Biosafety and the Environmental Uses of Micro-Organisms

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Foreword

This publication constitutes the proceedings of the OECD conference on the "Environmental Uses of Micro-Organisms: An Overview of the State-of-the-Art and Implications for Biotechnology Risk/Safety Assessment". This event, organised under the auspices of OECD's Working Group on the Harmonisation of Regulatory Oversight in Biotechnology (WG-HROB), was held on 26-27 March 2012. A total of 100 participants attended the conference, which was open to OECD delegates as well as external scientists, regulators and interested individuals. It was developed in collaboration with the OECD Co-operative Research Programme under the Trade and Agriculture Directorate.

OECD has undertaken activities related to the safety of the environmental uses of transgenic organisms (also known as genetically-modified organisms) since the mid-1980s. The Working Group was established in its current form in 1995. Since that time, its programme of work has included activities related to the environmental risk/safety assessment of transgenic organisms with a major focus on transgenic plants.

In addition to this core activity on agricultural crops, the Working Group has long had a number of projects related to the safety of environmental applications involving micro-organisms. This has resulted in a number of publications, some of them dealing with specific groups of micro-organisms which have been used in environmental applications such as *Pseudomonas*, *Acidithiobacillus*, *Acinobacter* and Baculoviruses. Other publications have addressed issues of importance in the risk/safety assessment of bacteria such as taxonomy, detection methods, horizontal gene transfer and pathogenicity factors.

There have been many scientific developments in recent years related to the use of micro-organisms in the environment and the conference was an opportunity to provide an overview of the current situation, focusing on concrete or expected developments involving the use of transgenic micro-organisms in the environment.

Following the conference, the Working Group discussed the outcomes and identified a number of areas in which the use of transgenic micro-organisms is either already underway or is likely to be forthcoming in the near future. At the time of writing, the Working Group has agreed to launch a project for developing a guidance document on the use of micro-algae. This document will aim to provide background information which could be used in an environmental risk assessment. As for the other topics addressed at the conference, some may be included in the programme of work of the Working Group at a later stage, but this will depend on the resources available.

Acknowledgements

This publication includes the outputs of the OECD conference on the Environmental Uses of Micro-Organisms: An Overview of the State-of-the-Art and Implications for Biotechnology Risk/Safety Assessment, held in March 2012. The conference was organised by OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology with the support of Co-operative Research Programme. The OECD Secretariat is grateful to all members of the Working Group, including the Chair and co-Chairs, for their work in preparing and attending the event. The contributions made by both speakers and participants were invaluable. At the same time, special thanks must go to Hans Bergmans (former Chair of the Sub-Working Group on Micro-organisms) for his major contribution to the arrangements. Thanks are also due to OECD colleagues Carl-Christian Schmidt, Janet Schofield and Nathalie Elisseou Leglise from the Co-operative Research Programme who did much to support the conference. Jennifer Allain prepared the manuscript for the publication. The arrangements for the conference were managed by Peter Kearns, Bertrand Dagallier, Kazuyuki Suwabe and Takahiko Nikaido of the OECD Secretariat, who were also responsible for editing this document.

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Abbreviations and acronyms

AMF	Arbuscular mycorrhiza fungi
ANI	Average nucleotide identity
AWG	Algae Working Group (United States)
BCA	Biocontrol agent
CAPEX	Capital expenditure
CI	Cytoplasmic incompatibility
DHA	
DSL	Domestic Substances List (Canada)
EM	Effective micro-organisms
EMF	Ectomycorrhiza
EPA	Environmental Protection Agency (United States)
ERA	Environmental risk assessment
FAME	Fatty acid methyl ester
GA	Gibelleric acid
GHG	Greenhouse gas
GILSP	Good Industrial Large Scale Practice
GMM	Genetically modified micro-organism
GPCA	Great Plate Count Anomaly
GRAS	Generally recognised as safe
HGT	Horizontal gene transfer
HSP	High-scoring segment pair
ISB	*In situ* bioremediation
LCO	Lipo-chitin-oligosaccharides
LSF	Life support function
N	Nitrogen
NES	Normalised ecosystem strain
NGS	Next-generation sequencing
NOR	Normal operating range

OPEX	Operational expenditure
P	Phosphorus
PBR	Photo bioreactors
PGPB	Plant growth-promoting bacteria
PGPR	Plant growth-promoting rhizobacteria
QA	Quality assurance
QC	Quality control
QPS	Qualified presumption of safety
R&D	Research and development
YOPI	Young, old, pregnant, immune compromised

Executive summary

Micro-organisms including bacteria and viruses are probably best known as pathogens causing disease in plants, man and other animals. At the same time, many microbial species also play a fundamental role in the functioning of the environment, being instrumental, for instance, in mineralization processes, nitrogen fixation and an array of other geochemical processes. Life on earth would not be possible without micro-organisms and the services that they provide.

The precise role of specific micro-organisms is, however, not clear in many cases. In 1990, the first evidence became available that the micro-organismal environment is much more complex than had been thought until then. The years that followed saw the development of novel concepts about the population structure of micro-organisms and how micro-organisms co-operate in a community or "microbiome". These innovative ideas and developments in environmental microbiology have had large impacts on biotechnology.

The purpose of the OECD conference was to inform policy makers, regulators and specialists in the use of transgenic micro-organisms (also known as genetically modified micro-organisms) and of the environmental aspects (biosafety and risk assessment), by drawing an overview of the current situation and relevant developments in environmental microbiology, as well as its potential applications. This is important if policy makers and others are to be proactive in ensuring the responsible use of such organisms.

The conference covered the state-of-the-art of environmental microbiology as it is applied for biotechnological purposes, and the role of genetic engineering of micro-organisms intended for use in the environment, now and in the near future. The conference programme addressed the following themes: i) the Use of Micro-Organisms in Agriculture; ii) the Use of Microalgae for Production Purposes; iii) the Use of Micro-Organisms for Bioremediation; iv) the Use of Micro-Organisms in Cleaning Products; v) Environmental Applications of Microbial Symbionts of Insects; and vi) Environmental Risk Assessment of the Deliberate Release of Engineered Micro-Organisms.

