modifications to be made, in some cases to the determination of specific alterations of bases in the DNA nucleotide sequence. Greater precision in the construction and characterization of desired genotypes allows scientists to make better judgements about the safety of genetically engineered microorganisms (GEMs). Second, they permit the formation of novel combinations which are impossible to obtain using classical methods, allowing scientists to bypass natural barriers to

genetic exchange. However, the biological effects of novel combinations are unknown and, before release of GEMs into the environment can be contemplated, the risk of a specific gene combination and the hazards associated with a GEM must be evaluated. The long history of introductions of naturally occurring microorganisms into the environment for agricultural purposes provides extensive data on which assessments of the risk can be based. While it is clear that certain microorganisms can be used safely in the environment without knowing their precise roles in the community or ecosystem, it is difficult to assess all potential risks when unfamiliar organisms are being used.

The Use of Rhizobia and Mycorrhizas in Agriculture

Nitrogen Fixation

It has long been known that bacteria in the genera *Rhizobium* and *Bradyrhizobium* enter into symbiotic relationships with leguminous plants, forming nodules on their roots. They fix atmospheric nitrogen in a form which can be used in plant growth, thereby increasing yield. Consequently, nitrogen-fixing bacteria have attracted considerable attention regarding genetic improvement (Schmidt & Robert, 1985). Indeed, twenty applications involving *R. meliloti* were received by the regulatory authority in the United States of America from 1983 to 1989, which comprised 55% of all requests for releases of microorganisms in that period (Levin & Strauss, 1991).

Rhizobia are an indigenous component of the soil microbiota and live independently before interacting with plant roots. However, they are mostly inactive: they are merely surviving in a nutrient-poor environment and are subject to predation by other soil microorganisms. The root nodule provides a protected environment enhancing microbial growth and survival, and, following plant death, the bacteria are returned to the soil when the nodule decays.

However, some soils have low natural populations of rhizobia, or contain rhizobia which will not nodulate an introduced crop, e.g. pasture legumes introduced to Australia. It is impractical to raise the numbers of naturally occurring rhizobia or change the species present by direct addition to the soil, and so inoculants containing appropriate rhizobia are added during sowing to seeds of forage (e.g., alfalfa, clover, medic) and grain legumes (beans, peas and soybean). Any new rhizobia applied as inoculants must function and compete successfully in the complex and dynamic soil ecosystem, and the largest increases in productivity have occurred following introduction of a non-indigenous *Rhizobium* with a non-indigenous legume, e.g., *Bradyrhizobium* sp. with soybean in the United States of America and Eastern Europe.

The most economical way of providing the nitrogen needed for optimum plant growth in legumes is to inoculate with selected strains of rhizobia which have improved nitrogen-fixing characters (Ham *et al.*, 1971). The nitrogen fixing efficiency of rhizobia varies greatly with a host plant (Batzli *et al.*, 1992), providing a source

of genetic diversity for strain improvement. There have been many attempts to improve the symbiosis by using selected strains of rhizobia, but the main problem in producing a suitable inoculum is competition from indigenous strains in the soil. Selections must be made which will compete with indigenous strains so that they can occupy a majority of nodules on legume plants in the field. Genes and phenotypes of rhizobia which influence competitive nodulation have been identified, e.g. competitiveness is related to serotype in soybean bradyrhizobia (Robert & Schmidt, 1985).

Phosphate and Micronutrient Capture

Symbiotic mycorrhizal associations are formed between fungi in the Basidiomycota, Zygomycota or Ascomycota and the roots of about 85% of all herbaceous and woody plants (Harley, 1989), including nearly all economically important crops, both in temperate countries and in the tropics. Mycorrhizas are associated with the concentration and transport of phosphate and other micronutrients, principally copper, zinc and manganese to the plant, in return for which they receive carbohydrates from the plant. Consequently, they are often found in plants that grow in low phosphate or other nutrient-poor soils. They thus have great potential to improve plant growth, productivity and survival in agricultural, horticultural and natural ecosystems, particularly in areas of marginal productivity, where they also have a role in soil stability and erosion control. However, improvements in plant growth are not universal for all mycorrhizal species involved. Individual species differ significantly in their effectiveness to promote growth with changes in soil fertility, pH and drought (Bethlenfalvay, 1992a). Ecotypes adapted to high concentrations of heavy metals, aluminium or salt have also been identified.

Fungi in the Basidiomycota and Ascomycota typically form two types of mycorrhizal associations with the roots of woody trees and shrubs. In the first type, called an ectomycorrhiza, the mycelium grows on the surface of the roots and between the outer cortical cells and spreads out into the surrounding soil and litter layer forming a many branched network. When environmental and physiological conditions are suitable, the mycelium forms a fruiting body (a structure bearing sexually produced spores) either above ground, as mushrooms and toadstools, or below ground, as truffles. Spores produced by asexual means are seldom produced in these fungi, although some may form vegetative structures such as cords, rhizomorphs and sclerotia. Ectomycorrhizas are a major feature of temperate and boreal forests and over 5000 species have been recorded. Ectomycorrhizal fungi are not very host specific and green plants are also not very selective in the fungi with which they form mycorrhizas (Harley, 1989). Consequently each tree species is typically infected by one of several different mycorrhizal species.

Some fungi in the Basidiomycete genus *Rhizoctonia* form associations with the seedling roots of orchids in which the mycelium invades the inner cortical tissues, termed an endomycorrhiza. The interaction is precarious for the seedling, as the fungal partner may overcome and destroy it. However, the symbiosis is essential

for the early growth and establishment of the plant, and the fungal partner disappears as the plant matures. Endomycorrhizas are also formed between many tropical trees and basidiomycete fungi, many trees having up to 30 mycorrhizal species.

Fungi in the Zygomycota mostly form associations with woody and herbaceous plants in which hyphae grow between the root cortical cells, forming large sac-like structures called vesicles and also branched feeding structures called arbuscules which penetrate root cells. This association is termed a "vesicular-arbuscular" mycorrhiza (VAM fungi) and is an extremely common and widespread type of root infection. VAM fungi seldom reproduce sexually and typically only produce resting spores, called chlamydospores. They are also the most common mycorrhizal type formed with nodulated, nitrogen-fixing legumes (Barea *et al.*, 1992). Soils typically contain several species of VAM fungi, all of which may colonize the roots of most crop plants, but which have strain-dependent responses to host and soils, suggesting that a combination of fungi is required at the plant-soil interface for maximum nutrient capture (Bethlenfalvay, 1992b).

Several genera of ectomycorrhizal fungi have been cultured on artificial media in the laboratory, e.g., *Tuber* (Fontana, 1977; Mischiati & Fontana, 1993), *Pisolithus, Laccaria, Hebeloma, Suillus, Scleroderma, Thelephora* and *Paxillus* (Jeffries & Dodd, 1991), which facilitates inoculum production and would permit genetic manipulation.

Despite their many benefits, and the variety of techniques available for evaluating and manipulating them (Hayman, 1984), VAM fungi have not been widely used in crop production for several reasons.

Each VAM isolate potentially elicits different host responses, and so selection of the best inocula from the large number of existing populations is difficult (Bethlenfalvay, 1992a). The difficulty and high cost of producing inoculum and the poor development of application technologies exacerbate this problem. All VAM fungi are obligately biotrophic symbionts, and so axenic cultures are extremely difficult to produce. Dual cultures can be produced with roots or root organ cultures of living host plants, usually on solid, or in liquid media, from which inoculants may be prepared (Sylvia & Jarstfer, 1992). Although it has not yet been possible to culture mycorrhizal fungi in quantity in pure culture, *Gigaspora magaritae* has been grown in a medium containing 2% CO_2 and flavonols (Bécard *et al.*, 1992). Development and improvement of such methods have promise for improving yield in axenic culture.

Land Regeneration

The focus of agricultural research in developed countries has moved from crop production above all else, and attention has been given to the effects of agricultural practice on the environment, both in temperate and tropical ecosystems. In arid, semiarid and sub-humid areas of the world, over-grazing, deforestation and unregulated cultivation techniques contribute to soil erosion which has led to desertification in many areas. Re-vegetation of such ecosystems with low water

and nutrient (nitrogen and phosphorus) availability can be started using woody legumes. These plants develop symbiotic associations with both rhizobia and mycorrhizas which help the plants to grow in stressed conditions. Attempts to recover desertified ecosystems with native legumes inoculated with selected VAM fungi and rhizobia increased outplanting performance, plant survival and biomass development in a desertified Mediterranean ecosystem (Herrera *et al.*, 1993). The symbiosis has also been exploited to reclaim poor quality land (Skujins & Allen, 1986; Allen, 1989; Morgan *et al.*, 1990) and phosphate-polluted soil (Sylvia, 1990), and in forest regeneration both in temperate (Perry *et al.*, 1987) and tropical ecosystems.

Means of Genetic Modification

Genetic modification usually involves either the removal of a trait by mutational inactivation of the encoding gene or by deletion of the DNA region encoding these determinants, or addition of a new trait, by gene insertion into the chromosome or indigenous plasmids or by introducing a new plasmid encoding a trait. Plasmids are circular, double-stranded, autonomously replicating DNA molecules which are widely distributed among bacteria. They do not often encode genetic characters (phenotypes) essential for the more routine aspects of cell growth and survival, and so are dispensable to the cell.

The genetic material of *Rhizobium* consists of a static chromosome and several plasmids of high molecular weight (10^2–10^3 kilobase pairs) which can be exchanged by conjugation. In *Rhizobium*, the nitrogenase structural genes, the nodulation genes and elements of the host-specific determinants are found on a single large plasmid, the symbiotic (*sym*) plasmid. Other genes relevant to nodulation and nitrogen fixation have been found in the chromosome and in other plasmids. Most genetic modifications have involved additions of foreign genes to, or deletions of native genes from these plasmids, mostly the *sym* plasmid.

Ectomycorrhizal fungi have been modified by transformation, a process which involves making protoplasts (cell-wall free protoplasmic units) by incubating mycelium with a lytic enzyme in the presence of osmotic stabilizers such as sorbitol or KCl. Plasmids containing foreign genes, or intact genes, can then be added and in the presence of polyethylene glycol and $CaCl_2$ become incorporated into the fungus genome. Transformed protoplasts are spread onto selective culture media, where transformants regenerate cell walls and grow hyphae. There have been no attempts to genetically modify VAM fungi or endomycorrhizal fungi.

Genetically Modified Rhizobia and Mycorrhizas

The introduction of genetically engineered strains of rhizobia with enhanced nitrogen fixing capacities will be important for meeting the future demands of agriculture. Inputs of, and dependence on, manufactured nitrogenous fertilizers could be greatly reduced and the fossil fuels used in fertilizer production could be conserved.

Substantial environmental benefits would be obtained from the ensuing reduction of nitrate pollution in runoff water fed to lakes and streams. Genes and phenotypes of rhizobia which influence competitive nodulation have been identified, e.g., motility and chemotaxis, cell surface polysaccharides involved in recognition-adhesion mechanisms, bacteriocin production, rate of infection, a gene product which blocks nodulation by other strains, substrate responsiveness and growth rates on soil substrates. Modifications to enhance competitiveness, investigate host specificity (the *hsn* gene), and increase nodulation (the *nod* gene), nodule occupancy, and nitrogen fixation (the *nif* gene) have been attempted. Marker genes containing rifampicin resistance, the Tn5 transposon, nodulation characters, nitrogen-fixation characters and then pTA2 plasmid have also been inserted as tracking devices before release (Wellington *et al.,* 1993). *Rhizobium* plasmids have been transferred by conjugation to the non-fixing species *Agrobacterium tumefaciens,* which subsequently acquired the ability to initiate a symbiotic relationship with an appropriate host legume (Martinez *et al.,* 1987). Another long-term objective is to construct rhizobia capable of infecting non-nodulating plants such as wheat and barley which normally require the addition of much nitrate fertilizer for healthy growth. The potential cost savings in cereal crop production are enormous.

Methods for the genetic manipulation of fungi have advanced greatly in recent years, but inability to grow all types of mycorrhizas in axenic culture has hindered progress in this field. Consequently, there is only one report of an attempt to genetically manipulate a mycorrhizal fungus, the endomycorrhizal Basidiomycete *Laccaria laccata,* which was transformed for resistance to the antifungal compound hygromycin B (Barrett *et al.,* 1990). The transformant contained genes from *Escherichia coli* and *Aspergillus nidulans* which were able to function in a taxonomically unrelated fungus. Barrett *et al.* (1989) reported conditions for the transformation of ten species of ectomycorrhizal fungi, and Meinharat & Esser (1987) characterized the plasmids of morel fungi (*Morchella* spp.) which could be used for vector transport. The potential to improve the ectomycorrhizal symbiosis is now available, provided specific genes beneficial to the symbiosis can be identified.

Releases of Native and Genetically Modified Strains

The deliberate, large-scale release of bacteria into the environment in many climatic regions and soil types has occurred for almost a century by the inoculation of legume seed with rhizobia (Catroux & Armarger, 1992). Inoculants consist of pure cultures of *Rhizobium* species and strains from different geographical origins, or mixtures of known and unknown microorganisms, adapted to local soil and climate conditions, and to plant genotypes. Non-indigenous species have been introduced, e.g., *Bradyrhizobium japonicum,* in the United States of America and in Europe, and improved strains of a single species have been introduced into preexisting

populations. Consequently, many non-indigenous rhizobia with new genotypes have been established in soils worldwide. Several releases of engineered rhizobia into soils have taken place using marked strains of *Bradyrhizobium japonicum* and *Rhizobium meliloti* (Wellington *et al.*, 1993).

There have been many deliberate releases of ectomycorrhizas, endomycorrhizas and VAM fungi in temperate and tropical soils, particularly to increase food crop production and to establish forest trees, and for environmental land reclamation (Jeffries & Dodd, 1991; Bethlenfalvay, 1992a). Examples are: inoculation of chickpeas in Syria (Weber *et al.*, 1991); of *Vigna parkeri* in Florida, United States of America (O'Donnell *et al.*, 1992); of cassava in Colombia (Sieverding & Toro, 1988; Dodd *et al.*, 1990); of pigeonpea in India (Sivaprasad & Rai, 1991); and of *Pinus* and *Eucalyptus* in the Philippines (Jeffries & Dodd, 1991). Occasionally dual inoculation with mycorrhizas and nitrogen-fixing bacteria is done, e.g., inoculation of the plants *Eleagnus* and *Spheherida* in Canada (Visser *et al.*, 1991). Inoculum of ectomycorrhizas is normally added by incorporating small quantities of raw soil containing the fungus, as inoculum production in the laboratory requires much space and is labour-intensive. There have been no releases of genetically engineered mycorrhizas to date.

Risk Assessment

The biological consequences of the release of novel genotypes produced by either classical or molecular manipulation methods into an uncontained ecological situation are only partially understood and so a risk assessment must be made for each release. The traditional framework for risk assessment is a methodical progression via analytical steps which has the following aims.

1. First, to identify potential hazards which may arise from a novel genetic combination.
2. Second, to estimate the probability that a hazard will cause actual harm (i.e., the risk) by assessment of exposure to the hazard and its consequences, and by assessment of the level of risk by consideration of the magnitude of harmful consequences and the likelihood of their realization.
3. Third, to select and assign appropriate containment and control measures (also termed risk management).

This approach is sometimes difficult to apply to risk assessments of GEM release, because the possible outcomes of an event are numerous and complex. Although scientists have previously relied on judgement, analogy and results from a few contained-release experiments, more quantitative information is now available to predict the risk associated with each experiment. Since the origin and function of each gene, the cloning method used and the ecosystem into which it will be released are usually specific to a given product, most of the world's regulatory authorities have adopted a case-by-case approach for risk assessment. Once com-

mon themes appear, a general basis for analysis, applicable worldwide might be adopted. It is important to remember that risk assessment should balance perceived hazards against potential benefits.

Information Requirements in Filing and/or Reviewing Applications for Field Trials of Rhizobia and Mycorrhizas

Familiarity with particular microorganisms, their functions and their target environments is important when assessing potential environmental effects. Risk assessment should include information on:

1. The type of genetic modification and the properties it confers upon the microorganism, including its host range.
2. The potential for gene transfer from the introduced microorganism to other microbiota.
3. The biological properties of the microorganism, its persistence and survival.
4. The functional role of the organism in the ecosystem.
5. Transport of the microorganism within the site by deliberate means (application method) and from the site by natural means (climatic effects) and mechanical and animal vectors.
6. The possible effects on ecosystem structure and function should a genetic trait persist longer than intended or spread to a non-target environment or organism.
7. The means to ensure the health of personnel involved in the application.
8. Containment and control measures for the protection of human health and safety.
9. A contingency plan in the event of an unplanned release, including termination procedures.
10. Names of staff responsible for the release and the location and date of the site, including the number of site visits to be made.
11. Plans to monitor the health of workers carrying out the experiments, to include an occupational record of experiments done, organisms used, and gene products expressed.

This list does not reflect an order of priority - all information requirements are equally important. Risk assessment is best carried out locally by a Genetic Manipulation Safety Committee which should include representatives from all staff involved in the experiments including culture collection and waste disposal, and administrative staff as well as researchers. Expert advice on risk assessment should also be available from a competent, national (governmental) body, if required. Each experiment involving a GEM should have a separate risk assessment and a record of the risk assessment for each experiment should be retained for 10 years after the work has ceased. An assessment should be reviewed if there is any reason to suspect that assessment is no longer valid because of a significant change in activity.

Potential Hazards Arising from Releases of Native and Genetically Engineered Rhizobia and Mycorrhizas

Release of Native Rhizobia into the Soil

Strains of *Rhizobium* may be highly stable once introduced into soils (Jansen van Rensburg & Strijdom, 1985; Brunel *et al.*, 1988), and much care must be taken before introducing a non-indigenous species or selected strain. The introduction of a weakly nodulating, but highly successfully competitive, soil saprotrophic strain of *Rhizobium* may preclude the introduction of a strain with improved nodulating ability, but less competitive ability, at a later date. Also, the introduction to soils of a strain with high nodule occupancy, but variable or low nitrogen-fixing ability may be difficult to eliminate later (Weber *et al.*, 1989). In the United States of America, *Bradyrhizobium japonicum* serotype USDA 123 is dominant in eight midwestern states because it is more competitive, although it is an inefficient nitrogen fixer and excludes more efficient nitrogen fixers. This problem may be compounded if the strain is later shown to injure plants or reduce yields. Examples of deleterious rhizobia–legume interactions are chlorosis induction in soybean by many serogroup 76 strains of *Bradyrhizobium japonicum* (Minamisawa, 1989), leaf-roll in pigeon pea by *Rhizobium* strain IHP324, and chlorosis in *Phaseolus vulgaris* induced by *Rhizobium tropici* type B strains (O'Connell & Handelsman, 1993). Similarly, serotype USDA 76 is dominant in seven southern states, although these strains have a high incidence of rhizobitoxine (Fuhrmann, 1990). The example of *Rhizobium tropici* illustrates the caution that must be applied to inoculation studies, as it tolerates high temperatures, acidity and high concentrations of aluminium and is believed to be a suitable candidate for inoculation into tropical soils (Graham *et al.*, 1982).

Release of GEMs into the Soil

It is important to remember that the presence of foreign DNA in a GEM is not hazardous in itself, but its influence on the expression of phenotypic traits and mobility of the genetic material might generate hazards. Consequently, the proposed release of genetically engineered strains of microorganisms has generated much concern both within the scientific community and the general public. Concern has been centred on: the survival of the GEMs, particularly their persistence and carry-over to subsequent crops; their transport from the site of application; their potential to disseminate genetically engineered DNA to the indigenous microbial population; and their potential to disrupt microbially-mediated ecological processes, and hence, wider ecosystem function and stability. The exposure of endangered species and alteration of host range and use of antibiotic resistance genes as markers have also been considered. Methods and approaches for investigating

gene transfer between different species (horizontal gene transfer) and ecosystem effects *in situ* have been recently developed and results are now becoming available which can help to allay most of the concerns expressed.

Gene Transfer in Bacteria

Gene transfer between bacteria in the environment occurs by one or more of three mechanisms. **Conjugation** or **transduction**, in which DNA is protected during transfer, have a reasonably high probability of occurrence in the environment. **Transformation**, which involves uptake of naked DNA, unprotected from the environment, has a relatively low probability of occurrence in the environment.

Conjugation

This is a parasexual process (there is no random reassortment of genes) requiring cell-to-cell contact between donor and recipient cells, which occurs widely in both Gram-positive and Gram-negative bacteria. During conjugation, plasmids are transferred from one cell to another either via a long, flexible or short, rigid pilus in Gram-negative bacteria, or via a DNA transport pore in Gram-positive bacteria. Plasmids which can code for all the functions needed for conjugation, including sex pilus formation, are termed conjugative. These may also mediate the transfer of other, non-conjugative plasmids when they are both in the same donor cell - a process called mobilization. The integration of a conjugative plasmid into the donor chromosome may also effect transfer of chromosomal genes. The degree to which any organism participates in gene transfer depends on the host and recipient, the plasmid involved and environmental factors.

There are two variations on this theme. *Transposon-mediated conjugation*, in which a transposon (a specific mobile DNA sequence) changes its position within the bacterial genome, and moves between a plasmid and the chromosome. Transposons are capable of conjugal transfer between cells with or without plasmids and are probably the most important means of gene transfer between distantly-related microorganisms. They are often associated with antiobiotic-resistance genes. In *retrotransfer*, donor cells inherit markers from the recipient at frequencies similar to those transferred from the donor - a process of reciprocal genetic exchange.

Transduction

Bacteria may be parasitized by viruses called bacteriophages (or phages) which infect a cell, multiply inside it, burst it and then infect new cells. This type of phage life cycle is termed lytic. Another type of phage infects a cell, but its DNA is incorporated into the host genome and replicated along with it, until it is excised and multiplies inside the host cell, finally bursting it to release more infective phages This type of life cycle is called a lysogenic (or temperate) life cycle. The transfer of genetic information between cells by bacteriophage particles acting as vectors is

called transduction, of which two types are recognized. Generalized transduction is where any genetic element within the host cell has an equal probability of being transduced by the phage vector. It occurs at low frequencies when pieces of the disintegrating donor chromosome are taken up by the maturing phage particles. Both chromosomal and plasmid genes can be transduced. Specialized transduction is the second type, where only specific genetic elements are transferred.

Transformation

Transformation is the process by which a piece of double-stranded DNA is taken up by a bacterium, integrated with the cell genome and replicated with it. Cells must shift from a non-competent to a competent (i.e. transformable) state to be able to absorb exogenous DNA. In bacterial cultures, DNA is released from cells by random lysis and by controlled release from a fraction of the population and may be taken up by the same or different species. Free DNA is present in the environment from decaying microbial cells (incidental or passive release), or is excreted from living cells (deliberate or active release) (Stewart *et al.*, 1983). Gene transfer by free DNA in the soil can be envisaged as a multi-step process involving release of the functional chromosomal and plasmid DNA, persistence of the released DNA, competence development in potential recipient cells, uptake of free or particle-associated DNA, and propagation of the DNA followed by expression of the newly acquired trait. Following uptake and expression, exogenous DNA could be transferred to other bacteria by conjugation, transduction after phage attack or transformation after autolysis.

Factors Affecting Gene Transfer in Bacteria and Evidence for Gene Transfer Among Rhizobia in Soil

Horizontal gene transfer does occur between bacteria in the soil (Stotzky, 1989), but field soils are highly variable in composition and are subject to uncontrolled climatic change and so are difficult to study. Experimental research has therefore used model systems, mostly soil microcosms, to study the effects of environmental factors on gene transfer, which also satisfies restrictions on the release of GEMs into the environment. Only recently has the transfer of genetic material from GEMs to indigenous microorganisms been studied in natural environments, under realistic conditions of substrate concentration and population densities, which is essential if the data are to have predictive value.

Initially, the dangers of accidental release of genetically engineered bacteria into the environment drew attention to conjugation as the means of intercellular transmission of genetic information, as the diversity of bacteria in which plasmids have been identified indicated widespread occurrence of this phenomenon. Soil habitats support dense and active (if often transitory) microbial communities so that cell-to-cell contact readily occurs, but barriers such as entry exclusion, host and foreign plasmid incompatibility, and restriction modification systems which recognize and degrade incoming donor DNA may act to prevent transfer. Conjuga-

tion requires a high metabolic state in both donors and recipients, which is often low in soil due to the scarcity of growth substrates, but may be high in the rhizosphere or on the root surface.

Early studies to determine the frequency of plasmid transfer within soil used model systems and sterile soil. It was shown that plasmids could be exchanged among strains of single species of common soil bacteria (Weinberg & Stotzky, 1972; Graham & Istock, 1978) and between a common soil bacterium and *Rhizobium fredii,* (Richaume *et al.,* 1989). Clay, organic matter, soil pH and temperature all affected transfer frequency (Richaume *et al.,* 1989). Studies carried out in non-sterile soil demonstrated transfer of the pea plasmid pJB5JI between strains of fast-growing rhizobia (Kinkle & Schmidt, 1991) and of the plasmid r68.45 from *Bradyrhizobium japonicum* to several strains of *Bradyrhizobium* sp. (Kinkle *et al.,* 1993). However, plasmid pJP4 was only transferred to two recipient strains of *Bradyrhizobium* sp. indicating some specificity. Evidence shows that gene transfer by conjugation does occur in the soil despite the many physiological and environmental barriers.

Evidence for DNA transfer within nodules is uncertain. In sterile systems, plasmids were transferred between rhizobia in pea nodules (Johnston & Beringer, 1975) and *Rhizobium meliloti* in alfalfa nodules (Pretorius-Gruth *et al.,* 1990). In non-sterile soil, Kinkle *et al.* (1993) found transconjugants of *Bradyrhizobia* in soybean nodules, but the absence of both parental strains suggested colonization of sterile nodules by transconjugants, and that conjugation had occurred in the soil, in the rhizosphere or on the root surface. Additions of organic matter or the presence of plant roots stimulated plasmid transfer (Kinkle & Schmidt, 1991; Kinkle *et al.,* 1993).

Transduction occurs in a large number of environmentally significant bacteria and bacteriophages, and may be the major mechanism of both chromosomal and plasmid DNA dissemination in some species (Novick *et al.,* 1986). Transductional transmission of DNA occurs between Gram negative bacteria in the soil (Germida & Khachatourians, 1988; Zeph *et al.,* 1988), although genes are only transmitted at low levels in *E. coli,* the most studied organism. However, the very strict host range of most phages limits their potential to act as vectors for gene spread (Reanney *et al.,* 1983). Bacteriophages have been isolated from all of the major groups of rhizobia (Staniewski, 1987) and their potential to act as vehicles for genetic exchange has been demonstrated *in vitro*. Generalized transduction type occurs for several phages of *Rhizobium meliloti, R. leguminosarum* and *Bradyrhizobium japonicum* and specialized transduction in phages of *R. meliloti*: all have a lysogenic (temperate) development (Sik *et al.,* 1980; Buchanan-Wollaston, 1979; Shah *et al.,* 1981; Svab *et al.,* 1978). As the lysogenic mode of development has been found in several groups of rhizobia and also *Bradyrhizobium japonicum* (Abebe *et al.,* 1992), there is a large potential to transfer genetic information by transduction between different rhizobia.

To date, transformation between rhizobia has not been demonstrated in the soil, however, the potential for its occurrence does exist. Studies using soil microcosms (either pure sand or sand-clay models or natural soils) indicate that transfor-

mation may occur in the soil. *Pseudomonas stutzeri* and *Acinetobacter calcoaceticus* can be transformed with high frequency in soil extracts (Lorenz & Wackernagel, 1991; Lorenz *et al.*, 1992), and *Bacillus subtilis* can take up mineral-associated DNA (Lorenz & Wackernagel, 1991). Soils and surfaces probably stabilize DNA by binding it to clay particles and protecting it from nuclease digestion, thereby forming potential microenvironments for natural transformation, and increasing the probability of its uptake (Greaves & Wilson, 1970). Many bacterial species can develop natural competence for DNA uptake in the soil (Lorenz & Wackernagel, 1988), and plasmid DNA may persist long enough to be available for uptake by competent recipient cells (Romanowski *et al.*, 1992). In the soil, continuous production and release of DNA would constitute an extracellular gene pool, available for sampling by bacteria. Although transformation can occur between bacteria in soil (Stotzky, 1989), lack of data makes it difficult to predict the probability of gene absorption and expression by bacteria. Low cell densities may further reduce the opportunity for transformation in the soil.

These three processes probably all act in concert between members of a soil microbial community. The potential for gene exchange between distantly related microbes within the soil ecosystem depends on the following processes; insertion (restriction/modification of the foreign DNA may occur in the host cell), integration into the host genome, establishment and expression of the gene. It is likely to be more frequent among clones of the same species than among more distantly-related organisms. Factors influencing successful transfer include cell densities, number of phages, concentration of available DNA, the activity of nucleases, temperature, density, nutrient status and the physiological state of the host. In principle, organisms in the laboratory manipulated to cross genetic barriers may be able to transfer genes to other microorganisms in the field, but transfers in nature are infrequent and infrequently documented.

The problem that many soil organisms cannot be cultured, or have not yet been cultured, on artificial media because they are stressed or dormant has yet to be addressed. Under conditions of starvation, bacteria may produce very small cells (ultramicrocells) which are viable, but which cannot be detected by culturing on standard media (Colwell *et al.*, 1985; Roszak & Colwell, 1987). In fact, it has been estimated that the majority of genetic diversity lies amongst the unculturable fraction of microbial communities. This fraction might act as a reservoir into which particular engineered genes could escape and subsequently fail to be recognized by conventional culture techniques, although it would be recognized by a specific gene probe.

The Persistence of Rhizobia in the Soil: Ecological Considerations

Persistence can be viewed as either that of the introduced organism, or of the genetic material incorporated during its modification, in new genetic combinations resulting from gene transfer. The soil supports a complex, indigenous microbiota so that advantageous traits such as rapid utilization of substrates, high maximum

growth rate and antibiotic production may be needed if the microorganism is to colonize successfully in competition with indigenous microorganisms. The survival, persistence, replication and success of both natural and recombinant DNA in bacterial communities will depend on many factors, including the host and its genotype (competitiveness, substrate utilization, environmental range and host range), the novel gene and its effect on the cell's phenotype, its frequency of transfer, its mutability and the selective pressure imposed by the cellular environment. Only a new combination with greater fitness[1] than the indigenous genotypes has the likelihood of persisting. In most cases, GEMs are disadvantaged due to the metabolic burden associated with carriage and expression of additional functions, although some have shown enhanced fitness[1] (Hartl *et al.*, 1983; Edlin *et al.*, 1984) in the laboratory. However, a modification could enhance persistence if it changed fitness, by, e.g., enhancing survival in the presence of an environmental toxin or increasing its ability to metabolize a substrate. Environmental factors which affect persistence of the GEM and stability and expression of its genome include soil type, nutrient and moisture availability, pH, temperature, inhibitory chemicals and biological factors such as predation and competition (Stotzky & Babich, 1984). Long-established microbial communities resist invasions by non-indigenous organisms (Liang *et al.*, 1982), and proliferation is unlikely to occur in environments such as the plant rhizosphere, being more likely in restricted density or diversity environments.

Species of *Rhizobium* present in soil are unlikely to be active, merely surviving in a low nutrient, adverse environment. When they interact with plant roots, they enter an environment which is both nutrient rich and which protects them from predation by flagellates and other eukaryotes, and from attack by viruses or other bacteria. Thus, occupation of a specific niche (the nodule) enhances survival in the face of competition with the result that non-indigenous species of rhizobia are often highly stable once introduced into soils (Brunel *et al.*, 1988). Environmental factors which enhance survival of rhizobia introduced into soils include the plant rhizosphere, clay content, temperature and inoculum size: those reducing survival include predation, and raised water content (Wellington *et al.*, 1993).

Natural selection favours isolated, but genetically advantaged populations, thus improving the chance of the establishment of novel phenotypes (and genotypes) in existing communities. However, simple genetic changes can debilitate a bacterium when faced with competition from other members of the microbial community. In spite of these considerations it has been found that the introduction of DNA as plasmids, transposons or chromosomal rearrangements, or the deletion of DNA has no consistent effect on the survival of rhizobia in soil microcosms (Wellington *et al.*, 1993).

Spread and Dispersal of Rhizobia

Rhizobia may be dispersed from the plots during application, leached through the soil, run off in surface water or particles, disseminated by wind, and transported by

animals, humans or machinery. In practice, transport is often very limited for soil microbes as most are firmly attached to soil particles. Controlling dispersal by insects may be difficult in a field situation, but is often ignored because of the small scale of field trials. However, both the wild type and engineered strains of a fluorescent *Pseudomonas* were detected both on and in the foliar tissue and herbivorous insects after seed inoculation at planting (Klüpfel & Tonkyn, 1990), which suggests that spread by vectors may be important.

Gene Transfer and Dispersal in Mycorrhizas

Dispersal of genetic information may occur through vegetative growth, spore dispersal and hyphal anastomosis. The hyphae of ectomycorrhizas and VAM fungi are not restricted to the host plant and grow out into the surrounding soil, sometimes infecting other plants nearby. This process may be repeated until several, sometimes many, plants become connected together by a network of hyphae between their roots. Carbohydrates may flow from plant to plant through hyphal networks formed by ectomycorrhizas (Read *et al.*, 1985). When new nuclei are formed during hyphal growth, genes will spread throughout the area covered by the host plant and further out into the surrounding soil.

The hyphae of many ectomycorrhizal Basidiomycetes can fuse with each other to form complex networks by a process known as anastomosis. In the soil, compatible hyphae meet, the hyphal walls break down and fuse, and their respective protoplasms and subcellular organelles mix, thereby effecting gene transfer from one mycelium to another. However, only some mycelia within a species are able to fuse with each other. Some show incompatibility reactions, which has led to the creation of anastomosis groups for these fungi, which may be useful for identification (e.g., in the plant pathogenic fungus *Rhizoctonia*). The existence of anastomosis groups is poorly known in other Basidiomycetes and deserves considerable research attention before release of genetically engineered mycorrhizas can be contemplated.

Ectomycorrhizal fungi produce their fruiting bodies above ground from which spores are dispersed by air currents or by attachment to insects. Fruiting bodies may also be eaten by a variety of animals (mammals, molluscs and man). Some Ascomycete mycorrhizas form subterranean fruiting bodies (truffles) and produce volatile chemicals which act as attractants to foraging animals, which excavate the fungi, eat them and disperse their spores in their faeces. Genes may be dispersed a long way from the original site of the Ascomycete or Basidiomycete fruiting body, and also have the potential to be incorporated into bacteria in the animal's gut by spore lysis or damage followed by transduction.

Anastomosis and the formation of wind-dispersed sexual spores does not occur in VAM fungi, which only form chlamydospores in the soil or in root tissues. These may be dispersed by wind if they are brought to the surface, but have frequently been found in the guts of rodents which may bring about dispersal (Silver-Dowding, 1955). The hyphae of VAM fungi also contain bacteria and bacteria-like

objects (Scannerini & Bonifante, 1991), and their chlamydospores are parasitized by several species of soil-inhabiting Chytridiomycete fungi (Paulitz & Linderman, 1991), although the potential for gene transfer to these organisms is unknown.

Persistence of Mycorrhizas in Soil

Mycorrhizas can persist for long periods of time as dormant spores, as mycelium in plant tissues (especially in wood), as hyphal cords in soil, and as sclerotia (resistant aggregations of hyphae). Any of these could be formed away from the inoculation site following hyphal growth in soil, thereby dispersing widely a genetically engineered mycorrhiza.

Effects of Introduced Genetically Engineered Rhizobia and Mycorrhizas on Ecological Processes

Introduced engineered rhizobia might disrupt the nitrogen cycle locally, if they were capable of substantial growth in soil. Although there is no information on the effect of rhizobia on the nitrogen cycle, the effect of five introduced model GEMs in a soil perfusion system showed that four had no effect on ammonification, nitrification or de-nitrification, or on the population dynamics of the microorganisms responsible for these processes (Jones *et al.*, 1991). In only one GEM, a strain of *Enterobacter cloacae* carrying a plasmid, were the rates of nitrification and NO_3^- slightly lower than the unmodified host control, but this was not considered to be large enough to constitute an ecologically significant effect.

The association of engineered rhizobia with non-target hosts might lead to enhanced fitness[1] and displacement of indigenous plants. Similarly, if a recombinant genotype conferred a competitive advantage on an ectomycorrhizal fungus, the plant associated with it might gain a competitive advantage over other mycorrhizal or non-mycorrhizal species and displace them. Following spread in the soil, the genotype might also be able to interact with other mycorrhizal species improving their performance, or induce a mycorrhizal relationship in previously non-mycorrhizal plants. Unfortunately, most studies following release of native mycorrhizas or native and engineered rhizobia have not measured changes in the composition of plant communities, and so effects on host vigour are unknown. This area deserves considerable research attention.

Means of Mitigating Potential Risk

Risk mitigation can be achieved by decontamination procedures, environmental gene control, reducing the occurrence of environmental gene transfer, containment by disabling, and monitoring their spread in the environment. Planned introductions must include appropriate methodology for monitoring the released microor-

ganisms around the test site. Monitoring provides data on the organism's effectiveness, survival, spread and genetic stability, and permits detection of any unexpected spread or biological effects.

Decontamination

Decontamination of contained environments into which GEMs have been released for initial trials can be achieved by autoclaving or disinfectant application. However, these methods cannot be used in field sites, either at the end of a trial or in an emergency, because of the heterogeneous nature of the area and the potential for adverse effects on crops and associated organisms. Decontamination methods for field sites have, instead, been based on methods developed for the control of plant pathogens released at field sites. These mostly employ burning, tilling or removal and autoclaving of the crop residue, and biocide application (Smitley & McCarter; 1982; National Research Council, 1989). Although several methods have been proposed, few have been tested and fully evaluated. Results show that conventional plant disease control methods may not provide satisfactory control of released GEMs and may adversely affect indigenous microorganisms (Donegan *et al.*, 1992), indicating the need to develop more effective and selective control methods at field sites.

Gene Construction

Because gene exchange does occur in bacteria it is desirable to use gene combinations which minimize the possibility of transfer. Generally, genes should be introduced onto the chromosome of the target microorganism or non-conjugal and immobilizable plasmids (either indigenous or introduced).

Environmental Gene Control

Genes can be placed under the control of a promotor that responds to inducing stimuli in a manner most appropriate for the intended function in the environment. In *Rhizobium*, it is possible to induce gene expression by adding secondary plant metabolites, such as flavonoids present in root exudates (Firmin *et al.*, 1986). Consequently, the rhizobia only express their genes when in the presence of a legume root.

Reduction of Environmental Gene Transfer

In bacteria, this can be achieved by deletion of genes required for self-transfer, use of small, non-self-transmissible, poorly-mobilizable plasmids as cloning vectors and placement of the novel material on the bacterial chromosome. Short-term survival is essential for the released *Rhizobium* to compete successfully with indigenous populations in the soil ecosystem to perform their desired functions. But if it

persists beyond the intended period of usefulness, it may be necessary to use containment, e.g., by suicide genes (Molin *et al.*, 1987).

Gene transfer in mycorrhizal fungi under natural conditions needs much investigation before control methods can be devised. While it is likely that the potential for gene transfer between VAM fungi is small, the potential for transfer within species of ecto- and endomycorrhizal species may be great. Genetically modified mycorrhizas would have to be disabled to ensure that hyphal anastomosis and basidio- or ascospore production did not occur. Strains engineered for host specificity would ensure that spread into non-target plant hosts did not occur.

Reducing the Risk of Survival of GEMs After Release

All native and indigenous strains of *Rhizobium* can survive in temperate soils, but little attempt has been made to reduce their survival, because it is more important to ensure survival to enhance plant growth. The long-term survival of deliberately released GEMs in the environment is undesirable, although limited survival is essential for most released organisms to carry out their allotted tasks. Consequently, safeguards to ensure that survival does not occur after an accidental release must be built into a development strategy.

Complete physical containment of organisms released for agricultural purposes is impractical and so strains with reduced survival, reduced reproductive capacity, low resistance to a predictable environmental change (seasonal heat or cold), or a tendency to lose the specific function of concern are selected. The incorporation of additional nutritional requirements is also possible. Lethal genes for cold- or heat-sensitivity, or toxin accumulation could be inserted into the genome or plant genotypes which restrict nodulation by a given strain of bacteria might be used, but these may cause the elimination of the strain before it has had any effect.