Micro-organisms are important in agriculture especially as biofertilizers, which are growth-promoting micro-organisms. Presently, there are about a hundred companies involved in the development of growth-promoting micro-organisms, and around 500 products have been registered. Another agricultural application involves the use of micro-organisms as biocontrol agents, that is, as plant protection products to control disease and attack by insects and other herbivores. It is likely that transgenic micro-organisms will appear in these types of application in the future.

A second application discussed in this publication is the use of microalgae for production purposes, including the production of biofuels. Algae, with photosynthetic cyanobacteria, offer ideal solutions because they can be cultivated year round,

on non-arable land, alleviating the pressure on farmland and freshwater resources that would be exerted by crops grown for biofuel purposes. Many algal strains are suitable for producing renewable fuels (biodiesel, bioethanol and kerosene). They may also become a promising source of food and feed. The production of algae, in particular microalgae, has therefore become a focus area in biotechnology development. Research on the use of transgenic algae is expanding, and commercial applications are likely in the coming period.

The use of micro-organisms for bioremediation is also addressed in this document. Bioremediation uses living organisms for removing contaminants from the environment, for example polluted land. To date, there have been few cases of bioremediation involving transgenic micro-organisms. This is probably due to the current lack of knowledge concerning the risks and benefits of releasing them into contaminated soils.

The development of cleaners, detergents and other similar products containing micro-organisms has increased over recent years. In many cases, detailed knowledge of their composition is lacking. As far as is known, it is unlikely that any such products currently available contain transgenic forms of micro-organisms, though this remains a possibility for the future.

A more surprising use of micro-organisms discussed in this publication is the use of *Wolbachia* species to control the spread of disease transmitted by insects. For example, cases are described of the potential control of dengue fever by mosquitoes as well as the control of malaria. While these techniques may be promising, no transgenic organisms have been used in large scale practice yet.

In terms of the **major findings and conclusions** of the conference, the following should be noted:

- In recent years, there has been a considerable increase in knowledge as to how micro-organisms function in nature and co-operate in a communities or the "microbiome".
- Certain micro-organisms are currently used (or have the potential to be used) in a variety of products and applications such as biofertilisers, plant protection products, biofuel production, bioremediation, cleaners, detergents as well as in the control of disease transmission.
- To date, there have been few uses of transgenic micro-organisms in such products and applications, though that might change in the future.
- In order that products involving transgenic micro-organisms are used in a responsible way, it is important that regulatory oversight involves a rigorous risk/ safety assessment.

It is clear that the uses of micro-organisms in agriculture and of microalgae for production purposes are significantly increasing. These developments could involve important economic impacts in their respective sectors. At the same time, they could well contribute to the OECD's work on green growth and sustainable development. As a final recommendation, therefore, it would be timely to consider the development of tools which would assist in a scientifically-sound approach to risk assessment of transgenic organisms in these two areas of applications.

Introduction: Micro-organisms, public health and the environment

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This chapter looks back at the results of the OECD conference on the "Environmental Uses of Micro-Organisms: An Overview of the State-of-the-Art and Implications for Biotechnology Risk/Safety Assessment", as well as forward, to what this could mean for the future.

Outcomes of the OECD conference on the "Environmental Uses of Micro-Organisms"

The role that micro-organisms, in particular bacteria, play in the environment can hardly be overestimated. This is often summarised in the concept put forward in 1934 by Baas Becking (italics by him): "*Everything is everywhere,* but, *the environment selects*" (see De Wit and Bouvier, 2006). Ever since it was shown that there is also much more heterogeneity in the prokaryotic world (e.g. Torsvik et al., 1990), one could add: and there is much more bacterial diversity in the soil than we ever thought possible. One could also add: and much of this diversity is still unknown to us. The micro-organisms in soil perform a vast array of intricate biogeochemical processes without which life on earth would not be possible. It is hardly feasible that such complex multi-step processes are performed by single organisms. It stands to reason, and it has become obvious, that these processes are performed by consortia of organisms, working together (de Lorenzo, 2008; and Chapter 7). Is the time of pure cultures gone, then? No, it is not, not in the sense that it would no longer make sense to study the environmental role of individual species. This is, of course, particularly the case when the role is obvious, as it is for pathogenic species. But even in those cases, the role of the complex microbial environment around the pathogen is important, as is shown in Part I.

Man has turned the resources and processes in the macroscopic environment to his use, to start with in agriculture. It stands to reason that the agricultural use of micro-organisms has been among the first uses of the microscopic environment. Further study of the microbial environment has lead to insights that can be used in a variety of applications, in biotechnology as well as in medicine. An underlying presumption is that we understand the ways of the microbial world. The contributions in this volume all relate to this underlying theme, that the microbial world can be used, but that its use should be prudent and sustainable. In our regulations, a particular case is made for the prudent use of modern biotechnology and genetically engineered micro-organisms. This use should be subsequent to a risk/safety assessment which takes into account the (potential) adverse environmental effects of the micro-organisms under their conditions of use. This risk/safety assessment is not straightforward, as we have seen, man's knowledge of the microbial environment and the processes occurring in it is still far from complete or even satisfactory. The contributions in this volume provide a further underpinning of these introductory statements, which are summarised below.

Part I: Use of micro-organisms in agriculture

Two chapters in this part focus on the use of micro-organisms as biofertilizers and for phytosanitary purposes respectively. For biofertilizers, it is pointed out that there have been extensive developments since the 1995 OECD consensus document on this subject, which have resulted in a much better understanding of the processes that are ongoing in plant-bacteria interactions. This knowledge is already used for strain improvement by genetic engineering, and suggests that the use of transgenic organisms such as biofertilizers will be an area of multiple and diverse possibilities of action in the near future. Presently, there are some 100 companies involved in the development of growth-promoting micro-organisms, and some 500 products have been registered. Like biofertilizers, the use of micro-organisms as biocontrol agents has become of vital importance for agricultural practice. Farmers are challenged to grow more, but with less fertilizer, pesticides and fumigants and more sustainable practices, such as no-till, precision farming, biocontrol. To meet these challenges, all types of traditional and new

pest management technologies are needed. Transgenic biocontrol agents (BCAs) will need to be a part of these technologies. The development of transgenic BCAs has already been ongoing for more than two decades. These are examples for the development of strains and traits that can be incorporated into strains by biotechnological means, that can be used directly for agricultural purposes, and where knowledge of other soil functions may help but is not a direct necessity for understanding the way to proceed.

The application of transgenic strains to be released into the environment as biofertilizers and BCAs requires a thorough risk/safety assessment to be carried out to determine any potential adverse environmental impacts that these strains might have. The baseline for such risk/safety evaluations is the understanding that we have of environmental microbiology. Over the last two decades, our knowledge of processes that occur in the microbial environment has increased enormously (see also Chapter 15 and 16). The contribution on risk/safety assessment points out the necessity of improving our understanding of the normal operating ranges of micro-organisms, in order to interpret the environmental impacts that may be observed as the result of releases of transgenic biofertilizers and biocontrol agents, and indeed any micro-organisms that are released into the environment.

Part II: Use of microalgae for production purposes

There is a global need for energy, and a growing conviction that more energy should be generated in a renewable way. Algae, including photosynthetic cyanobacteria, offer ideal solutions to this problem because they can be cultivated year round, on non-arable land, in various wastewater streams or brackish to marine waters, alleviating the pressure on arable land and freshwater resources that would be exerted by crops grown for biofuel purposes. Many algal strains are suitable for producing renewable fuels (biodiesel, bioethanol and kerosene). They may also become a promising source of food and feed. The production of algae, in particular microalgae, has therefore become a focus area in biotechnology development.

Chapter 5 in this part presents an overview of developments in the production of microalgae. Emphasis is given to the need to understand algal taxonomy and the consequences for strain selection for production purposes. Also, an integrated approach and complete lifecycle analyses still need to be conducted to evaluate any potential large-scale environmental implications.

Chapter 4 and 6 point out that research on transgenic algae is rapidly expanding, while large investments stimulate research on transgenic algae. Within two years, applications for outdoor, confined (e.g. in raceway ponds) cultivation of transgenic algae are expected to be actual in Europe, and earlier than that in the United States. The successful application of large-scale production of microalgae, be it in (confined) releases or in contained use, will require a science-based approach to the risk/safety assessment. Approaches to this risk/safety assessment have already been discussed in a number of workshops in the United States, and their results are available for further discussion. The development of this risk/safety assessment may furthermore require, for instance in the United States, harmonisation of the existant regulatory oversight, which in the United States is included within laws.

Part III: Use of micro-organisms for bioremediation

Bioremediation involves the application of micro-organisms for the removal of contaminants from the environment. Bioremediation competes effectively with other remediation approaches, such as thermal desorption and incineration. The development of genetically engineered stains with enhanced biodegradability capabilities looks like a promising way for further innovation of this technology. At present, however, there have been very few reported examples where genetically engineered micro-organisms have been successfully released into commercial bioremediation. One of the main reasons for this is the lack of knowledge of the environmental risks and benefits that may be caused by releasing genetically modified organisms into a contaminated area. In this respect, non-specialist stakeholder support remains a crucial area that should be further improved, concomitant as part of the sustainable development of bioremediation.

The contributions in this part make it clear that with the evolving knowledge of micro-organisms and their roles in the environment, it will be possible to design bacteria for environmental use in a focused way instead of by trial and error.

Part IV: Use of micro-organisms in cleaning products

This part focuses on the emerging problems with the regulatory oversight of microbial cleaning products. The development of microbial cleaners has occurred over the past few years, relatively unnoticed by the field, resulting in a situation where a large number of products are on the market that are more or less successful in cleaning, but where knowledge of the actual composition of the product in terms of active ingredients is lacking. This has led to concerns and regulatory approaches in the United States and in the European Union. Although it unknown whether any transgenic micro-organisms are present in these products – and this is expected not to be the case – the regulatory developments in this field may show parallels with as well as pitfalls for the developing regulatory oversight commercialisation of transgenic micro-organisms for other applications.

Part V: Environmental applications of microbial symbionts of insects

The use of micro-organisms in the control of disease transmission by insects has become very promising over the last few years. The use of *Wolbachia* symbionts for the control of dengue transmission by mosquitoes and fighting malaria with engineered mosquito symbiotic bacteria by "paratransgenesis" are two areas that are dealt with in this part.

For the case of controlling the transmission of dengue, no transgenic micro-organisms are used (yet), but it is an interesting and important example of how to deal with this type of approach in regulatory oversight and information of and co-operation with the general public. The case of paratransgenesis is a new – and to most people unexpected – way of gene delivery to insects, that is still in its infancy, but has a strong potential to become an important and valuable tool in public health. It would be important to anticipate how to deal with this in regulatory oversight. This probably requires a fuller understanding of the very specialised microbiomes in insects.

Part VI: Environmental risk/safety assessment of the deliberate release of engineered micro-organisms

The analysis of microbial populations in the environment is a major underlying theme of environmental microbiology. The continuously developing art of DNA sequencing has proven to be a powerful and indispensable method to unravel the genetic diversity of micro-organisms in the environment. In recent years, revolutionary next-generation sequencing technologies have become widely used in various microbiological disciplines, including microbial taxonomy and ecology. New views have evolved on the species concept of prokaryotes, including bacteria and archaea. The new techniques can be used as a comprehensive methodology for monitoring microbes in soil. Next-generation sequencing-enabled metagenomics should be useful and can be widely applied to modern microbiology and biotechnology. A good understanding of these techniques, and the interpretation of the results that are gathered by these means, are vital for performing meaningful and reliable risk/safety evaluations in this field.

The environmental use of micro-organisms offers a large number of extremely interesting and promising applications that may or will involve the use of transgenic organisms. Regulatory oversight will require rigorous risk/safety assessment, based on scientific knowledge about the role of micro-organisms in the environment. When the results of the OECD conference and the contributions in this volume are placed in the context of risk/safety evaluation, some questions arise. A major question in all debates on environmental risk/safety assessment, that is also prominent in the risk/safety assessment of releases of transgenic micro-organisms, is the question of what constitutes an "adverse environmental effect". With the present knowledge of the microbial environment, this question requires careful consideration. The OECD conference and the contributions in this volume have shown approaches to acquire the necessary knowledge, and have highlighted the knowledge and experience gained already.

Prospects and potential new biosafety projects for the OECD

A major aim of the OECD conference on the "Environmental Uses of Micro-Organisms: An Overview of the State-of-the-Art and Implications for Biotechnology Risk/Safety Assessment" was to provide a state-of-the-art overview of the environmental uses of micro-organisms, focusing on concrete or expected developments in the field of transgenic organisms, as a support for the development of the work programme of the Sub-Working Group on Micro-Organisms of the Working Group on the Harmonization of Regulatory Oversight in Biotechnology (WG-HROB).