Bacteria may be rendered incapable of replication by including lethal genes (suicide genes) activated under certain environmental conditions triggering cell death. The activation time can be chosen by the investigator and they can be transferred quickly *in vitro* between bacterial hosts. Other genes may be used which are effective when expressed at altered levels, or times, or without expression of a protecting gene. Examples are, the genes encoding bacteriocins, restriction endonucleases or plasmid-encoded lethal genes, such as the *hok* (host killing gene) on plasmid R1 which is lethal to a wide range of bacterial cells (Molin *et al.*, 1987). Another type of suicide plasmid is one which permits survival in the presence of a toxin, when it has maximum toxin-degrading activity, but which imposes a metabolic burden in its absence, thus putting the microorganism at a competitive disadvantage. The disadvantage of control of a GEM by lethal genes is that it may result in recombinant DNA being available transiently for transduction. However, rates of degradation in soil are high (Greaves & Wilson, 1970) and the residence time for dissolved DNA is very short, so it is unlikely that this will occur under field conditions.

Experience has shown that killing of bacterial populations has been incomplete with all of the constructs used to date (Cuskey, 1992). Survivors have been isolated which have lost the containment plasmid, or have deleted, mutated or rearranged plasmids which no longer confer a conditional phenotype which suggests that further research into lethal genes is required before they can be recommended unreservedly.

Similarly, suicide genes could be inserted into mycorrhizas, but as yet none are known, although it might be possible to clone in genes from other organisms such as bacteria. VAM fungi could be engineered to ensure that they did not produce chlamydospores.

Detection of Introduced Organisms

Great emphasis has been placed on the detection and enumeration of soil bacteria released in field inoculation studies as an essential requirement of risk assessment for introduced GEMs. Therefore, much attention has been given to developing methods for investigating the fate of bacteria in the soil. Traditional methods relied on identification by selective plating and fluorescent antibody techniques, but these are not sensitive enough for the detection or enumeration GEMs. Attention has focused on methods for the detection of released microorganisms at very low concentrations in the soil and for *in situ* detection.

Bacteria can be detected and identified by the use of cloned marker genes. Such genes should have normal expression and stable inheritance, no effect on strain survival, no ability to be transferred to other members of the soil ecosystem and should not affect plant growth. Genes for antibiotic resistance, heavy metal tolerance, bioluminescence (the prokaryotic *lux*A and *lux*B or eukaryotic *luc* genes), red pigmentation (prodigiosin), catechol 2,3-dioxygenase (the *xyl*E gene) and polygalacturonidase (the *pgl*A gene) production, or the chromogenic β-galactosidase *lac*Z and *lac*Y genes can be cloned into donor, recipient and transconjugant bacteria. It is desirable to avoid using antibiotic-resistance genes as markers, as they often impose substantial metabolic burdens on their hosts (Lee and Edlin, 1985) and they may contribute to the proliferation of antibiotic-resistant microorganisms. But, the presence of many multiple antibiotic-resistant bacteria in pristine environments suggests that caution may be exaggerated.

Detection of ectomycorrhizal fungi usually involves isolation and culture on agar media in the laboratory followed by attempts to induce fruiting bodies to form, or reaction with known tester strains. VAM fungi are obligately biotrophic and so methods for detection involve examination of infected roots. Most methods involve clearing and staining roots with trypan blue, chlorazol E or fuchsin, which kills the fungal hyphae (Phillips & Hayman, 1970) but autofluorescence of arbuscules, cytofluorimetry (Bianciotto & Bonfante, 1992) and histochemical staining for fungal alkaline phosphatase hold much promise for detection *in situ* (Tisserant *et al.*, 1993). In both ectomycorrhizas and VAM fungi, the use of specific gene probes would greatly benefit tracking.

As only a small proportion of soil microorganisms is culturable, analysis of directly extracted DNA has the potential to detect specific genes of otherwise cryptic organisms and the persistence of a recombinant gene under natural conditions. Techniques usually require the extraction of DNA from soil, but others may be used *in situ*. Examples of techniques available include transformation assays, immunological methods, specific DNA or RNA probes homologous to synthetic oligonucleotide sequences inserted into chromosomes or plasmids (Holben *et al.*, 1988; Hahn *et al.*, 1989), which may also be fluorescently labelled (Hahn, *et. al.*, 1992), and the PCR technique (Steffan & Atlas, 1988) which may be used *in situ* (Tebbe & Vahjen, 1993), although interfering humic acids must be removed. Stable tagging of *Rhizobium meliloti* with the firefly luciferase gene using a mini-Tn5 delivery vector permitted the detection of *R. meliloti* in the presence of more than 10^5 CFU per plate with no effect on growth rate or survival between marked and wild-type strains. This system could also be used for cell biomass determinations (Cebolla, *et al.*, 1993). Yang *et al.* (1991) localized *Rhizobium* RNAs in pea root nodules, thus providing a method for localization in plant tissues.

The great variation between microbial communities, soil types and moisture contents, and the dynamic interaction between a microbe and its host during the growing season indicate the importance of field data when predicting environmental risk. These molecular techniques can also be used to monitor the spread of bacteria from field sites. Mathematical models may also be useful in predicting the spread and dispersal of microorganisms, e.g., fate and transport models (Corapcioglu & Haridas, 1984; Strauss & Levin, 1991) and multimedia models (e.g., MICROBE-SCREEN), developed to assess the dispersal and fate of microorganisms released into air, surface water or soil. Fate and transport models combined with dose–response models, or epidemic models which focus on host population characteristics, may also have value in providing preliminary assessments of environmental risk from release of GEMs (Teng & Yuen, 1991). Spread into birds, insects and other plants is seldom examined and requires more research attention.

Field Tests

Small-scale field tests are useful for assessing risk when potential ecological effects are great,but their scale has been used as an excuse to avoid comprehensive risk assessments, e.g., on transfer to birds, insects and mammals. Field releases may also reveal consequences that were overlooked or incorrectly analysed.

Future Trends

Benefits from advances in genetic engineering have been slow to appear because of the lack of knowledge regarding the fate and effects of engineered microorganisms in the environment. This has been a direct result of poor research funding for basic

microbial ecology worldwide. However, modern methods based on molecular biology are now available for estimating intra- and interspecific gene exchange among rhizobia, for detecting very small numbers of microbes in the soil, for tracking and monitoring the spread of microbes in soil and for estimating their impact on the environment.

In many cases, there are now quantitative data about the release of both native and GEM rhizobia which is required for risk assessment. There have been no reports of gene transfer to indigenous populations by conjugation and neither transformation nor transduction of species in the genus *Rhizobium* have been demonstrated in the environment in field experiments to date. Consequently, many of the earlier fears about spread, genetic exchange and disruption of nutrient cycling are being allayed. This is not unexpected as commercial legume inoculants have been added to soil in enormous numbers for about a century without causing adverse environmental effects.

The less-well known bacterial genus *Frankia* is generating much interest as a potential partner for nitrogen-fixation, because it can interact with a much wider range of host plants than *Rhizobium*, covering species as diverse as alders and *Ceanothus*. So, it may be relatively easy to engineer *Frankia* to infect plants outside its usual host range, such as conifers which would allow planting density to be increased.

Genetic engineering will allow greater precision in tailoring nitrogen-fixing bacteria to specific crops and will produce a more carefully characterized and safer product than hitherto. Most applications involving the release of GEMs have required the organism to disappear or self-destruct after its function has been completed. In contrast, one of the goals of nitrogen fixation research is to make nitrogen-fixing bacteria persist in a soil community despite intense competition from the indigenous soil bacteria. Persistence as a requirement rather than a disadvantage has not been considered by regulatory frameworks and attention must be given to this aspect.

The potential for manipulation to improve mycorrhizas is immense (Hirsch, 1984) and has been suggested for several crops as diverse as cassava (Pistorius & Verschuur, 1989) and pine and eucalypt trees (Swart & Theron, 1990). However, most research on mycorrhizas has concentrated on nutrient cycling and crop improvement. Consequently, there is a dearth of information about their capacity to exchange genetic material with other fungi in the field, their interactions with other fungi and other soil organisms, the spread of native and introduced mycorrhizas, and their survival and persistence in the environment. Methods for detection in the soil are only in their infancy compared with those developed for rhizobia, although some, such as DNA probes, should be suitable for detection of mycorrhizas. Since this information is a prerequisite for assessing the risk of a GEM release, there have been no releases of genetically engineered mycorrhizas, and it is unlikely that release will occur in countries with well developed regulatory frameworks, until substantial basic research has been undertaken. The procedures adopted for bacteria are tried and tested and the principles can be applied to mycorrhizas.

Conversely, endomycorrhizas would most likely be used in the laboratory specifically for inoculation onto orchid seeds. It would be relatively easy to contain a fungus engineered to improve seedling establishment without parasitism of its host. Gene transfer from fungus to plant (if it were to occur) could be monitored, and the persistence of the fungus prior to release of the plant (commercial sale or reintroduction into the wild) could be tested.

The Future of Releases and Regulation

Irrational concerns which ignore the significant body of case histories indicating a lack of adverse effects on the environment should be ignored. A series of case histories has established the correctness of the current approach to risk assessment and regulatory frameworks in developed countries, and has done much to reduce concern among the general public. However, public concern has persisted despite growing scientific assurances that GEMs can be safely produced. Risk assessments alone will not solve the problems of public acceptance, because scientists and the general public have different perceptions of harm. Scientists should consider the impact of biotechnology on social and economic systems if their work is to receive widespread acceptance.

Regulation and Public Concern

The choice of regulatory framework for risk assessment will affect the type of data required for the risk assessment and thus the type of data required for permit applications. An important aspect of early (in a technology's) life risk analyses is their value as precedent, as future risk analyses will usually be based on them. Much experience has been gained since the first risk assessment by the United States Environmental Protection Agency (USEPA) for the planned release of genetically modified ice-nucleation negative (Ina^-) bacteria in California in the early 1980s. Quantitative knowledge, screening criteria and logical deduction can now be applied to a proposed introduction to assess risk. Although the chances of an epidemic are low, if it occurs, it will reduce public confidence and breed mistrust. So, it is important to include legislative, financial and emergency response procedures to deal with potentially unique risks. The regulatory framework should flow from the risk assessments and include public debate and information about site selection for trials, although commercial considerations may preclude this.

International Regulation of GEM Release

Strategies for regulating GEM research are especially well developed in North America (United States of America and Canada), Europe (particularly the EC countries) and Australasia. This is due to the historical science base of these countries and the rapid development of these technologies in relatively rich countries. Development strategies should ensure the most rational use of finite resources while also

ensuring that products can be safely delivered to consumers. Complication surrounding the establishment of biotechnology regulations in developing countries are due to several factors, including the diverse array of agencies involved in biotechnology research and testing (e.g., the International Agricultural Research Centres, donor agencies [the World Bank, United States Agency for International Development, European Community, Food and Agriculture Organization of the United Nations, United Nations Industrial and Development Organization etc.]), governments and research institutes of developing countries, private industry and various environmental groups. Lack of regulatory structure and finances, of confidence in decision-making expertise, of coordination with international organizations, of funds and technical expertise for risk assessment, and the fear that regulations will stifle scientific innovation, all constrain the development of responsible regulatory policy (Cohen & Chambers, 1991). Regulatory infrastructure may be non-existent or underdeveloped or be unenforced because of cash constraints. Public consultation may be infeasible and the means to collect data or assess environmental impact are unavailable. Therefore approval is often granted without national regulatory systems being in place (Cohen & Chambers, 1991). Consequently, donor countries usually insist that research involving genetic engineering sponsored by a host country should be conducted under conditions that meet the standards for health and environmental protection of that country, and be approved by its regulatory body. Field-testing cannot easily be done in the host country due to the wholly different nature and unknown behaviour of different ecosystems. However, developing countries which are embarking on biotechnology can benefit greatly from the experience and expertise of developed countries without the accompanying start-up costs.

Note

1. "Fitness" is used here in an ecological context to mean "ability to survive and persist in competition with other organisms".

References

Abebe, H.M., Sadowsky, M.J., Kinkle, B.K. & Schnidt, E.L. (1992) Lysogeny in *Bradyrhizobium japonicum* and its effect on soybean nodulation. *Appl. Environ. Microbiol.*, 58, 3360-3366.

Allen, E.B. (1989) The restoration of disturbed arid landscapes with special reference to mycorrhizal fungi. *J. Arid Environ.*, 17, 279-286.

Barea, J.M., Azcón, R. & Azcón-Aguilar, C. (1992) Vesicular-arbuscular mycorrhizal fungi in nitrogen-fixing systems. *Methods Microbiol.*, 24, 319-416.

Barrett, V., Lamke, P.A. & Dixon, R.K. (1989) Protoplast formation from selected species of ectomycorrhizal fungi. *Appl. Microbiol. Biotechnol.*, 30, 381-387.

Barrett, V., Dixon, R.K. & Lamke, P.A. (1990) Genetic transformation of a mycorrhizal

fungus. *Appl. Microbiol. Biotechnol.*, 33, 313-316.

Batzli, J.McC., Graves, W.R. & van Berkum, P. (1992) Diversity among rhizobia effective with *Robinia pseudoacacia*. *Appl. Environ. Microbiol.*, 58, 2137-2143.

Bécard, G., Douds, D.D. & Pfeffer, P.E. (1992) Extensive *in vitro* hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO_2 and flavonols. *Appl. Environ. Microbiol.*, 58, 821-825.

Bethlenfalvay, G.J. (1992a) Mycorrhizae and crop productivity. In: *Mycorrhizae in Sustainable Agriculture*, Bethenfalvay, G.J. & Linderman, R.G. (eds.), pp. 1-27. American Society of Agronomy Special Publication No. 54. ASA Publications, Madison, USA.

Bethlenfalvay, G.J. (1992b) Mycorrhizae in the agricultural plant-soil system. *Symbiosis*, 14, 413-425.

Bianciotto, V. & Bonfante, P. (1992) Quantification of the nuclear DNA content of two arbuscular mycorrhizal fungi. *Mycol. Res.*, 96, 1071-1076.

Brunel, B., Cleyet-Marel, J.C., Normand, P. & Bardin, R. (1988) Stability of *Bradyrhizobium japonicum* inoculants after introduction into soil. *Appl. Environ. Microbiol.*, 54, 2636-2642.

Buchanan-Wollaston, V. (1979) Generalized transduction in *Rhizobium leguminosarum*. *J. Gen. Microbiol.*, 112, 135-142.

Catroux, G. & Armarger, N. (1992) Rhizobia as soil inoculants in agriculture. In: *Release of Genetically Engineered and Other Microorganisms*, Fry, J.C. & Day, M.J. (eds.), pp. 1-13. Cambridge University Press, Cambridge, UK.

Cebolla, A., Ruiz-Berraquero, F. & Palomares, A.J. (1993) Stable tagging of *Rhizobium meliloti* with the firefly luciferase gene for environmental monitoring. *Appl. Environ. Microbiol.*, 59, 2511-2519.

Cohen, J.I. & Chambers, J.A. (1991) Biotechnology and biosafety: perspective of an international donor agency. In: *Risk Assessment in Genetic Engineering*, Levin, M. & Strauss, S. (eds.), pp. 378-392. McGraw-Hill Inc., New York.

Colwell, R. R., Brayton, P.R., Grimes, D.J., Roszak, D.B., Huq, S.A. & Palmer, L.M. (1985) Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. *Bio/Technology*, 3, 817-820.

Corapcioglu, M.Y. & Haridas, A. (1984) Microbial transport in soils and ground-water: a numerical model. *Adv. Water Res.*, 8, 188-200.

Cuskey, S.M. (1992) Lethal genes in biological containment of released microorganisms. In: *Release of Genetically Engineered and Other Microorganisms*, Fry, J.C. & Day, M.J. (eds.), pp. 94-99. Cambridge University Press, Cambridge, UK.

Dodd, J.C., Arias, I., Koomen, I. & Hayman, D.S. (1990) The management of vesicular-arbuscular mycorrhizal fungi in acid-infertile soils of a savanna ecosystem. II. The effects of pre-crops on the spore populations of native and introduced VAM-fungi. *Plant and Soil*, 122, 241-247.

Donegan, K., Fieland, V., Fowles, N., Ganio, L. and Seidler, R. (1992) Efficacy of burning, tillage and biocides in controlling bacteria released at field sites and effects on indigenous bacteria and fungi. *Appl. Environ. Microbiol.*, 58, 1207-1214.

Edlin, G., Tait, R.C. & Rodriguez, R.L. (1984) A bacteriophage lambda cohesive ends (cos) DNA fragment enhances the fitness of plasmid-containing bacteria growing in energy-limited chemostats. *Bio/Technology*, 2, 251-254.

Firmin, J.J., Wilson, K.E., Rossen, L. & Johnston, A.W.B. (1986) Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. *Nature*,

324, 90-92.

Fontana, A. (1977) Mycelia of Tuberales in pure culture. *Proc. 2nd North Am. Conf. on Mycorrhizae,* p. 200.

Fuhrmann, J. (1990) Symbiotic effectiveness of indigenous soybean bradyrhizobia as related to serology, morphology, rhizobitoxine and hydrogenase phenotypes. *Appl. Environ. Microbiol.*, 56, 224-229.

Germida, J.J. & Khachatourians, G.G. (1988) Transduction of *Escherichia coli* in soil. *Can. J. Microbiol.*, 34, 190-193.

Graham, J.B. & Istock, C.A. (1978) Genetic exchange in *Bacillus subtilis* in soil. *Mol. Gen. Genet.*, 166, 287-290.

Graham, P.H., Viteri, F., Mackie, F., Vargas, A.T. & Palacios, A. (1982) Variation in acid soil tolerance among strains of *Rhizobium phaseoli*. *Field Crops Res.*, 5, 121-128.

Greaves, M.P. & Wilson, M.J. (1970) The degradation of nucleic acids and montmorillonite-nucleic-acid complexes in soil microorganisms. *Soil Biol. Biochem.*, 2, 257-268.

Hahn, D., Dorsch, M., Stackebrandt, E. & Akkermans, A.D.L. (1989) Synthetic oligonucleotide probes for identification of *Frankia* strains. *Plant and Soil,* 118, 210-219.

Hahn, D., Amann, R.I., Ludwig, G., Akkermans, D.L. & Schleifer, K.-H. (1992) Detection of microorganisms in soil after in situ hybridization with rRNA targeted fluorescently labelled oligonucleotides. *J. Gen. Microbiol.,* 138, 879-887.

Ham, G.E., Fredrick, L.R. & Anderson, I.C. (1971) Serogroups of *Rhizobium japonicum* in soybean nodules. *Agron. J.,* 63, 69-72.

Harley, J.L. (1989) The significance of mycorrhiza. *Mycol. Res.,* 92, 129-139.

Hartl, D.L., Dykhuizen, D.E., Miller, R.D., Green, L., & DeFramond, J. (1983) Transposable element IS50 improves growth rate of *E. coli* cells without transposition. *Cell,* 35, 503-510.

Hayman, D.S. (1984) Methods for evaluating and manipulating vesicular-arbuscular mycorrhiza. In: *Microbiological Methods for Environmental Microbiology,* Grainger, J.M. & Lynch, J.M. (eds.), pp. 95-117. Academic Press Inc., London.

Herrera, M.A., Salamanca, C.P. & Barea, J.M. (1993) Inoculation of woody legumes with selected arbuscular mycorrhizal fungi and rhizobia to recover desertified Mediterranean ecosystems. *Appl. Environ. Microbiol.,* 59, 129-133.

Hirsch, P. (1984) Improved crop plant productivity through genetic manipulation of mycorrhizal fungi. *Chem. Ind.,* 23, 833-838.

Holben, W.E., Jansson, J.K., Chelm, B.K. & Tiedje, J.M. (1988) DNA probe methods for the detection of specific microorganisms in the soil. *Appl. Environ. Microbiol.,* 54, 703-711.

Jansen van Rensburg, H. & Strijdom, B.W. (1985) Effectiveness of *Rhizobium* strains used in inoculants after their introduction into soil. *Appl. Environ. Microbiol.,* 49, 127-131.

Jeffries, P. & Dodd, J.C. (1991) The use of mycorrhizal inoculants in forestry and agriculture. In: *Handbook of Applied Mycology,* Arora, D.K., Rai, B.R., Mukerji, K.G. & Knudsen, G.R. (eds.), pp. 155-185. Marcel Dekker Inc., New York.

Johnston, A.W.B. & Beringer, J.E. (1975) Identification of the *Rhizobium* strains in pea root nodules using genetic markers. *J. Gen. Microbiol.,* 87, 343-350.

Jones, R.A., Broder, M.W. & Stotzky, G. (1991) Effects of genetically engineered microorganisms on nitrogen transformations and nitrogen-transforming microbial populations in soil. *Appl. Environ. Microbiol.,* 57, 3212-3219.

Kinkle, B.K. & Schmidt, E.L. (1991). Transfer of the pea symbiotic plasmid pJB5JI in non-sterile soil. *Appl. Environ. Microbiol.,* 57, 3264-3269.

Kinkle, B.K., Sadowsky, M.J., Schmidt, E.L. & Koskinen, W.C. (1993) Plasmids pJP4 and r68.45 can be transferred between populations of bradyrhizobia in nonsterile soil. *Appl. Environ. Microbiol.,* 59, 1762-1766.

Klüpfel, D.A. & Tonkyn, D.W. (1990) Release of soilborne genetically modified bacteria: biosafety implications from contained experiments. In: *Biological Monitoring of Genetically Engineered Plants and Microbes,* MacKenzie, D.R. & Henry, S.C. (eds.), pp. 55-65. Agricultural Research Institute, Bethesda, USA.

Lee, S.W. & Edlin, G. (1985) Expression of tetracycline resistance in pBR322 derivatives reduces the reproductive fitness of plasmid-containing *Escherichia coli. Gene,* 39, 173-180.

Levin, M. & Strauss, H.S. (1991) Introduction: overview of risk assessment and regulation of environmental biotechnology. In: *Risk Assessment in Genetic Engineering,* Levin, M. & Strauss, S. (eds.), pp. 1-17. McGraw-Hill Inc., New York.

Liang, L.N., Sinclair, J.L., Mallory, L.M. & Alexander, M. (1982) Fate in model ecosystems of microbial species of potential use in genetic engineering. *Appl. Environ. Microbiol.,* 44, 708-714.

Lorenz, M.G. & Wackernagel, W. (1988) Impact of mineral surfaces on gene transfer by transformation in natural bacterial environments. In: *Risk Assessment for Deliberate Releases,* Klingmüller, W. (ed.), pp. 110-119. Springer Verlag, Berlin.

Lorenz, M.G. & Wackernagel, W. (1991) High frequency of natural genetic transformation of *Pseudomonas stutzeri* in soil extract supplemented with a carbon/energy and phosphorous source. *Appl. Environ. Microbiol.,* 57, 1246-1251.

Lorenz, M.G., Reipschläger, K. & Wackernagel, W. (1992) Transformation of naturally competent *Acinetobacter calcoaceticus* in non-sterile soil extract and ground-water. *Arch. Microbiol.,* 157, 355-36.

Martinez, E., Pardo, M.A., Palacios, R. & Cevallos, M.A. (1987) Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harbouring *Rhizobium phaseoli* plasmids. *J. Bacteriol.,* 169, 2818-2834.

Meinharat, F. & Esser, K. (1987) The plasmids of morels, characterisation and prerequisites for vector development. *Appl. Microbiol. Biotechnol.,* 27, 276-282.

Minamisawa, K. (1989) Comparison of extracellular polysaccharide production, rhizobitoxine production and hydrogenase phenotype among various strains of *Bradyrhizobium japonicum. Plant Cell Physiol.,* 30, 877-884.

Mischiati, P. & Fontana, A. (1993) In vitro culture of *Tuber magnatum* mycelium isolated from mycorrhizas. *Mycol. Res.,* 97, 40-44.

Molin, S.P., Klemm, P., Poulsen, L.K., Biehl, H., Gerdes, K. & Andersson, P. (1987) Conditional suicide system for containment of bacteria and plasmids. *Bio/Technology,* 5, 1315-1318.

Morgan, R.C.P., Rickson, R.J. & Wright, W. (1990) Regeneration of degraded soils. In: *Soil Degradation and Rehabilitation in Mediterranean Environmental Conditions,* Albaladejo, J., Stocking, M.A. & Diaz, E. (eds.), pp. 68-85. Murcia, Spain: Consejo Superior de Investigaciones Cientificas.

NRC (1989) *Field Testing Genetically Modified Organisms. Framework for Decisions.* National Academy Press, Washington, DC.

Novick, R.P., Edelman, I. & Lofdahl, S. (1986) Small *Staphylococcus aureus* plasmids are transduced as linear multimers that are formed and resolved by replicative processes. *J. Mol. Biol.,* 192, 209-220.

O'Connell, K.P. & Handelsman, J. (1993) Foliar chlorosis in symbiotic host and non-host

plants induced by *Rhizobium tropici* type B strains. *Appl. Environ. Microbiol.*, 59, 2184-2189.

O'Donnell, J.J., Sylvia, D.M., Pitman, W.D. & Rechigl, J.E. (1992) Inoculation of *Vigna parkeri* and mycorrhizal fungi in an acid Florida spodosol *Trop. Grassl.*, 26, 120-129.

Paulitz, T.C. & Linderman, R.C. (1991) Mycorrhizal interactions with soil organisms. In: *Handbook of Applied Mycology. Volume 1. Soil and Plants.* Arora, D.K., Rai, B., Mukerji, K.G. & Knudsen, G.R. (eds.), pp. 77-129. Marcel Dekker Inc., New York.

Perry, D.A., Molina, R. & Amaranthus, M.P. (1987) Mycorrhizae, mycorrhizospheres and reforestation: current knowledge and research needs. *Can. J. For. Res.*, 17, 929-940.

Phillips, J.M. & Hayman, D.S. (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.*, 55, 158-161.

Pistorius, R. & Verschuur, G. (1989) Biotechnological developments and cassava. *Biotechnol. Dev. Monit.*, 1, 11-13.

Pretorius-Gruth, I.-M., Puhler, A. & Simon, R. (1990) Conjugal transfer of megaplasmid 2 between *Rhizobium meliloti* strains in alfalfa nodules. *Appl. Environ. Microbiol.*, 56, 2354-2359.

Read, D.J., Francis, R. & Finlay, F.D. (1985) Mycorrhizal mycelia and nutrient cycling in plant communities. In: *Ecological Interactions in Soil*, Fitter A.H. (ed.), pp. 193-217. Blackwell Scientific, Oxford, UK.

Reanney, D.C., Gowland, P.C. & Slater, J.H. (1983) Genetic interaction among microbial communities. *Symp. Soc. Gen. Microbiol.*, 34, 379-421.

Richaume, A., Angle, J.S. & Sadowsky, M.J. (1989) Influence on *in situ* plasmid transfer from *Escherichia coli* to *Rhizobium fredii*. *Appl. Environ. Microbiol.*, 55, 1730-1734.

Robert, F.M. & Schmidt, E.L. (1985) Response of three indigenous serogroups of *Rhizobium japonicum* to the rhizosphere of pre-emergent seedlings of soybean. *Soil Biol. Biochem.*, 17, 579-580.

Romanowski, G., Lorenz, M.G., Sayler, G. & Wackernagel, W. (1992) Persistence of free plasmid DNA in soil monitored by various methods, including a transformation assay. *Appl. Environ. Microbiol.*, 58, 3012-3019.

Roszak, D.B. & Colwell, R.R. (1987) Survival strategies of bacteria in the natural environment. *Microbiol. Rev.*, 53, 958-965.

Scannerini, S. & Bonifante, P. (1991) Bacteria and bacteria-like objects in endomycorrhizal fungi (Glomaceae). In: *Symbiosis as a Source of Evolutionary Innovation,* Margulis, L. & Fester, R. (eds.), pp. 273-278. MIT Press, Cambridge, MA, USA.

Schmidt, E.L. & Robert, F.M. (1985) Recent advances in the ecology of *Rhizobium*. In: *Nitrogen Fixation Research Progress,* Evans, H.J., Bottomley, P.J. & Newton, W.E. (eds.), pp. 279-385. Martinus Nijhoff, Dordrecht, The Netherlands.

Shah, K., de Sousa, S. & Modi, V.V. (1981) Studies in transducing phage M-1 for *Rhizobium japonicum* D211. *Arch. Microbiol.*, 130, 262-266.

Sieverding, E. & Toro, T.S. (1988) Influence of soil water regimes on VA mycorrhizae. V. Performance of different VAM fungal species with cassava. *J. Agron. Crop Sci.*, 161, 322-332.

Sik, T., Horvath, J. & Chatterjee, S. (1980) Generalized transduction of *R. meliloti*. *Mol. Gen. Genet.*, 178, 511-516.

Silver-Dowding, E. (1955) *Endogone* in Canadian rodents. *Mycologia*, 47, 51-57.

Sivaprasad, P. & Rai, P.V. (1991) Synergistic association between *Glomus fasciculatum* and *Rhizobium* species and its effect on pigeonpea (*Cajanus cajan*). *Indian J. Agric. Sci.*,

61, 97-101.

Skujins, J. & Allen, M.F. (1986) Use of mycorrhizae for land rehabilitation. *MIRCEN J.*, 2, 161-176.

Smitley, D.R. & McCarter, S.M. (1982) Spread of *Pseudomonas syringae* pv. tomato and role of epiphytic populations and environmental conditions in disease development. *Plant Dis.*, 66, 713-717.

Staniewski, R. (1987) Morphology and general characteristics of phages active against *Rhizobium*. *J. Basic Microbiol.*, 3, 155-165.

Steffan, R.J. & Atlas, R.M. (1988) DNA amplification to enhance the detection of genetically engineered bacteria in environmental samples. *Appl. Environ. Microbiol.*, 54, 2185-2191.

Stewart, G.J., Carlson, C.A. and Ingraham, J.L. (1983) Evidence for an active role of donor cells in natural transformation in *Pseudomonas stutzeri*. *J. Bacteriol.*, 156, 30-35.

Stotzky, G. (1989) Gene transfer among bacteria in soil. In: *Gene Transfer in the Environment*, Levy, S.B. & Miller, R.V. (eds.), pp. 165-222. McGraw-Hill Inc., New York.

Stotzky, G. & Babich, H. (1984) Fate of genetically engineered microbes in natural environments. *Recomb. DNA Tech. Bull.*, 7, 163-188.

Strauss, H.E. & Levin, M.A. (1991) Use of fate and transport models in microbial risk assessment. In: *Risk Assessment in Genetic Engineering*, Levin, M.A. & Strauss, H.S. (eds.), pp. 240-271. McGraw-Hill Inc., New York.

Svab, Z., Kondorosi, A. & Orosz, L. (1978) Specialized transduction of a cysteine marker by *Rhizobium leguminosarum* phage 16-3. *J. Gen. Microbiol.*, 106, 321-327.

Swart, W.J. & Theron, J.M. (1990) Future needs of mycorrhizal research in South African forestry. *South Afr. For. J.*, 153, 31-35.

Sylvia, D. (1990) Inoculation of native woody plants with vesicular-arbuscular mycorrhizal fungi for phosphate mine land reclamation. *Agric. Ecosyst. Environ.*, 31, 253-261.

Sylvia, D.M. & Jarstfer, A.G. (1992) Sheared-root inocula of vesicular-arbuscular mycorrhizal fungi. *Appl. Environ. Microbiol.*, 58, 229-232.

Tebbe, C.C. & Vahjen, W. (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and yeast. *Appl. Environ. Microbiol.*, 59, 2657-2665.

Teng, P.S. & Yuen, J.E. (1991) Epidemic models: lessons from plant pathology. In: *Risk Assessment in Genetic Engineering*, Levin, M. & Strauss, S. (ed.), pp. 272-296. McGraw-Hill Inc., New York.

Tisserant, B., Gianinazzi-Pearson, V., Gianinazzi, S. & Gollotte, A. (1993) *In planta* histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. *Mycol. Res.*, 97, 245-250.

Visser, S., Danielson, R.M. & Parkinson, D. (1991) Field performance of *Eleagnus communatata* and *Spheherida canadensis* (Eleagneaceae) inoculated with soil containing *Frankia* and vesicular-arbuscular mycorrhizal fungi. Can. *J. Bot.*, 69, 1321-1328.

Weber, D.F., Keyser, H.H. & Uratsu, S.L. (1989) Serological distribution of *Bradyrhizobium japonicum* from United States soybean production areas. *Agron. J.*, 81, 786-789.

Weber, E., George, E., Saxena, M.C. & Marschener, H. (1991) VA mycorrhiza increase phosphate uptake and vegetative growth, but not seed yield of chickpeas in northern Syria. *Mitt. Dtsch. Bodenkd. Ges.*, 66, 749-752.

Weinberg, S.R. & Stotzky, G. (1972) Conjugation and genetic recombination of *Escherichia coli* in soil. *Soil Biol. Biochem.*, 4, 171-180.

Wellington, E.M.H., Herron, P.R. & Cresswell, N. (1993) Gene transfer in terrestrial envi-

ronments. In: *Monitoring Genetically Manipulated Microorganisms in the Environment*, Edwards, C. (ed.), pp. 156-158. John Wiley, Chichester, UK.

Yang, W.-C., Horvath, B., Hontelez, J., van Kammen, A. & Bisseling, T. (1991) *In situ* localization of *Rhizobium* mRNAs in pea root nodules: *nif*A and *nif*H localization. *Mol. Plant-Microbe Interact.*, 4, 464-468.

Zeph, L.R., Onaga, M.A., Stotzky, G. (1988) Transduction of *Escherichia coli* by bacteriophage P1 in soil. *Appl. Environ. Microbiol.*, 54, 1731-1737.

Chapter six
Microbial Pesticides: Safety Considerations

Morris Levin

Maryland Biotechnology Institute, Centre for Public Issues in Biotechnology, University of Maryland, College Park, Maryland, United States of America.

Introduction

Insects, like humans, are susceptible to a whole range of disease microorganisms. The control of insects by disease agents has long been a goal of agricultural research. As a result, microbial insect-control agents are widely used, and genetic engineering techniques are being applied to broaden their usefulness in terms of range and overall effect.

Many estimates of the commercial value of microbial pesticides are available. Meadows (1990) estimated a market value of US$100 million per year. Jutsum (1988) estimated US$160 million per year. Both point out that microbial pesticides make up a very small part, 1–2%, of total expenditures on pesticides of approximately US$20 billion annually.

The toxicity of chemical pesticides has necessitated safety controls by most countries, and there exists an international code of conduct on the distribution and use of pesticides (FAO, 1986). Although this code does not specify that it is applicable only to chemical pesticides, biological considerations, such as growth, survival and infectivity, are not mentioned.

Microbial pesticides are used because they are more selective and biodegradable, hence environmentally friendly, and it is these factors that are seen as increasing their use. For example, *Bacillus thuringiensis* is an effective pesticide because it produces toxins that are active against particular insect pests. Currently, products containing some form of *B. thuringiensis* comprise 80–90% of the microbial pesticides, both "natural" and engineered, that are purchased and used. These products contain live or killed bacteria suspended in a variety of media and at different concentrations, including native strains which have been conjugated to contain a number of different *B. thuringiensis* toxin genes, and bacteria of different species and genera (killed or live) which contain *B. thuringiensis* toxin genes.

Growing pressure to reduce the use of chemical pesticides, combined with increased research and advances in production techniques, will inevitably result in a larger market share and an increased diversity of microbial pesticides. As the market share increases, the releases into the environment will increase in scale, and more types of microorganisms will be used. The application of the DNA technology promises new strains with increased potency and a wider host range. Research has been reported on the addition of extra toxin genes to *B. thuringiensis*, the use of viruses as pesticidal agents, the addition of new toxin genes (scorpion, mite) to selected bacteria, and the direct introduction of microbial toxin genes into crop plants.

Much consideration has been given to the putative impact of large-scale use of microbial control agents - "natural" and engineered - on the environment (Brill, 1985; Chandler, 1985; Halvorson *et al.*, 1985; Lenteren, 1986; Frommer *et al.*, 1989; Tiedje *et al.*, 1989; USEPA, 1989). This chapter will focus on safety considerations involved in the field testing and large-scale use of such microbial pest-control agents. To do so calls for a discussion of the differences between natural and engineered pesticides, and a brief survey of the elements of risk assessment.

Natural versus Engineered Microbial Pest-Control Agents

Natural Agents

Natural microbial pest-control agents (MPCAs) have been used for many years. The first to be applied on a large scale, *Bacillus popilliae*, was registered for use with the United States Government in 1948, and was employed to control the Japanese beetle. *Bacillus thuringiensis* (variety berliner) was registered in 1961. It was isolated in Japan following an outbreak of disease in silkworms. Since 1948, seven bacteria, four viruses, three fungi, and one protozoan have been registered in the United States of America. Innumerable pesticides, each containing a mixture of organisms (for example, a mixture of several species of *B. thuringiensis*) or the same organism in a different type of suspension medium or concentration, have been registered for use. Over the years, researchers have improved these products. For example, they have developed, by simple laboratory procedures, more potent strains or strains which are more resistant to environmental stress.

The improvement procedures involved intensive efforts to isolate new strains (Meadows, 1990) evolved by natural selection through exposure to artificial, laboratory environments, a process similar to the development of microbes for industrial use in contained facilities (e.g., fermenters). These naturally produced strains have improved the usefulness of MPCAs and have not resulted in significant changes in regulatory scrutiny. This type of genetic manipulation takes advantage of natural forms of genetic recombination. These are conjugation, transduction, and transfor-

mation (Tortora *et al.*, 1989). Each of these phenomena has been the subject of intensive investigation.

Transformation was the first form of genetic change in nature to be identified by microbiologists; it was first reported in 1934. Transformation involves the death and subsequent lysis of a bacterial cell, followed by the release of DNA which can, under certain conditions, be taken up and utilized (i.e., inserted into the host genome) by surrounding bacteria. This allows the engineering of microorganisms, under controlled laboratory conditions, the result being new strains which are more suited to particular tasks (Watson *et al.*, 1983; Stewart, 1992).

Transduction is the outcome of viral infection in bacteria. After infection, the viral genes are expressed, resulting in the production of viral DNA and viral coat protein. This is followed by the assembly of the mature virus particles and the release of the particles from the cell. Transducing particles are formed by inappropriate packaging of host DNA in place of viral DNA (Miller, 1992).

Conjugation is the transfer of DNA between donor and recipient bacteria that requires cell-to-cell contact and is resistant to the action of deoxyribonuclease (an enzyme which digests any DNA with which it comes into contact). The process is usually plasmid-controlled, but may also be controlled by transposons. Conjugation was first identified in the early fifties (McIntire, 1992). Plasmids may be removed from a cell by a technique known as curing, the result being the loss of plasmid-associated genes. Replacement with alternate plasmids results in a different spectrum of genetic capability, such as pesticidal action. Some plasmids are easily exchanged (capable of self-transfer), while others require the presence of transfer-proficient plasmids (Tra^+) before genetic transfer can occur.

It has been shown that all of these processes occur in nature, where genetic exchange between related species is a common phenomenon. Several researchers have demonstrated that it occurs both in water and soil environments, and that the exchanged information is expressed (Stotzky & Babich, 1984; Miller, 1992; Saye & O'Morchoe, 1992; Walter & Seidler, 1992).

Genetically Engineered Agents

Molecular biology has increased the potential of improving MPCAs. The term "genetic recombination" has taken on a different meaning. Detailed knowledge about plasmids, enzymes involved in DNA metabolism, genes, and gene structure has made it possible to isolate genes and transfer them in a functional state to other organisms (Tortora *et al.*, 1989). The transfer is accomplished using a vector (e.g., plasmid, virus) into which foreign DNA has been inserted (recombinant DNA). DNA from any source can be inserted into a plasmid for use with a specific host. The plasmid can then be transferred into the host, where expression of the DNA usually occurs.

This enables the transfer of genetic information at will between diverse species, genera and kingdoms. The Ti plasmid of the genus *Agrobacterium* is an example of a plasmid used to transfer recombinant DNA from bacteria to plants. This

method, which has enabled the production of pest-resistant plants, is presented in detail in Chapter 4 by Dale and Kinderlerer. Similarly, bacteria which yield mammalian proteins or hormones have been produced using plasmids engineered to contain the corresponding mammalian genes.

Risk assessment data requirements for genetically engineered microorganisms generally include data intended to enable the assessors to determine the probability of dissemination of the genetic material to the indigenous microbial community. The issue is that, if the genetic material is transferred to another microorganism, it may be expressed, thus multiplying any effect, and/or it may be expressed differently, posing new problems (e.g., the level of expression may change). This may occur due to mobilization of transfer-deficient plasmids or self-transfer.