Based on the results of the conference, it can be concluded that there are two clear areas where research has progressed to an extent that use of transgenic organisms actually occurs or is forthcoming, and could be very important for further economic developments: the use of micro-organisms in agriculture and the use of microalgae for production purposes (see Parts I and II). Projects in these areas would fit very well with the OECD's work on green growth and sustainable development. The specific issues that would be tackled in these projects would be the scientifically sound approach of risk assessment of environmental releases of transgenic organisms for these purposes.

The ideas that were put forward in the considerations on the use of micro-organisms in bioremediation (see Part III) are conceptually very important, as are the considerations on the analysis of microbial populations in the environment that is a major underlying theme in discussions on the environmental uses of micro-organisms (see, for instance, Part VI). These ideas would be a good basis for a conceptual document on how

knowledge on microbial populations is gathered, and how this knowledge can be applied for predicting the behavior of soil micro-organisms, for optimising the (wanted) activity of micro-organisms in the soil (e.g. in bioremediation), as well as for optimising risk assessment of released micro-organisms. It has become clear that these underlying considerations are not always straightforward. The further development of these lines of thought, for instance in a guidance document, would be a very important complement to the projects mentioned above.

Environmental applications of microbial symbionts of insects are a specific niche within environmental microbiology that holds large promises for future developments, developments that could be very important from an economic as well as from a public health point of view. The scientific underpinning of the developments in these fields is not generally known even to all microbiologists, and they are not straightforward. A project to draft a conceptual document about the developments in this field could therefore be very important to help the development of regulation in this field proactively.

These considerations and the proposed projects will be further discussed in the Sub-Working Group on Micro-Organisms. The sub-working group will submit its conclusions to the WG-HROB, which will decide on whether and which project(s) will be initiated, while taking into account the available resources.

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Part I

The use of micro-organisms in agriculture

Chapter 1

Biofertilizers: Present and future use of transgenic micro-organisms

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Biofertilizers are living microbial preparations which enhance or promote plant growth, relatively to a control without inoculation. A huge amount of research literature has been produced in the last 20 years concerning plant growth-promoting rhizobacteria (PGPR) related subjects, describing different micro-organisms acting on different plants, and proposing different mechanisms to explain the plant growth promotion effect. However, we still do not know which of the different in vitro *mechanisms of biofertilizer action are responsible for the positive effects in the field. Biofertilizer technology has significantly developed in the market. The nature of multiple mechanisms discovered for PGPR actions and the possibility of genetically modifying a particular strain concerning a particular plant growth-promoting activity suggest that the use of genetically modified organisms such as biofertilizers will be an area of multiple and diverse possibilities of action in the near future. The study of the microbial ecology of this scenario and its dynamics will certainly improve the development of biofertilizer technology for the future of agriculture.*

Introduction

One of the big challenges for the future of humanity is to produce enough food in a sustainable way. Another big challenge is to produce bio-fuels to replace those non-renewable types of fuels for which resources will be exhausted some day. Crop plants play a key role for solving both of these challenges. Besides water, crop production is limited by the availability of the main nutrients in soil, such as nitrogen (N) and phosphorus (P). Soil micro-organisms are key elements in biogeochemical cycles of elements on our planet (Buscot and Varma, 2010).

From an evolutionary point of view, many plant/micro-organisms interactions have been selected which produced mutual benefits for the interacting organisms. Plants are primary biomass producers through photosynthesis and those photosynthates can be partially released into the soil via root exudates or via root and/or plant debris degradation. In this way, soil organic matter is increased and can be used by heterotrophic organisms as substrates to grow. It is reasonable to think that some microbes have succeeded in the history of evolution because of their capacities to improve plant growth, to assure their own source of food or substrates needed to grow. Most of these kinds of microbes live in the rhizosphere, the part of the soil which is influenced by the release of substances from the plant (Dessaux et al., 2010).

From a utilitarian point of view, these kinds of micro-organisms can be used to improve plant growth to assure food production. If these micro-organisms facilitate plant nutrition, their action would be valuable in terms of sustainability of the processes, because it would diminish the need for chemical fertilizers, whose production depends on non-renewable energy sources.

Taking all of these ideas into account, biofertilizers are defined as industrial products based on culturable micro-organisms that were isolated from the soil or rhizosphere of plants and which have been proven capable of modifying, and improving, plant development through a collection of different mechanisms of action. A product is characterised as a biofertilizer following an experimental test where the behaviour of a plant inoculated with a suspension containing a huge amount of cells of a particular micro-organism is compared to a control situation where the plant grows without the addition of this micro-organism. This experimental test can be performed either *in vitro* or *in vivo*. *In vitro* means growing plants hydroponically or using a controlled substrate, in pots in growth chambers or in greenhouses. *In vivo* means that the test is performed in soil, either in greenhouses or in field conditions. *In vivo* results may differ from *in vitro* results because the microbial background is different, and it is almost impossible to verify and compare the microbial background in soils with experimental *in vitro* conditions, simply because we still do not know precisely how to characterise the total microbial diversity existing in soil. Culturable micro-organisms are about 1% of the total existing micro-organisms in soil (Staley and Kanopa, 1985; Torsvik and Ovreas, 2002). So, regardless of the characterisation result of a micro-organism as a biofertilizer after different *in vitro* tests, the biofertilizer activity should be proven in soil, in field conditions, because the plant microbe interaction must function in the presence, and influence, of the huge diversity of other micro-organisms living in soil.

There are different modes of interactions between biofertilizers and plants (Gray and Smith, 2005), considering the degree of association between micro-organisms with plant roots in a gradient of root proximity and intimacy as follows:

1. micro-organisms living in the soil near the root, utilising nitrogen and carbon metabolites leaking from the root (rhizosphere)
2. micro-organisms colonising the rhizoplane (root surface)
3. micro-organisms colonising the root tissue inhabiting intercellular spaces (endophytes)
4. micro-organisms living inside cells in specialised root structures or nodules (symbionts).

Cases 1-3 do not induce any particular root structure and the micro-organisms are considered to be in a looser associative interaction with the plant compared to a more complex integrated association including the development of specialised root structures as in the last case of symbiotic associations. In all cases, biofertilizers should reach and colonise the rhizosphere to act on the plant through interaction with its root. It has been shown that root colonisation is part of the mechanism needed to produce plant growth promotion (Lugtenberg and Kamilova, 2009).

Besides the degree of association with the root tissue, at least two different modes of action can be recognised in biofertilizers' activity: direct and indirect mechanisms. Direct mechanisms imply the supply of a nutrient or the release of microbial substances which enhance plant growth or development. Indirect mechanisms are those which suppress or inhibit a deleterious situation for regular plant development, as for instance, a disease caused by a pathogen (Vessey, 2003; Glick et al., 1999).

For study purposes and organisation of the available knowledge on this subject, considering the different scenarios of plant-microbe interactions and the different mechanisms of plant growth promotion, different definitions are used to organise the concept of biofertilizer:

1. Free-living/non-symbiotic micro-organisms considered to be plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). These biofertilizers are also considered extracellular (Gray and Smith, 2005). To clarify different kinds of PGPR, different authors have proposed different definitions which try to separate them according to their mode of action:
   - Plant growth-promoting bacteria (PGPB). These microbes promote direct plant growth by enhancing different mineral nutrition or by regulating plant development implying a phytohormone-like way (Bashan and de-Bashan, 2010; Verma et al., 2010).
   - Biocontrol PGPB. These microbes are mainly antagonists to different plant pathogens. They improve indirectly plant growth by releasing the disease state of the plant (Bashan and Holguin, 1997). This particular group of micro-organisms has received some attention in the last years because of their economic implications (see Chapter 2).
   - Plant stress homeostasis-regulating bacteria (PSHB). This group of micro-organisms was quite recently proposed to highlight cases were the plant growth promotion takes place within an abiotic stress condition (i.e. water stress, salt stress) (Cassan et al., 2009; Bashan and de-Bashan, 2010)
2. Micro-symbionts or intracellular plant growth-promoting micro-organisms, as defined by Gray and Smith (2005). These associations are more visible in the

plant because they induce specialised root structures where the plant microbe interaction takes place. All of these interactions are at least important for a main nutrient supply for the plant (nitrogen or phosphorus), but other positive concurrent mechanisms for plant growth promotion cannot be disregarded as part of the activity of these micro-organisms:

- N_2-fixing rhizobacteria:
  - rhizobia-legume simbiosis
  - *Frankia*-actinorhizal plant simbiosis.
- Micorriza fungi:
  - arbuscular mycorriza fungi (AMF)
  - ectomycorrhyza fungi (EMF).

What is new about this since the previous OECD consensus document dedicated to biofertilizers, published in 1995? Back then, 48 out of 51 pages referred to symbiotic biofertilizers, describing mostly rhizobia, *Frankia* and mycorrhiza, which are symbiotic cases. The remaining three pages of the document referred to free-living micro-organisms as "future biofertilizers". Since 1995, many articles have been published related to these new groups of micro-organisms generally named or referred to as PGPR. Different mechanisms of action have been described and are still a matter of intensive research. Most of this knowledge has been recently reviewed (Glick et al., 1999; Vessey, 2003; Bashan et al., 2004; Gray and Smith, 2005; Barriuso et al., 2008; Lugtenberg and Kamilova, 2009; Verma et al., 2010; Bashan and de-Bashan, 2010).

Symbiotic biofertilizers

As stated above, these symbiotic micro-organisms induce new structures in plants or infect the root tissue in quite a visible way under the microscope. It is because of this characteristic that these micro-organisms have been known for a long time, discovered by the end of 19th century (Werner, 1992). In many cases, the application of molecular biology tools allows the discovery of the genes and signals involved in the beneficial interaction between the micro-organism and the plant. The main symbioses concerning agricultural application as biofertilizers are considered below.

Rhizobia

The most advanced studied system corresponds to the nitrogen-fixing symbiosis between Gram negative bacteria generally called rhizobia, and leguminous plants, including many important crops for forage and food production (Werner, 1992). The molecules involved in the signal exchange between the bacteria and the plant which determines the recognition control the development of the infection and nodule structure in the root have been described in many model legumes (Schultze and Kondorosi, 1998). Different species of root nodule endophytes are included in the group of alpha-proteobacteria but some beta-proteobacteria were also found. The different genus and species inhabiting legumes root nodules are usually referred to as rhizobia. Within these endophytes genera are alpha-proteobacterias such as: *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium (Ensifer)*, *Mezorrhizobium*, *Azorhizobium*, *Allorhizobium*, *Agrobacterium*; and beta-proteobacteria such as *Burkholderia*. The best model describing the interaction between rhizobia and legume roots includes flavonoids/isoflavnoids molecules released

by the plants which induce bacteria genes and consequently the synthesis of the LCO (lipo-chitin-oligosaccharides) molecules, which in turn control infection and nodule development in the root tissue (Schultze and Kondorosi, 1998; Madsen et al., 2010). Besides this Nod factor model characterised by the LCO molecules, a Nod factor independent nodulation of some legumes (Giraud et al., 2007) has been described. As a consequence of the knowledge developed over the last decades, the new generation of inoculants for legumes based on specific rhizobia strains has been improved by the use of new technologies, including the addition of those signals involved in the early interactions between the bacteria and the plant.

Mycorrhiza

The symbiosis between mycorrhiza fungi and the plant is not only the most ancient symbiosis for which we have fossil records, but it is also one of the most studied beneficial plant-microbe interactions (Smith and Read, 2008). Besides the kind of mycorrhizal association that the fungi establish with the plant, ecto- or endo-mycorrhiza, in all the cases the external fungi hyphae create a net outside the root that extend the exploration capacity of the plant root, improving the interaction of the plant with the soil, and the uptake of nutrients. It is well documented that mycorrhiza association improves water, nitrogen and phosphorus uptake by the plant, and probably other micronutrients.

Ectomycorrhiza (EMF) induce the formation of short modified roots where the fungus creates a net in the outer cell layers of the plant cortex without invading the plant cell but establishing a huge surface of plant-fungus interaction. The EMF are more specific in the association with plant species, and generally this symbiosis is present in woody plants. The EMF belong to culturable fungi species and there are different examples of biofertilizers based on this kind of micro-organism, whose market is orientated to tree and shrub production in nursery conditions.