Experimental data derived from the diverse systems available for transferring DNA between microbes is difficult to interpret. Any of a large number of unrelated conjugal plasmids, cloning vectors, transducing phages, or transposons may be used. The data may reflect properties peculiar to the method, the environment, or the organism. Researchers recognized that some of this confusion could be alleviated if a series of plasmids, "benchmark plasmids", could be employed in different laboratories and under various environmental conditions (Zylstra *et al.*, 1992). To this end, two series of benchmark plasmids have been created: self-transmissible and non-self-transmissible. These are both based on the R388 plasmid, which carries resistance to trimethoprim and sulphonamide, making it easy to detect. The benchmark plasmids have been of value in assessing survival and transfer of genetic material in environmental situations.

If one releases large numbers of genes into new environments, bypassing the process of natural selection, one may affect the path and rate of evolution. This is due to the relationship between the genetic material, the environment, and the process of natural selection. If genetic material is added in large enough amounts, the likelihood of mutation is increased, which leads in turn to an increased likelihood of a successful mutant surviving. In addition, if the genetic material is exchanged with indigenous microbes, it may multiply and mutate. Meadows (1990) states that genetic exchange is the only risk factor unique to engineered organisms. All other factors are related to existing characteristics which have been transplanted (cross-breeding simply changes the location of genes within a species; genetic engineering results in many more copies of the genetic material in significantly different species and environments, increasing the probability of transfer and mutation).

However, to limit genetic exchange, plasmids have been produced, by altering the nucleotide sequences, that are not able to function in the exchange process (disarmed plasmids). Once inserted into select hosts, they prevent the latter from passing genetic material to other microbes. Nevertheless, under certain circumstances, helper plasmids can enter the cell, resulting in the mobilization of the disarmed plasmids (Zylstra *et al.*, 1992).

These developments have led to increased regulatory scrutiny.

Rationale for Regulatory Control

It is widely accepted that determining the risk associated with the use of biopesticides requires identification of the hazard involved and of the exposure level (NAS, 1983, 1987; NRC, 1989; OECD, 1986; United Kingdom, 1993). These two factors must be estimated, and the results must be combined in order to produce a risk assessment (NAS, 1983). On the basis of the risk assessment, regulatory officials can make decisions about the feasibility of using a particular agent. The term "risk management" refers to regulatory action taken by the governmental body involved. Management varies depending on the particular case. Different options are open to the regulator depending on the particular statute and set of regulations involved. Thus, in the United States of America, regulators operating under the Toxic Substances Control Act (TSCA) can allow applicants a one-time use for experimental purposes, or a limited use in particular locations or for specific purposes, a new application being required for each different use or location (Giamporcaro, 1993).

Hazard refers to the properties of the agent itself (e.g., virulence, potency, host range). Exposure refers to characteristics of the agent such as stability, resistance to environmental factors, and transportability, and, of course, to the amount to be used in the field. The data requirements for the risk assessment of a given product or activity reflect the components of these two factors.

Data requirements also reflect the purpose of the statute they support and the special interests of the governmental body which developed the statute. Thus, in the United States of America, the Federal Insecticide, Fungicide and Rodenticide Act specifies the type of agent which is covered (pesticide), and the protection required (human health and the environment). In some instances, in a tropical country, for example, emphasis may be placed on protecting biodiversity.

Potential Risks Concerning the Release of Biological Control Agents: General Issues

Microbes are used by man for a variety of purposes, such as the production of fermented foodstuffs, baking, and the production of speciality chemicals. Microbes developed and used for these purposes are generally benign and highly adapted to industrial settings (Brill, 1985). MPCAs differ greatly. These organisms have one role: to control a pest by inhibiting its growth or reproduction, or by causing immediate death. Also, they have been selected and/or engineered for their ability to survive in nature. Thus, the possibility of adverse effects is built in. In addition, chemical agents differ from MPCAs in that they do not reproduce in nature. That being the case, risk assessment procedures for chemical agents need not take into account the growth and survival issue. Therefore, guidelines and protocols have been developed (e.g., USEPA, 1989) for assessing risks associated with the environmental use of microbial pesticides. The guidelines (Table 6.1) describe in

Table 6.1. Major components of points to consider[a]

I SUMMARY OF TRIAL
- Objective
- Feasibility
- Benefits and risks
- Justification

II GENETIC CHARACTERISTICS OF ORGANISMS (PARENT AND RECIPIENT)
- Identification
  - Taxonomic description
  - Methods used for taxonomy
- Genotype
  - Characterization of genetic material:
    Chromosome, transposon, plasmid
- Potential for gene transfer
  - Capability of transduction, transformation, conjugation
  - Evidence for exchange in nature
- Phenotype
  - Rationale for selection
  - Anticipated changes in host
  - Culture requirements, life cycle, habitat
  - Pathogenicity data (type, virulence)
  - Antibiotic resistance and production
  - Survival and persistence data
  - Control mechanisms:
    Natural agents, effective disinfectants

III INTRODUCTION OF GENETIC MATERIAL
- How modified
  - Source and function of inserted DNA
  - Methods used for identifying, isolating and inserting the DNA
- Vector
  - Identification
  - Site of gene insertion
  - Method of introduction to host
  - Characterization of inserted genes:
    Location, amount, stability, remaining vector DNA
    Comparison of MPCA with parent
  - Laboratory data describing relative survival, persistence, multiplication and dissemination

Table 6.1. cont.

IV ENVIRONMENTAL CONSIDERATIONS (PARENT AND RECIPIENT)

Organism

- Habitat
- Survival factors:
  - Microcosm data, environmental conditions favouring or adversely affecting survival and growth
- Data describing survival, replication, dissemination and potential for biological interaction
- Identification of specific potential adverse effects

Trial

- Conditions of trial
- Location
- Site characteristics:
  - Probability of dissemination, description of target and non-target population present
- Containment
- Procedures to be employed:
  - Onsite containment procedures (physical, biological), transportation procedures, employee training, security procedures

Monitoring

- Procedures to be employed:
  - Description of techniques (discuss sensitivity and reliability)
  - Discuss available data on recovery, sensitivity and reliability of techniques with MPCA and parent

Mitigation procedures

- Termination procedures
- Disposal procedures
- Disinfection procedures

[a] Table summarizes points that should be considered from Australian, Canadian, EC, New Zealand, OECD and United States (EPA, USDA) sources.

general terms the type of data which could be required. These are known as "points to consider". It is assumed that the biological agent will be dispersed on a large scale and at a high concentration. Also, that it is subject to environmental effects in the sense of drift due to high winds and/or possible re-growth and subsequent secondary spread. Not all of the parameters identified in the "points to consider" document would be relevant for all MPCAs. The guidelines are meant to identify all possible issues and provide a menu for the selection of items which are relevant to a particular case. The parameters identified in Table 6.1 appear in the guidelines or requirements (if no guidelines are available) of Australia (1990),

Canada (Agriculture Canada, 1993), the European Community (CEC, 1990), New Zealand (1992), OECD (1990) and the United States of America (USEPA, 1989; USDA, 1990). This commonality reflects the scientific basis for evaluating the hazard and exposure factors involved in releasing MPCAs into the environment. In all cases, the lists of parameters were prepared by panels of academic, government and industry scientists. These factors are most clearly elucidated in a special feature article by Tiedje *et al.* (1989). The article discusses the parameters with regard to the potential for environmental impact. The Australian guidelines, published shortly after the article appeared, were developed independently, but are very close in content and structure.

Other experts (Kalmakoff & Miles, 1980; Day & Fry, 1990; Fry & Day, 1990; Meadows, 1990) have discussed the potential interactions between released organisms and the environment in terms of the importance of various environmental parameters. Meadows lists the known environmental considerations (e.g., temperature, moisture, nutrient availability) and discusses their significance as factors in the persistence and survival of microbes. Fry and Day examine the issue in general and use *B. thuringiensis* as an example for an extensive discussion of specific effects with actual data. They point out that there is no evidence of adverse ecological or health effects despite widespread and heavy utilization of the organism as a pesticide. More recently, it has been reported that pests have developed resistance to the *B. thuringiensis* toxin (USDA, 1992). Although this is not an adverse ecological effect, it has been suggested that the potential loss of this MPCA as an effective pesticidal agent be considered as part of the risk assessment.

The Tiedje article and the Australian guidelines are excellent examples of attempts to provide a guide describing the amount of information required for a credible risk assessment. In both cases, four attributes are identified:

1. The genetic alteration.
2. The phenotype of the wild-type organism.
3. The phenotype of the engineered organism.
4. The specific environment involved.

Each attribute is defined by seven to nine items which delineate particular characteristics. The level of consideration required for each item under each attribute is specified. The level is determined by the specific alteration made, the degree of knowledge available about the attribute, or some inherent characteristic of the attribute. Genetic alteration, for example, is defined as requiring information about the character and stability of the added DNA, the nature of the alteration (e.g., deletion, alteration), the function and source of the DNA, the vector and its source, as well as information about any vector RNA or DNA which remains in the altered genome. In all cases, the more information available (either from the general scientific literature or from experiments done to specifically develop data for the organism in question), the less the need for risk assessment consideration. For example, if the source of the vector or transferred DNA is a pathogen, additional information

will be required as to the relationship of the vector or DNA to the pathogenicity of the donor.

The phenotype attributes specify characteristics such as level of domestication, ease of control, pest status, survival, range and prevalence of gene exchange for the wild-type organism and infectivity, changes in substrate utilization, resistance to disease or natural enemies, as well as changes in susceptibility to antibiotics, changes in environmental limits, and similarity to previously released phenotypes for the altered organism. Thus, if the environmental limits have been broadened, additional scrutiny is called for. If there has been no change or the limits have been narrowed as a result of genetic alteration, no additional scrutiny is needed, and the category may be ignored if literature data or experimental data support such a conclusion.

Table 6.2 identifies data requirements for an MPCA and indicates which are necessary in all cases and which may be called for under particular circumstances. Thus, a detailed analysis of the product and some information about the toxicology of the MPCA, as well as information about anticipated and known effects on non-target organisms are required in all cases, while in-depth toxicity testing and hazard information may be required if early data warrant it. The requirements listed in Table 6.2 are taken from the USEPA Subdivision M document (USEPA, 1989). However, the requirements established by USDA (1990), Australia (1990), OECD (1990), European Community (CEC, 1990), New Zealand (1992), and Canada (Agriculture Canada, 1993) are similar. In 1973, the World Health Organization proposed a tentative scheme for evaluating the efficacy and safety of MPCAs for pest control (WHO, 1973; see also Kalmakoff & Miles, 1980). Five stages were proposed, in a manner similar to the USEPA tier system. Information about the organism (identification, characterization, non-target as well as target effects), and vertebrate infectivity tests were suggested as initial data requirements. If a review of the initial data warranted it, field data and more detailed infectivity and host-range data would be requested. In the USEPA scheme, the "may be required" items are called for if the required items indicate a significant potential for adverse impact. It must be pointed out that the required items are not mandatory in all cases. The USEPA pesticide office has a waiver procedure for particular data requirements if it can be shown that they are not germane to the risk assessment. An applicant may request a waiver if sufficient justification can be provided. For example, a product containing *B. thuringiensis* as the active ingredient might have the toxicology requirements waived on the basis of a long history of safe utilization.

The data required in the areas listed in Table 6.2 would provide regulators with an indication of the "probability of occurring" and the potential effects. It would offer information concerning all aspects of the organism involved, possible interactions with the environment at the test site, long-term effects, genetic exchange, and containment and monitoring issues. Information about these parameters has been requested by most of the countries promulgating risk assessment procedures. Some countries request or develop information about social, cultural and economic

Table 6.2. Summary of requirements for registration of an MPCA[a]

Product analysis	Toxicology	Hazards to non-target organisms
Required:		
Product identity	Acute toxicology	Effects on:
Manufacturing process	Hypersensitivity incidents	Terrestrial and aquatic wildlife
Properties (physical and chemical)	Results of tissue culture tests (for viral agents)	Non-target plants and insects
Inactive ingredients		
Submission of sample		
May be required:		
	Acute toxicity	Chronic pathogenicity testing
	Reproductive effects	Fish life cycle studies
	Oncogenicity	Simulated or actual field testing

[a] In addition, if the required data indicate a potentiol problem, *environmental expression data* may be required. These data would document the MCPA's ability to survive and replicate in the environment proposed for use. Based on USEPA requirements (USEPA, 1989).

impact. There is no agreement concerning the advisability of including risk assessment data requirements for these parameters. There is no doubt, however, that social, cultural and economic issues will play a role in the final decisions made by regulatory agencies about particular products, but this will depend on the product and the country where its use is applied for. What may be acceptable in one location may not be acceptable elsewhere. The rationale for rejection may be based on "hard science" information (differences in climate, diversity of endogenous species in the new environment), or on economic or social issues - the product may threaten a segment of the local agro-industry, or it may be a potential threat to a natural resource which is so important that even a low-probability effect is not tolerable.

Protocols describe methods to develop the data, and thus assure data quality and reliability. They describe specific assays and techniques to develop required information about the product, its toxicology, the hazard to non-target organisms, environmental considerations (expression *in situ*), and product performance. They provide detailed information about the assay procedures (e.g., number of samples, replicates, conditions of the test, duration, etc.). Protocols were initially developed

Table 6.3. Data points for hazard assessment

1.	TEST PRINCIPLES
	Test at multiple of anticipated dose
	Maximum hazard dose used if positive results obtained
2.	SINGLE SPECIES TESTS
	Avian single dose oral toxicity
	Avian dietary toxicity
	Freshwater fish acute bio-assay
	Freshwater invertebrate test
	Non-target plant
	Non-target insect
	Honey bee
	Estuarine non-target species

because data produced by different techniques and using different standards were not sufficiently reliable and comparable to permit a credible risk assessment. They are developed by the regulatory body and are tested before being issued or recommended. Tables 6.3 and 6.4 list protocols available for determining hazard (Table 6.3) and exposure (Table 6.4). These protocols are listed and fully described in the USEPA Subdivision M (USEPA, 1989).

In 1991, the Royal Commission on Environmental Pollution issued a report which described potential effects of engineered organisms and regulatory mechanisms, as well as control and mitigation techniques. The report defined a proposed computerized scheme for risk assessment, GENHAZ, which would provide re-

Table 6.4. Data points for exposure assessment

SPECIFIC DATA REQUIRED:
Biological fate of gene
• Habitat of MPCA
• Survival and replication factors
• Gene flow
• Gene construct, probability of transfer and expression
• Expression level
Chemical fate of gene
• Fate of gene/gene product in soil
• Fate of gene/gene product in water

viewers with a structured list of information requirements relating the proposed test, use, and organism to potential impacts. It also provided a format for discussing and identifying the specific data in order to estimate exposure and hazard in a particular case. In general, this scheme required more data input than procedures currently in use elsewhere. In 1993, the Commission decided to terminate the project.

Containment and Mitigation

Two types of containment of microorganisms have been proposed: physical and biological. Physical containment implies the use of structures (greenhouses) or netting (in the case of engineered insects) in order to eliminate the possibility of dispersal of the engineered organisms. Biological containment can be achieved in two ways. The organisms can be used or tested in a location inimical to its survival and/or dispersal (e.g., tested on plots in a climate which requires continuous alteration in order to permit survival - a desert climate may be suitable if enough water is supplied), or the organisms can be altered to preclude survival outside a limited area. The latter can be accomplished by the inclusion of a suicide gene or the alteration of the organism's ability to utilize nutrients. For example, *Escherichia coli* K12 requires specific nutrients not normally present in environmental situations.

The use of chemicals, heat treatment, or other forms of sterilization is not considered confinement, but rather mitigation, that is, removing the organism from the site and minimizing effects.

Confinement means controlling and minimizing spread. Dissemination can be minimized, and effects can be managed. Quarantine standards have been applied with success and permit the testing of organisms in varied geographical locations. It is necessary to understand the organism involved, its mode of replication, its reactions to environmental stress, and, in particular, its host range. A judicious choice of location can effectively confine an organism to a particular area. Pathogens require specific hosts in order to survive and persist. Organisms which cannot tolerate extreme cold are better tested in regions with severe winters. While a small percentage of the population may survive, the level will be low enough to preclude significant adverse effects.

Only one disease, smallpox (Fenner *et al.*, 1988), has been eradicated in the history of mankind, and this was done at great expense and after many years of concerted effort. However, it is possible to control microbial population levels by using specific methods. These controls can have effects in the short, intermediate or long term (Table 6.5). The selection of a method depends on the particular situation, feasibility, and costs involved. In the case of a microbe which has infected animals or plants, attempts at confinement by incineration or deep burying of the infected material may be called for.

Chemicals may be employed to decontaminate fields (Table 6.6), but they will not completely eradicate the target population. The total population will be re-

Table 6.5. Controlling unwanted microorganisms

Immediate[a]	Short term[b]	Long term[c]
Fumigation	Fumigation	Fumigation
Flooding	Flooding	Flooding
Chemicals	Chemicals	Erosion control
	Erosion control	Soil amendments
	Soil amendments	

[a] Hours to several days to achieve effect.
[b] Up to three years to achieve effect.
[c] Longer than three years to achieve effect.
Source: Adapted from Vidaver and Stotzky (1992).

duced, perhaps to undetectable levels, but total eradication is not assured. The indigenous populations of microbes will be replenished from edges of the field, or by tilling in soil from adjacent fields, and this will limit the re-growth of the target microbe. Care must be used in applying chemicals since all are toxic.

Biological control has been suggested, and ongoing research indicates a possibility for containment and control (Cuskey, 1992). Plasmids containing environ-

Table 6.6. Common control chemicals

Common name	Chemical name	Dosage (litres/ha)	Plant toxicity[a]	LD_{50}[b]
Methyl bromide	Bromomethane	450–900[c]	Toxic	1
Ethylene dibromide (EDB)	1,2-Dibromomethane	19–94	Toxic	150
Chlorinated hydrocarbons	1,2-Dichloropropane, 1,3-dichloropropene and others	100–500	Toxic	140
Methylisothiocyanate	Same	600–1200	Toxic	280–650
Dibromochloropropane (DBCP)	1,2-Dibromo-3-chloropropane	19–38	Toxic	172
Chloropicrin	Trichloronitromethane	300–500	Toxic	1

[a] Pertains to crop plants.
[b] Mammalian.
[c] kg/ha.
Source: Adapted from Vidaver & Stotzky (1992).

mentally-controlled lethal genes provide a mechanism to eradicate the altered microbe if the selected environmental stimulus changes. Two types of lethal genes have been used: one which attacks the cell's DNA and one which attacks the cell wall (i.e., lyses the cell). Control can be achieved by the presence of a repressor gene which is activated by a particular compound in the environment. Alternatively, the lethal gene is controlled by a convertible promoter gene, such as *fimA*, which is active in one orientation which it achieves on a random basis. Any MPCA containing this combination of lethal and promoter genes would ultimately die off. The use of these techniques would provide safety assurance to risk assessors. However, these methods have not been tried in the field. There is a possibility of mutation of the lethal gene or of the control gene. There is also the issue of the lytic enzyme or DNA-degrading enzyme affecting other microbes.

Summary

The issues involved in assessing the risk of using microbial pesticides have been considered by many regulatory bodies and by panels of scientists convened by regulators or scientific organizations. In general, the recommendations are similar, regardless of the origin or make-up of the group. This similarity speaks for the science base for assessing risks. The application of critical thinking to determine data requirements, and the use of population-theory and ecological principles as foundations have resulted in agreement as to the basic information needs for decision making.

The important parameters and the need for measurements are clearly delineated. However, some authors have pointed out the need for additional research to provide better means for developing necessary data (McCormick, 1986; Levin *et al.*, 1987). McCormick points out the need for greater sensitivity in field-test methodology. Levin and colleagues describe the research being conducted at USEPA to provide more sensitive and reliable techniques.

The application of the findings, or data, derived for risk assessment purposes is subject to interpretation based on the regulatory framework in which it is perceived. The same product may not be acceptable in all situations or locations. As indicated above, factors relevant to a particular product, geographical area, or economic situation may affect the decision.

There has been much discussion about whether the focus should be on the process or the product. In general, one cannot evaluate a product without some knowledge of the process. Thus, in all regulatory schemes, information about the process is required. However, this requirement is not intended to imply that, simply because a product is the result of biotechnology research, it is hazardous and requires risk assessment. Rather, as it has been illustrated, regulatory attention is paid to all releases into the environment, with similar risk-based decisions made for all products.

References

Agriculture Canada (1993) Regulation of Agricultural Products. Plant Industry Directorate, Ottawa.

Australia (1990) Australian Guidelines for Large Scale Work with Genetically Manipulated Organisms. Australian Government Public Service, Canberra.

Brill, W. (1985) Safety concerns and genetic engineering in agriculture. *Science*, 227, 381-384.

CEC (1990) Council Directive of 23 April 1990 on the deliberate release into the environment of genetically modified organisms. Reference no. 90/220/EEC. *Official Journal L117*, Volume 33, 8 May 1990. Commission of the European Communities, Brussels.

Chandler, J.L.R. (1985) New mechanistic models for risk assessment. *Fundam. Appl. Toxicol.*, 5, 634-652.

Cuskey, S.M. (1992) Biological containment of genetically engineered organisms. In: *Microbial Ecology, Principles, Methods and Applications*, Levin, M.A., Seidler, R.J. & Rogul, N. (eds.), pp. 911-918. McGraw-Hill Inc., New York.

Day, M.J. & Fry, J.C. (1990) Microbial ecology, genetics and risk assessment. In: *Release of Genetically Engineered and Other Microorganisms*, Fry, J.C. & Day, N.J. (eds.), pp. 160-166. Cambridge University Press, Cambridge, UK.

FAO (1986) International Code of Conduct on the Distribution and Use of Pesticides. FAO, Rome.

Fenner, F., D.A. Henderson, N.J. Arita, L., Jezek, Z. & Laduyi, I.D. (1988) *Smallpox and its Eradication*. WHO, Geneva.

FIFRA (1984) 40CFR 158 and 162. Washington, DC.

Frommer, W., Ager, B., Archer, L., Brunius, G., Collins, C.H., Donikian, R., Frontali, C., Hamp, S., Houwink, E.H., Kuenzi, M.T., Kramer, P., Lagast, H., Lund, S., Mahler, J.L., Normand-Pleisler F., Sargeant, K., Tuijnenburg, G., Vranch S.P. & Werner, R.G. (1989) Safe biotechnology. *Appl. Microbiol. Biotechnol.*, 30(6), 541-552.

Fry, J.C. & Day, M.J. (1990) Release of genetically engineered and other microorganisms. In: *Release of Genetically Engineered and Other Microorganisms*, Fry, J.C. & Day, N.J. (eds.), pp. 120-136. Cambridge Press, Cambridge, UK.

Giamporcaro, D. (1993) Law and policy on the frontier: the regulation of bioremediation of hazardous waste. In: *Biotreatment of Industrial Waste*, Levin, M.A. & Gealt, M. (eds.), pp. 137-167. McGraw-Hill Inc., New York.

Halvorson, H., Pramer, D. & Rogul, M.R. (1985) *Engineered Organisms in the Environment*. ASM, Washington, DC.

Jutsum, A.R. (1988) Commercial application of biological control: status and prospects. *Philos. Trans. R. Soc. Lond.*, B318, 357-373.

Kalmakoff, J. & Miles, J.A.R. (1980) Ecological approaches to the use of microbial pathogens in insect control. *Bioscience*, 30(5), 344-347.

Lenteren, J.C. (1986) Evaluation, mass production, quality control and release of entomopathogenous agents. In: *Biological Plant and Health Protection*, Franz, J.M. (ed.) pp. 378-403. Gustav Fischer Verlag (VCH Pubs.), New York.

Levin, S.A. (1988) Safety standards for the environmental release of genetically engineered organisms. *Trends Biotechnol.*, 6(4), 547-549.

Levin, M.A., Bourquin, A.P., Barkay, T. & Fowle, J. (1987) EPA developing methods to

assess environmental release. *Bio/Technology*, 5, 33-37.

McCormick, D. (1986) Detection technology. *Bio/Technology*, 4, 419- 421.

McIntire, S. (1992) Analysis of conjugation in bacteria. In: *Microbial Ecology, Principles, Methods and Applications*, Levin, M.A., Seidler, R.J. & Rogul, M. (eds.), pp. 199-228. McGraw-Hill Inc., New York.

Meadows, M. (1990) Environmental release of *Bacillus thuringiensis*. In: *Release of Genetically Engineered and other Microorganisms*, Fry, J.C. & Day, N.J. (eds.), pp. 120-136. Cambridge University Press, Cambridge, UK.

Miller, R.V. (1992) Methods for evaluating transduction. In: *Microbial Ecology, Principles, Methods and Applications*, Levin, M.A., Seidler, R.J. & Rogul, N. (eds.), pp. 229-251. McGraw-Hill Inc., New York.

NAS (1983) *Risk Assessment*. National Academy Press, Washington, DC.

NAS (1987) *Introduction of Recombinant DNA-Engineered Organisms into the Environment: Key Issues*. National Academy Press, Washington, DC.

New Zealand (1992) Checklist for Researchers Submitting a Proposal to Field Test a Genetically Modified Organism (GMO). GMO Interim Assessment Group, Ministry for the Environment, Wellington.

NRC (1989) *Field Testing Genetically Engineered Organisms: Framework for Decisions*. National Academy Press, Washington, DC.

OECD (1986) Recombinant DNA Safety Considerations. OECD, Paris.

OECD (1990) Good Developmental Principles: Guidance for the Design of Small Scale Field Trials with Genetically Modified Plants and Microorganisms. DST1/STP/BS (90)1. OECD, Paris.

Royal Commission on Environmental Pollution (1991) 14th Report: GENHAZ. HMSO, London.

Saye, D. & O'Morchoe, S.B. (1992) Evaluating the potential for genetic exchange in natural environments. In: *Microbial Ecology, Principles, Methods and Applications*, Levin, M.A., Seidler, R.J. & Rogul, N. (eds.), pp. 283-311. McGraw-Hill Inc., New York.

Stewart, G.J. (1992) Natural transformation and its potential for gene transfer in the environment. In: *Microbial Ecology, Principles, Methods and Applications*, Levin, M.A., Seidler, R.J. & Rogul, N. (eds.), pp. 253-282. McGraw-Hill Inc., New York.

Stotzky, G. & Babich, H. (1984) Fate of genetically engineered microbes in natural environments. *RDNA Tech. Bull.*, 7(4), 163-189.

Tiedje, J.M., Colwell, R.K., Grossman, Y.L., Hodson, R.E., Lenski, R.E., Mack, R. N. & Regal, P.J. (1989) The planned introduction of genetically engineered organisms: Ecological considerations and recommendations. *Ecology*, 70(2), 298-315.

Tortora, G.J., Funke, B.R & Case, C.L. (1989) *Microbiology*, pp. 201-228. Cummings, New York.

United Kingdom (1993) *A Guide to the Genetically Modified Organisms (Contained Use) Regulations 1992*. HMSO, London (ISBN 0 11 882049 4).

USDA (1990) Guidelines for Research with Genetically Engineered Microorganisms Outside Contained Facilities. USDA, Washington, DC.

USDA (1992) Scientific evaluation of the potential resistance to the *Bacillus thuringiensis* delta endotoxins. USDA, Washington, DC.

USEPA (1989) Subdivision M of the pesticide testing guidelines. NTIS PB89-211676.

Vidaver, A. & Stotzky, G. (1992) Overview: confinement, decontamination and mitigation. In: *Microbial Ecology, Principles, Methods and Applications*, Levin, M.A., Seidler, R.J. & Rogul, N. (eds.), pp. 781-797. McGraw-Hill Inc., New York.

Walter, M.V. & Seidler, R.J. (1992) Measurement of conjugal gene transfer in terrestrial ecosystems. In: *Microbial Ecology, Principles, Methods and Applications*, Levin, M.A., Seidler, R.J. & Rogul, N. (eds.), pp. 311-326. McGraw-Hill Inc, New York.

Watson. J.D., Tooze, J. & Kurtz, D.T. (1983) *Recombinant DNA: A Short Course*. W.H. Freeman, New York.

WHO (1973) The uses of viruses for the control of insect pests and disease vectors. WHO Tech. Report, Ser. No. 531. WHO, Geneva.

Zylstra, G.J., Cuskey, S.M. & Olson, R.H. (1992) Construction of plasmids for use in survival and gene transfer research. In: *Microbial Ecology, Principles, Methods and Applications*, Levin, M.A., Seidler, R.J. & Rogul, N. (eds.), pp. 363-371. McGraw-Hill Inc., New York.

Chapter seven
Safety in the Contained Use and Release of Transgenic Animals and Recombinant Proteins

Don Powell

Cell Determination Laboratory, Department of Development and Signalling, The Babraham Institute, Babraham, Cambridge, United Kingdom.

Introduction

In one sense, genetic manipulation has been practised for thousands of years in the breeding of various species of domestic animals. It has taken but a few hundred years for humans to derive, from a common wolf stock, dogs as diverse as the Pekinese and the Great Dane. In these breeding efforts, whether "scientifically" designed or not, animals displaying the "desirable" traits are allowed to breed, and the "undesirable" traits are bred out. This type of genetic improvement, upon which all classical breeding experiments are founded, relies on chromosomal recombination and the random assortment of chromosomes to bring together desirable traits - a lengthy and costly business. Modern technologies in animal breeding represent a dramatic change: nuclear transfer, cloning, sexing, and transgenic biology may generate dramatic shifts in the phenotypes of animals. These changes may bring new benefits - but what problems to they pose? This chapter discusses the nature of transgenic animals and recombinant proteins within the framework of the impact of these technologies on the environment. It will include references to existing regulations, especially those of the United States of America and the United Kingdom, which have taken different approaches to the same problems. It is interesting to note that, whereas the United States Department of Agriculture (USDA) has approved over 300 releases of transgenic plants, only a single contained release of a transgenic animal (carp) has been fully implemented. This is accounted for by the fact that the great complexity of the traits being introduced into animals has raised numerous questions at the scientific level and has been frequently the source of public concern.

Scope of Definitions

The remit begs the question: what defines a genetically manipulated organism (GMO), a transgenic animal, or a recombinant protein? A transgenic animal is a simple concept; in essence, it is any animal whose genome contains DNA sequences (a transgene) not found in either parent. In research experiments, this DNA may direct the synthesis of a functional protein, such as growth hormone or α_1-antitrypsin; it may direct synthesis of a marker protein, such as ß-galactosidase; or it may simply serve by itself as a DNA marker in the genome. Each of these is an example of transgenesis, although only the first category is designed to alter the physiology of the animal. This is the category on which this chapter will concentrate. The transgenic animal may be derived by one of several routes (see "Production of recombinant genomes" below), each of which will bestow different characteristics on the organism.

Recombinant proteins are derived from DNA that has been manipulated *in vitro* (rDNA), and may be produced by joining together "natural" gene sequences, by deleting gene sequences, or by adding synthesized DNA sequences. A narrow definition of a recombinant protein is that it contains sequences that differ from those found in nature. A broader definition, and one that is usually applied, is that a recombinant protein is a protein synthesized from an exogenous gene or transgene, whether in *Escherichia coli*, yeast, animal cells in culture, or a transgenic animal. The following section argues that this definition is an unfortunate one for those concerned with safety.

Are Recombinant Genes and Proteins Special?

Recombinant genes and genomes are special because of their means of production. They are founded on technologies that are only about 15 years old. But what consequences does this have for their safety? The safety of any product, whether biological, chemical, or physical, is defined by its *behaviour*, or *properties*, and not by its method of production (Miller, 1991). The safety of an automobile is defined by its behaviour in safety tests, and not by whether it is made by hand or on a production line. In this sense, GMOs do not form a special category because of their means of production, and it is widely accepted that this means of production is not associated with special risk categories. If the behaviour or properties of a recombinant product (gene or protein) differ from those of natural products, then it is important to assess the implications of that novel behaviour.

For example, a transgene may be less stable than an endogenous gene. This is an example of a property that must be addressed in assessment. However, it is the biological properties of the novel genotype that determine behaviour in the environment. This applies as much to novel "natural" genomes as to recombinant genomes. The release of the rabbit, an entirely novel, but also an entirely "natural" genome, in Australia has had wide-ranging consequences for the ecosystem, and has caused damage on a scale that is unlikely to result from most planned releases of transgenic animals.

In a similar way, recombinant proteins do not necessarily represent a special risk category because of their means of production. Although genetic manipulation may be used to produce a protein with properties that are not found in nature, in order to determine the hazards associated with that protein, one will largely follow the same guidelines used to assess any novel food, drug, or industrial component. The environmental consequences of recombinant genomes thus do not differ in kind from the consequences of natural, but exogenous genomes.

Production of Recombinant Genomes

Transgenic Animals

The history of producing transgenic animals is a little over ten years old (Brinster *et al.*, 1981). During this time, several mechanisms by which DNA can be incorporated into an animal have been developed (Fig. 7.1, Table 7.1).

Microinjection. DNA is injected into the pronucleus of the fertilized egg (usually into the larger, male pronucleus); this mechanism has been used to generate the vast majority of transgenic mouse lines, as well as the transgenic lines of almost all livestock species. Microinjection has also been used to generate transgenic fish and poultry (Chen & Powers, 1990; Love *et al.*, 1994). In these cases, because the nucleus is either poorly visible or not detectable at all, the DNA is often deposited in the cytoplasm. Although this method is largely reliable, the site of integration is random, the number of copies of DNA that integrate can be unpredictable (though this can be targeted towards a range of zero to two or three copies by using low concentrations of DNA), and a proportion of animals will be mosaic (not all cells contain the transgene). However, breeding programmes meant to establish the transgenic line can be used to select individuals containing only one copy, to determine that expression is at appropriate levels, and to establish that the homozygous transgene is stable in the host genome. Once these parameters are determined, the transgene will behave as any "normal", endogenous, host gene.

Embryonic-Stem (ES) Cells. Embryonic-stem (ES) cells are cells derived from the early embryo that can be grown in culture and then returned to a recipient embryo, where their progeny will contribute to all the tissues of the developing organism (Evans & Kaufman, 1981). During the culture period, foreign DNA can be introduced into the cells using simple methods, and the expression and stability of the transgene can be verified. The major advantage of ES cells is that very subtle modifications can be introduced into the host genome (Thomas & Capecchi, 1990). Constructs can be designed that are homologous with a host gene, but contain an interruption or modification of that gene's sequence. In rare cases, the DNA transfection *in vitro* will lead to integration into the endogenous gene, based on the homology between the transgene and the endogenous gene. In this way, those gene sequences can be altered at any level of subtlety, from a single base-pair change to

(a) Microinjection

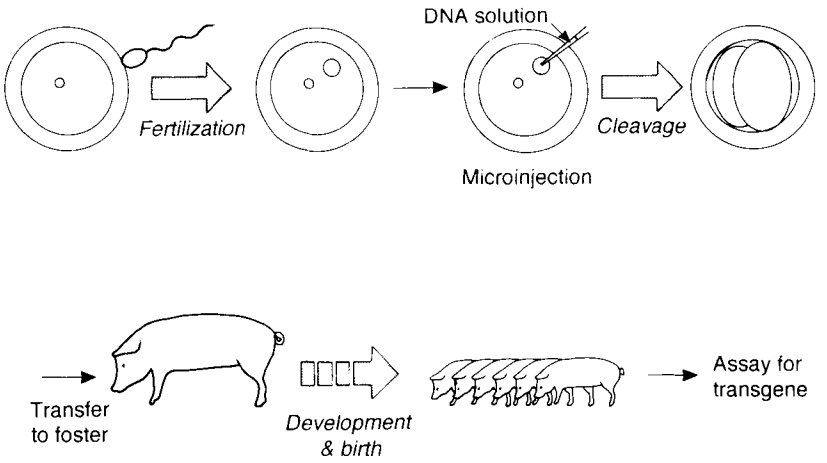


Fig. 7.1. Four major techniques for generating transgenic animals.
(a) DNA is most commonly introduced into embryos by **microinjection.** Shortly after fertilization, a glass needle is inserted into the larger male pronucleus and 10^{-12} ml injected. The injected embryos are transferred to foster mothers and allowed to develop to term. Approximately one in eight manipulated embryos survives the treatment; of these, about one in five contains integrated transgene copies.

(b) ES cells

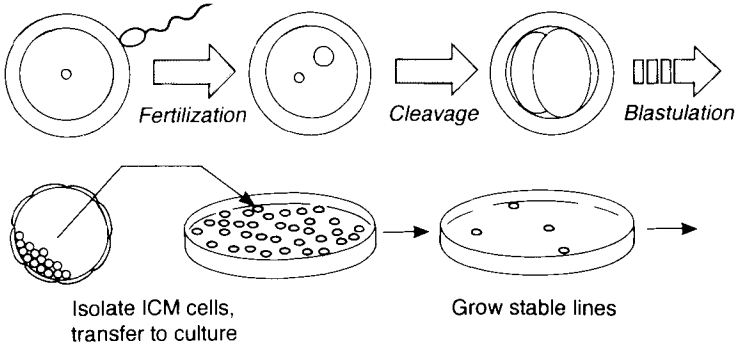


Fig. 7.1. cont.

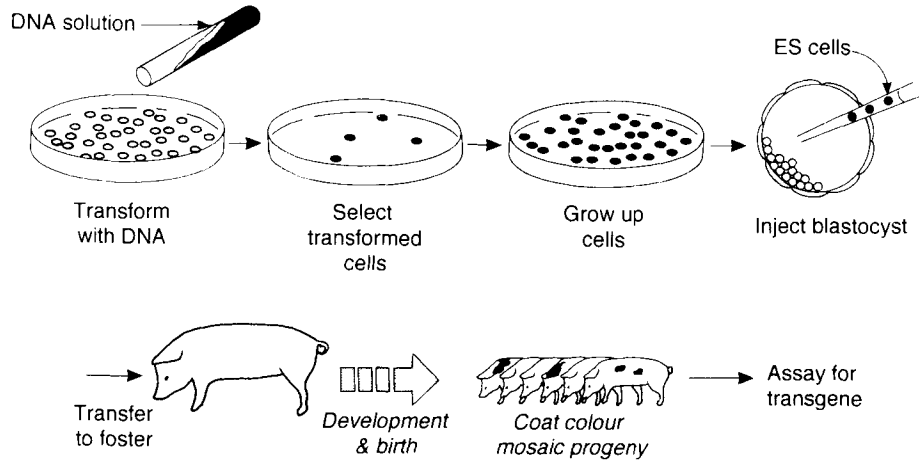


Fig. 7.1. cont.
(b) In a more recent technique, early **embryonic cells** derived from the inner mass cells of the blastocyst are established in culture dishes. DNA can be introduced simply onto these embryonic stem (ES) cells *in vitro* and appropriately transformed cells containing the transgene selected, isolated and grown up. A small number of selected cells is injected into a recipient blastocyst where they will divide and may contribute to all the tissues of the resulting animal. If the animals from which donor and recipient blastocysts are derived have different coat colours, then mosaic individuals containing transgenic cells can be identified by patches of donor cell coat colour. The success rates of such manipulations are high - routinely one in two mice contains some transgenic cells.

(c) Retroviral vectors

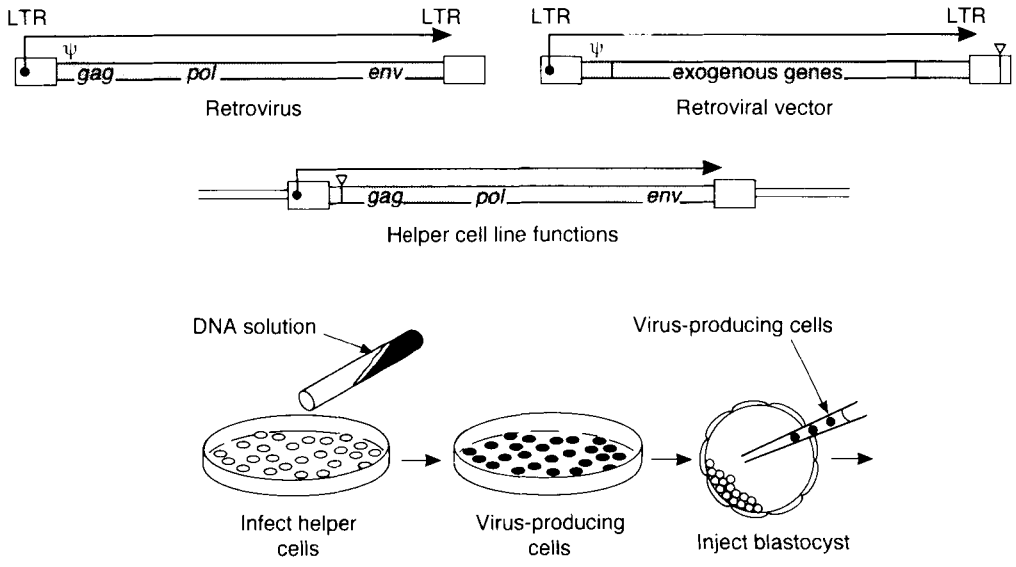


Fig. 7.1. cont.