Endomycorrhiza, also referred as arbuscular mycorrhiza fungi (AMF), is the most ancient documented symbiosis which is actually present in about 90% of plant species. Different fungi species are able to induce arbuscular mycorriza, the most abundant ones being fungi of the order Glomales. It has not been possible to cultivate any of the AMF in the lab. This fact is a very important limitation for the industrialisation of biofertilizers based on endomycorrizal fungi. Although the AMF do not appear to be active in conventional agriculture with tillage soil, they become important in soil conservative agricultural management, as direct sowing with no till or reduced till practices. Thus, from a sustainable point of view, the availability of pure AMF inoculum would be needed for good agriculture practices. The possibility of cultivating AMF strains in carrot root organ culture is good news and provides new possibilities in this area (Declerck et al., 2010). Quite recently, the chemical nature of the signals involved in the early recognition between the AMF and host plants has been described; the signals have been called Myc factors (Maillet et al., 2011). Curiously, the Myc factors are also lipochitinoligosacharide in nature as rhizobia Nod factors are. These signals appear to stimulate arbuscular mycorrhizal formation and also lateral root development. Most probably, these kind of signals will be part of the future generation of biofertilizers, as it was the case in rhizobia-Nod factors based inoculums.

Free-living or non-symbiotic biofertilizers

Since the description of PGPR by Kloepper and Schroth (1978), many different bacteria genera have been described as PGPR: *Pseudomonas*, *Azospirillum*, *Azotobacter*,

Gluconacetobacter, *Herbaspirillum*, *Bacillus*, *Burkholderia*, *Erwinia*, *Caulobacter*, *Azotobacter*, *Chromobacterium*, *Serratia*, *Micrococcus*, *Flavobacterium*, *Actinobacter*, *Enterobacter*, *Arthrobacter*, *Agrobacterium*, *Hyphomycrobium*, and fungus such as *Trichoderma*, among others (Bashan and de-Bashan 2010, Verma et al., 2010; Richardson and Simpson, 2011).

Many PGPR have been described as endophytic bacteria. It is not clear if the plant growth promotion effects are a consequence of plant-microbe interaction in the external part of the rhizophere or if an endophytic state is necessary. Many different mechanisms have been claimed to be responsible for the plant growth promotion effect after *in vitro* experiments under controlled conditions (Glick et al., 1999). In some cases, the use of appropriate mutants helps in the definition of these mechanisms. But since different mechanisms are always present in a single strain, it is almost impossible to know which are the main mechanisms operating and driving the plant growth promotion. Irrespective of the real mechanisms operating in PGPR with a positive effect in field, the use of these micro-organisms has dramatically increased in recent years and will probably continue to grow because biofertilizers appear as a valuable opportunity for future sustainable agriculture. Many commercial products already exist which are based on *Pseudomonas* or *Azospirillum* strains in the market.

The different mechanisms operating in PGPR can be classified and discussed as: N (nitrogen) and P (phosphorus) nutrition effects, and plant root development and fitness mediated by phytohormones.

Nitrogen nutrition

One of the historically misleading cases in N nutrition mediated by PGPR was *Azospirillum* spp. Different strains of *Azospirillum* were initially characterised as free living diazotrophs able to fix nitrogen in micro-aerobic conditions (Döbereiner and Day 1976). In different experiments it was shown that inoculation of plants, mainly grasses, with *Azospirillum* enhanced plant growth, and this was initially attributed to N fixation/assimilation mediated by the bacteria. N balance measurements and *in situ* determination of acetylene reduction activity have shown that N fixation was not certainly the main reason of the plant growth stimulation mediated by *Azospirillum*, but an effect on root development and architecture appears to be the main mechanism responsible for the stimulatory effect (Bashan et al., 2004). Nevertheless using ^{15}N isotope techniques it was demonstrated that plants inoculated with diazotrophic PGPR (i.e. *Azospirillum*, *Herbaspirillum Gluconacetobacter*) benefited from N derived from fixation (Saxena and Tilak, 1998, Baldani and Baldani, 2005). The problem with N nutrition via free living diazotrophs is that fixed nitrogen is not released by the bacteria but assimilated for its own growth. The use of glutamine synthetase (GS) mutants of *Azospirillum* as plant inoculum improved plant growth compared to the parental strains (Van Dommelen et al., 2009).

Phosphorus nutrition

Micro-organisms are part of the soil phosphorus cycle and as such play an important role in mediating the availability of P to plants (Richardson and Simpson, 2011). Microbial enhancement of P availability is mediated by at least two different mechanisms: P solubilisation and P mineralisation.

Solubilisation of inorganic P from an insoluble chemical form is usually mediated by the ability of the micro-organism to acidify growth medium, to release organic anions such as citrate, gluconate, oxalate and succinate, and consequently to increase free phosphate in the medium or environment. This bacterial characteristic is usually tested in an agar plate medium with precipitated tricalcium phosphate which is clarified by the acid released from the bacterial colony. This microbial activity can also be measured and quantified in a liquid medium (Fernández et al., 2012). The ability to solubilise P in a culture medium is a potential activity and does not always guarantee biofertilizer activity in the field. Field experiments should be done with the amendment of insoluble P source to test if these bacteria can enhance P availability in field conditions and consequently improve plant growth, behaving as true biofertilizers.

Alternatively, P can be released from organic matter in the soil by mineralisation procedures mediated by enzymatic activities released by the micro-organism. Different enzymes have been characterised to mediate this activity such as phosphatases, phytases and phospholipases, which are key drivers in this transformation, independently of organic matter turn over. Again, the problem of the destiny of the released phosphorus appeared as in the case of fixed N; there would be competition between the bacteria and the plant for the released P. Thus, the final effect of these bacteria in plant growth promotion should be tested in field conditions. Since the availability of released P is independent of the plant species which can make use of it, the biofertilisation concerning P nutrition has been developed in different commercial products based on different bacteria and fungi species, orientated to a broad spectrum of plant species.

Phytohormones mediated mechanisms of plant growth promotion induced by micro-organisms

One of the most visible effects on plants after inoculation with PGPB is the huge development – and sometimes changes in the architecture – of the root of the plant. This general improvement of root growth, including root hairs development, is one of the characteristic phenotypes of the interaction plant-PGPB.