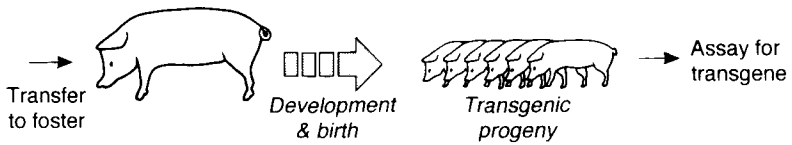


Fig. 7.1. cont.
(c) Another method is to use disabled **viral vectors**; by genetic engineering, vectors have been generated that contain only the sequences (called ψ sequences) required for integration of viral DNA into the host chromosome. These deleted viral vectors can carry about 7000 base-pairs of foreign DNA. Helper cells provide the viral functions but contain no ψ sequences. Using these cultured cells, the foreign DNA/viral vector can be packaged into virus particles and these defective virus particles injected into early embryos where they enter the cell and allow the transgene to be integrated into the host chromosome with high efficiency.

(d) Sperm-mediated transfer

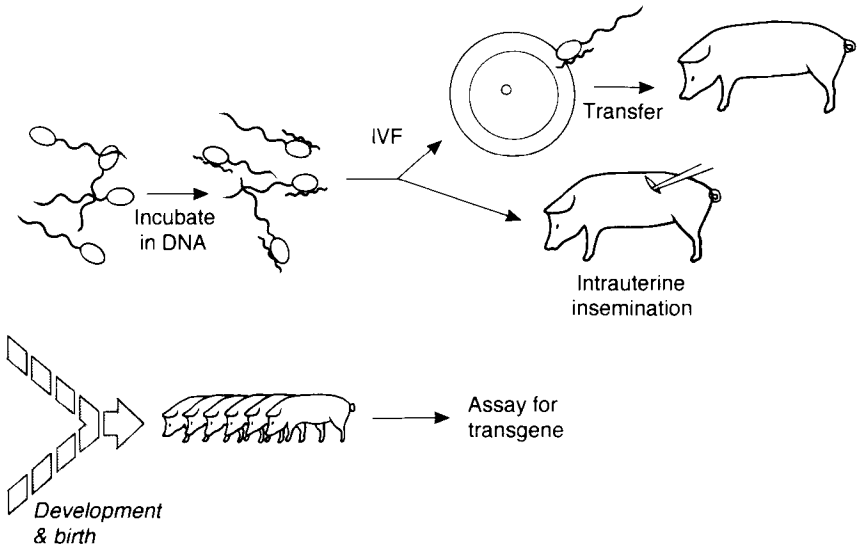


Fig. 7.1. cont.
(d) In **sperm-mediated transfer**, DNA is mixed with sperm in a specially formulated medium. The DNA becomes attached to the sperm (and some may enter). The treated sperm may be used for *in vitro* fertilization or for surgical insemination by intrauterine implantation.

deletion of much of the protein-coding region (Bradley, 1993). So far, this sophisticated technology applies only to mammalian species, and principally to rodents. Much effort and considerable expense is devoted around the world to obtaining reliable ES cell cultures from domestic species in order to use these invaluable techniques with agriculturally important species (Notarianni *et al.*, 1990).

Retroviral Vectors. Retroviral vectors have held much promise since their development in the early 1980s (Miller *et al.*, 1983). Retroviruses are RNA viruses that make a DNA copy of themselves during replication; this DNA copy then integrates into the genome of the host cell. Using recombinant DNA technology, the genes that code for viral proteins can be removed, leaving only those sequences that are necessary for the integration of DNA into the host genome. Foreign DNA can then be added to these viral sequences and transferred to recipient cells using a "helper" cell line that provides the deleted viral functions. Because of problems regarding expression stability and fears about the safety of viral vectors, such experiments have been limited in animal biotechnology. However, they have been used to produce transgenic poultry (Salter & Crittendon, 1989; Chen *et al.*, 1990) (in which retroviruses were first discovered), and they have also been the only tool used to date for human gene therapy (Anderson, 1992; Fox, 1993), and may be resurrected for livestock species.

Sperm-Mediated DNA Transformation. Sperm-mediated DNA transformation is the technique of potentially widest application, but is also that with the most unreliable history (Brinster *et al.*, 1989; Gandolfi *et al.*, 1989; Lavitrano *et al.*, 1989). Because it is a very simple technique (it basically consists of mixing DNA with sperm and then performing *in vitro* fertilization), it could be used in laboratories throughout the world. However, its low reliability, coupled with questions about the stability of transgenes, keeps this technique at the research stage.

Other techniques, such as electroporation, have been tried and have found application in some areas in which the more "conventional" methods have proved unreliable (Xie *et al.*, 1993).

Recombinant Proteins

Many therapeutic proteins are used in human and animal medicine. The majority of these are derived from animal or human tissue. It has been possible to purify extremely low concentrations of, for example, growth hormone from porcine or human pituitaries, and Factor IX from human blood has also been purified to acceptable levels. Recent concern has focused on the possibility that such human or animal tissues might be contaminated with slow viruses or retroviruses. Fortunately, such concerns have evolved contemporaneously with recombinant DNA technologies, and a variety of synthetic systems have become available (Goeddel, 1990). Table 7.2 lists a selection of those currently in use as well as some that are in development.

Table 7.1. Routes to transgenesis in animal species

Method	Outline	Advantages	Disadvantages	References
1. Microinjection	DNA is microinjected into a pronucleus (mammals) or into the region of the nuclei (fish, birds)	Established technique Low frequency of mosaics Expression well established Usually stable integration	Complex, expensive equipment	Brinster *et al.*., 1981; Pursel *et al.*, 1989; Archibald *et al.*, 1990; Ebert *et al.*, 1991; Wright *et al.*, 1991; Clark *et al.*, 1992; Love *et al.*, 1994
2. ES[a] cells	Embryonic cells established *in vitro*, are transfected with a DNA construct, analysed and introduced into a recipient embryo	Established technique (in rodents: livestock still experimental) Reliable, stable integration Expression well established Subtle modifications or gene ablation possible	Very complex Fastidious cells All transgenics of first generation are chimeric Very expensive	Evans & Kaufman, 1981; Notarianni *et al.*, 1990; Thomas & Capecchi, 1990; Bradley, 1993

Table 7.1. cont.

3. Viral vector	An engineered vector is made into packaged virus using a helper cell line. Resulting defective virus particles used to infect host embryo cells	Established technique Major technique if nuclear injection or ES cells not possible	Expression can be unstable Concern about recombination with endogenous viruses Concern about contamination with helper virus	Huszar *et al.*, 1985; Yu *et al.*, 1986; Salter & Crittendon, 1989; Chen *et al.*, 1990
4. Sperm-mediated transfer	DNA is mixed with sperm, to which it binds. DNA transferred to eggs during *in vitro* fertilization (IVF)	Remarkably simple, no specialist equipment or culture	Unverified Unstable integration? Unstable expression?	Brinster *et al.*, 1989; Gandolfi *et al.*, 1989; Lavitrano *et al.*, 1989
5. Electroporation	Cells in DNA solution are expressed to transient, high-voltage pulse, during which DNA enters the cell	Can be used for a variety of cell types Reliable for cultured cells	Unverified in most animal cells, though some unpublished reports of success	Chen & Powers, 1990; Xie *et al.*, 1993

[a] ES cells: embryonic stem cells; for further explanation see Glossary.

Table 7.2. Production of recombinant proteins

Host organism	Advantages	Disadvantages	Examples	References
Escherichia coli	Well-characterized, easy manipulation Good expression systems Simple culture	Protein usually remains in cells Protein may be aggregated or degraded Little modification	Insulin Interferon Growth hormone	Goeddel *et al.*, 1979a, b; Strueli *et al.*, 1982
Yeast	Long history of use Simple culture Good expression systems	Protein modification may not be accurate Protein may aggregate	Hepatitis antigen	Valenzuela *et al.*, 1982
Cultured cells	Export of modified proteins Good expression systems	Risk of contamination of culture Expensive culture	Erythropoietin Tissue plasminogen activator	Kaufman *et al.*, 1985; Goto *et al.*, 1988
Animals	Simple "culture": self-replicating Accurate modification Some good expression systems	Difficult manipulation Little experience	α_1-antitrypsin Tissue plasminogen activator	Archibald *et al.*, 1990; Ebert *et al.*, 1991; Wright *et al.*, 1991; Swanson *et al.*, 1992; Carver *et al.*, 1993

Escherichia coli. *Escherichia coli* was used for efficient production of recombinant proteins more that 15 years ago (Genentech produced human insulin in 1978), and continues to be a popular method today: there is a wide variety of expression vectors, and many simple purification procedures have been developed. Glutamate-*S*-transferase (GST) fusion vectors are one example; the fusion protein is readily isolated because the GST component will bind to a column containing reduced glutathione. Such vectors also include a recognition site for a protease (usually thrombin or Factor XA) situated between the GST and the foreign protein. The GST component can be removed from the purified fusion protein by proteolytic cleavage.

The most common problems with these systems are poor solubility of the product or inappropriate post-translational modification. It is quite common to find that the expressed products are deposited as insoluble complexes. Inappropriate modification is a legacy of evolution: glycosylation, sulphation, and proteolytic cleavage are often different in bacterial and eukaryotic cells. To produce a heavily modified polypeptide, it is often best to choose a eukaryotic system.

Yeast Systems. Yeast systems have the advantage that yeast culture has been a popular method for fermentation for many years, and the technology is well established. Many yeast vector systems have been established, and most of them rely on the use of yeast regulatory sequences for efficient expression. Both sulphation and glycosylation can be efficiently obtained (Moir & Dumais, 1987), although glycosylation in yeast may not accurately mimic glycosylation in higher eukaryotic cells.

Cultured Cells. Cultured cells have been the hosts for probably the greatest array of expression systems: DNA can be introduced using electroporation, chemical transformation, microinjection, or viral vectors, and mammalian cell cultures are used extensively in research for the production of foreign proteins and for therapeutic monoclonal antibodies.

In insect cells, the main vector systems are based on baculoviruses which, during their normal life cycle in insect larvae, produce large amounts of the protein polyhedrin. This product is dispensable for the lytic cycle. Many groups have used the regulatory sequences from the polyhedrin gene to drive expression of foreign protein (Luckow & Summers, 1988). The baculovirus genome is large and difficult to manipulate, and a variety of vector systems have been developed to maximize the efficiency of protein production (Davies, 1994).

Animals. Animals have been used as "bioreactors". The surplus protein synthetic capacity of the mammary gland and the haematopoietic system can be recruited to the production of recombinant proteins. Such experiments are discussed below in "Applications of Transgenic Animals".

Table 7.3. Selected transgenic animals

Species	Transgene	Desired/anticipated phenotype	Comments	References
Mouse	MT/hGH	Increased growth	First dramatic demonstration of transgenic phenotype Fertility impaired	Palmiter *et al.*, 1983
Mouse	MT/GHRH	Increased growth	Fertility improved More physiological?	Hammer *et al.*, 1985a
Pig	MT/GH	Increased growth	Increased growth rate Severe pathology, similar to that found with injection of GH protein	Hammer *et al.*, 1985b; Vize *et al.*, 1988; Pursel *et al.*, 1990
Sheep	BLG/α_1-AT	Production of pharmaceutical in milk	35 to 60 g/l of protein	Wright *et al.*, 1991; Clark *et al.*, 1992; Carver *et al.*, 1993
Goat	WAP/LAtPA	Production of pharmaceutical in milk	3 mg/l of protein Purified 8000-fold	Ebert *et al.*, 1991
Chicken	RSV–MT/bGH	Increased growth	100 µg/l of protein	Chen *et al.*, 1990

Table 7.3. cont.

Chicken	ALV *env*	Viral resistance	Retrovirus-mediated RSV resistance	Salter & Crittendon, 1989
Fish (carp)	RSV/rtGH	Increased growth	Effective in approximately 20% of transgenic fish	Zhang *et al.*, 1990

Abbreviations used: ALV, avian leukosis virus; bGH, bovine growth hormone; BLG, β-lactoglobulin; *env*, gene encoding the envelope glycoprotein; GHRH, growth hormone releasing hormone; hGH, human growth hormone; LAtPA, long-acting tissue plasminogen activator; MT, metallothionein; RSV, Rous sarcoma virus; rtGH, rainbow trout growth hormone; WAP, whey acidic protein; α_1-AT, α_1-antitrypsin.

Applications of Recombinant Genomes

Transgenic Animals

Classical breeding has sought, since the domestication of animals, to alter the characteristics of animals in order to make them more "desirable" to humans. One of the first steps was to reduce the "wild" behaviour patterns; a more recent example is the 2.5-fold increase in milk production per animal between 1945 and 1983 (Seidel, 1986). Transgenesis may also seek to produce such changes: it offers methods to generate either subtle or dramatic changes in phenotype.

1. It may induce the "improvement" of an existing trait in an animal. In these cases, transgenesis is used to circumvent or to improve upon the alternative of existing breeding programmes designed to fix a desirable trait in an already valuable line. The transgenic approach might thus be used to make a livestock species grow faster (Pursel *et al.*, 1989).
2. It may introduce entirely new properties into a species. In this approach, a foreign protein might be produced in a transgenic animal (for example, pharmaceutical proteins in the milk of ungulates [Archibald *et al.*, 1990; Ebert *et al.*, 1991; Wright *et al.*, 1991]), or it may confer new disease-resistant properties (Salter & Crittendon, 1989; Erickson & Izant, 1992), or alter the immune system to generate new histocompatibility antigens or new antibodies (Brüggemann *et al.*, 1991), or confer new digestive capabilities to improve the calorific use of less digestible feedstuffs (Hazlewood & Teather, 1988).

Some examples of transgenic animals are listed in Table 7.3.

Recombinant Proteins

The range of application of recombinant proteins is as wide as the current use of "natural" proteins (Table 7.4). Any industrial process, from brewing or cheese-making to the production of pharmaceuticals, may, potentially, be modified by the use of either recombinant proteins or of microorganisms producing such proteins (Enari, 1991; Gill & Zaworski, 1991). Indeed, in the United Kingdom, genetically engineered rennin is used in the production of cheese, and engineered yeast in the production of bread and in the pilot-scale production of beer. By 1991, the United States Food and Drug Administration (FDA) had approved more than 12 therapeutic agents and vaccines, and had permitted the clinical testing of some 800 more, derived from rDNA (Miller, 1991). By mid-1992, some 40 versions of these pharmaceutical products were approved for use in one or more country (Bienz-Tadmor, 1993). Recombinant proteins have thus become well established in industrial and clinical life.

The use of recombinant proteins as vaccines or as biopesticides offers many new opportunities. Research into the responses of the organism to recombinant

Table 7.4. Selected recombinant proteins

Effective protein	Disease/pathogen	Production/vector system	References
Insulin	Diabetes	Plasmid/*E. coli*	Goeddel *et al.*, 1979a
(Deletion)	Cholera	Deletion mutant of *Vibrio cholerae*	Levine *et al.*, 1988
O-antigen (*Vibrio cholerae*)	Cholera	*Salmonella typhi*	Tacket *et al.*, 1990
HIV antigen	AIDS	Vaccinia virus	Moss, 1991
HIV antigen	AIDS	BCG vaccine	Stover *et al.*, 1991
Neurotoxin	Plant pests	Baculovirus	Tomalski & Miller, 1992

vaccines is providing a better understanding of the relative contributions of cell-mediated and humoral immune systems, as well as identifying the optimal methods of presenting recombinant antigens to the immune systems. The two main approaches to vaccine production are to delete genes that confer pathogenicity from the pathogenic organism, or to insert genes encoding major antigens of the pathogen into a non-pathogenic vector (such as vaccinia virus or adenovirus) (Moss, 1991; Jacobs, 1993).

Live attenuated vaccines are commonly used for prophylaxis in human and animal populations; indeed, such use can be regarded as the largest introduction of GMOs, with an enormously successful history (Miller, 1991). In Europe, rabies is commonly carried in the fox populations. In campaigns using attenuated virus strains, it was found that the proposed vaccine remained pathogenic in rodents (and the raccoon, a major vector in the United States of America) and could revert to virulence. In a major environmental test, a recombinant vaccinia-rabies surface glycoprotein vaccine appeared to be highly effective in eradicating rabies from the fox population in a 2200 km^2 region of southern Belgium (Brochier *et al.*, 1991). This programme followed the recommendations of the World Health Organization (WHO) for research and trials with oral rabies vaccines (WHO, 1989).

Destruction of crops by arthropod pests causes a major loss of production throughout the world. The possibility of control by viral pathogens of these pest species has focused on baculoviruses, because they are stable, effective and species-specific. Normal virus infection, however, allows the host to live and to continue feeding for several days while the virus replicates. Virus vectors similar to

those described above can be modified to express genes that paralyse or kill the host more quickly (Tomalski & Miller, 1992). An alternative approach has been to remove the viral genes that allow the host to continue feeding after infection (O'Reilly & Miller, 1991). These elegant systems provide a great deal of hope for efficient, safe pest management.

Existing Regulations

History of Regulations

There are as many sets of regulations as there are countries that practise genetic manipulation. In 1991, the United Nations Industrial Development Organization (UNIDO) published its *Voluntary Code of Conduct for the Release of Organisms into the Environment* (UNIDO, 1991), one of the intentions of which was to harmonize global recombinant DNA guidelines. In the United Kingdom, in addition to two European Community (EC) Directives (CEC, 1990a,b) and the Genetic Manipulation Regulations, with 11 Notes of Guidance (ACGM/HSE, 1989), there are nine Acts of Parliament on the production and release of GMOs. Australia labours under the weight of 23 regulations. Many countries have attempted to use existing legislation (such as for the regulation of animal health, the environment, foods, or chemicals) to cover many aspects of the use of GMOs, and have adopted additional regulations to supplement these. In many ways, this is a reasonable course of action. As propounded above, transgenic products will in most cases represent no risk different in kind from those posed by other novel products derived by conventional technology. If one accepts this concept, it may be that the existing legislation will cover the safety aspects at issue - the release of the organism, the safety of the individual, the safety of the food or of the drug product. The important requirement is that each country addresses the question and develops a system of assessment.

Regulation has fallen into the two major camps of process versus product. Many countries have chosen to regulate genetic manipulation at the laboratory level (i.e. the process of producing the GMO) and have subsequently extended this regulatory oversight to large-scale use and release. This has largely been the experience in the United Kingdom, where assessment (see below) is applied specifically to GMOs. By contrast, the United States of America has developed, after considerable and still rumbling debate, a risk-based oversight of environmental experiments that addresses risk by examining the nature of the organism (with less emphasis on the means of production) and its intended release site (Medley, 1992; Miller *et al.*, 1990; Miller, 1991).

Safety in the Production of GMOs

The area where legislation must be most carefully studied is the production and assessment of GMOs, for it is at this stage that the organism is novel and uncharacterized. In the United Kingdom, the regulations, together with the associated Notes of Guidance, were originally framed in the 1970s and early 1980s. With a greater perception of the risks of laboratory-scale work, many of the original Guidelines were modified (ACGM/HSE, 1989). Nevertheless, these regulations still maintain that, if there is a risk of expression of the foreign DNA, then the categorization is raised to a higher level. Similarly, if the protein product has toxic properties, then a still higher level may be required. Clearly then, a fundamental understanding of the DNA sequences being manipulated is crucial to properly assessing, and therefore perhaps relaxing, the safety criteria imposed. If an arbitrary scale of factors that indicate "access" to the protein (i.e. the likelihood that the GMO or its DNA will enter the human organism and survive) is used, the "expression" of the protein (defined by whether the site in the vector is designed to make the cloned protein) and "damage" by the protein (a measure of the risk to the health of the worker) give a combinational assessment of the relative risk; this then allows categorization of the experiment into one of four classes. This categorization is based on work with non-recombinant pathogenic organisms.

Two of the Notes of Guidance (ACGM/HSE, 1989) adopted in the United Kingdom (Notes 1 and 5) govern the use of oncogenic nucleic acid sequences and eukaryotic viral vectors. Because of the possibility of human infection, the restrictions on the use of such systems are tighter. However, it should be noted that no human infection has clearly been shown to be due to laboratory work with a GMO.

Contained Use and Release

Safety and Assessment Committees

As far as possible, the proponents of a release should ensure that they exercise the maximum amount of care in planning their intended action. There is thus a need for an expert body with a range of interests to examine releases that are sufficiently novel to cause concern, whether these releases are of transgenic or non-transgenic species. The role of such a body is to bring to bear its expertise on the question of release. In the United Kingdom, such a committee would include many of those who had planned the release as well as those in control of the research establishment, since the responsibility for safety lies with them (Royal Commission on Environmental Pollution, 1991). In addition, it might be important for such a body to include experts in:

1. Genetics
2. Ecology

3. Safety
4. Molecular biology
5. Botany
6. Entomology
7. Environmental health

In the United States of America, it is normal to include in such a committee an ethicist (with a background in philosophy or religion).

The task of the committee is to investigate the security of the release site, to use their skills to apply lateral thinking in order to envisage risks, and to attempt to estimate their significance and consequences. It is important that, at an early stage, the public is made aware of the intended release. Openness in discussing proposed releases is an important part of informing the public about perceiving hazards and the methods of estimating their likelihood, and of persuading people that all reasonable precautions have been taken.

Contained Use of Recombinant Genomes

For many transgenic species, containment at the research level is already practised as part of good animal husbandry. Special care will normally be taken to exclude access to research animals by their wild-type relatives. Similarly, care will be taken, especially since each of the animals is extremely "valuable" at this stage, to ensure that no escape into the environment is possible. Also, conventional pest-control methods should be used to prevent the access of common pests to the research site. Such restrictions apply to all transgenic species. Those organisms, for example, insects or fish, that are not easily contained should be held with particular care. The assessment of the research on transgenic carp at the Alabama Agricultural Experiment Station in Auburn (Auburn, AL, USA) found that there is no significant impact of the release of modified fish into a contained facility (USDA, 1990). In its 67-page assessment, the Station considered five alternative approaches to containment and took into consideration such aspects as flooding leading to the release of the carp. A similar proposal for the contained release of transgenic catfish was subject to an equally or even more rigorous assessment (Fox, 1992).

Release of Recombinant Genomes

Potential Hazards of Transgenic Animals

The release of novel genotypes into the environment has a history of several hundred years. In the area of biological pest control, hundreds of organisms have been released into new locations in an attempt to eradicate pests of agronomically important crops (DeBach & Rosen, 1991). One of the first successful releases was that of the vedalia ladybeetle, with the intention to control cottony-cushion scale in California in 1888-9. A long examination of ladybeetles finally led to the selection

of a suitable Australian species. More recently, a large-scale attempt to control the cassava mealybug in Africa has been undertaken. In this case, the predator is a South American organism, *Epidinocasis lopezi.*

The history of such releases has seen many great successes. There have also been failures, most often when the relationship between predator and prey - or host and parasite was understood only poorly. The major lesson from these experiments (many of which have been carried out over the last hundred years) is that a sound understanding of the ecology of the new location is essential. The ecology of the host population (be it plant or animal) and its relationship with the pest are most important. An assessment of the validity of the new control agent in contained facilities is also crucial. Steps such as these have proven essential if the risks are to be minimized.

The successful introductions (as well as the unsuccessful ones) emphasize the requirement for a background understanding of such ecosystems. The introduction of an entirely novel (exotic) genotype into the environment will, in most cases, have much more extreme consequences than the introduction of a transgenic organism. Information about hazards arising from the release of transgenic animals can be sought under several categories (Table 7.5). Through laboratory and research-site assessment, many of the characteristics of the transgenic organism can be compared with those of homologous, non-transgenic individuals - its feeding behaviour, its sexual and aggressive behaviours, and its movement can be assessed under containment. It is clear that some of these hazards will be of minor relevance to some experiments; however, the regulations of most nations require that they be considered.

Potential Hazards of Recombinant Proteins

The hazards attached to recombinant proteins are most often of the same type as those attached to the parent products. In the case of humans, these are risks of allergic reactions or of side-effects of a type that occurs with currently used therapeutic products such as insulin. These risks are exacerbated by the use of recombinant gene technology.

Food safety has been addressed recently by several groups (WHO, 1991; OECD, 1992). Among recent concerns have been areas as diverse as the use of bovine growth hormone (or bovine somatotropin, bST) to increase milk yields, and the possibility of activating "toxic" genes in fish. Bovine somatotropin injected into dairy cattle can increase milk production by 10–15%. The concern over human health focuses on the possibility of the effects of bST consumed. In fact, growth hormones are inactivated when ingested, this particular concern seems to be well answered (Juskervich & Guyer, 1990).

The question over fish is more complex: could fish toxin genes be activated by the effects of transgene integration or expression? The fear is founded on the suspicion that fish may contain toxin genes, whereas current evidence indicates that they do not (Berkowitz & Sørenson, 1994). Indeed, toxins have never been detected in

Table 7.5. Potential hazards of release of genetically modified organisms (GMOs)[a]

1. The organism:	The nature of the host The stability and nature of the genetic modification Laboratory testing and verification of the organism
2. The environment:	The size and location of site, including ownership and security Proximity to humans and other animals The ecosystem of the release site and predicted effects Release of any target biota (e.g. predators), the known effects of the non-manipulated organism and known effects of manipulated organisms Numbers released at the site, the frequency of release and duration of release Effects of the manipulation in behaviour of the organism in its natural habitat Monitoring - how are the animals traced and for how long
3. Survival and spread:	Susceptibility to artificial stress Any details of modification designed to affect its ability to survive and to transfer genetic material Potential for transfer of inserted DNA to other organisms and methods for monitoring that transfer Elimination of superfluous organisms
4. Safety:	Safety of the workers on site and their education Contingency plans for unexpected effects of the transgenic organism Physical containment and contingency plans in the event that this containment may be breached (for example, flooding of fish ponds) Procedures for the termination of the experiment and disposal of manipulated organisms

[a] Based on the recommendations of GENHAZ (ACGM/HSE, 1989).

common food fish species, and even in toxic fish (such as the pufferfish) it appears to be bacterial commensals that produce the toxin.

It is important to consider that undesirable outcomes or side-effects may be produced in novel ways, but also to approach these possibilities in a rational way that examines the level of risk in the context of our current practices.

Safety and Risk Evaluation - Transgenic Animals

Transgenic Animals

In the United Kingdom, the procedure of preparing for a release is currently covered by the GENHAZ system (Royal Commission on Environmental Pollution, 1991). This is based on the chemical industries' assessment scheme and is designed to force the assessors to examine potential consequences of the release, no matter how unlikely, and, more important, to make them predict outcomes as precisely as possible (Table 7.6). At the time of writing, this process is under review; UK regulation of biotechnology has recently been criticized by the House of Lords Science and Technology Select Committee as being "excessively precautionary, obsolescent, and unscientific, [imposing] an unnecessary burden to academic researchers and industry alike" (UK House of Lords' Science and Technology Select Committee, 1993). New proposals suggest that *experience* and *familiarity* play a much larger part in a streamlined approach and that stringent review is reserved for potentially hazardous or the most novel of applications. It is still instructive, however, to look at the principles that could apply to the release of novel genomes, those for which no local experience exists. With increased understanding and familiarity, such a stringent approach will not be necessary. The essential components are a set of keywords and guide words designed to help frame these questions. One such guide word is "WHERE ELSE"; it may be necessary to ask: What happens if the DNA is detected somewhere other than at its original integration site? What happens if expression occurs in tissues other than the intended site? What happens if the organism is found at a location other than its intended release site? By considering these combinations of keywords and guidewords against the background of a wide range of understanding, the large majority of possible (as well as unlikely) outcomes must be defined.

Clearly, the implications of the release of a GMO are more severe if the organism cannot readily be recovered. Transgenic fish are a case in point. Growth hormone (GH) and cold-tolerance genes have been introduced into lines of fish. It appears that both experiments have been successful at the laboratory level: transgenic fish containing GH genes have been produced that grow larger than their non-transgenic relatives; similarly, more cold-tolerant fish have been obtained by using the antifreeze gene.

At an intuitive level, it might be thought that both such populations could pose a threat to natural ecosystems, the larger fish feeding more aggressively, the cold-

Table 7.6. A Summary of GENHAZ

Components of the genetically modified system

Construct	-	the components of the rDNA
Recipient	-	the host organism
Product	-	the GMO

Stages of the release

MAKE or SELECT the recipient, prepare the construct and generate the product
RELEASE of the product into the environment
ESTABLISH: the period during which the product either establishes itself in the release environment or fails to do so
POPULATION: the pattern of growth, spread and reproduction that follows the initial period of establishment; the interaction of the product and the release environment
GENETIC TRANSFER: the unintended transfer of DNA
MONITOR: the monitoring of the release
TERMINATE AND CLEAN UP: plans for when the trial is completed or if it must be terminated early

GENHAZ procedure

Apply guide words to generate DEVIATIONS
Develop CONSEQUENCES of each DEVIATION
Examine each CONSEQUENCE:
    Decide whether it requires ACTION and to avoid it
    Decide whether it has a realistic cause
Decide what ACTION to take

Table 7.6. cont.

Guide words	
NO or NOT	a complete negation of the intention (e.g. a gene fails to insert into a vector)
MORE	a quantitative increase (e.g. the level of expresson of a gene is greater than had been expected); could also be applied to time in terms of duration or frequency
LESS	a quantitative decrease (e.g. intended sterility of a transgenic animal is incomplete); could also be applied to time in terms of duration or frequency
AS WELL AS	a qualitative increase: something additional to the design intention happens (e.g. insects other than those targeted by a gene product are killed)
PART OF	a qualitative decrease: something less than the design intention happens (e.g. one of the genes inserted into the recipient fails to express)
OTHER THAN	something quite different from the design intention happens (e.g. the wrong construct is inserted)
WHERE ELSE	an intended event takes place in a location other than that planned (e.g. genetic material or the product of its expression occurs elsewhere than was planned)
WHEN ELSE	some effect appears at a time different from the expected (e.g. a modified animal reaches sexual maturity earlier or later than its unmodified form even though this was not the purpose of modification)

Based on the recommendations contained in GENHAZ[65].

tolerant fish displacing natural species from cooler waters. In the contained releases of transgenic carp, several factors argue against this (USDA, 1990; Fox, 1992).

1. Escape of any fish is an unlikely event, and an escape could not involve the large numbers required to establish a new genotype in the environment.
2. The mirror carp chosen for the growth experiments is less hardy than the naturally-occurring domestic carp - it is at a selective disadvantage.
3. Small numbers of escaping fish are unlikely to become established (fixed) in the environment; they may well be geographically isolated and their breeding patterns may differ from those of their non-transgenic relatives.
4. The transgene is unlikely to become fixed in the natural population unless it is under positive selection pressure.
5. Even if the transgenic fish became fixed in the local environment, it would still be subject to the biological control (disease, predators, food shortage) that affects the natural carp.

Therefore, in a well-designed release, it is possible to identify not only artificial factors influencing the ecological balance, but also the (perhaps more important) natural biological barriers. Because the GMO is usually a weaker, domesticated strain of natural relatives, the types of barriers that might operate can, if necessary, be studied in some detail.

The risk assessment should be examined by a competent body that is independent of the research group. Invariably, national review bodies will be established, but it is important now, and will become more important in the future, that such national bodies communicate with one another (see below). Communication between national bodies will allow the shared knowledge to act in a streamlining of review through past experience. Each body will have many of the specialized skills of the committee members preparing the release assessment and, in addition, will include competent persons who will examine national regulations and laws.

Safety and Risk Evaluation - Recombinant Proteins

Many countries throughout the world have established guidelines for the assessment of novel foods, drugs, and biology-based industrial components. These assessment protocols, which can take many years to complete for new therapeutic agents, provide most or all of the tools for the assessment of proteins produced by genetic manipulation. Indeed, recombinant proteins are often more similar to their parental products than are novel products generated by classical means. The EC, however, currently proposes a new level of oversight for food products obtained by genetic manipulation. By contrast, the US FDA and the OECD have determined that new foods should be assessed on the basis of the novelty of their characteristics. It seems that this approach is the more valid, relying, as it does, on the characteristics of the product and on the effects of the product on the individual(s).

Recently, in the United States of America, a new set of guidelines for evaluating novel drugs has been introduced. Prompted in part by the educated and frank criticism coming from the AIDS community, the FDA has attempted to expedite the release of new drugs through accelerated testing and approval. This example served to remind the developed world that a regulatory programme designed for careful, highly scrutinized approval can seem harshly inappropriate to the individual facing death. Regulation and risk assessment are invariably balancing acts between cost and benefit, and there is a need to recognize that the cost to the individual may sometimes be absolute.

Impact After Release

A controlled release allows the time to determine the properties of the GMO through several seasons of breeding (Fig. 7.2). Laboratory experiments will reveal many of the alterations that may have occurred in the behaviour of the GMO, but it is in more natural environments that behaviour may be fully assessed. If it is felt to be necessary, a contained release will allow the study of behaviour in competition with natural species. Many of these concepts would be part of normal good agricultural practice. New strains of domestic animals or fish will normally be tested before being used on a large scale to replace a current organism. Such an assessment, for example of increased milk production through non-transgenic means in cattle, would include several generations in order to determine the stability of the phenotype and to detect any deleterious effects on the organism. These steps are essential in all classical breeding programmes before an acceptable breeding line can be established. In a similar way, a controlled release of GMOs that are sufficiently novel, or sufficiently distinct in their properties from their parent animals, provides the opportunity to investigate the impact of these differences.

The classical common sense of breeding programmes, then, applies as much to new strains produced by the interbreeding of selected animals as it does to GMOs. In aquaculture, the introduction of a novel food species requires as much attention and care as the use of a genetically modified fish stock.

Conclusions

Biology, Common Sense and Release

One of the difficulties in preparing a chapter on transgenic animals or recombinant proteins is that it quickly becomes clear (as clear, I hope, to the reader as to the author) that these categories of recombinant genomes encompass an enormous array of technologies, designs, hopes and hazards. These methods are often less similar to one another than they are to classical breeding and selection techniques. When considering the risks attached to such manipulated organisms, the biological

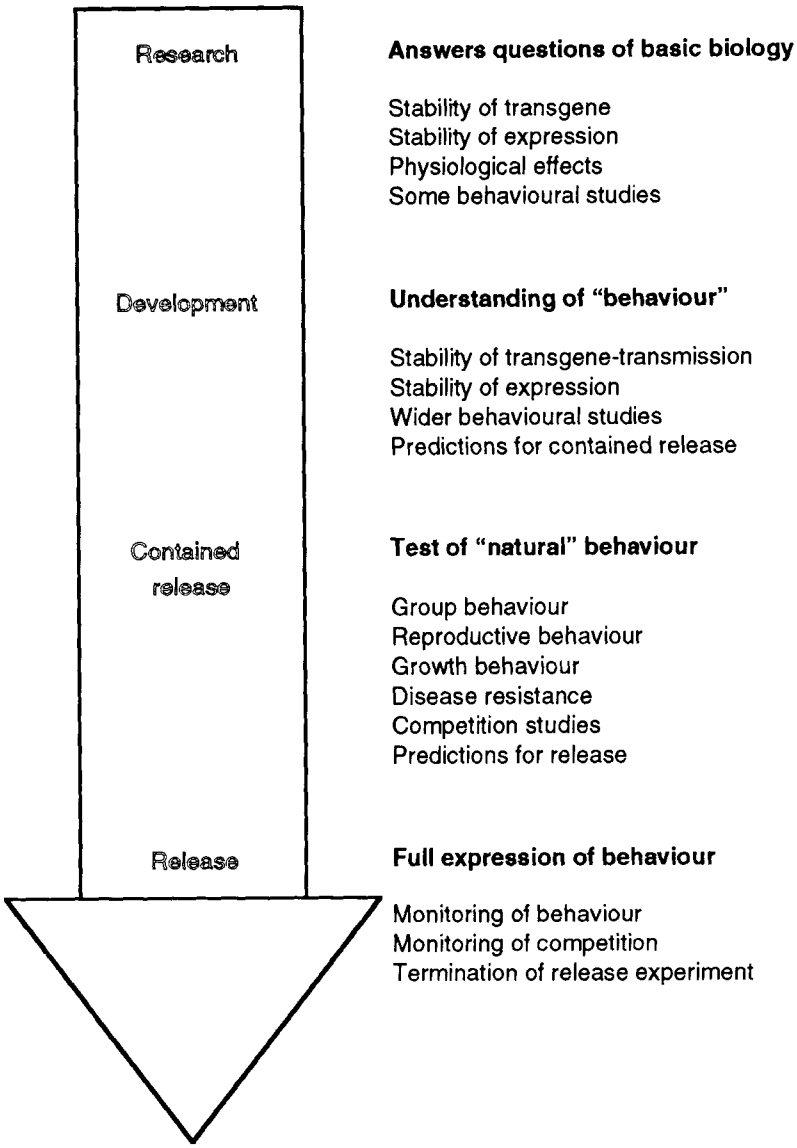


Fig. 7.2. Levels of assessment of novel genomes and proteins.
Assessment is a continual process, as indicated by the arrow at the left. Major uncertainties about GMOs exist at the levels of research and development, particularly in relation to stability of the genetic modification and stability of the phenotype. By the time a GMO has been characterized sufficiently to consider release, these questions, largely unique to GMOs, should have been answered. The remaining questions at the stages of contained and full release are questions that pertain to all novel genotypes. With GMOs, familiarity can play a large part in such assessments.

principle and guiding force should be that it is the properties of the animal or protein that primarily dictate the hazards, not the means by which that animal or protein was derived. Acceptance of this argument may well mean that existing legislation can be used to regulate recombinant DNA releases. The products of genetic manipulation can, in most cases, be compared with novel foods, proteins, or crops produced by traditional means, except that we often understand far more clearly the genetic changes in the GMO than we do in the "traditional" product. Most countries have legislation that covers hazardous, pathogenic or exotic organisms, unapproved drugs, and chemicals, as well as their testing. Such statutes may well provide sufficient regulatory oversight. The extent of the difficulty in controlling exotic (i.e., non-manipulated) organisms is a problem that should not be underestimated. Each year, approximately 11 potential pests enter the United States of America through all modes of unintentional transfer, and seven of these are likely to be injurious (DeBach & Rosen,1991). There is also the human dimension: in 1986, the USDA intercepted, at ports of entry, nearly 50 000 attempted introductions of exotic organisms. It is important to remember that some "traditional" practices in agriculture or industry are less sophisticated and may be more hazardous than biotechnological solutions to the same problem.

Against this must be set the public perception of genetic manipulation. It is important that the public - the consumer - is aware that the type of change wrought by genetic manipulation is often more subtle, more predictable, and more defined than the changes produced by classical means. It is important to remember, however, that there are many groups who object on ethical grounds to the use of all animals or of specific animals. For some of these people, certain types of genetic manipulation may never be acceptable. Many devout religious groups in the United Kingdom would not countenance food products containing DNA based on pig sequences - even if the DNA used were synthesized *in vitro*. However, education about the nature of traditional and recombinant methods and about the presence of foreign DNA (actually most often made in *E. coli*) can play a part in reassuring many people that recombinant DNA does not alter the fundamental nature of the host organism.

However, the arguments about regulation have recently increased. In the United States of America, the experience gained with the release or movement of about 1000 genetically modified plants has led to a proposal that regulatory "...oversight should be more commensurate with scientific indications of potential risk ... comparable with that historically applied to conventional plants" (Hunter *et al.*, 1992). However, the policies pursued by the US FDA are still being formed, and Jeremy Rifkind's Pure Food Campaign pressure group and, more recently, the Union of Concerned Scientists have attempted to revive the public debate and to direct it against transgenic products (Hoyle, 1992, 1994). Criticism of the European regulations was raised in 1989 (Miller, 1991), and the debate about the need for stringent regulations and the influence they have on investment has raged since (Young & Miller, 1989; Hodgson, 1992; Kathuri *et al.*, 1992). It appears now that many releases will fall under the influence of the new, less process-based regula-

tions, rather than under those originally proposed. Nevertheless, only six of the twelve member states of the European Community have even partially ratified these proposals.

It is important to appreciate that regulatory decisions are very significant factors in the industrial view of investment opportunities. Bayer AG of Germany and NOVO Industry of Denmark have both established major research and development facilities outside their home centres in part as a reaction to the rigorous regulations imposed. There are, therefore, two sides to the regulatory question. Regulations that are very stringent may discourage investment: conversely, biotechnology companies are also reluctant to invest in countries that do *not* have a regulatory framework in place. The need is to establish such a framework in each country that addresses local needs as well as taking a global view - a familiar political and social problem. Various aspects of the social, economic and technological implications of biotechnology for the developing world have also been discussed recently (DaSilva *et al.*, 1992).

Framing Legislation

With these social and economic constraints in mind, it may be valuable to ask oneself how to frame legislation and what is the need for new legislation (Table 7.7). The starting point is to examine existing legislation, in order to determine whether most or even all aspects of the release of GMOs can be covered. All countries hold that the safety and risk assessment of the production of GMOs forms a special category that requires legislation. This is based on the view that in many cloning experiments the precise nature of products generated cannot be predicted. In the case of the release and use of transgenic animals or proteins, many countries have made use of the existing (perhaps modified) legislation. In the United Kingdom, such legislation includes the Environmental Protection Act and the Food and Environment Protection Act; in the United States of America, the Coordinated Framework for the Regulation of Biotechnology establishes that existing laws (such as the Animal Quarantine Laws) are sufficient to regulate the products of biotechnology.

In the absence of appropriate existing legislation, the discussion in this book and the guidance supplied by the *Voluntary Code of Conduct for the Release of Organisms into the Environment* (UNIDO, 1991) provide a framework for general principles.

International Resources

There are several bodies that seek to harmonize and integrate international biosafety, for this is inevitably a transnational concern. UNIDO was instrumental in bringing together a panel to draw up the *Voluntary Code of Conduct for the Release of Organisms into the Environment* (UNIDO, 1991) and has established research institutes, under the International Centre for Genetic Engineering and Biotechnology

Table 7.7. Checklist for legislation on release

1. What controls already exist for:
   - novel exotic organisms
   - new strains of animals produced for food
   - new food or drug products?

2. What is legislation designed to protect:
   - the consumer
   - the worker
   - the GMO
   - species that interact with the GMO
   - the environment?

3. How novel are the products of genetic manipulation:
   - growth and reproductive regulation
   - disease resistance
   - increased efficiency (e.g. stress tolerance, feed efficiency)
   - production of novel proteins?

4. What risks do such products imply:
   - genomic risks common to all breeding programmes, e.g. more aggressive behaviour, wider ecological range
   - special risks arising from the nature of the rDNA, e.g. stability, gene transfer, novel product, novel expression patterns?

5. New legislation for recombinant genomes:
   - include all releases of novel or unfamiliar organism, not only GMOs
   - establish natural body of experts, independent of proposers
   - use international experience (databases, previous releases) and expertise
   - legislate to be flexible, to simplify and generalize wherever possible on the basis of experience
   - engage public (the consumer) in the debate
   - respecting commercial confidentiality, keep assessment open
   - establish monitoring and termination protocols

(ICGEB), in Trieste and New Delhi, designed to assist technology transfer between the industrial and the developing world. ICGEB, which is an autonomous intergovernmental institution, is "owned" by its member states, and with its flexibility, is responsive to the changing needs of those countries. It also maintains a computer network, ICGEBnet, that can give access to many of the databases worldwide (Simon & Pongor, 1992). In addition, UNIDO has set up an international Biosafety Information Network and Advisory Service (BINAS), which has designated national

nodes in a number of countries. These BINAS national nodes are meant to provide expertise and information support to biosafety authorities. In this way, an efficient network of information is planned to be established at the pivotal level of those involved in the formulation and administration of biosafety regulation. Both information and expertise could be formally and, perhaps even more important, informally exchanged through these channels. Lists of contacts in regulatory authorities as well as in companies intending to carry out releases will lead rapidly to the dissemination of information, the rationalization of procedures, and the elimination of duplicated effort. The emphasis of biosafety regulation must be on maintaining a safe posture founded on sound biological principles, and on using the information we have and will gain in the coming years around the world to refine regulation through the removal of unnecessary legislative burdens that address nonexistent or unimportant conjectural risks.

Acknowledgements

While they may not necessarily share my views, I would like to thank many people who have helped in the preparation of this chapter. In particular, my thanks go to Henry I. Miller of the United States Food and Drug Administration and Tony Medley of the Agriculture, Pest and Health Inspectorate Service for the very helpful information they generously supplied. I also thank Gilbert Howe, Morris Levin, Phil Dale and George Tzotzos, lecturers on the UNIDO course at ICGEB, Trieste in December 1992, and Professor Brian Heap for his encouragement. I am also indebted to Dianne Styles and Linda Notton for transforming my writing into a typescript.

References

ACGM/HSE (1989) Genetic Manipulations and Supplementary Notes of Guidance (1-11). Health and Safety Executive, London.

Anderson, W.F. (1992) Human gene therapy. *Science*, 256, 808-813.

Archibald, A.L., McCleneghan, M., Hornsey, V., Simons, J.P. & Clark, A.J. (1990) High level expression of biologically active human α_1-antitrypsin in the milk of transgenic mice. *Proc. Natl. Acad. Sci. USA*, 817, 5178-5182.

Berkowitz, D.B. & Sørenson, I.K. (1994) Transgenic fish - safe to eat? *Bio/Technology*, 12, 247-252.

Bienz-Tadmor, B. (1993) Biopharmaceuticals go to market: patterns of worldwide development. *Bio/Technology*, 11, 168-172.

Bradley, A. (1993) Site-directed mutagenesis in the mouse. *Recent Prog. Horm. Res.*, 48, 237-251.

Brinster, R.L., Chen, H.Y., Trumbauer, M., Senear, A.Y., Warren, R. & Palmiter, R.D. (1981) Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell*, 27, 223-231.

Brinster, R.L., Sandgren, E.P., Behringer, R.M. & Palmiter, R.D. (1989) No simple solution for making transgenic mice. *Cell*, 59, 239-241.

Brochier, B., Kieny, M.P., Costy, F., Coppens, P., Bauduin, B., Lecoq, J.P., Languet, B., Chappuis, G., Desmettre, P., Afiademanyo, K., Libois, R. & Pastoret, P.-P. (1991) Large-scale eradication of rabies using recombinant vaccinia-rabies vaccine. *Nature*, 354, 520-522.

Brüggemann, M., Spicer, C., Buluwela, L., Rosewall, I., Barton, S., Surani, M.A. & Rabbits, T.H. (1991) Human antibody production in transgenic mice: expression from 100 kb of the human IgH locus. *Eur. J. Immunol.*, 21, 1323-1326.

Carver, A.S., Dalrymple, M.A., Wright, G., Cottom, D.S., Reeves, D.B., Gibson, Y.H., Keenan, J.L., Barrass, J.D., Scott, A.R., Colman, A. & Garner, I. (1993) Transgenic livestock as bioreactors: stable expression of human α_1-antitrypsin by a flock of sheep. *Bio/Technology*,11, 1263-1270.

CEC (1990a) Council Directive of 23 April 1990 on the contained use of genetically modified microorganisms. Reference no. 90/219/EEC. *Official Journal L117*, Volume 33, 8 May 1990. Commission of the European Communities, Brussels.

CEC (1990b) Council Directive of 23 April 1990 on the deliberate release into the environment of genetically modified organisms. Reference no. 90/220/EEC. *Official Journal L117*, Volume 33, 8 May 1990. Commission of the European Communities, Brussels.

Chen, H.Y., Garber, E.A., Mills, E., Smith, J., Kopchick, J.J., DiLella, A.G. & Smith, R.G. (1990) Vectors, promoters and expression of genes in chick embryos. *J. Reprod. Fertil.*, (suppl.) 41, 173-182.

Chen, T.T. & Powers, D.A. (1990) Transgenic fish. *Trends Biotechnol.*, 8, 209-215.

Clark, A.J., Cowper, A., Wallace, R., Wright, G. & Simons, J.P. (1992) Rescuing transgene expression by co-integration. *Bio/Technology*, 10, 1450-1454.

DaSilva, E.J., Ratledge, C. & Sasson, A. (1992) (eds.) *Biotechnology: Economic and Social Aspects. Issues for Developing Countries*. Cambridge University Press, Cambridge, UK.

Davies, A.H. (1994) Current methods for manipulating baculoviruses. *Bio/Technology*, 12, 47-50.

DeBach, P. & Rosen, D. (1991) *Biological Control by Natural Enemies*, (2nd edn.). Cambridge University Press, Cambridge, UK.

Ebert, K.M., Selgrath, J.P., DiTullo, P., Denman, J., Smith, T.A., Memon, M.A., Schindler, J.E., Monasterski, G.M., Vitale, J.A. & Gordon, K. (1991) Transgenic production of a variant of human tissue-type plasminogen activator in goat milk. *Bio/Technology*, 9, 835-838.

Enari, T.-M. (1991) Genetic modification of food and beverage yeast. *Ann. N. Y. Acad. Sci.*, 646, 181-192.

Erickson, R.P. & Izant, J.G. (eds.)(1992) *Gene Regulation: Biology of Anti-sense RNA and DNA*. Raven Press, New York.

Evans, M.J. & Kaufman, M.H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature*, 292, 154-156.

Fox, J.L. (1992) USDA snarls at transgenic catfish. *Bio/Technology*, 10, 292.

Fox, J.L. (1993) NIHRAC okays gene therapy. *Bio/Technology*, 11, 1227.

Gandolfi, F., Lavitrano, M., Camaioni, A., Spadafora, C., Siracusa, G. & Lauria, A. (1989) The use of sperm-mediated gene transfer for the generation of transgenic pigs. *J. Reprod. Fertil.*, Abstr. Ser. 4, 10.

Gill, G.S. & Zaworski, P.G. (1991) Use of yeasts in production and discovery of pharmaceuticals. *Ann. N. Y. Acad. Sci.*, 646, 173-180.

Goeddel, D.V. (1990) *Gene Expression Technology*. Goeddel, D.V. (ed.) *Methods Enzymol.*,

vol. 185. Academic Press, San Diego, CA.

Goeddel, D.V., Heyneker, H.L., Hozumi, T., Arentzen, R., Itakura, K., Yasura, D.G., Ross, M.J., Miozzari, G., Crea, R. & Seeburg, P.H. (1979a) Direct expression in *Escherichia coli* of a DNA sequence coding for human growth hormone. *Nature*, 281, 544-548.

Goeddel, D.V., Kleid, D.G., Bolivar, F., Heyneker, H.L., Yansura, D.G., Crea, R., Hirose, T., Kraszewski, A., Itakura, K. & Riggs, A.D. (1979b) Expression in *Escherichia coli* of chemically-synthesized genes for human insulin. *Proc. Natl. Acad. Sci. USA*, 76, 106-110.

Goto, M., Akai, K., Murakami, A., Hashimoto, C., Tsuda, E., Ueda, M., Kawanashi, G., Takahashi, N., Ishimoto, A., Chiba, H. & Sasaki, R. (1988) Production of a recombinant erythropoietin in mammalian cells: host-cell dependency of the biological activity of the cloned protein. *Bio/Technology*, 6, 67-71.

Hammer, R.E., Brinster, R.L., Rosenfield, M.G., Evans, R.M. & Mayo, K.E. (1985a) Expression of human growth hormone releasing factor in transgenic mice results in increased somatic growth. *Nature*, 315, 413-416.

Hammer, R.E., Pursel, V.G., Rexroad, C.E. Jr, Wall, R.J., Bolt, D.J., Ebert, K.M., Palmiter, R.D. & Brinster, R.L. (1985b) Production of transgenic rabbits, sheep and pigs by microinjection. *Nature*, 315, 680-683.

Hazlewood, G.H. & Teather, R.M. (1988) the genetics of rumen bacteria. In: *The Rumen Microbial Ecosystem*, Hobson, P.N. (ed.), pp. 323-341. Elsevier Applied Science, Cambridge, UK.

Hodgson, J. (1992) Europe, Maastricht and biotechnology. *Bio/Technology*, 10, 1421-1426.

Hoyle, R. (1992) FDA's slippery food policy. *Bio/Technology*, 10, 935-939.

Hoyle, R. (1994) A quixotic assault on transgenic plants. *Bio/Technology*, 12, 236-239.

Hunter, S.L., Arntzen, C., Beachey, R., Breuning, G., Qualset, C. & Vidaver, A. (1992) Revising oversight of genetically modified plants. *Bio/Technology*, 10, 967-971.

Huszar, D., Balling, R., Kothary, R., Magli, M.C., Hozumi, N., Rossant, J. & Bernstein, A. (1985) Insertion of a bacterial gene into the mouse germline using an infectious retroviral vector. *Proc. Natl. Acad. Sci. USA*, 82, 8587-8591.

Jacobs, S.C. (1993) A novel recombinant adenovirus vector expressing a flavivirus nonstructural protein protects against lethal flavivirus challenge. *Clin. Sci.*, 85, 117-122.

Juskervich, J.C. & Guyer, C.G. (1990) Bovine growth hormone: human food safety evaluation. *Science*, 249, 875-884.

Kathuri, C., Polastro, E.T. & Mellor, N. (1992) Biotechnology in an uncommon market. *Bio/Technology*, 10, 1545-1547.

Kaufman, R.J., Walsey, L.C., Spiliotes, A.J., Gossels, S.D., Latt, S.A., Larsen, G.R. & Kay, R.M. (1985) Co-amplification and co-expression of human tissue-type plasminogen activator and murine dihydrofolate reductase in Chinese hamster ovary cells. *Mol. Cell. Biol.*, 5, 1750-1759.

Lavitrano, M., Camaioni, A., Fazio, V.M., Dolci, S., Farace, M.G. & Spadafora, C. (1989) Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. *Cell*, 57, 717-723.

Levine, M.M., Herrington, D., Losonsky, G., Tall, B., Kaper, J.B., Ketley, J., Tacket, C.O. & Cryz, S. (1988) Safety, immunogenicity and efficacy of recombinant live oral cholera vaccines CVD-103 and CVD-103-HGR. *Lancet*, ii, 467-470.

Love, J., Gribbin, C., Mather, C. & Sang, H. (1994) Transgenic birds by DNA microinjection. *Bio/Technology*, 12, 60-63.

Luckow, V.A. & Summers, M.D. (1988) Trends in the development of baculovirus expres-

sion systems. *Bio/Technology*, 6, 47-51.

Medley, T.L. (1992) Status of regulatory approval of biotechnology-derived plants and animals. *Crit. Rev. Food Sci. Nutr.*, 32, 151-155.

Miller, A.D., Jolly, J.D., Friedman, T. & Verma, I.M. (1983) A transmissible retrovirus expressing human hypoxanthine phosphoro ribosyltransferase (HPRT): Gene transfer into cells derived from humans deficient in HPRT. *Proc. Natl. Acad. Sci. USA*, 80, 4709-4713.

Miller, H.I. (1991) Regulation. In: *The Genetic Revolution*, Davis, B.D. (ed.), pp. 196-211. The Johns Hopkins University Press, Baltimore, MD.

Miller, H.I., Burris, R.H., Vidaver, A.K. & Wivel, N.A. (1990) Risk-based oversight of experiments in the environment. *Science*, 250, 490-491.

Moir, D.T. & Dumais, D.R. (1987) Glycosylation and secretion of human α_1-antitrypsin by yeast. *Gene*, 56, 209-217.

Moss, B. (1991) Vaccinia virus - a tool for research and vaccine development. *Science*, 252, 1662-1667.

Notarianni, E., Laurie, S., Moor, R.M. & Evans, M.J. (1990) Maintenance and differentiation in culture of pluripotential embryonic cell lines from pig blastocysts. *J. Reprod. Fertil.*, (suppl.) 41, 51-56

OECD (1992) *Safety of Foods Derived by Modern Biotechnology: Concepts and Principles*. OECD, Paris.

O'Reilly, D.R. & Miller, L.K. (1991) Improvement of a baculovirus pesticide by deletion of the *egt* gene. *Bio/Technology*, 9, 1086-1089.

Palmiter, R.D., Norstedt, G., Gelinas, R.E., Hammer, R.E. & Brinster, R.L. (1983) Metallothionein-human GH fusion genes stimulate growth of mice. *Science*, 222, 809-814.

Pursel, V.G., Hammer, R.E., Bolt, R.E., Palmiter, R.D. & Brinster, R.L. (1990) Integration, expression and germline transmission of growth-regulated genes in pigs. *J. Reprod. Fertil.*, (suppl.) 41, 77-87.

Pursel, V.G., Pinkert, C.A., Miller, K.F., Bolt, D.J., Campbell, R.G., Brinster, R.D., Palmiter, R.L. & Hammer, R.E. (1989) Genetic engineering of livestock. *Science*, 244, 1282-1288.

Royal Commission on Environmental Pollution (1991) 14th report: GENHAZ. HMSO, London.

Salter, D.W. & Crittendon, L.B. (1989) Artificial insertion of a dominant gene for resistance to avian leukosis virus into the germline of the chicken. *Theor. Appl. Genet.*, 77, 457-467.

Seidel, G.E. Jr. (1986) Characteristics of future agricultural animals. In: *Genetic Engineering of Animals*, Evans, I.W. & Hollander, A. (eds.), pp. 299-310. Plenum Press, New York.

Simon, G. & Pongor, S. (1992) ICGEBNet: the UNIDO computer resource for molecular biology. *Bioinformatics*, 1, 12-19.

Stover, C.K., de la Cruz, V.F., Fuerst, T.R., Burlein,J.E., Benson, L.A., Bennett, L.T., Bansal, G.P., Young, J.F., Lee, M.H., Hatfull, G.F., Snapper, S.B., Barletta, R.G., Jacobs, J.R.Jr & Bloom, B.R. (1991)New use of BCG for recombinant vaccines. *Nature*, 351, 456-460.

Strueli, M., Hall, A., Boll, W., Stewart, W.E.II, Nagata, S. & Weissman, C. (1982) Target cell specificity of two species of human interferon-a produced in *Escherichia coli* and of hybrid molecules derived from them. *Proc. Natl. Acad. Sci. USA*, 78, 2848-2842.

Swanson, M.E., Martin, M.J., O'Connell, J.K., Hoover, K., Lago, W., Huntress, V., Parsons, C.T., Pinckert, C.J., Pidler, S. & Logan, J.D. (1992) Production of functional human haemoglobin in transgenic swine. *Bio/Technology*, 10, 557-559.

Tacket, C.O., Forrest, B., Morona, R., Attridge, S.R., LaBrooy, J., Tall, B.D., Reymann, M., Rowley, D. & Levine, M.M. (1990) Safety, immunogenicity and efficacy against cholera challenge in humans of a typhoid-cholera hybrid vaccine derived from *Salmonella typhi* TY21a. *Infect. Immun.*, 58, 1620-1627.

Thomas, K.R. & Capecchi, M.R. (1990) Targeted disruption of the murine *int*-1 protooncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature*, 346, 847-850.

Tomalski, M.D. & Miller, L.K. (1992) Expression of a paralytic neurotoxin gene to improve insect baculoviruses as biopesticides. *Bio/Technology*, 10, 454-549 .

UK House of Lords' Science and Technology Select Committee (1993) Regulation of the United Kingdom Biotechnology Industry and Global Competitiveness. HMSO, London.

UNIDO (1991) Voluntary Code of Conduct for the Release of Organisms into the Environment. UNIDO, Vienna.

USDA (1990) Availability of an environmental assessment and finding of no significant impact relative to USDA funding of research on transgenic carp. *Federal Register* 55, 48661-48662.

Valenzuela, P., Medina, A., Rutter, W.J., Ammerer, G. & Hall, B.D. (1982) Synthesis and assembly of hepatitis B virus surface-antigen particles in yeast. *Nature*, 298, 347-350.

Vize, P.D., Michalska, A.E., Ashman, R., Lyody, B., Stone, B.A., Quinn, P., Wells, J.R.F. & Seamark, R.F. (1988) Introduction of a porcine growth hormone fusion gene into transgenic pigs promotes growth. *J. Cell. Sci.*, 90, 285-300.

WHO (1989) Report of WHO Consultants on Requirements and Criteria for Field Trials on Oral Rabies Vaccination for Dogs and Wild Carnivores. WHO/Rab. Res./89.32.

WHO (1991) Strategies for Assessing the Safety of Foods Produced by Biotechnology. FAO/WHO, Geneva.

Wright, G., Carver, A., Cottom, D., Reeves, D., Scott, A., Simons, P., Wilmut, I., Garner, I. & Colman, A. (1991) High level expression of active human α_1-antitrypsin in the milk. *Bio/Technology*, 9, 830-834.

Xie, Y.F., Liu, D., Zou, J., Li, G.H.& Zhu, Z.Y. (1993) Gene-transfer via electroporation in fish. *Aquaculture*, 111, 207-213.

Young, F.E. & Miller, H.I. (1989) "Deliberate releases" in Europe: over-regulation may be the biggest threat of all. *Gene*, 75, 1-2.

Yu, S.-F., von Rüden, T., Kantoff, P.W., Garber, C., Seiberg, M., Rüther, U., Anderson, W.F., Wagner, E.F. & Gilboa, E. (1986) Self-inactivating retroviral vectors designed for the transfer of whole genes into mammalian cells. *Proc. Natl. Acad. Sci. USA*, 83, 3194-3198.

Zhang, P., Hayat, M., Joyce, C., Gonzalez-Villagenor, L.T., Lin, C.M., Durham, R.A., Chen, T.T. & Powers, D.A. (1990) Gene transfer, expression and inheritance of pRSV-rainbow trout GH cDNA in the common carp *Cyprinus carpio* (Linnaeus). *Mol. Reprod. Dev.*, 25, 3-13.

Chapter eight
Safety Aspects of Aquatic Biotechnology

Raymond A. Zilinskas

Centre for Public Issues in Biotechnology, University of Maryland Biotechnology Institute, College Park, Maryland, United States of America.

Introduction

As biotechnology develops and advances, scientists will employ genetic engineering techniques to develop ever-increasing numbers and varieties of new animate and inanimate products. Most of these products will be tested and used on land, but some will be applied in the aqueous environment. Inevitably, two questions arise. Do genetically modified aquatic organisms pose special or unique problems to risk assessors and regulators? Do inanimate biotechnology products present special risks when applied in the sea? The answers to these questions are important not only for the sake of securing public and environmental health, but also because, if the answers are affirmative, appropriate management procedures and regulations will have to be developed and instituted.

The objective of this chapter is to answer these questions, that is, to try to determine whether genetically modified (hereafter transgenic) aquatic animals and plants or inanimate biotechnology products pose special risks to humans or the environment. In order to make this determination, four issues have to be considered. First, what are the potential applications of transgenic aquatic macroorganisms or inanimate products obtained from transgenic organisms? Second, are there standards, guidelines, or procedures pertaining to the use or release of transgenic aquatic animals or plants and, if so, what are their content and reach? Third, have containment or other mitigating approaches been developed that aim to manage the use or release of transgenic aquatic animals or plants and, if so, how effective are they? Fourth, have risk assessment schemes pertaining to the use or release of transgenic aquatic macroorganisms or genetically engineered products been developed and, if so, how effective are they?

The four sections that follow, accordingly, address each issue in turn. In the concluding section, the findings from the preceding sections are amalgamated in

order to determine whether biotechnology as applied to the aquatic environment poses, or is likely to pose, especially vexing safety problems to researchers, risk assessors and regulators.

Possible Applications of Transgenic Aquatic Macroorganisms and Inanimate Products from Transgenic Organisms

Aquatic biotechnology, which may be defined as "the application of scientific and engineering principles to the processing of materials by aquatic biological agents to provide goods and services", is one of the more rapidly developing and growing fields of general biotechnology (Zilinskas *et al.*, 1995). As discussed in a World Bank study, this field has especially promising applications for coastal and island developing countries (Zilinskas & Lundin, 1993). Most applications of genetically modified aquatic animals and plants, at least in the short and medium term, will be in aquaculture. Inanimate products from transgenic organisms are likely to have a wider range of uses in the aquatic environment, in applications related to animal health, bioremediation and antifouling.

Applications of Transgenic Aquatic Macroorganisms

Many authors have written about the research, development, and possible applications of transgenic aquatic macroorganisms (Renn, 1986; Colwell, 1987; Chen & Powers, 1990; Powers *et al.*, 1991; Chen *et al.*, 1992; Devlin & Donaldson, 1992; Hallerman & Kapuscinski, 1992; Donaldson & Devlin, 1993). An analysis of the contents of this body of work indicates that transgenic aquatic macroorganisms may be developed to enhance six sets of properties.

1. **Metabolism** - the genetic control over metabolic pathways may be modified to speed up maturation, attain larger adult growth, increase reproduction rates, lower the amount of fat in body tissues, and/or improve food utilization. For example, research in this area already has led to the development of transgenic carp and catfish containing growth hormone genes from trout, and these transgenic organisms grow faster and larger than their wild relatives (Chen & Powers, 1990).
2. **Physiology** - the physiological characteristics of organisms may be altered so that they are better able to tolerate colder or warmer water, saltier or less salty water, higher concentrations of metals or pollutants, and/or smaller concentrations of dissolved oxygen. An example of relevant research is the attempt in Canada to develop transgenic salmon containing genes from the Arctic flounder coding for an antifreeze protein which will enable it to grow in cold water of 10°C or less (Shears *et al.*, 1991).
3. **Biochemistry** - aquatic plant species may be engineered so that they overproduce substances valuable as pharmaceuticals or speciality chemicals. For example,

several projects are under way to develop transgenic macroalgae that produce larger quantities of carrageenans than do wild species (Robinson, 1985), or to improve the yields of β-carotene and other speciality chemicals from aquacultured microalgae (Brown *et al.*, 1989).

4. **Settling** - the spat from most molluscan species require specific chemical signals before they will settle and grow on a surface (Morse, 1991). If the spat of species valuable to aquaculture could be engineered to settle where and when the aquaculturist desires, the efficiency of aquaculture operations would increase significantly.

5. **Disease resistance** - research can be aimed at increasing an organism's ability to resist infections by engineering its immune defence system to produce more or different cytokines, higher concentrations of antibodies against common bacterial, viral and fungal pathogens, and/or immune enhancers. These research goals as yet are theoretical because little is known about the immunology of aquatic vertebrates and invertebrates.

6. **Behavioural biology** - since some species being raised in aquaculture have the destructive trait of eating their eggs and fry, research may be directed at changing behaviour by, for example, genetically altering the quantity or type of hormones being secreted in aquacultured animals. Before applications can be envisaged in this area, basic research will have to clarify the biological basis of behaviour in fish and shellfish species.

Applications of Products from Genetic Engineering in the Aqueous Environment

Inanimate products obtained from genetically engineered organisms for applications in the terrestrial environment are used for many purposes, such as to improve health in humans and animals, remediate toxic pollutants, and enhance agriculture. Since the development of aquatic biotechnology lags behind "terrestrial" biotechnology by at least a decade, only three sets of applications from research in this field can be foreseen in the short term: aquatic animal health, bioremediation, and biofilm/bioadhesion.

Animal Health

Through biotechnology, unique vaccines may be developed against bacterial and viral diseases that commonly afflict aquatic organisms (Meyer, 1991). Vaccines will protect fish, shrimp, and other aquaculture organisms from diseases that now periodically decimate stocks, causing enormous economic damage in Asia and Latin America (Arthur & Sheriff, 1991). For example, research seeking to develop a vaccine that will protect salmon from infectious haematopoietic necrosis (IHN) is quite advanced. Prototypes of three kinds of vaccines have been developed and tested in the laboratory (Powers, 1990). When injected, all three types protected fish against the IHN virus. However, the first (a conventional type of killed vac-

cine) proved ineffective when administered in water. The second, a live attenuated type, was effective via water-borne inoculation, but questions regarding its safety have not been resolved. The third type, which is a recombinant vaccine, shows most promise in terms of efficacy, safety, and price. It stems from research done by scientists at the Oregon State University in the United States of America, who have identified, characterized, and cloned several genes coding for proteins that elicit antibody formation in fish. High levels of some proteins have been expressed (Engleking & Leong, 1991). The candidate vaccine is now being scaled up, and approval is being sought from the United States Department of Agriculture (USDA) to test it in the field.

Substances other than vaccines may have protective functions. For example, an extract from the shellfish *Ecteinascidia turbinada* protects eel from infection by the bacterium *Aeromonas* and in general enhances the immunological defences of blue crab, crayfish, and prawn (Colwell, 1986). More actual, the Phillips Petroleum Company Norway claims that its product, called Macrogard, a glucan produced by yeast, improves the efficiency of vaccines and helps farmed fish to resist disease (Hoffman, 1990).

Bioremediation

Bioremediation is the use of microorganisms or their products to break down pollutants and wastes in soil or water into harmless or less toxic end-products. Because bioremediation is relatively harmless to the environment, it holds significant advantages over conventional techniques, which usually rely on harsh physical or chemical treatment procedures to clean contaminated sites. As its techniques are perfected, bioremediation may become the preferred approach for cleaning polluted harbours, waterways, and other structures, as well as for decontaminating estuaries, mangroves, and similar sensitive coastal communities (Holloway, 1991).

Microorganisms are not covered in this chapter, so readers interested in microbial bioremediation are referred to Chapter 9 on biotreatment operations by Morris Levin, as well as to a publication on bioremediation for marine oil spills by the United States Office of Technology Assessment (OTA, 1991). However, two types of inanimate microbial products may be used in bioremediation procedures - dispersants and surfactants. Dispersants act to separate oil spills into small particles of oil, which then are easily transported from the surface to the water column and the sea bottom. Dispersants enhance microbial bioremediation because dispersed oil is more susceptible than massed oil to attack by microorganisms. Surfactants reduce the surface tension of the oil-water interface, allowing the oil to emulsify in the water. Surfactants produced by bacteria generally are non-toxic and biodegradable. For instance, a biological surfactant produced by *Pseudomonas aeruginosa*, when tested in the Prince William Sound, was found to increase the rate of oil removal from sand and rocks on beaches (Harvey *et al.*, 1990). Another biological surfactant, named Emulsan, has been isolated from the marine bacterium *Acinetobacter calcoaceticus*. It is widely used to clean oil-holding tanks in tankers and other

ships. Emulsan is also being tested in applications for enhanced oil recovery from oil wells and for pollution control (Weiner, 1985).

Biofilm/Bioadhesion

Whenever an object is immersed in natural water, aqueous microorganisms settle and secrete adhesive substances that allow them to adhere to it, forming a film. Soon, marine plants and invertebrates are attracted to the colonized surface, so they also settle, in the process creating a conspicuous crust. Organisms enmeshed in this crust produce acids, which corrode piers, derricks, and other structures. Encrustation also increases hull drag in ships, raising operating costs by 20–40 % (Costerton & Lappin-Scott, 1989). At present, paints containing heavy metals are used to coat exposed surfaces in order to repel organisms and prevent them from settling. However, these paints are toxic to workers and pollute seawater.

Aquatic biotechnology research seeks to clarify the molecular basis of the settling and adhesion process; findings may be used to develop clean methods for preventing the settling by marine organisms on ships and marine structures. For example, research could lead to the development of a non-toxic biological film that repels colonizers or interferes with settling. Conversely, the adhesives that encrusting organisms secrete and use to attach themselves to surfaces are interesting to industry because they can be used as powerful glues that set underwater or in other wet sites (Strausberg & Link, 1990). Indeed, genes in mussels and clams that code for the production of adhesives have been cloned and expressed in industrial microorganisms. Such marine adhesives have already been used to bond underwater structures and to fasten immobilized microorganisms on media in glass columns. Marine adhesives are also undergoing testing for use in humans, to bond bones in orthopaedic surgery, and to implant teeth in dental procedures (Strausberg & Link, 1990).

Standards, Guidelines, or Procedures Pertaining to the Use or Release of Transgenic Aquatic Animals and Plants or Inanimate Products in the Aqueous Environment

It can be said with certainty that as yet there are no standards, guidelines, or procedures pertaining to the use or release of transgenic aquatic animals or plants into the aquatic environment. This is probably due to the fact that aquatic biotechnology is at an early stage of development, with only a few of its products approaching the application stage. Therefore, there has been little reason for anyone to try to assess the risk of an activity that might take place at some indeterminate time in the future, and further, an activity that cannot as yet be fully defined. However, this situation will undoubtedly change in the next few years since, as mentioned above, products from genetically engineered organisms will soon be used in applications

that may affect lakes and oceans, and scientists will seek permission to test transgenic fish in the open aqueous environment. A sign of the future is that two species of transgenic fish already are being tested in closed, open-air ponds. If these tests prove successful, it would seem that the next step is for their developers to strive for more realistic testing in the open aquatic environment.

Since one cannot refer to standards, guidelines, or procedures specific to aquatic biotechnology or to biotechnology developments related to the aqueous environment, the history and experience of general biotechnology must be examined in order to identify the biosafety problems that such activities have generated and to consider regulatory measures that were taken to alleviate them.

Advanced biotechnology research can engender two types of products - inanimate products and transgenic organisms. Each of these two categories poses important questions to regulators: Do inanimate products yielded by genetically engineered organisms present risks above and beyond those posed by conventionally obtained products to humans, other animals, or plants? Would the deliberate release of transgenic animals and plants into the aqueous environment create hazards to existing life forms or to the environment itself?

With respect to inanimate products from genetic engineering, two main lessons have been learned by national regulatory agencies and international organizations since 1981, when the first genetically engineered product was presented for review. First, the process whereby a product is obtained does not present regulatory problems above or beyond those raised by conventional processes. In other words, whether a particular industrial fermentation process employs genetically engineered microorganisms or microorganisms developed through classical breeding and selection techniques matters little; the same standards of good manufacturing and safety practice apply in both instances. Second, the testing of genetically engineered products need not differ from that of conventionally obtained products; the same criteria of safety and efficacy apply equally to both. Thus, the regulatory situation is similar wherever in the world governments have promulgated or adopted regulations for inanimate products obtained through genetic engineering. No country, apparently, has enacted regulations aimed specifically at inanimate biotechnology products. Further, biosafety regulations, schemes, or guidelines developed by international agencies, such as the institutions of the European Community, the Organisation for Economic Co-operation and Development (OECD), and the World Health Organization (WHO), treat inanimate products from advanced biotechnology the way they treat products from conventional research and development (OECD, 1986). To illustrate the point, OECD has formulated biotechnology guidelines based on these criteria for its member nations (OECD, 1986, 1992). Similarly, an inter-agency working group, established jointly by the Food and Agriculture Organization of the United Nations (FAO), the United Nations Environment Programme (UNEP), the United Nations Industrial Development Organization (UNIDO), and WHO, has developed a set of guidelines that are intended to be used by governments of developing countries as models for local laws (UNIDO, 1992). However, to reiterate, no set of guidelines specifically addresses risks associated

with aquatic biotechnology or the application of inanimate products in the aquatic environment.

The second issue, which concerns the deliberate release of living transgenic organisms, is currently receiving much attention from the scientific community and the public. The two major risks of deliberate release are that the introduced organism may harm directly the environment or any of its inhabitants, and that a gene or genes from the introduced organism's genome may disperse and become integrated in the genome of a non-target organism. These concerns, as well as risk assessment schemes, are described and discussed on pp. 158-166.

Preventing or Controlling the Release of Transgenic Organisms into the Aqueous Environment

Apparently, no government or international agency has developed containment or other mitigating approaches to prevent or control the release of transgenic plants or animals into the aqueous environment. This being the case, it is useful to see whether an analogous situation exists from which one might draw lessons pertinent to future schemes relevant to transgenic aquatic plants or animals.

The world's experience with managing the dispersals of exotic marine organisms, i.e., aquatic animals or plants that have been transported from their normal habitat to a new environment, seems to suggest possible problems related to future introductions of another type of exotic organism: transgenic aquatic plants or animals. Therefore, information can be drawn from past instances in which aquatic organisms were introduced into new sites, and on the basis of this information one can identify risks that may be inherent to the future field testing of transgenic aquatic organisms, assess the likelihood of the risks being realized, and devise methods whereby risks may be lowered or eliminated. Accordingly, one must consider past natural and mediated dispersals and discuss what may be learned from them, and then review national and international attempts to prevent or control damaging introductions.

Dispersals of Aquatic Species

The most usual mechanism whereby species disperse naturally is range expansion (Mann & Rosenfield, 1992). Because little scientific effort has been directed at clarifying this phenomenon, it is not well understood. Therefore, no predictions can be made about the range-expansion possibilities of an aquatic organism considered for introduction into a new site. The lack of scientific data regarding range expansion is certain to cause problems for investigators attempting to assess the risks associated with a proposed deliberate introduction of an aquatic species into a new locale. It probably would make little difference whether the organism in question came from a foreign location or were genetically engineered, since the same uncertainties regarding range expansion would be present in both cases.

Mediated dispersals of aquatic species are the deliberate or accidental introductions, by human actions, of species into a habitat where they have never existed before. The accidental introductions of exotic species by human actions probably commenced when mankind took up sailing, with ships carrying organisms from one place to another in their ballasts (Aquatic Nuisance Species Task Force, 1992; Carlton, 1992a), or encrusted on or bored into their wooden hulls (Peterson, 1992). The construction of inter-oceanic and inter-lake canals extended the range of ships, and also opened new migratory paths for organisms. Traders have shipped crustaceans, fish, and molluscs to markets far away from the fishing grounds. Pathogens that afflict these fishery products have been carried along (Carlton, 1989). Owners of personal aquaria have discarded ornamental fish and other organisms by releasing them into local waterways (Andrews, 1992). It is easy to see how aquatic species numbering in the thousands have accidentally been transported across the globe in innumerable patterns since transoceanic trading commenced and, as a consequence, how an unknown but significant percentage of them have been able to successfully colonize new sites.

Besides being responsible for accidental introductions and transfers, traders have removed aquatic species from territories where they were indigenous and transported them to new sites for some preconceived purpose. Such deliberate introductions of exotic aquatic species, like the deliberate introductions of animals and plants on land, most often have been done to develop aquaculture and fisheries, but in some cases, the aim was to affect the environment (Welcomme, 1986; Stickney, 1992). In the 1950s and 1960s, there were large-scale deliberate introductions of fish and shellfish throughout the Third World to improve or establish aquaculture, including the African Tilapia into Asia and Latin America, Indian major carps into Southeast Asia and Latin America, and the black tiger shrimp (*Penaeus monodon*) and the white shrimp (*P. orientalis*) into many Asian and some Latin American countries. Examples of deliberate introductions for environmental aims include the introduction of the fish species *Gambusia affinis* and *Lebistes reticulatus*, which preferentially feed on mosquito larvae, into parts of the world where malaria is endemic, and the introduction of the grass carp (*Ctenopharyngodon idelle*), which is used to control the over-growth of aquatic plants, into hundreds of canals and other waterways throughout the world's tropical and subtropical regions.

In the late 1970s and early 1980s, large-scale introductions included striped bass (*Morons saxatilis*) into the United States west coast region, the Pacific oyster (*Crassostrea gigas*) into the United States of America and Canadian west coast regions and into France, the Pacific salmon (*Oncorhynchus* species) into Atlantic waters, the pink salmon (*O. gorbuscha*) into the Arctic Sea coastal waters of the former Soviet Union, a shrimp species from Panama (*P. stylirostris*) into Hawaii, and the Pacific seaweed (*Undaria pinnatifida*) into France (Sindermann, 1986; Welcomme, 1986). More recently, in 1989, the macroalgal species *Euchema spinosum* was transported from the Philippines to Zanzibar, where it is now cultured, harvested, dried, and exported to Europe, where polysaccharide is extracted for use as a food conditioner (Zilinskas & Lundin, 1993).

Many of the deliberate introductions have benefited local populations and improved the economies of recipient countries. However, like introduced species on land, some aquatic introduced species have caused damage ranging in severity from barely discernible to serious. For example, while *G. affinis* prefer to eat mosquito larvae, they will also devour eggs and fry of other fish. The grass carp transmits a cestode capable of causing disease in other species of fish. The Pacific seaweed *Sargassum muticum*, which was inadvertently introduced with *C. gigas*, eventually grew so dense along the English and French coasts of the English Channel that it started to interfere with transport and recreational activities. The widely introduced shrimp *P. vannamei* carries a pathogen called infectious hypodermal and haematopoietic necrosis virus, which has spread in aquaculture facilities throughout the Pacific rim countries, decimating shrimp stocks.