It is likely that water and mineral uptake is consequently improved because of the increase in the root system, although the specific mechanism is not completely clear. Changes in hormone balance, enhancement of proton efflux activity extrusion and modification in a wide range of related enzymatic activities would be part of the mechanisms behind this phenotype (Bashan and de-Bashan, 2010; Cohen et al., 2009). Most of the existing data is, however, descriptive.

Auxins

This general root improvement phenotype can be reproduced by replacing phytohormones with PGPB, and phytohormones-like substances have been detected in bacterial culture supernatants so it is likely that this phenotype is mediated by phytohormones synthesised by the bacteria (Costacurta and Vanderleyden, 1995). Auxin-related substances, such as indole acetic acid (IAA), appear to be involved in one of the

most important mechanisms regarding this general root development improvement. Nevertheless, bacterial production of IAA *in planta* has not yet been demonstrated. There are no IAA completely deficient mutants, but IAA attenuated mutants were ineffective as PGPB, compared to parental strains (Bashan and de-Bashan, 2010).

Gibberellins

Gibbelleric acids (GAs) are produced by some PGPB species *in vitro* and have also been shown *in planta* since those PGPB strains capable of producing GAs *in vitro* were able to complement GA-deficient mutant dwarf rice by inoculation (Bottini et al., 2004). PGPB producing GAs were also active in improving seed germination. *In vitro* results support the hypothesis that PGPB effect would be a combination of GA production and GA-glucoside/glucosyl ester deconjugation by the PGPB.

Cytokinins

The adenine-type cytokinins represented by kinetin, zeatin and 6-benzylaminopurine which occur in plants have also been produced in a defined culture medium by many PGPB (Strzelczyk et al., 1994). The role of citokinins in the promotion of root development is not clear, but cytokinin-producing PGPB stimulate nodulation in legumes when co-inoculated with rhizobia, and it was recently demonstrated that there is a Nod factor independent mechanism for infection and nodulation (Giraud et al., 2007), probably mediated by rhizobial cytokinin (see helper bacteria, below). This particular area deserves more attention in the future.

Ethylene/ACC deaminase

Ethylene is a plant hormone related to general plant responses when a stress condition appears, even if it is a very low stress situation (Glick, 2004). When this happens, the plant synthesises ethylene and stops its growth temporarily because of the regulatory effects of ethylene on different cell functions. 1-aminocyclopropane-1-carboxylate (ACC) is a precursor of ethylene synthesis. The enzyme ACC deaminase is present in some bacteria which can even use ACC as C (carbon) and N sources. When ACC deaminase is expressed by a rhizospheric bacteria root growth and development is enhanced, it is probably because of the elimination of the inhibitory concentrations of ethylene produced by the plant (Glick, 2004). This enzyme is not ubiquity present in bacteria and its activity is codified by a single gen *acdS*. The introduction of this gene from *Pseudomona putida* into other bacteria species confers plant growth-promoting functions to the recipient bacteria that were absent in the parental strain (Glick et al., 2007). This represents a potential biotechnological tool to improve micro-organisms to be used as biofertilizers.

Nitric oxide

Nitric oxide (NO), a plant regulator volatile phytohormone, is also produced by some PGPB as *Azospirillum* spp. (Molina-Favero et al., 2008). Bacterial NO is an intermediary in IAA-induced root development. NO can also mediate plant growth-promoting activity in *Azospirillum brasilense* Sp245 inducing morphological changes in tomato roots regardless of the full bacterial capacity for IAA synthesis.

Polyamines

Azospirillum spp. can produce different ployamines in culture (Perrig et al., 2007, Cassan et al., 2009). Cadaverine is synthesised by these bacteria from lysine mitigated osmotic stress in rice seedlings, based on improved water status and decreased production of ABA in inoculated seedlings (Cassan et al., 2009).

Helper bacteria

In the studies of plant microbe interaction which induced some kind of plant growth promotion, there are other cases that do not fit into the previous definitions but which can be considered as another kind of biofertilizer. That is the case of bacteria which improve a plant-microbe interaction as a third partner in the interaction. An example can be found in rhizospheric actinomycetes isolated from legumes or actinorhizal nitrogen-fixing nodules (Solans, 2007) which are able to stimulate nodulation, consequently nitrogen fixation in the plant, and finally plant growth (Solans et al., 2009). This tripartite plant-microbe interaction is not well known yet in terms of mechanisms, but clearly shows that biofertilizers can be improved by the use of more than one micro-organism at a time.

Conclusion

Although not all the different bacterial mechanisms that have been claimed to be responsible for the plant growth promotion phenomenon are present in a single strain, it is also true that each single strain usually shows more than one characteristic activity related to plant growth promotion. Thus, it has been almost impossible to prove with certainty the relevance of each and every mechanism described as plant growth-promoting activities in selected micro-organisms. This is especially true when the plant growth-promoting activity is tested in field conditions. Despite this uncertainty, the positive results are reproducible and no harmful effects have appeared. Thus, practical application of biofertilizers is increasing worldwide.

The nature of multiple mechanisms discovered for PGPR actions and the possibility of genetic modification of a particular strain to enhance its PGPR activity, suggest that the use of genetically modified organisms is not needed to implement this technology but could be a way to improve what can be found in nature.

In addition to all these descriptions which try to give an overview of the current state of the art in biofertilizers, it must be pointed out that nowadays the picture of soil microbial ecology is completely different from what it was when biofertilizers were discovered and began to be studied. Microbial soil ecology appears as a very complex and mostly unknown scenario where all these PGPR-plant interactions take place. The study of the soil microbial ecology and its dynamics will certainly improve the development of new and better biofertilizer technology for the future of agriculture. Since the same plant growth-promotion function or mechanism could be driven by many different bacteria or micro-organisms, this functional redundancy in soil microbial diversity may be managed in favour of plant development.

As this chapter has shown, the mechanisms that are at the basis of plant growth promotion by micro-organisms are beginning to be unravelled at the molecular level. This knowledge is already used for strain improvement by genetic modification, and there are several areas, e.g. introducing an ACC deaminase gene in PGPB strains which lack this

particular activity (Glick et al., 2007), creating overproducing IAA strains (Bashan and de-Bashan 2010), genetically modified strains which release the fixed ammonium (Van Dommelen et al., 2009), where important improvements of the potential for plant growth stimulation of bacterial strains may be achieved. Environmental risk assessment of the use of such strains will require a solid knowledge about the mechanisms behind plant growth stimulation. For instance, horizontal gene transfer of ACC deaminase genes in rhizospheric bacteria has been suggested (Hontzeas et al., 2005). It is clear that quite some new insights and knowledge have become available in this area since the previous OECD publication (OECD, 1995).