An extensive literature addresses the causes and effects of damaging dispersals. Some authors discuss and analyse problems pertaining to aquaculture generally (Carlton, 1992b; Courtenay & Williams, 1992; Davidson *et al.*, 1992); others write about more specific problem areas, including those pertaining to aquatic plants (Neushul *et al.*, 1992), molluscs (Farley, 1992), shellfish (Kern & Rosenfield, 1992; Lightner *et al.*, 1992), and finfish (Ganzhorn *et al.*, 1992; Thorgaard & Allen, 1992). By analysing the effects of past mediated dispersals, whether accidental or incautious deliberate introductions, six lessons may be derived (Zilinskas *et al.*, 1995):

1. An introduced animal may disrupt local fauna through competition or predation. In the worst case, the introduced exotic species may annihilate one or more wild species.
2. The introduced species may damage or disrupt some aspect of the habitat into which it is introduced, thereby upsetting natural balances, which can lead to the degradation or destruction of the local environment.
3. In some cases, a host stock introduced into a new locale will suffer genetic degradation. In other words, when an introduced species breeds with wild species inhabiting the new locale, some of the favourable genetic characteristics that it possesses may be lost or degraded.
4. Conversely, wild species inhabiting the locale into which the exotic species has been introduced may undergo genetic degradation. For example, if the introduced species were to breed with indigenous wild species, the adaptations for survival that the wild species have evolved may become diluted or may disappear in hybrid progeny. Even worse, important genes may be lost if the exotic species displaces or replaces the wild species.
5. In addition to posing risks themselves, introduced species may carry or contain exotic disease agents capable of infecting susceptible indigenous species. The newly introduced disease agent can cause damage that differs in both quantity and quality from that caused by the host organism.
6. Once an introduced species has successfully colonized a locale, it may become endemic and impossible to eliminate.

If a general lesson can be derived from the history of introductions of aquatic species, it would be that, while accidental introductions, such as that of the zebra mussel into North America, probably have caused more damage than deliberate introductions, the latter also have significantly harmed local and regional habitats, as the plight of the African lakes demonstrates (Baskin, 1992). Further, our predictive powers are not sufficiently sophisticated to predetermine whether or not the candidate species for introduction indeed will directly or indirectly cause damage to native species or the environment; nor is it possible to predetermine whether the cost of damages stemming from introductions will ultimately outweigh benefits. Therefore, a certain degree of risk will always accompany introductions.

Controlling the Dispersal of Exotic Aquatic Organisms

Due to the problems that dispersals of aquatic organisms have engendered throughout the world, many governments have adopted and implemented control measures. At times, guidelines developed by intergovernmental and non-governmental organizations have formed the basis for national legislation. It is useful to review briefly national and international measures that seek to control the dispersal of aquatic organisms.

National Measures to Control Introductions

After having reviewed available information about introductions of exotic fish into Australia (McKay, 1984), Canada (Crossman, 1984), Mexico (Contreras-B & Escalante-C, 1984), New Zealand (McDowall, 1984), Oceania (Maciolek, 1984), Puerto Rico (Erdman, 1984) and the United States of America (Zilinskas *et al.*, 1995), it is possible to draw three general conclusions about their effects and the response of governments to damaging introductions.

First, it is clear that every country and region has suffered some negative effects from deliberate or accidental introductions of exotic aquatic organisms. However, since some of the surveyed countries, such as Mexico, do not keep detailed records of introductions, and most states keep no such records, or record only rudimentary data, the full impact of introductions throughout the world cannot be determined.

Second, when faced with the unequivocal fact of one or more damaging introductions, governments usually react by promulgating rules or laws that seek to prevent further damaging introductions. These laws or regulations tend to be of two kinds. In the first instance, laws are passed that focus on deliberate introductions, and some time later, additional laws are promulgated that seek to prevent accidental introductions.

Third, national laws that seek to control and manage deliberate introductions list several criteria for rejecting a proposed introduction. Rejection criteria common to national laws include:

1. Whether the organism under consideration has a proven capacity for causing environmental damage by, for example, rapid reproduction, method of feeding, or having an unusual ability to survive or disperse at the new site.
2. Whether the organism is exceptionally voracious or aggressive. Whether the organism is capable of inflicting harm to man or other animals by, for example, being able to inflict wounds with sharp teeth, venomous spines, or electric organs.
3. Whether the organism is capable of carrying or spreading infectious or parasitic agents that may harm indigenous populations.

Attempts by governments to prevent accidental introductions are a more difficult endeavour. Usually, an accidental introduction with catastrophic consequences triggers legislative attempts to prevent recurrences. For example, in the United States of America, the disastrous accidental introduction of the zebra mussel prompted Congress to pass Public Law 101-646, also known as the Non-Indigenous Aquatic Nuisance Prevention and Control Act of 1990 (Zilinskas *et al.*, 1995). Although the primary impetus for the passage of this law was congressional concern about the zebra mussel infestation of the Great Lakes, it provides a framework for a set of actions addressing accidental introductions generally. It has five objectives: (i) to prevent, for example, through ballast water management, the introduction and dispersal of exotic species into United States waters; (ii) to coordinate federally supported research and prevention activities regarding aquatic nuisance species, especially the zebra mussel; (iii) to institute control measures to prevent and control non-intentional introductions of exotic species through means other than ballast water; (iv) to minimize impacts when exotic species become established; and (v) to establish a national program to help states control zebra mussels (Kern & Rosenfield, 1992). It is probable that other countries have adopted similar legislation to prevent accidental introductions.

International Measures

On the international level, several sets of codes and rules dealing with dispersal have been promulgated (Carlton, 1992c; Jacob, 1994). The seminal event was the adoption of the Revised Code of Practice to Reduce Risks for Adverse Effects Arising from Introductions and Transfers of Marine Species, a code designed by the International Council for the Exploration of the Sea (ICES) in 1973 (and revised in 1979). Other codes of practice, position statements, and conventions on the subject have been issued by the American Fisheries Society (1973), the UN Conference on the Law of the Sea (1982), the Council of Europe (1984), FAO's European Inland Fisheries Advisory Commission (1984), and the International Union for Conservation of Nature and Natural Resources (1987). The overriding objective of these codes and statements is to direct concerted international actions to avert future accidental introductions and to prevent adverse effects from deliberate introductions. None of the codes mentioned here are binding on states, however.

Risk Assessment Schemes Related to the Use or Release of Transgenic Animals or Plants into the Aquatic Environment

No risk assessment schemes have been developed as yet to determine specific risks pertaining to a proposed use or release of transgenic animals or plants into the aquatic environment. This being the case, the next two sections examine risk assessment schemes used in the two fields that constitute marine biotechnology (general biotechnology and marine biology), and then, a third section suggests how they might be amalgamated to encompass transgenic aquatic macroorganisms.

Risk Assessment Schemes Used in Biotechnology Applications

The United States National Research Council (NRC) has scrutinized the issues related to the field testing of genetically engineered microbes or plants in terrestrial situations and concluded that there are three essential criteria for evaluating the risks associated with a proposed release (NRC, 1989):

1. Is there sufficient knowledge about the properties of the organism and the environment into which it may be introduced?
2. Can the organism be confined or controlled effectively?
3. What are the probable effects on the environment, should the introduced organism or a genetic trait it carries persist longer than intended or spread to non-target organisms?

OECD, which already began to consider the biotechnology safety issue in 1983 (Teso, 1992), published its guidelines for the field testing of genetically manipulated organisms in 1992 (OECD, 1992). Its scheme is substantially the same as that of the NRC, including the three essential criteria for evaluating risks. However, for the purpose of evaluating the safety of testing aquatic organisms in the field, it is useful to review specific measures developed by OECD related to evaluating the field testing of plants. Although these measures pertain to the terrestrial environment, it is likely that similar measures will be developed when schemes for future field tests in the aquatic environment will be formulated. The specific measures refer to the biology of the organism and to the site where the proposed test is to take place (OECD, 1992).

Biology of the Organism

When evaluating possible risks associated with the field testing of a plant species, certain characteristics of that species must be considered, as follows:

1. The reproductive potential and biology of the plant, such as its flowers, pollination requirements, and seed characteristics, and the history of the plant's controlled reproduction in an environment similar to the test site.

2. The mode of action, persistence, and degradation of any newly acquired toxic property.
3. The characteristics of the biological vector used to transfer DNA to the plant.
4. The possible interactions with other species and biological systems.

Test Site

Certain characteristics of the proposed field testing site need to be taken into account for the safety evaluation, as follows:

1. Significant ecological and environmental considerations related to the site that might bear on the safe carrying out of the field test, such as the water runoff pattern, water table, wind patterns, and other meteorological and geophysical phenomena peculiar to the test site.
2. The size of the site, including a possible safety zone.
3. The site's geographic location as related to the nearby or distant presence of biota that could be affected by the organism being tested.

Specific methods for safely managing the field testing of genetically engineered organisms are in a state of flux. Referring to the experience of the United States of America in this area, proposals for testing genetically engineered organisms in the field are dealt with on a case-by-case basis by the USDA, specifically by its Animal and Plant Health Inspection Service (APHIS).

The first step inherent to any proposal considered by the USDA is the drafting of a thorough environmental-impact statement. The statement addresses health and safety concerns by considering both direct and indirect effects stemming from the proposed release. It must provide convincing evidence for a conclusion that the proposed release would probably not significantly alter or harm any aspect of the environment or its biota. If the USDA assesses the project as having a negligible impact on the environment, this finding is widely publicized before a final decision is made in order to give the public and its representatives the opportunity to scrutinize the environmental impact statement and to comment on it. The agency takes these comments, as well as statements made by other interested parties, into account before it makes its decision. USDA and the United States Environmental Protection Agency have given final approval to over 900 field trials of genetically engineered organisms, most of them plants. No negative effects have so far been observed, indicating that the United States scheme seems to be working, at least in the short term (Miller *et al.*, 1991).

Referring to the NRC and OECD criteria, the field testing of transgenic terrestrial animals is hardly mentioned. This is because they usually are easy to contain, and, even if set free or accidentally released, the transgenic animal undergoing testing is not likely to disperse easily or cause damage. However, one cannot be so complacent about the prospective field testing of aquatic animals. There are two

reasons for concern when dealing with aquatic organisms. First, an important reproductive characteristic of most aquatic animals is that they release thousands to millions of fertile eggs or spawn into the water that surrounds them. Second, water is a very good carrying medium, being gentle to living organisms and proteinaceous genetic material. Also, most water is not still; rivers flow into lakes and oceans, and ocean water is moved around by eddies, currents and wind. Thus, the barriers that researchers use to contain terrestrial animals cannot be used or duplicated to contain aquatic animals; instead, new containment systems must be designed and deployed.

Although several different species of aquatic macroorganisms have been transformed, only two of them are undergoing open-air testing: transgenic carp and catfish. The technical aspects of these tests are as follows.

The carp being tested is a scaleless variant of the common carp (*Cyprinus carpio*), called mirror carp, which has been genetically modified by the insertion of two types of foreign genetic material - a gene coding for a trout growth-hormone gene and a so-called Rous sarcoma virus promoter, which is a genetic marker. The transgenic carp was developed by a team including scientists from the Centre of Marine Biotechnology, Maryland, Stanford University, California, and Auburn University, Alabama (Chen & Powers, 1990; Chen *et al.*, 1992). In 1990, the team submitted a proposal to the United States Department of Agriculture (USDA), requesting permission to raise 50 000 fry that had been spawned from nine transgenic carp in ten outdoor pools. The team proposed that after three months the number of fry be reduced to 300 per pond; these were to be marked for identification and studied for the next 15 months. The fish were then to be destroyed before reaching sexual maturity. The ponds stocking the fish were to be well protected by fences, nets and filters, and there was to be no direct connection between the ponds and the existing waterways. Further, if a natural event such as a hurricane were to threaten the integrity of the testing site, the fish being tested could have been killed by toxic chemicals on very short notice.

After performing an environmental assessment of the proposed project (USDA, 1990) and presenting its findings at a series of public hearings, and after having received comments and critique from interested members of the public and from public interest groups, the USDA decided on a "Finding of No Significant Impact", that is, it determined that the... "experiment with transgenic carp presents no significant risks to the environment (Anonymous, 1990)". It gave approval for the experiment to proceed, beginning in the spring of 1991. Actual testing of the transgenic carp began in June 1991. A few months later, a similar open-air test protocol was proposed for a newly developed transgenic catfish, which had an inserted growth-hormone gene from rainbow trout (Anonymous, 1991). This proposal was approved by the USDA in early 1992 (Anonymous, 1992).

For the purpose of this chapter, it is useful to compare the testing conditions for the transgenic carp and catfish to the three criteria formulated by the NRC (and *de facto* sanctioned by OECD). First, in scientific terms, the carp and catfish have been extensively studied, so they are well characterized. The insertion of a trout growth-hormone gene will not change either fish's physical properties, except the one bearing on growth. Whether the alien gene would change their behaviour patterns is, of course, a question that can be answered through testing. Since the testing is being carried out in a closed, artificial system, the environment into which the transgenic fish is introduced is known. For these reasons, the first criterion is largely satisfied. Second, unless a deliberate, criminal attempt is made to release the fish, the conditions under which the testing of the transgenic carp and catfish is taking place preclude the possibility of escape. Thus, certainly, the second criterion is also met: the organism being tested is confined and controlled effectively. The third criterion is not applicable since the test conditions preclude persistence or spread.

Clearly, the open-air testing of the transgenic carp and catfish is so circumscribed and controlled that it resembles more closed-system testing than true field testing in the aquatic environment. Nevertheless, since scientists are entering a new realm of testing (the aqueous environment), and the test subjects themselves are unique, the approach that has been taken is a prudent one. If these tests go well, and there is no reason at this time to believe otherwise, the requirements for future open-air testing can be eased. Such a progression of relaxation occurred in the development of field testing in the terrestrial environment; it probably will be repeated in the aqueous environment.

Risk Assessment Schemes for Proposed Introductions of Aquatic Species

Due to the uneven implementation of the ICES code, Dr Carl Sindermann has developed several strategies for dealing with future proposals for introductions (Sindermann, 1986, 1992). The first of these strategies deals with the unsatisfactory state of international management measures related to controlling introductions of naturally occurring aqueous animals and plants. Here, UN agencies and non-governmental organizations should take the lead in educating the public, policymakers, and national regulatory agency personnel about the damage that the importation of a non-indigenous species can do to native stocks and the local environment. Once national authorities have become sensitized to the problems that might accompany or result from introductions, they would be in a good position to recognize that it is in the best economic interest of a country to have a strong regulatory regime in place in order to prevent unauthorized introductions and to delineate the conditions under which authorized introductions may proceed.

The strategy most relevant to this chapter emphasizes regional approaches to controlling the transfer of organisms, where the governments involved ensure uniformity and continuity. Whatever approach is adopted by a government, it should be implemented according to the general operating principles set forth in the ICES

code. These principles are based on the assumption that risks from introductions are never zero. This being so, national regulatory regimes should be designed so as to minimize risks from proposed introductions. Risk reduction includes, *inter alia*, a thorough study, in its natural habitat, of the organism proposed for introduction, assessing the possibility of developing native stocks as an alternative to introducing a new stock, stressing the introduction of non-migratory species over migratory species, and establishing a mechanism for the continuous monitoring of the introduced stock. It is particularly important that the scientific implications of a proposed introduction be analysed before the event through a review of ecological considerations, such as competition and predation; genetic considerations, including the potential for hybridization and change in gene frequency; behavioural considerations, including interactions between the introduced and native species; and pathological considerations, including the possibility that the introduced species will carry along with it new infectious diseases (Sindermann, 1986, 1992).

A more detailed and comprehensive protocol than that proposed by Sindermann for guiding the evaluation process for proposed exotic fish introductions has been developed by Kohler and Stanley (1984). Their approach is tiered, with five levels of review, each requiring progressively more information (see Fig. 8.1). The evaluation process is carried out by an "Exotic Fish Protocol Committee", which is set up to deal with a specific proposal of introduction.

The first review level is to determine the feasibility of the proposed introduction. Will the introduced species respond to some need not being met by indigenous species? Will the stock be sufficiently available to meet future needs? Will removal of members of the introduced species endanger it? Is escape likely from the testing site? If any of these criteria cannot be met, the proposed introduction should probably be disapproved.

The second review level deals with the introduced organism's ability to acclimatize itself to the new habitat (i.e., form a self-sustaining population), should it escape. If the proposed organism's acclimatization potential is high, it might be better to forego introducing it.

The third review level consists of a benefit-cost analysis. If it is likely that the organism proposed for introduction will cause unacceptable damage to the environment or present hazards to humans, it should not be introduced.

The fourth review level consists of researching the scientific literature and databases according to the format used for a FAO Species Synopsis. In addition, research should generate complete information on the environmental impacts of past introductions of a similar nature.

The fifth review level allows the Exotic Fish Protocol Committee to request that additional research be carried out, for example, to resolve remaining questions or clarify potential problems. Depending on what research is required, and on the findings of the additional research, the five-level evaluation process may have to be repeated.

Elements of a Future Risk Assessment Scheme for the Field Testing of Genetically Engineered Fish

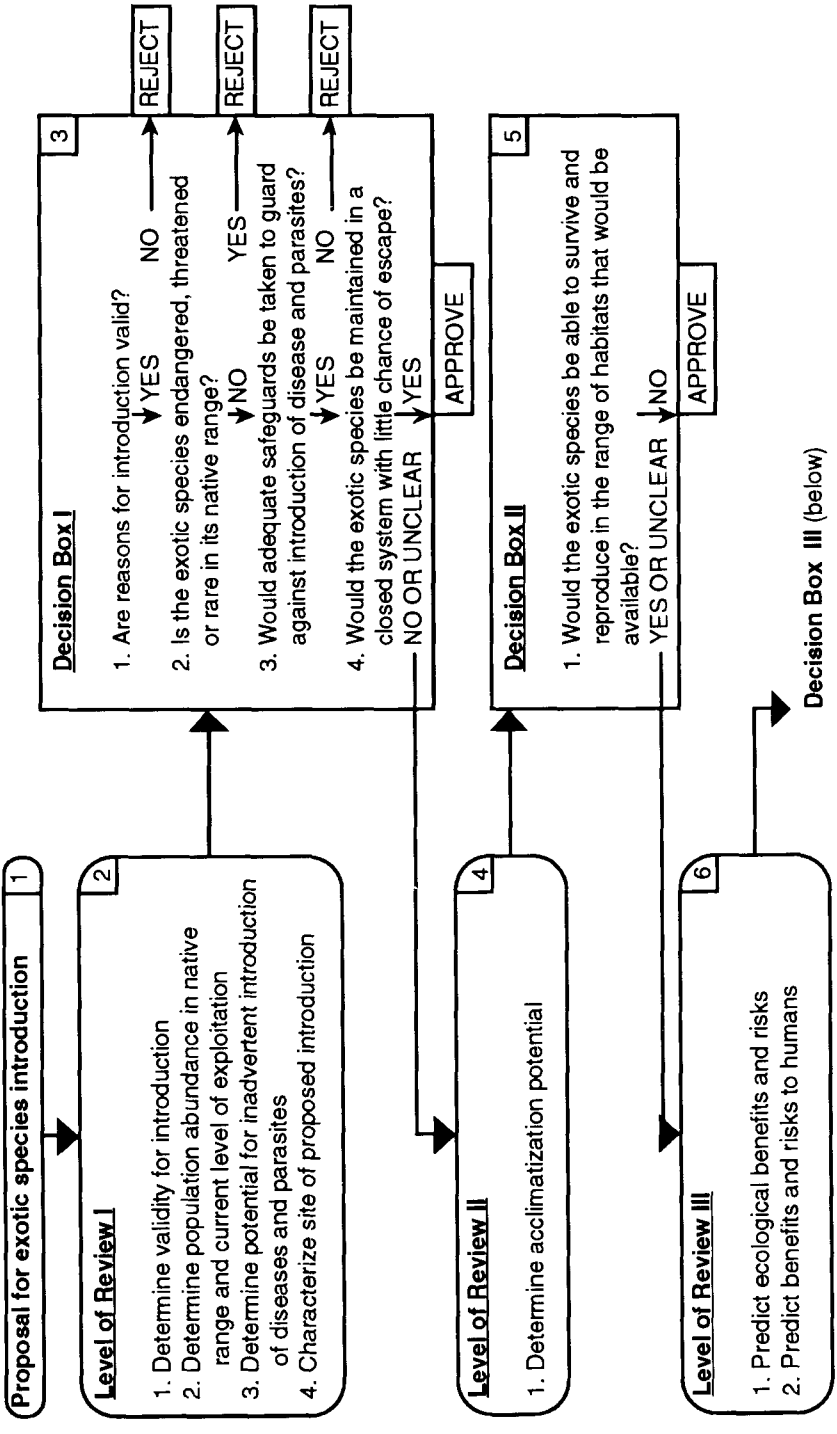
After a transgenic aqueous animal or plant is developed in the laboratory, its testing should begin in a contained, open-air facility like the ones used in Alabama to test transgenic carp and catfish. Such tests are likely to generate many data on the growth patterns of the transgenic species, its behaviour, and so forth, but since they are being carried out in closed systems, they cannot be considered more than prototypes of the future field testing in the open aquatic environment. Nevertheless, they do allow testing to proceed safely, while providing researchers with the opportunity to become familiar with testing procedures, permitting regulators to assess the risks pertaining to aqueous macroorganisms and enabling them to formulate guidelines or rules for testing based on these assessments. However, because such testing is elaborate and expensive, it is hardly an undertaking to be taken on lightly. Also, it should be recognized that the number of research units in the world that could carry out such a test is rather small.

No one so far has proposed to test genetically modified organisms in the open aquatic environment. Here, the tester, and the regulator, would face special problems, not encountered in closed-system testing, even if it is carried out in open-air facilities. Similar to field tests in the atmosphere (Stetzenbach *et al.*, 1992), tests performed in the open aquatic environment may not insure the biological isolation of the organisms being tested. Biological isolation cannot be guaranteed for two major reasons: first, due to the reproductive characteristics of marine macroorganisms, which often involve the emission of large numbers of eggs or fry into the aquatic environment, and, second, because of the three characteristics of the aquatic environment - the continuity of the aqueous space, the perpetual motion of water and particles suspended in it, and the potential existence of unfamiliar biological modes of gene dispersion (Zilinskas & Lundin, 1993). Further, the major lesson drawn from past introductions of exotic organisms is that, if and when members of the aquatic species being tested escape, neither the probability of their survival nor their subsequent dispersal via natural mechanisms can be predetermined; in other words, the consequences of escapes are incalculable. In view of these difficulties and uncertainties, the evaluation process for a future open-field test of aquatic macroorganisms must be designed with extreme care.

Such an evaluation process could be an adaptation of the one developed by Kohler and Stanley and described above. Thus, a five-tier protocol would be followed, with guidance provided by an *ad hoc* test committee set up by the research institute's biosafety committee.

Thus, the first review level would determine the feasibility of the proposed introduction, including the scientific feasibility of the testing, the possible value of the transgenic species to science or to industry (or both), and the likelihood of test subjects escaping from the test site.

The second review level would deal with the transgenic organism's ability to acclimatize itself to the new habitat, should it escape. If the transgenic organism's


Proposal for exotic species introduction
1
Level of Review I
2
1. Determine validity for introduction
2. Determine population abundance in native range and current level of exploitation
3. Determine potential for inadvertent introduction of diseases and parasites
4. Characterize site of proposed introduction
Decision Box I
3
1. Are reasons for introduction valid?
YES
NO
REJECT
2. Is the exotic species endangered, threatened or rare in its native range?
NO
YES
REJECT
3. Would adequate safeguards be taken to guard against introduction of disease and parasites?
YES
NO
REJECT
4. Would the exotic species be maintained in a closed system with little chance of escape?
NO OR UNCLEAR
YES
APPROVE
Level of Review II
4
1. Determine acclimatization potential
Decision Box II
5
1. Would the exotic species be able to survive and reproduce in the range of habitats that would be available?
YES OR UNCLEAR
NO
APPROVE
Level of Review III
6
1. Predict ecological benefits and risks
2. Predict benefits and risks to humans
Decision Box III (below)

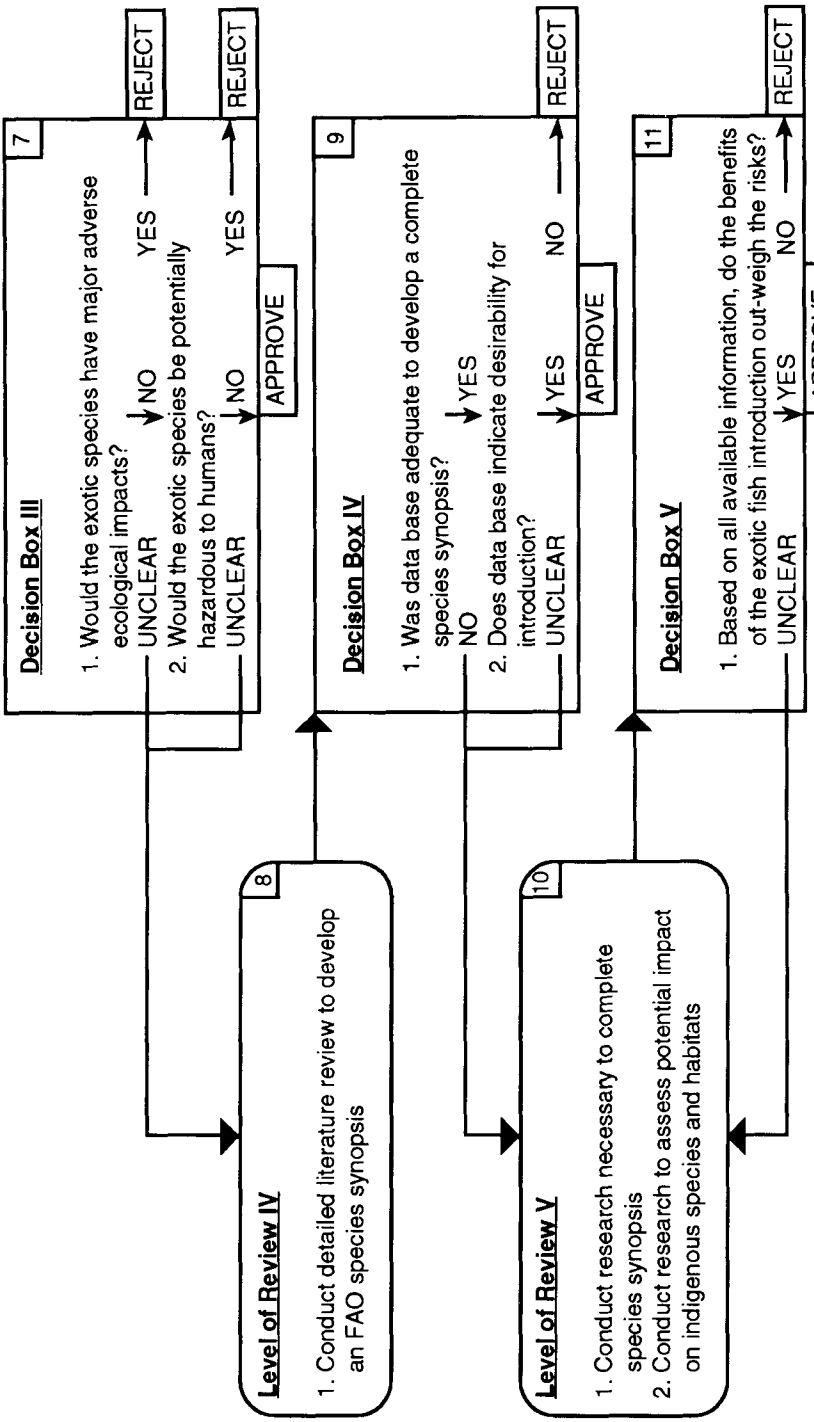


Fig. 8.1. Review and decision model for evaluating proposed exotic introductions.

acclimatization potential is deemed to be high, it should not be tested in the field unless it is first sterilized (see below).

The third review level would consist of assessing the risk of the testing. The risk assessment would seek to find out if it is likely that the transgenic organism proposed for testing will cause unacceptable damage to the environment or present hazards to humans. The risk assessment scheme that would be followed could very well be adapted from those developed by the NRC and/or OECD for use in field tests in the terrestrial environment, as described and discussed above.

(A factor that is not taken into account in the NRC/OECD risk assessment schemes is the reproductive potential of aquatic animals. As discussed above, the emission of large numbers of eggs by aquatic organisms and the ability of moving water to disperse the eggs over a wide area create a situation unlike that found on land. It would seem, then, that the best means by which risks related to the field testing of transgenic fish and shellfish can be reduced is to sterilize all test subjects slated for outgrowth in culture. Fish, for example, may be sterilized by two methods. First, certain hormones can be administered to fish embryos that render them sterile. Researchers do not favour this method since it cannot achieve 100% sterilization, and hormonal residues may contaminate food fish. Second, fish eggs can be treated so the progeny are triploid [each fish carries three sets of chromosomes rather than two]. Triploids are sterile. For added safety, triploid induction can be combined with further treatment that produces an all-female progeny. Triploid females are 100% non-fertile. However, non-sterile broodstock would have to be maintained in secure containment facilities apart from the test site [Kapuscinski, 1990].)

The fourth review level would consist of researching the scientific literature and databases according to the format used for an FAO Species Synopsis in order to generate complete information on the impacts of past introductions of a similar nature. Even though little or no information might be found pertaining to the transgenic organism itself, it should be remembered that this transgenic organism is genetically nearly identical to its wild relative, since only one gene (and its operon) has been added or changed. Thus, the consequences of introducing, say, a transgenic carp into a test site would most probably be about the same as those of past introductions of its wild relatives into new sites.

The fifth review level probably would consist of the test committee requesting additional research to be carried out, for example, in order to clarify site characteristics or the reproductive potential of the test subject. We can hypothesize that more information would be required to define the ecoregion containing the test site, clarify sub-regions of the ecoregion, delineate reference sites to be used for comparison purposes, and so forth. Depending on what this research finds, the five-level evaluation process might be repeated.

Conclusions

Does aquatic biotechnology or, more specifically, do inanimate products and aquatic organisms obtained through genetic engineering pose special or unique problems to risk assessors and regulators? From the descriptions, discussions, and analyses presented in the foregoing sections, it appears that the answer is both no and yes.

Natural inanimate products, whether of terrestrial or aquatic origin, are characterized in the same manner. For example, carbamates, lactones, and terpenes will have certain general characteristics in common whether they were isolated from a sponge or a terrestrial plant, although their structures may vary widely. Undoubtedly, as more organisms from extreme environments are collected, screened, and investigated, exceptional compounds, showing antibiotic, antiviral, antitumour, and other properties, will be found. However, if the experience of general biotechnology is a guide, no matter how unique the structure of an aquatic natural product, it will not create a novel situation, or uncommon hazard, demanding a new risk assessment scheme or regulatory regime. For example, if a unique marine toxin is discovered, its physiological action is not likely to differ markedly from that of a known toxin; nor is its toxicity likely to be significantly greater than that of known toxins. Therefore, testing done according to established procedures would elucidate the chemical structure of the new compound, explain its mode of action, and eventually assess its effectiveness and safety.

Similar to natural inanimate products, a cell-culture system using genetically modified microorganisms and developed through aquatic biotechnology techniques will not create an unusual situation demanding extraordinary control measures or regulations. For example, the development of a recombinant vaccine against a virus-caused fish disease would most probably be done using procedures similar to those used to develop vaccines for other animals; the field testing of the fish vaccine would most likely follow established animal vaccine testing procedures; and the whole developmental and testing process would be monitored adequately by existing national regulatory authorities. Consequently, current protocols for testing products yielded by conventional or advanced biotechniques are appropriate for use in the testing of aquatic biotechnology products. For these reasons, inanimate products of biotechnology employed in the aqueous environment do not present special or unique problems to risk assessors or regulators.

It would seem that, if anyone attempted to take advantage of the superior characteristics of a transgenic fish species by intensively culturing it in cages or pens emplaced in a pond, lake, river or brackish-water estuary, the possibility of some transgenic fish escaping would be high. The consequences of such an escape cannot be calculated, but could range from non-discernible or minimal to severely damaging to existing wildlife. If past experience of terrestrial field testing of organisms is a guide, no ill effects would be likely to result as long as the test subject has been minimally altered genetically by having a single alien gene inserted in its genome.

Today, the possible field testing of aquatic transgenic organisms resembles the situation that existed about ten years ago, when field tests involving terrestrial plants were imminent. However, one can argue that, in contrast to this earlier period, scientists now can access the experience of past field tests to draw lessons for future aquatic field testing. In addition, better methods for assessing risks have been developed, and sophisticated techniques for detecting and tracking genetic material are available. Today's scientists are thus better prepared in several ways than were yesteryear's researchers. First, they should be able to adopt risk assessment methodologies that have proven their worth in the terrestrial environment. Suggestions of how this may be done are presented above. Second, they should be better prepared to draft comprehensive environmental impact statements prior to testing, employing the newly adapted risk assessment methodologies. Third, these risk assessment schemes should prove adequate for designing safe test protocols and instituting efficient mechanisms for monitoring test events and the long-term effects of tests.

Having stated that today scientists are probably better prepared to consider undertaking field tests of transgenic aquatic organisms, it is still quite difficult to evaluate and determine the possible effects of field testing transgenic aquatic animals, plants, and microorganisms. Furthermore, as noted above, current risk assessment schemes either do not relate well to the aquatic environment or are inappropriate. Even the schemes employed to assess the risks related to the deliberate or accidental introduction of exotic aquatic animals or plants, which have a long history, are still being developed and perfected. In view of these shortcomings, it can be stated that no existing regulations or laws are adequate for managing the field testing of transgenic aquatic animals or plants. The conclusion is unavoidable - the present regulatory situation does not favour the testing of a transgenic aquatic macroorganism in the open environment. Due to the many uncertainties that would accompany such a field testing, regulatory agencies should defer making such decisions until research in biological oceanography, microbial ecology, and environmental toxicology have clarified in detail the mechanisms of dispersal of organisms and genes in the aquatic environment, and a satisfactory risk assessment methodology for the field testing of aquatic transgenic organisms has been developed.

Note

1. Terms relevant to dispersal used in this chapter are in accord with the definitions elaborated by the International Council for the Exploration of the Sea (ICES) (Mann & Rosenfield, 1992). Accordingly, an introduced species is one that has intentionally or accidentally been transported and released into an environment *outside* its present range. A transferred species is any species that by intent or accident has been transported and released within its present range. Species may be introduced or transferred through natural actions or in the course of human activities.

References

Andrews, C. (1992) The ornamental fish trade and fish conservation. *INFOFISH Int.*, No.2, 25-29.

Anonymous (1990) Transgenic fish experiment gets green light. *Biotechnol. Notes*, 3(10), 1-2.

Anonymous (1991) Transgenic catfish may be next in line. *Biotechnol. Notes*, 4(10), 2-3.

Anonymous (1992) ABRAC panel gives nod to catfish study. *Biotechnol. Notes*, 5(4), 1.

Aquatic Nuisance Species Task Force (1992) *Proposed Aquatic Nuisance Species Program*. United States National Fish and Wild Life Service, Alexandria, VA, USA

Arthur, R. & Sheriff, M. (1991) Towards international fish disease control in Southeast Asia. *INFOFISH Int.* No. 3, 45-48.

Baskin, Y. (1992) Africa's troubled waters. *BioScience*, 42(7), 476-481.

Brown, L.M., Dunahay, T.G. & Jarvis, E.E. (1989) Applications of genetics to microalgae production. In: *Developments in Industrial Biotechnology, Volume 31*, Pierce, G.E. (ed.), pp. 271-274. Elsevier, New York.

Carlton, J.T. (1989) Man's role in changing the face of the ocean: Biological invasion and implications for conservation of near-shore environments. *Conserv. Biol.*, 3(3), 265-273.

Carlton, J.T. (1992a) Marine species introductions by ship's ballast water: An overview. In: *Introductions and Transfers of Marine Species: Achieving a Balance Between Economic Development and Resource Protection*, DeVoe, M.R. (ed.), pp. 23-26. South Carolina Sea Grant Consortium, Milton Head Island, SC, USA.

Carlton, J.T. (1992b) Dispersal of living organisms into aquatic ecosystems as mediated by aquaculture and fisheries activities. In: *Dispersal of Living Organisms Into Aquatic Ecosystems*, Rosenfield, A. & Mann, R. (eds.), pp. 13-48. Maryland Sea Grant College, College Park, MD, USA.

Carlton, J.T. (1992c) An international perspective on species introductions: the ICES protocol. In: *Introductions and Transfers of Marine Species: Achieving a Balance Between Development and Resource Protection,* DeVoe, M.R. (ed.), pp. 31-34. South Carolina Sea Grant Consortium, Hilton Head Island, SC, USA.

Chen, T.T. & Powers, D.A. (1990) Transgenic fish. *Trends Biotechnol.*, 8(8), 209-215.

Chen,T.T., Lint C,M., Gonzalez-Villaseñor, L.I., Dunham, R., Powers, D.A. & Zhu, Z. (1992) Fish genetic engineering: A novel approach in aquaculture. In: *Dispersal of Living Organisms Into Aquatic Ecosystems*, Rosenfield, A. & Mann, R. (eds.), pp. 265-280. Maryland Sea Grant College, College Park, MD, USA.

Colwell, R.R. (1986) *Marine Biotechnology and the Developing Countries*. UNIDO report IS. 593, January 7. United Nations Industrial Development Organization, Vienna.

Colwell, R.R. (1987) Genetically engineered organisms in the ocean environment - risks and benefits. In: *Microbes in the Sea*, Sleigh, M.A. (ed.), pp. 182-189. John Wiley & Sons, New York.

Contreras-B., S. & Escalante-C., M.A. (1984) Distribution and Known Impacts of Exotic Fishes in Mexico. In: *Distribution, Biology, and Management of Exotic Fishes*, Courtenay Jr, W.R. & Stauffer Jr, J.A. (eds.), pp. 102-130. The Johns Hopkins Press, Baltimore, MD, USA.

Costerton, W. & Lappin-Scott, H.M. (1989) Behaviour of bacteria in biofilms. *ASM News*, 55, 650-654.

Courtenay Jr, W.R. & Williams, J.D. (1992) Dispersal of exotic species from aquaculture sources, with emphasis on freshwater fishes. In: *Dispersal of Living Organisms Into Aquatic Ecosystems*, Rosenfield, A. & Mann, R. (eds.), pp. 49-82. Maryland Sea Grant College, College Park, MD, USA.

Crossman, E.J. (1984) Introduction of exotic fishes into Canada. In: *Distribution, Biology and Management of Exotic Fishes*, Courtenay Jr, W.R. & Stauffer Jr, J.A. (eds.), pp, 78-101. The Johns Hopkins Press, Baltimore, MD, USA.

Davidson, J.R., Brook, J.A. & Young, L.G.L. (1992) Introduction of exotic species for aquaculture purposes. In: *Dispersal of Living Organisms into Aquatic Ecosystems*, Rosenfield, A. & Mann, R. (eds.), pp. 83-102. Maryland Sea Grant College, College Park, MD, USA.

Devlin, R.H. & Donaldson, E.M. (1992) Containment of genetically altered fish with emphasis on salmonids. In: *Transgenic Fish*, Hew, C.L. & Fletcher, G.L. (eds.), pp. 229-265. World Scientific, NJ, USA.

Donaldson, E.M. & Devlin, R.H. (1993) Aquaculture biotechnology in Canada including the development of transgenic salmon. In: *The Proceedings of the Symposium on Aquatic Biotechnology and Food Safety*, pp. 47-56. Organisation for Economic Cooperation and Development, Paris.

Engleking, H.M. & Leong, J.C. (1991) Sub-unit vaccine trials using glycoprotein and nucleoprotein constructs of the rhabdovirus, Infectious Haematopoietic Necrosis virus. In: *Proc. 14th Ann. AFS/FHS Meeting; 32nd Western Fish Disease Conference* . July 31 - August 3, Newport. p. 45. Oregon State University/Hatfield Marine Science Centre, USA.

Erdman, D.S. (1984) Exotic fishes in Puerto Rico. In: *Distribution, Biology, and Management of Exotic Fishes*, Courtenay Jr, W.A. & Stauffer Jr, J.A. (eds.), pp. 162-176. The Johns Hopkins Press, Baltimore, MD, USA.

Farley, C.A. (1992) Mass mortalities and infectious lethal diseases in bivalve Mollusca and associations with geographic transfers of populations. In: *Dispersal of Living Organisms Into Aquatic Ecosystems*, Rosenfield, A. & Mann, A. (eds.), pp. 139-154. Maryland Sea Grant College, College Park, MD, USA.

Ganzhorn, J., Rohovec, J.S. & Fryer, J.L. (1992) Dissemination of microbial pathogens through introductions and transfers of fish. In: *Dispersal of Living Organisms Into Aquatic Ecosystems*, Rosenfield, A. & Mann, R. (eds.), pp. 175-194. Maryland Sea Grant College, College Park, MD, USA.

Hallerman, E.M. & Kapuscinski, A.R. (1992) Ecological and regulatory uncertainties associated with transgenic fish. In: *Transgenic Fish*, Hew, C.L. & Fletcher, G.L. (eds.), pp. 209-228. World Scientific, New Jersey, USA.

Harvey, S., Elashvili, I., Valdes, J.J., Karnely, D. & Chakrabarty, A.M. (1990) Enhanced removal of Exxon Valdez spilled oil from Alaskan gravel by a microbial surfactant. *Bio/Technology,* 8, 228-230.

Hoffman, J. (1990) Provesta forms marine biology company. *Chem. Mark. Report.*, 238(22), 27.

Holloway, M. (1991) Soiled shores. *Sci. Am.*, 265, 102-116.

Jacob, M.T. (1994) Marine aquaculture growth faces regulatory and environmental obstacles. *Genet. Engineering News,* 14(1), 32-34.

Kapuscinski, A.R. (1990) Integration of transgenic fish into aquaculture. *Food Rev. Int.,* 6(3), 373-388.

Kern, F.G. & Rosenfield, A. (1992) Shellfish health and protection. In: *Dispersal of Living Organisms Into Aquatic Ecosystems*, Rosenfield, A. & Mann, A. (eds.), pp. 313-324.

Maryland Sea Grant College, College Park, MD, USA.

Kohler, C.C. & Stanley, J.G. (1984) A suggested protocol for evaluating proposed exotic fish introductions in the United States of America. In: *Distribution, Biology, and Management of Exotic Fishes*, Courtenay Jr, W.A. & Stauffer Jr, J.R, (eds.), pp. 387-406. The Johns Hopkins Press, Baltimore, MD, USA.

Lightner, D.V., Redman, R.M., Bell, T.A. & Thurman, R.B. (1992) Geographic dispersion of the viruses IHHN, MBV and HPV as a consequence of transfers and introductions of Penaeid shrimp to new regions for aquaculture purposes. In: *Dispersal of Living Organisms into Aquatic Ecosystems*, Rosenfield, A. & Mann, A. (eds.), pp. 155-174. Maryland Sea Grant College, College Park, MD, USA.

Maciolek, J.A. (1984) Exotic fishes in Hawaii and other islands of Oceania. In: *Distribution, Biology, and Management of Exotic Fishes*, Courtenay Jr, W.R. & Stauffer Jr, J.R. (eds.), pp. 131-161. The Johns Hopkins Press, Baltimore, MD, USA.

Mann, D.W. (1990) Seaweeds and biotechnology - inseparable companions. *Hydrobiologia*, 204/205, 7-13.

Mann, R. & Rosenfield, A. (eds.) (1992) *Dispersal of Living Organisms into Aquatic Ecosystems*. Maryland Sea Grant College, College Park, MD, USA.

McDowall, R.M. (1984) Exotic fishes: The New Zealand experience. In: *Distribution, Biology and Management of Exotic Fishes*, Courtenay Jr, W.A. & Stauffer Jr, J.R. (eds.), pp. 200-214. The Johns Hopkins Press, Baltimore, MD, USA.

McKay, R.J. (1984) Introduction of exotic fishes in Australia. In: *Distribution, Biology, and Management of Exotic Fishes*, Courtenay Jr, W.R. & Stauffer Jr, J.R. (eds.), pp. 177-199. The Johns Hopkins Press, Baltimore, MD, USA.

Meyer, F.P. (1991) Aquaculture disease and health management. *J. Anim. Sci.*, 69, 4201-4208.

Miller, H.I., Burris, R.H. & Vidaver, A.K. (1991) Regulation of biotechnology (letter). *Science*, 252, 1599-1600.

Morse, A.N.C. (1991) How do planktonic larvae know where to settle? *Am. Sci.*, 79, 154-167.

Neushul, M., Amsler, C.D., Reed, D.C. & Lewis, R.J. (1992) Introduction of marine plants for aquaculture purposes. In: *Dispersal of Living Organisms into Aquatic Ecosystems*, Rosenfield, A. & Mann, R. (eds.), pp. 103-138. Maryland Sea Grant College, College Park, MD, USA.

NRC (1989) *Field Testing Genetically Modified Organisms: Framework for Decisions*. National Academy Press, Washington, DC.

OECD (1986) *Recombinant DNA Safety Considerations*. Directorate for Science, Technology and Industry, Organisation for Economic Co-operation and Development, Paris.

OECD (1992) *Safety Considerations for Biotechnology-1992*. Directorate for Science, Technology and Industry, Organisation for Economic Co-operation and Development, Paris.

OTA (1991) *Bioremediation for Marine Oil Spills*. United States Congress, Office of Technology Assessment, Washington, DC.

Peterson, M.N.A.(ed.) (1992) *Diversity of Ocean Life: An Evaluative Review*, Centre for Strategic and International Studies, Washington, DC.

Powers, D.A. (1990) Marine and freshwater biotechnology: a new frontier. In: *Biotechnology: Perspectives, Policies and Issues,* Vasil, I. (ed.), pp. 41-70. University of Florida Press, Gainesville, FL, USA.

Powers, D.A., Chen, T.T. & Dunham, R.A. (1991) Gene-spliced fish experiments, *Science*, 254, 779.

Renn, D.W. (1986) Marine algae biotechnology: Possibilities and realities. In: Workshop on Marine Algae Biotechnology: Summary Record, Board on Science and Technology for International Development (ed.), pp. 53-67. National Academy Press, Washington, DC.

Robinson, P.K. (1985) Phycotechnology. *Ind. Biotechnol.*, 42(5), 73-78.

Shears, M.A., Fletcher, G.L., Hew, C.L., Gauthier, S. & Davies, P.L. (1991) Transfer, expression and stable inheritance of antifreeze protein genes in Atlantic salmon (*Salmo salar*). *Mol. Mar. Biol. Biotechnol.*, 1(1), 58-63.

Sindermann, C.J. (1986) Strategies for reducing risks from introduction of aquatic organisms: A marine perspective. *Fisheries*, 11(2), 10-15.

Sindermann, C.J. (1992) Role of the International Council for the Exploration of the Sea (ICES) concerning introductions of marine organisms. In: *Dispersal of Living Organisms into Aquatic Ecosystems,* Rosenfield, A. & Mann, R. (eds.), pp. 367-376. Maryland Sea Grant College, College Park, MD, USA.

Stetzenbach, L.D., Hern, S.C. & Seidler, R.J. (1992) Field sampling design and experimental methods for the detection of airborne microorganisms. In: *Microbial Ecology: Principles, Methods and Applications*, Levin, M.A,, Seidler, R.J. & Rogul, M. (eds.), pp. 543-555. McGraw-Hill Inc., New York.

Stickney, R.R. (1992) The importance of introduced marine species to the development of the marine aquaculture industry in the United States of America and Puerto Rico. In: *Introductions and Transfers of Marine Species: Achieving a Balance Between Economic Development and Resource Protection*, DeVoe, M.R. (ed.), pp. 17-22. South Carolina Sea Grant Consortium, Milton Head Island, SC, USA.

Strausberg, R.L. & Link, R.P. (1990) Protein-based medical adhesives. *Trends Biotechnol.*, 8(2), 53-57.

Teso, B. (1992) International harmonization of safety principles for biotechnology. In: *Proceedings of the 2nd International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms*, May 11 - 14, 1992, Goslar, Germany. Casper, R. & Landsmann, J, (eds.), pp. 7-15. Biologische Bundesanstalt fur Land- und Forstwirtschaft, Braunschweig, Germany.

Thorgaard, G.H. & Allen, S.K. (1992) Environmental impacts of inbred, hybrid and polyploid aquatic species. In: *Dispersal of Living Organisms Into Aquatic Ecosystems*, Rosenfield, A. & Mann, A. (eds.), pp. 281-290. Maryland Sea Grant College, College Park, MD, USA.

UNIDO (1992) Voluntary code of conduct: Release of organisms into the environment. *Biotechnol. Eur.*, 9(4), 218-221.

USDA (1990) *Environmental Assessment of Research on Transgenic Carp in Confined Outdoor Ponds*. Office of Agricultural Biotechnology, Office of the Secretary, United States Department of Agriculture, Washington, DC.

Weiner, J. (1985) Marine biotech in the Negev desert. *Bio/Technology*, 3, 39.

Welcomme, R.L.I. (1986) International measures for the control of introductions of aquatic organisms. *Fisheries*, 11(2), 4-9.

Zilinskas, R.A. & Lundin, C.G. (1993) *Marine Biotechnology for the Developing Countries*. World Bank, Washington, DC.

Zilinskas, R.A., Colwell, R.R., Lipton, D.W. & Hill, R.T. (1995) *The Global Challenge of Marine Biotechnology: A Status Report of Marine Biotechnology in the United States of America, Japan & Other Countries*. Maryland Sea Grant College, College Park, MD, USA.

Chapter nine
Safety Considerations in Biotreatment Operations

Morris Levin

Maryland Biotechnology Institute, Centre for Public Issues in Biotechnology, University of Maryland, College Park, Maryland, United States of America.

Introduction

The effects of pollution on our environment and ourselves are becoming increasingly evident in statistics describing changes in ecological and health indicators. These effects are manifested by their negative impact on tree stands in forests around the world, on water and air purity, on other specific indicators of ecological balance, and by their impact on human health. Some of these problems are associated with the production of goods used in our daily lives. Wastes generated by manufacturers of automobiles, petroleum products, industrial chemicals, pesticides, plastics, paper, etc., have been and are still being placed in dump sites around the world. Many of these effects are the result of emissions from industrial facilities, urban populations (automobiles, heating), and agricultural sources (pesticides, fertilizers). These pollutants have diverse impacts, ranging from the well-known (global warming, depletion of the ozone layer) to the not so well-known (ground-water depletion and spoilage) (Council on Environmental Quality, 1979). Many of the wastes are directly toxic to humans and hazardous to the environment.

Most of the waste is disposed of in landfills, stored in containers, or simply dumped on the ground. This practice has been going on for decades, resulting in the existence of many filled sites containing unidentifiable wastes. There are approximately 14 000 industrial sites in the United States of America, and about 265 million tons of hazardous waste are produced annually. Table 9.1 lists the common types of wastes found in typical waste dumps in the United States of America. The volume and type of waste material varies greatly, as does its toxicity. More than 6000 sites have been cleaned up since 1982. In the Netherlands, the costs of soil-rehabilitation efforts are estimated by the European Community to reach $10 billion by the year 2000 and $30 billion over the following decade (Porta, 1991).

Table 9.1. Sample of waste types at a Superfund NPL [a] site

Material	Quantity (gallons)
Oyster shells with copper	6 000
Oil and water	58 150
Paint	2 457 904
Perchloroethylene	800
Paint/Formaldehyde	4 250
Paint thinner/stripper	90 025
Paint and plastic sludge	251 885
Polychlorinated biphenyls	14 000
Paint sludge and epoxy	9 740(yd^3)
Pesticide-affected fabric	500(lbs)
Perchlorethylene, oil and alcohol	18 400
Pesticides	7 582
Phenolic resins	89 360
Phenol-formic acid and methylene	900
Phosphoric acid solution	2 940
Phosphorus	350(lbs)
Potassium cyanide and candy	168(lbs)
Poisoned cookies (arsenic)	2(boxes)

[a] USEPA National Priority List site, to be remediated with EPA Superfund monies.
Source: Superfund Innovative Technology Program, USEPA; EPA/540/5-91/004 (1991).

The solution to the problem is not clear. Choices must be made concerning the methodologies for site clean-up, and it has to be decided which sites are to be treated first and by what means. The available techniques are physical (such as incineration, immobilization), chemical (such as neutralization), or biological (use of natural or engineered microbes), each of them having specific advantages and disadvantages regarding, among other things, costs, safety issues, and the time needed to complete the task.

The use of microbes to degrade waste is not new. Man has been using microbes to treat sewage wastes for centuries, and the process is still being improved upon (Mckinney, 1962; Nicholas, 1987; Sterritt & Lester, 1988). With the advent of biotechnology, biological techniques are being re-examined and improved, and genetic engineering techniques allow the alteration of microbes that can degrade noxious materials more rapidly.

It must be kept in mind that it is not sufficient to develop technical solutions that can be demonstrated in the laboratory. Regulatory issues, safety considera-

tions, business and market issues, and social and political considerations - all play major roles in the application of technically possible solutions.

This chapter will describe the development and use of microbes to degrade hazardous waste, and will review briefly procedures for degrading common wastes. Potential health and ecological problems associated with commercial-scale applications will be identified, and mitigation and control methods discussed.

Biodegradation

Biodegradation is the process of mineralization of organic material by microbes. This environmental process has been known for centuries. Organic matter is cycled from organic to mineral material through the action of microbes (Marx, 1989). Organic compounds are reduced to CO_2 and H_2O via aerobic or anaerobic metabolism. In the anaerobic process, CH_4 is produced. In the carbon cycle, atmospheric carbon dioxide is incorporated into organic compounds by photosynthetic organisms. In the sulphur cycle, bacteria process inert sulphur and organic sulphur-containing compounds. In both cases, many tons of material are changed every year as a result of microbial action. For example, it has been estimated that 6000 tons of sulphur pass through the cycle annually.

Over the millennia that microbes, plants and mammals have coexisted, the microbial capability to degrade (decompose) organic matter has evolved in parallel to the ability of plants and animals to produce different types of organic matter. Nevertheless, the first synthesized organochloride compound, ethyl chloride, was prepared in 1940, while large-scale synthesis of chlorinated organic compounds at commercial levels has been made possible only during the past few decades. This short time frame has not permitted the development of microbial systems capable of coping easily and rapidly with the onslaught of xenobiotic chemicals (Hutzinger & Verkamp, 1981; Rochkind *et al.*, 1986). Many xenobiotic chemicals are resistant to microbial attack and/or are toxic to the microbes, hampering man's attempts to harness this resource. Nevertheless, microbes that can degrade many xenobiotic compounds with different degrees of ease and at different rates (Table 9.2) have been isolated from locations contaminated with various xenobiotic chemicals. The environmental isolates vary greatly in their ability to degrade congeners of chlorinated aromatic compounds. Some can degrade more than one compound and at different rates. Abramowicz (1989) demonstrated similar results in soils contaminated with polychlorinated biphenyl (PCB) compounds. Twenty-six isolates capable of degrading a wide variety of PCB congeners were found. He proposed combining their genetic capabilities to produce a single more useful microbe.

Some compounds are mineralized by a mix of organisms. This fact leads to the use of a mix (consortium) of natural isolates that has the capability of degrading a number of target compounds. In some cases, the identification of the microbes in the consortium has been accomplished, but in many cases, the consortium used

Table 9.2. Nutritional versatility of strains when selected hydrocarbons are present as the sole carbon and energy source

	Pseudomonas putida	*Pseudomonas oleovorans*	6Dp[a]	DI[a]	PB[a]
Toluene	+	+	+++	-	+
2-Chlorotoluene	++	++	+++	++	+
3-Chlorotoluene	+++	+++	++++	+++	+++
3,4-Dichlorotoluene	+++	+++	++++	+++	+++
2,6-Dichlorotoluene	++	++	+++	++	+
Xylenes	+	+	++	+	+
Benzoate	++++	++++	++++	++++	++++
3-Chlorobenzoate	-	-	++	-	-
4-Chlorobenzoate	+	+	+++	+	+
2,4-Dichlorobenzoate	++	+	+++	++	+
3,4-Dichlorobenzoate	++++	++++	++++	+++	++++
2,4-Dichlorophenoxyacetic acid	++	+++	++++	++	++
2,4-Dichlorophenol	+	+	++	+	-
2,4,5-Trichlorophenoxyacetic acid	++	++	++++	+++	+++

[a] Environmental isolate of unspecified genus and species.
Source: Adapted from Pierce (1982).

contains an unknown number of unidentified microbes. A consortium of microbes may be involved in the sense that specific microbes may be needed to act over the range of specific components of the waste mixture, or in the sense that a combination of microbes may be needed for a particular compound (Table 9.3). Some microbes are active only as members of specific pairs. In this process (co-metabolism), the compound being degraded serves as an energy or carbon source (Atlas & Bartha, 1987). The work of Pfaender & Alexander (1972) and that of Sakazawa and colleagues (1981) illustrate the fact that where co-metabolism is involved, the species designation of the organism(s) involved is often not known, although in most cases the genus is specified. When consortia of microbes are involved, the end products of metabolism are identified, but the microorganisms are often not specified (Nielson *et al.*, 1987; Fliermans *et al.*, 1988). It has been suggested that whatever man can make, nature can degrade (Sterritt & Lester, 1988).

Microbes are sensitive to environmental conditions. In general, acidity or alkalinity in the neutral range and temperatures close to normal body temperature are

Table 9.3. Degradation by microbial consortia

Degradative activity	Microorganism(s)	References
Degradation of DDT [a]: *p*-chloro-phenyl acetic acid produced and then utilized by *Arthrobacter* spp. (co-metabolism)	*Hydrogenomas* spp. and *Arthrobacter* spp.	Pfaender & Alexander, 1972
Degradation of polyvinyl alcohol: degradation by *Pseudomonas putida* provided growth factors for co-metabolism to occur (co-metabolism)	*Pseudomonas putida* and other *Pseudomonas* spp.	Sakazawa *et al.*, 1981
Degradation of Kepone [b] (co-metabolism)	*Pseudomonas aeruginosa*	Orndorff & Colwell, 1980
Degradation of Silvex [c]: a pair of microbes grew using Silvex; no growth when separated (co-metabolism)	*Pseudomonas* and *Achromobacter* spp.	Ou & Sikka, 1977
Consortia, but not pure cultures were able to degrade trichloro-ethylene	Aerobic degradation yielding HCl and CO_2	Fliermans *et al.*, 1988
Demonstrated "concurrent metabolism" of xenobiotics (present at environmental concentrations) by resting cells (consortia)	Wood pulp wastes degraded by stable consortia anaerobically	Nielson *et al.*, 1987

[a] Complex chemical mixture in which *pp'*-dichlorodiphenyltrichloroethane predominates: a highly toxic synthetic insecticide.
[b] Kepone: Decachloro-octahydro-1,3,4-metheno-2H-cyclobuta[cd]pentalen-2-one (insecticide).
[c] Silvex: 2-(2,4,5-Trichlorophenoxy)propionic acid (herbicide).
Source: Adapted from Johnston & Robinson (1983).

optimal. However, some microbes are active at extreme temperatures (psychrophilic and thermophilic bacteria), and these characteristics are being exploited in specific waste-treatment situations. It has been estimated that the efficiencies of psychrophilic bacteria are 60–70% of those of the mesophiles (Bioremediation Report, 1991). It must be kept in mind that microbial metabolism is susceptible to shifts in environmental conditions, that a buildup of intermediate metabolic products may occur, and that some of these products may be more toxic than the original material. Tetrachloroethylene (a known animal carcinogen) can lead, through degradation, to the accumulation of vinyl chloride (a known human carcinogen) under conditions of anaerobic degradation (Barrio-Lage *et al.*, 1986). McCall and colleagues (1981) reported that during the degradation of 2,4,5-trichlorophenoxyacetic acid, in addition to CO_2, concentrations of 2,4,5-trichlorophenol and 2,4,5-trichloroanisole were found in the soil.

Research is being conducted in many laboratories with the aim of enhancing the degradative ability of natural microbes (Rojo *et al.*, 1988), while other researchers are attempting to create altered microbes with enhanced degradative scope and rates. Rojo has demonstrated the integration of enzymes from five different catabolic pathways of three different, distinct soil bacteria into one strain. Attention is also focusing on isolates that can survive and flourish after being released at a waste site (Neidle *et al.*, 1987; Dwyer *et al.*, 1988). Some researchers are attempting to understand better the environmental parameters that control the metabolic rates and genetic composition of microbial flora *in situ* (Olson & Goldstein, 1988) with the objective of manipulating environmental parameters to enhance selected degradative characteristics of the natural flora. The United States National Science Foundation conducted a workshop to discuss the feasibility of field applications of environmental biotechnology (Sayler *et al.*, 1988). Although no large-scale field applications of engineered microbes have been conducted, tests with mutated isolates have been made, and other tests are planned for engineered strains (Bioremediation Report, 1991).

Attempts to exploit more fully the degradative ability of microbes on a commercial scale take several forms. The oldest and most direct is the enhancement of sewage treatment by a modification of the treatment process (Hall & Melcer, 1983). Mizrahi (1989) reviewed the various treatment methods and the modifications in biogas digestors, the anaerobic digestion technology, and the managerial aspects that lead to a more efficient operation of sewage plants. All of the sewage-treatment methods involve three components: the physical manipulation of the environment, the chemical augmentation of the microbial nutrient mixture, and the augmentation of the microbial population (by adding natural organisms, by engineering a microbe with superior performance characteristics, or by stimulating the growth of indigenous microbes by adding appropriate nutrients). The development of biotreatment began with the development of methods of accelerating the rate of degradation of sewage and generating effluents less harmful to human health and the environment. Early treatment of sewage consisted of sprinkling it on large areas of land (an area of about 0.4 ha was required for each 100 persons). Studies at

the Lawrence Experimental Station in Massachusetts in 1889 led to the use of gravel as a percolating filter. This work was followed by the development of anaerobic digestion and then aerobic digestion of sewage at Davyhulme, Manchester (United Kingdom). Aerobic digestion, which is simply adding air to the digestion mixture, when combined with the use of an inoculant from previous digestions, shortened the digestion period from five weeks to twenty-four hours (Sterritt & Lester, 1988). This is perhaps the earliest example of using naturally adapted microbes to enhance the degradation of waste material.

Many different procedures have been developed to permit and enhance contact between microbes and the target pollutant. Table 9.4 describes some of the most common procedures and the safety issues associated with each. Clearly, the use of immobilized microbes or fixed-film bioreactors will result in minimizing the release of microbes into the environment, thus minimizing the possibility of adverse environmental or health effects. In addition, any type of reactor can be combined with appropriate systems for disinfecting the effluent to assure containment of the microbes involved in the process. Soil-treatment systems and applications involving subsurface reclamation or land farming will lead to an extensive dispersal of microbes. In these situations, emphasis must be placed on assuring that the microbe(s) are innocuous.

In general, experience has shown that no single component is ideal for all sites and that some combination is essential to obtain optimal degradation. In addition, at some polluted sites, some form of physical or chemical pre- or post-treatment may be necessary.

In the United States of America, over 100 companies are actively engaged in applying scale-up procedures for biodegradation techniques to clean up waste sites. Most are also involved in research that seeks to improve the biodegradation process without the use of engineered organisms. Major firms, such as Dow Chemical and General Electric, are involved in developing and implementing methods for the biotreatment of wastes. A number of companies have formed an association and produced a compendium describing successful instances of biotreatment on a commercial scale (Applied Biotreatment Association, 1989). Microbes have been employed successfully to clean up some of the Alaskan coastline after the Exxon Valdez spill (Crawford, 1990) as well as at other locations in the United States of America and in Europe (Stone, 1984; Bluestone, 1986; Savage, 1987; Keeler, 1991). They have also been used to control odours from treatment plants (Grubbs & Molnaa, 1987). To date, only non-engineered isolates have been used as inocula. In many cases, such as the Exxon Valdez spill, treatment consists of adding nutrient material to enhance the growth of indigenous microbes. The use of engineered microbes offers the possibility of faster degradation of a broader range of compounds. However, the engineered organism may not persist as well in environmental situations (Lenski, 1991) and may not survive long enough to accomplish the objective. In addition, there is public resistance and hence governmental reluctance to use engineered microbes in environmental applications. When these considerations are balanced against the availability of naturally occurring mi-

Table 9.4. Types of biotreatment processes

Type	Principle	Primary application
Sequencing batch reactor	Microbial digestion in liquid suspension	Control of reaction conditons; release of microbes to environment
Aqueous treatment system	Immobilized microbes or enzymes in flow-through system	Requires soluble organic material. No microbial release
Soil treatment system	Wash procedure to solubilize adsorbed contaminants	Necessary pre-treatment to maximize efficacy
Fixed film bioreactor	Microbes or enzymes on plastic media in column to maximize surface area and nutrient exchange	Can treat low concentrations of organic material
Soil slurry (tank or lagoon)	Soil and water agitated together in reactor	No temperature control
Land farming	Soil mixed with nutrients and tilled *in situ*	Requires lining to contain microbes and material
Subsurface reclamation	Water, nutrients and oxygen (electron acceptor) pumped through soil	Enhanced growth of entire indigenous population. Oil and gasoline spills; organic contamination of ground water

crobes, alone or in consortia, capable of mineralizing most target compounds, the basis for a commercial emphasis on the use of naturally occurring microbes is clear.

Procedures Involved in On-Site Biodegradation

It is now generally accepted that the application of biotreatment must include a thorough hydrological and physical analysis of the site involved, as well as laboratory and field studies to determine the appropriate strategy and the possible need for some form of physical or chemical pre- or post-treatment. The physical aspects of the site must be determined from the perspective of the effect on the metabolism of the microbes to be added or nurtured. The native microflora must be examined for degradative capability and for nutrient requirements. Finally, the degradative process must be successfully demonstrated in the laboratory, and it must also be shown to be effective on a large scale (Wick & Pierce, 1990). This holds whether the treatment is to be *in situ*, in which case the material to be treated is not moved from its location and is treated by altering the moisture content, nutrients, or microbial flora at the site and under natural conditions, or whether the treatment is to take the form of transferring the material to reactors in which exposure to microbes under controlled conditions will occur. If the treatment is to be conducted *in situ*, the monitoring procedures, including the selection of the compounds to be monitored, the sampling times and locations, and the duration of the monitoring period must be established before the project begins. Cost and regulatory considerations must also be taken into account at this stage.

Site Examination

A complete survey leading to a thorough understanding of the waste site is essential for the success of the project. This includes a characterization of both waste and site. The type of waste material will govern the choice of microbes and the need for physical or chemical treatment. The type of soil and hydrology involved at the specific site will govern both the schedule for addition and the need for nutrients and moisture. One to two years can be required for site evaluation. Keystone Environmental Resources spent two years studying the soil beneath and immediately adjacent to a contaminated area (Campbell *et al.*, 1989). During this time, the physical aspects, such as site hydrology, soil type, subsurface conditions, and climate characteristics, were defined, while at the same time laboratory studies to determine the characteristics of the microbial flora and the impact of the pollutants on the flora were carried out.

Results of feasibility trials showed that the microbes present at the site could degrade the contaminating material if appropriate nutrients and moisture were supplied. The Keystone project involved the addition of nutrients (nitrogen, phosphorus,

minerals) as well as nitrate as an alternate electron acceptor. Typically, to degrade approximately 1000 gallons of hydrocarbon material, 10 000 lbs of oxygen and 875 lbs of ammonia nitrogen would be required. Approximately 7000 lbs of bacteria are produced in the process.

A sampling procedure was developed to provide monitoring of both the success of the treatment and the level of nutrient available. In this procedure, the chloride content was monitored as an indication of mineralization, and direct pollutant measurements were made at three upstream and three downstream wells. After 12 weeks of treatment, approximately 90% of the contaminant had been removed. In other field applications, a 98–99% reduction in the levels of carbon tetrachloride, chlorobenzene, ethyl benzene, toluene, 1,1,1-trichloroethylene and xylene was achieved.

Identification of the microbes involved is not commonly attempted at the species level. The degradation process often involves a consortium of microbes, including strains in the genera *Nocardia, Pseudomonas, Acinetobacter,* and *Flavobacterium.* Biodegradation is often the result of the metabolic activity of a group or consortium of microbes. One company reports that as many as thirty-two different microbes were involved in degrading a specific gasoline spill (Bluestone, 1986). In general, the more complex the mixture, the more complex the consortium of microbes (Bluestone, 1986; Olson & Goldstein, 1988). Research seeking a better understanding of the relationship between the genetic capability of the entire microbial population at a given site and the phenotypic expression of biodegradation (Olson & Goldstein, 1988) is ongoing. The intent is to develop methods to identify and augment, *in situ*, the specific genes that contribute to the degradation of specific compounds, rather than provide enough nutrients to result in general microbial growth. This would require a much deeper understanding of the factors controlling gene expression and multiplication under environmental conditions, but could lead to a less expensive and more rapid degradation of wastes, with less potential for adverse environmental effects. For more recalcitrant wastes, modified organisms may be developed, or otherwise the use of some form of bioreactor would be required.

To date, no engineered organisms have been used in *in situ* situations involving release into the environment, because of regulatory considerations. Modified microbes have been used in bioreactors. Bioreactors provide containment of the microbes, and thus their use permits avoiding some of the environmental issues. In addition, they provide control over the physical conditions of the biodegradation process. The temperature, time of contact with the microbes, nutrient levels, and concentration of the material to be degraded can be optimized. The use of Sequencing Batch Reactors (SBRs) to treat leachate is described by Irvine *et al.* (1982) and by Wick & Pierce (1990). The efforts of Irvine's group focused on a leachate from a contaminated industrial site. Initially, the leachate was placed in storage tanks in contact with "non-sterile raw waste feed" from a wastewater plant for up to 19 days prior to being filtered through granular activated carbon (GAC) columns. Modified organisms were added to the reactors.

Table 9.5. Parameters and compounds monitored

For regulatory compliance [a]	For monitoring the process
pH	pH
Benzene	Benzoate
Hexachlorocyclobutadiene	Biological oxygen demand
Hexachlorocyclopentadiene	Chlorendic acid
Monochlorobenzene	*o*-, *m*- and *p*-Chlorobenzoic acids
Monochlorobenzotrifluoride	Oxygen consumption rate
Monochlorotoluene	Phenol
Phenol	Suspended solids
Tetrachlorobenzenes	Total organic halide
Tetrachloroethylene	
Total organic carbon	
Trichlorobenzenes	
Trichloroethylene	
2,3,5-Trichlorophenol	

[a] Must meet compliance levels set by USEPA.
Source: Adapted from Levin & Gealt (1993).

Ultimately, a neutralization step coupled with the augmentation of the microbial population by the addition of pure cultures isolated from the indigenous population was instituted (Wick & Pierce, 1990). A unique strain of *Pseudomonas putida* that was adapted to the SBR environment and possessed degradative abilities not found in the original strains was isolated, cultivated, and added to the existing microbial mix in the SBRs. The SBRs were operated as closed systems. All volatile organic material was trapped on GAC and recycled through the SBRs.

Table 9.5 lists the parameters monitored to permit evaluation of the efficacy of the process and to assure regulatory compliance. Intensive monitoring requires a careful selection of locations and times, and an equally careful handling of sample material. The analytical methods used to estimate the concentrations of compounds under regulatory control must be acceptable to the regulatory agency, and appropriate quality-assurance procedures are required. This is an essential and expensive part of any biotreatment project.

The SBRs were operated on a 24 hour cycle. The annual treatment volume was in excess of 10^4 cubic metres. The reduction of monitored compounds varied greatly. Chlorobenzoic acid (*ortho-* and *meta-*) was not detectable (sensitivity of measurement: 3.5 mg/l) with starting levels of 763 and 219 mg/l, respectively. Total organic halide levels were reduced from 1062 to 319 mg/l (70%). The SBR process had the greatest effect on total organic carbon and phenol, achieving a reduction of more than 99% from starting levels of 10 575 and 1553 mg/l, respectively. The SBR-

treated leachate still required GAC treatment to meet discharge standards. However, because of the biotreatment, the amount of carbon needed was dramatically reduced. The replacement of carbon filters shifted from a daily procedure to approximately three times per year. The cost *reduction* was calculated at approximately US $30 per cubic metre of water treated.

Other types of batch reactors include the use of microbes and/or enzymes attached to a support material. In this procedure, the reactor serves as a packed column through which the liquid to be treated is passed. Figure 9.1 illustrates such a system, in this case developed by Biotrol Inc., and used in the Superfund Innovative Technology Evaluation programme of the United States Environmental Protection Agency (USEPA SITE, 1988; Ellis & Stinson, 1991). The units can be operated aerobically or anaerobically and permit control of the temperature, retention time, conditioning of the waste liquid (pH, nutrient adjustment), and monitoring of the influent and effluent. The microbial population can be altered to permit degradation to treat a broad spectrum of contaminants. Biotrol has also developed a soil-scrubbing procedure to release bound material to the liquid phase, thus permitting treatment in the bioreactor.

Many variations of a few basic procedures of biotreatment of contaminated soil and water have been developed and reported on. These include the use of proprietary equipment, cultures, and nutrient formulations. Table 9.4 describes the basic procedures that have been applied commercially to treat liquids and soils under contained, controlled conditions and *in situ*. These can be divided into two basic types: bioreactors that involve some type of liquid/microbe interaction, and soil treatments in which the contaminating material is treated while still adsorbed onto particles. The bioreactors generally involve soil washing, in which desorption of the target compounds is accomplished by treatment with solvents or a specific, often proprietary, washing solution. The liquid is then treated by exposure to microbes in digestion tanks (SBRs), or in aqueous treatment systems or fixed-film bioreactors, where the microorganisms are attached to some form of support and the liquid is passed through. Soil-slurry systems and land farming involve *in situ* mixing of soil, nutrients, and moisture in various proportions to achieve maximum contact between the microbe and the target compounds.

Methods to maximize contact between microbes and the material to be treated include solubilizing the material and increasing the exposure area by using any of a variety of physical media providing attachment surfaces. The use of enzymes in "immobilized" systems has also been proposed. The contaminated liquid would be pumped through a column containing an immobilized enzyme that would catalyse one step in the biodegradation process. The cost and efficacy of this approach have not yet been established.

Soil systems, such as that used in the Keystone project, rely on nutrient and moisture addition, with constant tilling to provide contact between the microbes and the material to be digested. Environmental parameters (pH, temperature) are manipulated to maximize the reaction rates and the end products obtained. Exist-

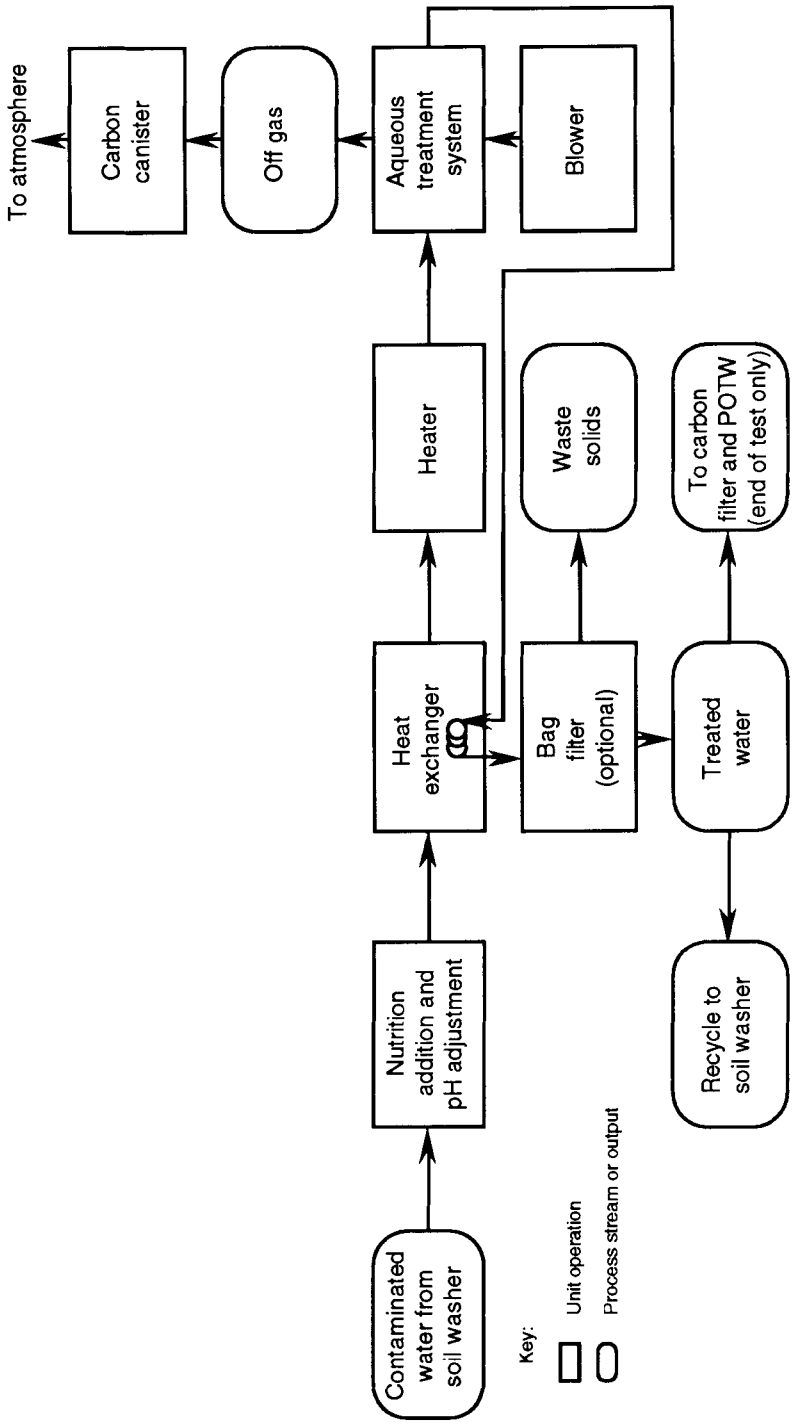


Fig. 9.1. Flow diagram of aqueous treatment systems (ATS) (Ellis & Stinson, 1991).

Table 9.6. Comparison of treatment methods

Type of treatment	Cost per cubic yard (US $)	Time (months)	Major problems
Incineration	250–800	6–9	Emissions; high energy consumption
Fixation	90–125	6–9	Decomposition; leaching
Landfill	150–250	6–9	Seepage; long-term containment
Biotreatment	40–100	18–60	Metabolic by-products; time factor; release of microbes

Source: Adapted from Levin & Gealt (1993).

ing microbial populations are augmented by adding cultures of microbes grown in the laboratory (either as taken from the site, or after selection for specific degradative characteristics).

Treatment rates vary greatly depending on the type of material, the physical characteristics of the site, the goal of the operation in terms of acceptable final concentrations of pollutants, and the scale selected for the operation. Reported rates range from 60 000 gallons of leachate per week to 1.7 tons of soil per month. One bioreactor is being used to treat daily 700–1000 lbs of cyanide residue from a steel-coking operation (McCormick, 1985).

The report on the proceedings of the Hazardous Materials Control Research Institute symposium on biotreatment (1989) and the USEPA SITE report (1988) contain detailed descriptions of specific techniques. Most of the techniques demonstrated significant removal of pollutants. Generally, 80–98% of the compound(s) being monitored was removed. Although in some cases complete removal is not achieved, the volume of material requiring treatment is significantly reduced, providing large cost and time savings.

Cost

Cost figures can be found in a number of sources (Rishel *et al.*, 1984; McCormick, 1985; Bluestone, 1986; Savage, 1987; Wick & Pierce, 1990). However, a comparison of costs between various modes of remediation is difficult because one must take into consideration more than the direct estimation of actual expenses. Each process has advantages and disadvantages, and costs vary greatly. The type of material and site characteristics are major factors. Table 9.6 compares the cost per cubic yard, the time required for a large project, and a few of the major problems involved. Biotreatment is the least costly if one considers only immediate cost. It requires the least energy and can result in mineralization of the waste material into innocuous products. However, biotreatment takes longer and does not necessarily result in clean-up to the levels required by national or local regulations. This fact may lead to the need for additional treatment and additional cost. But biotreatment does result in significant reductions in the volume of the waste, thus cutting the cost of follow-up treatments.

Health and Environmental Hazards

Under ideal conditions, all biodegradation attempts would result in the mineralization of the target compounds. Aerobic processes would yield carbon dioxide and water, and anaerobic processes would yield methane and inorganic ions. As indicated above, biodegradation is not a new process. However, the use of engineered microbes to enhance the process and a more widespread use of biological methods for the treatment of wastes have raised some risk-assessment issues not previously considered and have placed greater emphasis on existing issues.

Assessing the risks associated with environmental applications of engineered and natural microbes has been the subject of active research over the past decade. There is general agreement that the estimation of risk involves the identification and quantification of the hazards involved, and a coupling of that information with the exposure factor. Numerous authors and organizations have suggested procedures and protocols for evaluating the risks associated with the environmental application of engineered or natural microorganisms (OTA, 1985; Tiedje *et al.*, 1989; NRC, 1989; Levin & Strauss, 1991; Ginzburg, 1991). There have been debates over the issues involved in risk assessment (Sharples, 1987; Davis, 1987). Methods to monitor and control the microbes have been developed and reviewed in general (OECD, 1986; Levin *et al.*, 1987, 1992; Biotechnology Action Programme, 1990), and specifically (Katz & Marquis, 1991; Sharples, 1991; Vidaver & Stotzky, 1992; Vandenbergh, 1992; Lindow *et al.*, 1992).

There is general agreement that the methods of waste degradation involving biotechnology will result in a more complete mineralization of the target material

at less cost in terms of energy utilization. However, three types of problems are recognized when considering the environmental application of microbes for waste treatment:

1. Generic problems associated with the use of microbes (engineered or natural isolates);
2. Problems associated with the microbial process of waste degradation;
3. Specific problems associated with uncontained techniques used to enhance the rate of microbial degradation.

There is also general agreement as to the need to assess the risks associated with the environmental application of engineered or exotic (i.e., non-indigenous) microbes.

Generic Problems Associated with Using Microbes to Degrade Wastes

The environmental application of chemical products is well accepted, and methods to assure safety have been developed and proven over the past decades. Many of the concerns regarding the environmental application of microbes are similar, and initial attempts to deal with health and safety issues have been based on methodologies developed to assess the risks associated with the use of chemicals in environmental situations. Milewski (1985) defined the problems associated with the field-testing of engineered microorganisms, and presented a list of points to consider in evaluating a proposed field application. These included:

1. *Genetic considerations.* Identification of the parental organism, the host organism, and the genetic material to be transferred, as well as information describing the construction of the modified organism, the means of transfer, and the stability and expression of the introduced material.
2. *Environmental considerations.* Information about the organism to be modified, including habitat, general distribution, survival, reproduction, and dispersal characteristics; a discussion of biological interactions to indicate host range, interactions with other organisms, possible impact on biological cycling processes, and the likelihood of exchange of genetic information with other organisms in nature.
3. *Field-test information.* Description of the proposed test (objectives, significance, and justification) and of any relevant laboratory data regarding survival, replication, and dissemination of the modified organism; a description of the conditions of the field test, including numbers of organisms, location, specific target organisms that would be affected, and methods to contain and monitor the trial.

These points have been re-emphasized over the years (USGAO, 1988; Sharples, 1991), and they answer five main questions:

1. Will the organism survive?
2. Will it multiply?
3. Will it spread to other sites?
4. Will it be harmful?
5. Will it transfer genes to other non-target organisms?

The National Academy of Sciences (NAS, 1987) summarized the problem by stating that the "assessment of the risks of introducing engineered organisms into the environment should be based on the nature of the organism and the environment into which it is introduced". Subsequently, the issues of decontamination and mitigation have been raised (Vandenbergh, 1992).

Although most of the early emphasis was on agricultural applications, these generic safety issues apply equally to the introduction of microbes (engineered or natural) for waste-treatment purposes. Since risk is a function of hazard and exposure, the answers to the above questions provide a basis for assessing the risks involved in using a microbe in a particular environmental situation.

Problems Associated with Microbial Degradation of Waste

Health Issues

Two distinct health issues are involved when assessing risks associated with biotreatment. These are: (i) possible effects on workers; and (ii) possible public-health effects. These are related in terms of cause (incomplete mineralization and microbial growth), but are distinct in terms of means to control or avoid. These effects may be the result of exposure to compounds produced as a result of the treatment process, or to microbes used or augmented as a result of deliberate alterations of the environmental characteristics of the site.

Physical treatment methods will result in the transfer of material from one medium to another (for example, water to soil, or water to air). Microbial biodegradation will, in theory, result in complete mineralization. However, degradation may not be complete, and intermediate products of microbial metabolism may accumulate (i.e., biotransformation vs. biodegradation). These biotransformation products may be less, more, or as toxic as the initial material. They may be less, more, or as mobile as the initial material. And they may be less, more, or as persistent as the initial material. Differences in mobility and/or persistence will lead to changes in exposure levels, that could result in adverse effects. Longer exposure to higher levels of a less toxic material could result in an unanticipated expression of toxic-

ity. As indicated before, partial degradation of polyvinyl chloride can result in the accumulation of vinyl chloride, a known human carcinogen. Other examples include the conversion of amines into *N*-nitrosamines in the presence of nitrites or nitrogen oxides (Ayanba & Alexander, 1974; Greene *et al.*, 1981) and the accumulation of chlorobenzoate as a result of the partial biodegradation of PCB congeners (Sayler *et al.*, 1988).

If partial degradation (biotransformation) occurs, additional risk assessment issues are raised. One must ascertain the toxicity, mobility, and persistence of the accumulated metabolite. These will determine the potential for adverse effects on the environment, non-target organisms and humans. The extent and path of partial degradation will determine the type and quantity of compounds present. Many tests are available to determine the harmful effects of specific compounds on biological tissue (Loomis, 1978; Paustenbach, 1989).

However, the prediction of the specific metabolite and its concentration may not be possible. Environmental factors (pH, temperature, moisture content) and the presence of indigenous microbes may greatly affect the extent of degradation. Tests for individual compounds do not provide information on possible synergistic effects of mixtures of chemicals, although tests are available that attempt to measure the toxicity of complex mixtures of chemicals (Irvin & Akgerman, 1987; Irvin, 1989).

It can be assumed that the specific microbes selected (or engineered) for use in degrading the waste material will have been shown to be innocuous relative to human and other non-target animals or insects. However, as indicated above, biotreatment involves the addition of nutrients to support the metabolic activity of the desired microbes. This will not create concern in the case of closed systems; however, in non-contained systems, the growth of other microbes normally present may occur, including those pathogenic to humans or other non-target animals or insects. The exposure of workers or populations to these microbes would result in adverse effects.

Health issues related to incomplete mineralization would result from the exposure of populations via contaminated water or air. If it is demonstrated that groundwater contamination is possible, water safety can be assured by the use of test wells that permit the monitoring of effluent from the site. Similarly, discharge water can be monitored. It must be stressed that monitoring is necessary only if the metabolic intermediates are known to be hazardous, and an incomplete digestion is likely. Airborne contamination, wherein microbes are dispersed generally by dust particles, can be dealt with as described below (under Problems Associated With Non-Enclosed Systems).

Environmental Issues

There has been much public concern and speculation about the possibility of adverse environmental effects generated by runaway engineered organisms in environmental applications. The possibility of effects on non-target organisms, on biological cycles, and on human health has been discussed. To date, after almost

700 field tests of engineered microbes or plants, there is no record of such problems. In one instance (Short *et al.*, 1991), researchers evaluating the efficacy of *Pseudomonas* strains that had been engineered to degrade 2,4-dichlorophenoxyacetate found that 2,4-dichlorophenol (a toxic intermediate metabolite) accumulated in the soil. The accumulation of 2,4-dichlorophenol resulted in a loss of 90% of the fungal population in the soil.

The possibility of an adverse effect requires that environmental applications be reviewed for safety considerations. Cavalieri (1991) has proposed that microcosms be used to predict the environmental consequences of the application of engineered microorganisms. Microcosms can provide information about persistence, survival, and specific effects of the modified microbe in question relative to the unmodified host. While the information from microcosms may not be entirely representative of results under field conditions, it will provide a basis for deciding whether or how field-testing should proceed. Similarly, based on microcosm data, the process could be modified, safety precautions instituted, provisions for confinement or mitigation devised, and effective monitoring protocols designed.

Problems Associated with Non-Enclosed Systems

There is considerable variation in the methods for biotreatment due to the variable nature of the material to be treated, the physical characteristics of the site, as well as due to climate and regulatory considerations. Clearly, the more control the operator has over the bioremediation system, the greater the likelihood of a successful outcome, and the less the likelihood of adverse effects. The batch reactor provides the most control, followed by the various types of holding tanks or semi-enclosed bioreactors. Finally, natural or modified ecosystems provide the least control. Batch reactors are closed systems, and the microbes can be thought of as contained and not free to enter the environment. At the same time, the physical/chemical environment can be controlled to assure complete mineralization. Holding tanks and semi-enclosed reactors provide limited control. These vary from small to large, open (fenced to restrict entry) or covered lagoon-type enclosures, to greenhouse-type structures covering mounds of contaminated soil.

These semi-enclosed systems may employ an augmentation process in which additional microbes are introduced, or a treatment by which nutrients are added in order to enhance the growth of indigenous microbes. Very often, nutrients and microbes are added simultaneously. Most microbes are not identifiable in natural situations. Also, 5% of the microbes in a soil sample cannot be cultured in laboratory situations, generally due to the lack of an appropriate medium, and are considered to be in a viable, non-culturable state. With the addition of nutrients in uncharacterized field situations, bacteria, fungi, and protozoa will multiply. Many of these will have their associated viruses. Some of these microbes could be human, animal, or plant pathogens responding to the added nutrients and altered growth conditions.

These microbes may cause infection or allergic reactions (especially among workers on the site), or may produce toxins. Perhaps the best example of a normal soil bacterium that can cause infection is *Clostridium tetani*, which infects through a puncture wound. Other bacteria, such as *Bacillus subtilis*, are known to produce allergic reactions in workers. According to Emmons (1962), "the fungi that cause systemic mycoses are normal and more or less permanent members of the soil". In addition, exposure to fungi results in allergic reactions, and some fungi produce toxins.

The use of enclosed systems (i.e., covering the lagoon or reactor with canvas or plastic) is encouraged as a means of minimizing the dispersal of microbes. While enclosing the site will minimize exposure in the case of the general public, within the closed system, workers may be exposed to high concentrations of microbes via dust particles or spray. Moistening the surface of the soil being treated at sites will reduce the amount of dust in the air. In some cases, face masks may be advisable.

Containment and Mitigation

Total eradication of unwanted microorganisms is rare, but reduction to acceptable levels (i.e., below the level of unacceptable economic or health impact) is possible. Absolute containment of microorganisms is not possible and, based on experience with both beneficial and detrimental microorganisms, not essential (Vidaver & Stotzky, 1992). Vidaver and Stotzky propose the use of the more realistic term "confinement" in place of containment. Confinement does not imply that the microbe will not spread beyond the point of application, but rather that it can be effectively managed, the adverse effects being minimized. Most microorganisms are confined biologically by their individual requirements for nutrients and moisture, and by their sensitivity to environmental conditions (i.e., their ecological niche).

Additional strategies involve the use of debilitated organisms or the construction and use of safe cloning vectors with limited ability to transfer or survive outside the original host, and the use of replicons sensitive to temperature or other environmental factors (Cuskey, 1992). The use of debilitated microbes is not practical in environmental applications. However, several conditionally lethal systems for the control of released bacteria have been designed and tested. These include a temperature-sensitive system (where DNA repair does not occur at cold temperatures), a conditionally lethal construct wherein the organism has an inducible metabolic pathway which can be activated only by the presence of an innocuous chemical not normally present in the environment of the microbe, and which includes a "suicide" gene that interferes with a key metabolic feature essential for the survival of the cell. The gene is controlled by the presence (induced) or absence (derepressed) of the waste in question. If the waste concentration falls below a critical level, the gene is activated. Alternatively, the gene is always active, and a second gene provides protection. The activity of the second gene is controlled by the concentration of the waste being treated.

Table 9.7. Time frames and methods for controlling or eliminating unwanted effects of free-living microorganisms associated with plants and animals

Microorganism association	Immediate[a]	Short-term[b]	Long-term[c]
Free-living	Fumigation Flooding Chemicals[d]	Fumigation Flooding Chemicals Erosion control Soil amendments	Fumigation Flooding Erosion control Soil amendments
Plants	Burning (eradication) Quarantine Tillage Chemicals Irrigation/flooding Insect vector control Machinery sanitation Runoff water control Solarization	Quarantine Chemicals Crop rotation Cultivar rotation Irrigation/flooding Heat treatment Soil solarizaton Erosion control	Crop rotation Cultivar rotation Soil amendments Weed control Erosion control
Animals	Incineration Quarantine Slaughter Bird, rodent, insect control Runoff water control (insects) Physical security	Quarantine Antibiotics, drugs Bird, rodent, insect control Physical security	Antibiotics, drugs Bird, rodent, insect control Physical security

[a] Hours to several days to achieve effectiveness.
[b] Up to three years to achieve effectiveness.
[c] Longer than three years.
[d] Choice and availability of chemical for target microorganisms dictate feasibility and approach.
Source: Adapted from Vidaver & Stotzky (1992).

Decontamination (or mitigation) of the environment of microbes has been studied and is discussed by Vidaver & Stotzky (1992). It is important to keep in mind that each situation is different, and that procedures for decontamination will differ. A case-by-case approach is essential. The type of organism, the physical environment, the nature of the modification, and the season must all be considered. Knowledge about the organism, whether it is a wild type or has been

Table 9.8. Soil fumigants

Common name	Chemical name (some trade-names)	Formulation	Specificity	Dosage (amount per hectare)	Toxicities (plant)	Mammalian LD_{50}[a] (mg/kg)	Application consider-ations
Methyl bromide	Bromomethane (Dowfume MC-2)	98% + 2% chloropicrin	General biocide	450–900 kg	Toxic	1	Requires gas proof seal
Chloropicrin	Trichloronitromethane (Picfume, Larvacide)	100%	General biocide	300–500 litres	Toxic	1	Best activity with gas proof seal
Chlorinated hydrocarbons (1,3D)(DD)	1,2-Dichloropropane, 1,3-dichloropropene and other chlorinated hydrocarbons (Telone, Vidden D)	1,3D alone or with other chlorinated hydrocarbons	Nematicidal	100–500 litres	Toxic	140	Requires soil seal
Ethylene dibromide (EDB)	1,2-Dibromoethane (Dowfume W-84, Nematox 100)	60–85% liquid	Nematicidal	19–94 litres	Toxic	150	Requires soil seal

Table 9.8. cont.

Methyl isothiocyanide	Methylisothiocyanide is added directly or is the active breakdown product of several unstable compounds	30–40% liquid or wettable powder	General biocide	600–1200 litres or 300–400 kg	Toxic	280–650	Injected or rotovated in
Dibromochloro-propane[b] (DBCP)	1,2-Dibromo-3-chloropropane (Fumazone, Nemagon, etc.)	Liquid	Nematicidal	19–38 litres	Toxic to some plants	172	Injected or drenched
Hypochlorite	Chlorine	100 ppm in water	Micro-biocine	Variable: pH and temper-ature dependent	Toxic	0.03–0.2[c]	Applied as liquid

[a] LD_{50} is the dosage lethal to 50% of a test (usually rat) population.
[b] Because of toxicities, DBCP is no longer used. It is included here for comparisons only.
[c] Sensitivity range for continuous exposure of sensitive fish species: Le_{50} (i.e. lethal concentration for 50% of fish exposed to toxicant).
Source: Adapted from Vidaver & Stotzky (1992).

modified, is critical to designing a decontamination protocol. Table 9.7 lists methods of decontaminating soils, plants and animals, if they are contaminated with a hazardous microbe, and provides an indication of the time required to achieve effectiveness. Plants and animals are presented because of the possibility of contamination during a field application. Thus, in the event of contamination of animals (straying onto the site), incineration, quarantine, or slaughter could be employed immediately to minimize the spread of the microorganism. Birds, rodents, and run-off water must be considered as alternate sources of microbial dispersal. Plants that are growing on the site may be contaminated with the microbe. If the microbe is considered a hazard, the plants should be immediately destroyed (burning, tillage) or quarantined (if a future use is being considered). Long-term solutions are presented for use in the event the project has a long life span, and the problem is recurrent. The issue of physical security, especially with animals, cannot be overemphasized. Strong, tall fences will eliminate the presence of most unwanted mammals and will insure against trespassers.

Details of soil sterilization to decrease the bacterial levels at the site are given in Table 9.8. Specific soil fumigants in common use in the United States of America are identified. As can be seen, most have general effectiveness. All are toxic to plants and animals and must be used with care. Use of a fumigant will significantly lower the population densities of all microorganisms present in the soil. But sterilization is not achieved, and over time, the remnants of the microbial flora will reproduce, and densities will increase. The new population may be similar to the previous one in terms of types and relative numbers of individual types, or it may differ radically, depending on which portion of the population survived the fumigation and at what level. There is a possibility that the introduced microbe could be the dominant type. For this reason, it is recommended that the treated site be reinoculated with uncontaminated soil from the surrounding area. This will most likely result in the replacement of the original indigenous microbial flora and will significantly decrease the probability that the introduced microbe would flourish.

Lamptey *et al.* (1992) discuss methods of decontamination specifically oriented toward small- or large-scale field trials with *Bacillus*, which are generally more refractive. They suggest that if the problem area is small enough, the upper layer of soil (including plants and associated fauna) could be dug up and sterilized. They propose the use of steam (121 °C for 15 min) or irradiation using a ^{60}Co source (3000 krad/h for 3 h or 3 krad/h for 96 h). For larger sites, where excavation would be impractical, direct application of steam is recommended. This can be accomplished by burying steam pipes (80 cm apart), and supplying steam from a boiler (10^6 kcal/h). A more widely used system is "steam stripping": PVC sheets are spread over the area to be treated and weighted down, and steam is pumped under the sheets. Temperatures ranging from 54 to 100 °C have been observed. The process can be repeated at intervals to destroy germinated spores. These procedures could be used with any microorganism.

References

Abramowicz, D.A. (1989) Biodegradation of PCB contaminated soil using recombinant DNA bacteria. *Proc. A&WMA/EPA Symp.*, pp. 301-312. Cincinnati, OH, February, 1989.

Applied Biotreatment Association (1989) *Compendium of Biotreatment Applications*. ABTA, Washington, DC.

Atlas, R.M. & Bartha, R. (1987) *Microbial Ecology*. Benjamin Cummings Co., Menlo Park, CA.

Ayanba, A. & Alexander, M. (1974) Transformations of methylamines and formation of a hazardous product, dimethylnitrosamine, in samples of treated sewage and lake water. *J. Environ. Qual.*, 3, 83-89.

Barrio-Lage, G., Parsons, F.Z., Nassar, R.S. & Lorenzo, P.A. (1986) Sequential de-halogenation of chlorinated ethenes. *Environ. Sci. Technol.*, 20, 96-99.

Bioremediation Report (1991) Waste stream technology not slowed by winter. COGNIS, CA. December, 1991, 1-2.

Biotechnology Action Programme, Part 1 (1990) Economidis, I. (ed.), Commission of the European Community, Brussels.

Bluestone, M. (1986) Microbes to the rescue. *Chem. Week*, October 29, 34-40.

Campbell, J.R., Fu, J.K. & O'Toole, R. (1989) Biodegradation of PCP contaminated soils using *in situ* subsurface reclamation. *Proc. 2nd Natl. Conference on Biotreatment*, pp. 17-29. Hazardous Materials Control Research Institute, Washington, DC.

Cavalieri, L.F. (1991) Scaling up field testing of modified microorganisms. *BioScience*, 41, 568-574.

Council on Environmental Quality (1979) Global 2000 Report. Washington, DC.

Crawford, M. (1990) Bacteria effective in Alaska cleanup. *Science*, 247, 1537.

Cuskey, S.M. (1992) Biological containment of engineered microorganisms. In: *Microbial Ecology: Principles, Methods and Applications*, Levin, M.A., Seidler, R.A. & Rogul, M. (eds.), pp. 911-918. McGraw-Hill Inc., New York.

Davis, B. (1987) Bacterial domestication: underlying assumptions. *Science*, 235, 1329-1335.

Dwyer, D.F., Rojo, F., Timmis, K.N., Sussman, M., Collins, C.H., Skinner, F.A. & Stewart-Tull, D.E. (1988) Fate and behaviour in an activated sludge of a genetically-engineered microorganism designed to degrade substituted aromatic compounds. In: *The Release of Genetically Engineered Microorganisms*, Sussman, M., Collins, C.H., Skinner, F.A. & Stewart-Tull, D.E. (eds.), pp. 77-88. Academic Press, London.

Ellis, W.D. & M.K. Stinson (1991) Site demonstration of a soil washing system by Biotrol, Inc. at a wood preserving site in Brighton, MN. In: *Remedial Action, Treatment, and Disposal of Hazardous Wastes; Proc. 17th Ann. RREL Hazardous Waste Res. Symp.* April 1991, EPA/600/9-91/002.

Emmons, C.W. (1962) Natural occurrence of opportunistic fungi. *Lab. Invest. II*, 1026-1039.

Fliermans, C.B., Phelps, T.J., Ringelberg, D., Mikell, A.T. & White, D.C. (1988) Mineralization of trichloroethylene by heterotrophic enrichment cultures. *Appl. Environ. Microbiol.*, 54, 1709-1714.

Ginzburg, L.R. (1991) *Assessing Ecological Risks of Biotechnology*. Butterworth, Boston.

Greene, S., Alexander, M. & Legget, D. (1981) Formation of *N*-nitrosodimethylamine during treatment of municipal waste water by simulated land application. *J. Environ. Qual.*, 10, 416-421.

Grubbs, R.B. & Molnaa, B.A. (1987) Enhanced anaerobic digestion and odour control through

bioaugmentation. *Proc. Natl. Conference on Municipal STP Sludge Management*, pp. 2-7. HMCRI, Silver Spring, MD.

Hall, E.R. & Melcer, H. (1983) Biotechnology developments for the treatment of toxic and inhibitory wastewaters. *Biotechnol. Adv.*, 1, 59-71.

Hazardous Materials Control Research Institute (1989) *Proc. 2nd Natl. Conference on Biotreatment*. HMCRI, Washington, DC.

Hutzinger, O. & Verkamp, W. (1981) Xenobiotic chemicals with pollution potential. In: *Microbial Degradation of Xenobiotic and Recalcitrant Compounds*, Leisinger, T., Cook, A.M., Hutter, R. & Neusch, J. (eds.), pp. 3-46. Academic Press, London.

Irvin, T.R. (1989) Application of cell culture systems to monitor chemical toxicity during biodegradation. 7th Ann. Hazardous Materials Management Conference, May, 1989, Atlantic City, NJ.

Irvin, T.R. & Akgerman, A. (1987) Post-implantation rodent embryo culture systems to identify prenatal toxic components of complex environmental chemical mixtures. In: *Short-Term Bioassays in the Analysis of Complex Mixtures*, Sandhu,V.S., DeMarini, D., Mass, M., Moore, M. & Mumford, J. (eds.), pp. 367-372. Plenum, New York.

Irvine, R.L., Sojka, S.A. & Colaruotolo, J.F. (1982) Treating a landfill by pure strain inoculations of SBRs. In: *Impact of Applied Genetics in Pollution Control*, Kulpa, C.F., Irvine, R.L. & Sojka, S.A. (eds.), pp. 96-108. University of Notre Dame, Lafayette, Indiana.

Johnston, J.B. & Robinson, S.G. (1983) *Genetic Engineering and the Development of New Pollution Control Technologies*, p. 61. University of Illinois Press, ULU 830102.

Katz, L.S. & Marquis, J.K. (1991) Toxicology of genetically-engineered microorganisms. In: *Risk Assessment in Genetic Engineering*, Levin, M.A. & Strauss, H. (eds.), pp. 51-63. McGraw-Hill Inc., New York.

Keeler, R. (1991) Bioremediation: Healing the environment naturally. *R&D Magazine*, July 1991, 34-40.

Lamptey, J., Hendrick, C.A., Tomes, N.J., Brown, S. & Dean, D.H. (1992) Decontamination and mitigation: *Bacillus*. In: *Microbial Ecology: Principles, Methods and Applications*, Levin, M.A., Seidler, R.A. & Rogul, M. (eds.), pp. 827-841. McGraw-Hill Inc., New York.

Lenski, R.E. (1991) Quantifying fitness and gene stability in microorganisms. In: *Assessing Ecological Risks of Biotechnology*, Ginzburg, M. (ed.), pp. 173-190. Butterworth, Boston.

Levin, M.A. & Gealt, M. (1993) *Biotreatment of Industrial and Hazardous Waste*, p 4. McGraw-Hill Inc., New York.

Levin, M.A. & Strauss, H. (eds.) (1991) *Risk Assessment in Genetic Engineering*. McGraw-Hill Inc., New York.

Levin, M.A., Seidler R., Bourquin, A., Fowle, J.III & Barkay, T. (1987) EPA developing methods to assess environmental release. *Bio/Technol*, 5, 38-45.

Levin, M.A., Seidler, R.A. & Rogul, M. (eds.)(1992) *Microbial Ecology: Principles, Methods and Applications*. McGraw-Hill Inc., New York.

Lindow, S.E., Lindemann, J. & Haefele, D. (1992) Containment, decontamination and mitigation of *Pseudomonas* species in environmental settings. In: *Microbial Ecology: Principles, Methods and Applications*, Levin, M.A., Seidler, R.A. & Rogul, M. (eds.), pp. 891-909. McGraw-Hill Inc., New York.

Loomis, T.A. (1978) *Essentials of Toxicology*. Lea and Febiger, Philadelphia.

Marx, J.L. (1989) *A Revolution in Biotechnology*. Cambridge University Press, Cambridge, UK.

McCall, P., Vrona, S. & Kelly, S. (1981) Fate of uniformly carbon-14 ring labelled 2,4,5-trichlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid. *J. Agric. Food Chem.*, 29, 100-107.

McCormick, D. (1985) One bug's meat. *Bio/Technol*, 3, 429-435.

Mckinney, R. (1962) *Microbiology for Sanitary Engineers*. McGraw-Hill Inc., New York.

Milewski, E. (1985) Field testing of microorganisms modified by recombinant DNA techniques: Applications issues and development of points to consider. *Doc. Recomb. DNA Tech. Bull.*, 8 (3), 102-108.

Mizrahi, A. (1989) Biological waste treatment. *Adv. Biotechnol. Process.*, 1-310.

National Academy of Sciences (NAS) (1987) *Introduction of Recombinant DNA-Engineered Organisms into the Environment: Key Issues*. National Academy Press, Washington, DC.

Neidle, E.L., Shapiro, M.K. & Ornston, L.N. (1987) Cloning and expression in *Escherichia coli* of *Acinetobacter calcoaceticus* genes for benzoate degradation. *J. Bacteriol.*, 169, 5496-5503.

Nicholas, R. (1987) Biotechnology in hazardous waste disposal: An unfulfilled promise. *ASM News*, 3, 138-142.

Nicholas, R.& Giampocoro, D.E. (1989) Natures prescription. *Hazmat World*, June, 1989, 30-36.

Nielson, A.H., Allard, A.S., Lindgren, C. & Remberger, M. (1987) Transformations of chloroguaiacols, chloroveratroles, and chlorocatechols by stable consortia of anaerobic bacteria. *Appl. Environ. Microbiol.*, 54, 2511-2519.

NRC (1989) *Field Testing Genetically Modified Organisms. Framework for Decisions*. National Academy Press, Washington, DC.

OECD (1986) *Recombinant DNA Safety Considerations*. Organisation for Economic Cooperation and Development, Paris.

Olson, B.H. and Goldstein, R.A. (1988) Applying genetics to environmental management. *Environ. Sci. Technol.*, 22, 370-376.

Omenn, G. (ed.) (1987) *Environmental Biotechnology*. Plenum, New York.

Omenn, G. & Hollaender, A. (1984) (eds.) *Genetic Control of Environmental Pollutants*. Plenum, New York.

Orndorff, S.A. & Colwell. R.R. (1980) Microbial transformation of Kepone. *Appl. Environ. Microbiol.*, 39, 398-406.

OTA (1985) *New Developments in Biotechnology: 3. Field Testing Engineered Organisms*.. Office of Technology Assessment of the US Congress, Washington, DC.

Ou, L.T. & Sikka, H.C. (1977) Extensive degradation of Silvex by synergistic action of aquatic microorganisms. *J. Agric. Food Chem.*, 25, 1336-1339.

Paustenbach, D.J. (1989) *The Risk Assessment of Environmental and Human Health Hazards: A Textbook of Case Studies*. Wiley, New York.

Pfaender, F.K. & Alexander, M. (1972) Extensive microbial degradation of DDT *in vitro* and DDT metabolism by natural communities. *J. Agric. Food Chem.*, 20, 842-846.

Pierce, G. E. (1982) Diversity of microbial degradation and its implications in genetic engineering. In: *Impact of Applied Genetics in Pollution Control,* Kulpa C.F., Irvine, R.L. & Sojka, S.A. (eds.), pp. 20-25. University of Notre Dame, Lafayette, IN.

Porta, A. (1991) A review of European bioreclamation practice. *Biotreatment News*, 1, no. 9, 6-8.

Rishel, H.L., Boston, T.M. & Schmidt, C.J. (1984) *Costs of Remedial Response Actions at Uncontrolled Hazardous Waste Sites*. Noyes Publ., Park Ridge, NJ.

Rochkind, M.L., Blackburn, J.W. & Sayler, G.S. (1986) *Microbial Decomposition of Chlorinated Aromatic Compounds*. EPA/600/2-86/090.

Rojo, F., Ramos, J.L., Pieper, D., Engesser, K.H., Knackmuss, H.J., Timmis, K.N., Hollenberg, C.P. & Sahm-Heds, J.S. (1988) Laboratory evaluation of novel catabolic pathways. *Bio/Technology*, 6, 65-74.

Sakazawa, C., Shimo, M., Taniguchi, Y. & Kato, N. (1981) Symbiotic utilization of polyvinyl alcohol by mixed cultures. *Appl. Environ. Microbiol.*, 41, 261-267.

Savage, P. (1987) Bacteria pass a Houston cleanup test. *Chem. Week*, Nov. 11, 55-56.

Sayler G.S., Blackburn, J.W. & Donaldson, T.L. (1988) *Environmental Biotechnology of Hazardous Wastes*. National Science Foundation Project Report, no. CES 8714691.

Sayler, G.S., Kong, H. & Shields, M.S. (1984) Plasmid-mediated biodegradative fate of mono-halogenated biphenyls in facultatively anaerobic sediments. In: *Genetic Control of Environmental Pollutants*, Omenn, G.S. & Hollaender, A. (eds.), pp. 377-415. Plenum, New York.

Sharples, F.E. (1987) Regulation of products from biotechnology. *Science*, 235, 1329-1332.

Sharples, F. (1991) Ecological aspects of hazard identification for environmental uses of genetically-engineered organisms. In: *Risk Assessment in Genetic Engineering*, Levin, M.A. & Strauss, H. (eds.), pp. 18-31. McGraw-Hill Inc., New York.

Short, K.A., Doyle, J.D., King, R.J., Seidler, R.J., Stotzky, G. & Olsen, R.H. (1991) Effects of 2,4-dichlorophenol, a metabolite of a genetically-engineered bacterium and 2-4-dichlorophenoxyacetate on some microorganism mediated ecological processes in the soil. *Appl. Environ. Microbiol.*, 57, 412-418.

Sterritt R.M. & Lester, J.N. (1988) *Microbiology for Environmental and Public Health Engineers*. E. & F.N. Spon Ltd., New York.

Stone, M. (1984) Superbugs devour poisonous wastes. *Eur. Chem. News Chemscope*, November, 1984.

Tiedje, J.M., Colwell, R.K., Grossman, Y.L., Hodson, R.E., Lenski, R.E., Mack, R.N. & Regal, P.J. (1989) The planned introduction of genetically-engineered organisms: Ecological considerations and recommendations. *Ecology*, 70(2), 298-315.

USEPA Superfund Innovative Technology Profile (SITE) (1988) EPA/540/5-88/003. US EPA, Washington, DC.

USGAO (1988) *Biotechnology: Managing the Risks of Field Testing Genetically-Engineered Organisms*. GAO/RCED-88-27 USGAO, Washington, DC.

Vandenbergh, P.A. (1992) Degradative bacteria. In: *Microbial Ecology: Principles, Methods and Applications*, Levin, M.A., Seidler, R.A. & Rogul, M. (eds.), pp. 799-804. McGraw-Hill Inc., New York.

Vidaver, A. & Stotzky, G. (1992) Overview: containment, decontamination and mitigation. In: *Microbial Ecology: Principles, Methods and Applications*, Levin, M.A., Seidler, R.A. & Rogul, M. (eds.), pp. 781-797. McGraw-Hill Inc., New York.

Wick, C.B. and Pierce, G.E. (1990) An integrated approach to development and implementation of biodegradation systems for treatment of hazardous organic wastes. *Dev. Ind. Microbiol.*, 31, 81-93.

Glossary of Terms

cDNA: The complementary DNA copy of eukaryotic mRNA made by using the enzyme reverse transcriptase, a type of DNA polymerase found in retroviruses, whose function is to synthesize DNA from an RNA template. By virtue of its origin from mRNA, cDNA lacks the intron sequences which are usually present in the corresponding genomic DNA of eukaryotic organisms.

Embryonic-stem (ES) cell: An undifferentiated cell derived from an early mammalian embryo that is able, after culture *in vitro*, to contribute to the tissues of a developing, recipient embryo. Such cells can be used for gene transfer.

Gene flow: The movement of genes between populations or between different parts of the same population.

Gene library (gene bank): A very large number of recombinants from a cloning experiment which together contain a complete collection (or nearly all) of the DNA sequences in the entire genome of the particular organism whose DNA was cloned. (Also referred to as a **genomic library.**) A **cDNA library,** by contrast, is constructed using **cDNA**.

Genetic engineering (or gene manipulation): Broadly, the use of recombinant DNA (**rDNA**) techniques to alter the sequences of DNA molecules. Two unrelated DNA molecules may be joined together to produce a molecule with novel properties. In the UK, the legal definition of gene manipulation is "the formation of new combinations of heritable material by the insertion of nucleic acid molecules, produced by whatever means outside the cell, into any virus, bacterial plasmid or other **vector** system so as to allow their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation."[1]

Genome: The complete DNA complement of an organism comprising the sequences of all its DNA.

Genotype: The genetic content of an organism, defined by its DNA sequences.

The genotype determines many of the aspects of the **phenotype** of an organism. The genotype may be modified by classical breeding programmes or by **gene manipulation**.

GMO: Genetically modified organism. Any organism modified by the enormous variety of techniques of modern molecular biology, from a cell of the gut bacterium *Escherichia coli* modified by bacteriophage transformation, through plants modified by a biolistic gun, to animals modified by **ES cell** incorporation.

Homozygous: The state in which both copies of a gene (on the pair of chromosomes) are identical. Also may refer to pairs of chromosomes that are equivalent. If the two copies of the gene are dissimilar they are said to be **heterozygous**.

Introgression: The gene transfer between species mediated through repeated backcrossing of hybrid individuals to one of the parents.

Microinjection: The introduction of DNA (or, rarely, RNA) into the nucleus of a recipient cell. In animal transgenic biology, one of the pronuclei of the newly fertilized egg is microinjected with about 3 pl (3×10^{-9} ml) of a solution containing DNA.

Molecular cloning (gene cloning): The amplification of a recombinant DNA molecule and any gene product whose synthesis it directs by propagation of a line of genetically identical organisms all of which contain the recombinant DNA molecule.

Mosaic: An individual that contains cells of two or more genotypes. Such an individual results from the introduction of **ES cells** into an embryo, or from the integration of a **transgene** into only some of the cells of the very early embryo.

PCR: Polymerase chain reaction. An *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers.[2]

Phenotype: The expressed characteristics of an organism, determined by the interaction between its **genotype** and the environment. Thus, an organism that expresses growth hormone and is expected to grow more rapidly may not do so without an adequate supply of food.

Recombinant DNA (rDNA): Composite DNA molecules in which foreign DNA has been inserted into a **vector** molecule using **gene manipulation** techniques.

Restriction enzymes: Certain bacterial enzymes that can cleave DNA at specific points or restriction sites characterized by a specific sequence of bases (usually four or six base pairs long) in the DNA.

RFLP: Restriction fragment length polymorphism. Polymorphic restriction site resulting in production of differing lengths of DNA that distinguish individuals when their DNA is cut with the particular restriction enzyme. The frag-

ments of DNA can be detected with radioactive probes and used as markers in breeding and gene mapping experiments.

Somaclonal variation: Variability commonly found among plants that have been regenerated from tissue cultures.

Southern blotting: A method originally described by Southern[3] by which DNA restriction fragments separated by gel electrophoresis may be transferred from the gel to a nitrocellulose or nylon membrane. The DNA fragments can subsequently be screened with radioactively labelled, complementary DNA sequences or **probes** in order to detect the presence of those sequences.

T-DNA: The transforming DNA occurring as specialized sequences which border the disease-causing genes on the independently replicating plasmids of *Agrobacterium* spp.

Transfection (transformation): The process of altering the genetic constitution of a cell by introducing foreign DNA. Typically, transformation is used to describe such introductions into bacterial cells, and transfection for introductions into animal cells. *In vitro* experiments use simple methods to transfer DNA into such cultured cells.

Transgene: The DNA introduced into the **genome** of a recipient organism: typically used when the DNA is stably integrated into the host genome. A **transgenic organism** is one which has received a segment of DNA that has been manipulated by recombinant DNA techniques, and the foreign segment of DNA has been integrated into the organism's genome.

Vaccine: Classically, an attenuated form of a disease-causing organism that confers immunity against infection by the parent, virulent organism. Recombinant vaccines typically cause production of the crucial immunity-inducing protein components in a non-pathogenic vector, most commonly vaccinia virus, the organism used as a vaccine for smallpox.

Vector: A self-replicating carrier DNA (occasionally RNA) molecule into which foreign DNA can be inserted to allow its propagation and amplification.

Notes

1. Old, R.W. & Primrose, S.B. (1985) *Principles of Gene Manipulation.* Blackwell Scientific Publications, Oxford, UK.

2. Erlich, H.A. (ed.) (1989) *PCR Technology.* Stockton Press, New York.

3. Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, 98, 503-517.

Acronyms and Abbreviations

ABTA	Applied Biotreatment Association (USA)
ACGM	Advisory Committee on Genetic Manipulation (UK)
ACRE	Advisory Committee on Releases to the Environment (UK)
AFS	American Fisheries Society
APHIS	Animal and Plant Health Inspection Service (USA)
ASM	American Society for Microbiology
BBEP	Biotechnology, Biologics and Environmental Protection (USA)
BINAS	Biosafety Information Network and Advisory Service (UNIDO)
CEC	Commission of the European Communities
DOE	Department of the Environment (UK)
EC	European Community
EPA	Environmental Protection Agency (USA)
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration (USA)
FFDCA	Federal Food, Drug and Cosmetic Act (USA)
FHS	Fisheries Health Section (USA)
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act (USA)
GEM	Genetically engineered microorganism
GENHAZ	14th Report of the Royal Commission on Environmental Pollution (UK)
GLSP	Good large-scale practice
GMM	Genetically modified microorganism
GMO	Genetically modified organism

GNP	Gross national product
HMCRI	Hazardous Materials Control Research Institute (USA)
HSE	Health and Safety Executive (UK)
ICES	International Council for the Exploration of the Sea
ICGEB	International Centre for Genetic Engineering and Biotechnology
ISNAR	International Service for National Agricultural Research (The Hague, Netherlands)
LPS	Lipopolysaccharide
MPCA	Microbial pest control agent
NAS	National Academy of Science (USA)
NIH	National Institutes of Health (USA)
NRC	National Research Council
OECD	Organisation for Economic Co-operation and Development
RAC	Recombinant DNA Advisory Committee (NIH)
RREL	Risk Reduction Environmental Laboratory (USA)
SBR	Sequencing batch reactor
STP	Sewage treatment plant
TSCA	Toxic Substances Control Act (USA)
UN	United Nations
UNEP	United Nations Environment Programme
UNIDO	United Nations Industrial Development Organization
US HHS	US Department of Health and Human Services
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
USGAO	United States General Accounting Office
WHO	World Health Organization

Index

GENETICALLY MODIFIED ORGANISMS:
A GUIDE TO BIOSAFETY

Prepared by the Secretariat of the United Nations Industrial Development Organization (UNIDO) in cooperation with the International Centre for Genetic Engineering and Biotechnology (ICGEB), for the UNIDO / UNEP / WHO / FAO Informal Working Group on Biosafety.
Editor: George T. Tzotzos

For a number of years the promise of biotechnology has been dimmed by concerns over the intrinsic safety of transgenic organisms. Although considerable knowledge of the properties of recombinant systems and a vast volume of data gathered from different application of biotechnology are now available, these concerns are still evident. In the developing world, there are also fears that such countries might be used as testing grounds for recombinant products. Considerations of this nature have often overshadowed the benefits these countries might derive from the application of genetic engineering.

In response to these concerns, UNIDO, together with the United Nations Environment Programme and the World Health Organization, formed in 1985 the Informal Working Group on Biosafety. In 1991 the Food and Agriculture Organization of the United Nations also joined the Group. The present volume was commissioned by the Group and is intended to help scientists and regulators to conceptualize the major issues underlying biological safety as well as to understand how these affect policies to regulate biotechnology.

ISBN 0 85198 972 1

CAB INTERNATIONAL

Headquarters: Wallingford, Oxon OX10 8DE, UK.

North America: 845 North Park Avenue, Tucson, AZ 85719, USA.

Asia: PO Box 11872, 50760 Kuala Lumpur, Malaysia.

Caribbean: Gordon Street, Curepe, Trinidad and Tobago.

Cover designed by:
S Jayne Clements,
Watkiss Studios Limited,
Biggleswade, Beds.