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Chapter 2

Phytosanitation and the development of transgenic biocontrol agents

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By the year 2050, there will be at least 9 billion people on Earth to feed using the same amount or less land and water than is available today. Currently, about one-third of all potential agricultural commodities grown worldwide are lost to diseases, weeds, insects and other pests. Farmers will be challenged to produce more, but to do so using sustainable cropping practices and less fertilizer and pesticides. Biological control is an integral part of sustainable agriculture. This chapter provides an overview of the topics of the construction, activity and use of transgenic biocontrol agents (BCAs) and their future potential in 21st century agriculture.

Introduction

It is expected that by the year 2050 there will be more than 9 billion people on Earth to feed using the same amount or even less land and water than is now available for agricultural production. Currently, about one-third of all potential agricultural commodities grown worldwide are lost to diseases, weeds, insects and other pests, either before or after harvest. Farmers are being challenged to grow more, but with less fertilizer, pesticides and fumigants, and to use more sustainable practices such as direct seeding (no-till), precision farming and biological control. In the United States and elsewhere, farmers also are being asked to produce the biomass for 21st century biofuels. To meet these challenges to reduce losses from pests and to increase production, all types of traditional and new pest management technologies are needed. Genetically engineered biocontrol agents (BCAs) will need to be a part of these agricultural technologies. This chapter provides an overview of the topics of the construction, activity and use of transgenic BCAs and their potential in 21st century agriculture.

Mechanisms of plant defense

Plants defend themselves against pathogens and insects by several well-described mechanisms: *i)* innate (non-host) immunity; *ii)* localised race-specific resistance; *iii)* systemic resistance; *iv)* microbial-based mechanisms of defense (biological control). Microbial-based defense is especially important because plants lack genetic resistance to some of the most common pathogens and insects, especially organisms that are soilborne. For example in wheat production, the diseases Pythium root rot, Rhizoctonia root rot and Take-all cause billions of dollars in losses annually, yet no commercial variety has resistance. Thus, microbial-based mechanisms serve as the first line of defense against these and other diseases and insects. These mechanisms are modulated by the plant through processes of leaf exudation and rhizodeposition, which stimulate and support specific groups of antagonist microbes (Weller et al., 2007). Pathogen or insect suppression by antagonistic micro-organisms occurs through the mechanisms of competition/pre-emptive exclusion, parasitism/predation, induction of systemic resistance, and/or antibiosis/toxin production. Multiple mechanisms of antagonism can operate simultaneously, and in addition, a micro-organism may both suppress pathogens and/or insects and directly stimulate plant growth by enhancing the uptake of nutrients, producing phytohormones and/or degrading ethylene.

Biological control by indigenous and introduced micro-organisms

Disease-suppressive soils provide some of the best examples of indigenous micro-organisms protecting plants' roots against plant pathogens (Weller et al., 2002). "Suppressive soils are soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for awhile but thereafter the disease is less important, although the pathogen may persist in the soil" (Baker and Cook, 1974). In contrast, conducive (non-suppressive) soils are soils in which disease readily occurs. Suppressive soils occur globally and are known for many different pathogens (Weller et al., 2002).

Instances of natural pathogen and insect suppression have been rich sources of micro-organisms for development into BCAs. For example, crown gall caused by *Agrobacterium tumefaciens* is a disease of a wide variety of plant species, but it is especially serious in deciduous fruit nurseries. The observation four decades ago by

Allen Kerr (New and Kerr, 1972) that the incidence of crown gall on almond correlated with the ratio of pathogenic to nonpathogenic agrobacteria suggested the potential for biocontrol by bacterization with nonpathogenic strains. *Agrobacterium radiobacter* strain K84 (isolated from soil around a peach gall) applied to seeds or roots resulted in dramatic control of crown gall (Kerr, 1980). K84 and its transfer-deficient mutant (K1026, see below) (Jones et al., 1988) are used worldwide for crown gall control.

During the last four decades, thousands of putative BCAs have been isolated and then tested on hundreds of diseases, insects, weeds and other pests. Although the use of biocontrol technology remains only a small fraction of that of chemical pesticides, the number of new BCAs, their performance and acceptance by growers continues to increase steadily. *Bacillus* and *Trichoderma* spp. have been the micro-organisms of choice for development into commercial BCAs of plant diseases (Harman et al., 2010; Kloepper et al., 2004; McSpadden Gardener and Driks, 2004), and *Bacillus*, *Beauveria* and *Metarhizium* spp. have been the microbes of choice for development as insect BCAs.[1] These micro-organisms are appealing because they are easily mass produced and formulated. Interestingly, *Pseudomonas* spp. have been the microbes of choice for fundamental studies of biocontrol mechanisms because they are easily genetically modified and engineered. Although they are easily mass produced, they are harder to formulate because they do not produce a dormant spore like *Bacillus* spp. do.

Barriers to the wider use of biocontrol technology

There are several historic and chronic problems that need to be overcome before the use of biocontrol technology can reach its full potential as an integral component of sustainable agriculture in the 21st century. The first problem is inconsistent performance. Why a BCA suppresses a disease or kills an insect pest in one field or year but not the next is a fundamental unanswered question. In contrast, there is a perception that chemical pesticides are always effective, but chemicals also can perform inconsistently. A second problem is the narrow spectrum of activity of most BCAs. An agent may be highly effective against a single pest, but often a complex of pathogens or insects must be controlled. Most chemical pesticides have broader activity than BCAs, and thus one chemical often can be used for multiple pests. Thirdly, BCAs are thought to operate over a narrower range of environmental conditions and are much more sensitive to environmental extremes than chemical pesticides. For this reason, BCAs have been shown to be especially successful in the production of glasshouse-grown crops where the environment can be controlled.

The problem of inconsistent performance stems in part from lack of a fundamental understanding of the complex *in situ* interactions among the BCA, host plant, pathogen/insect, indigenous organisms and the environment. For example, what are the *in situ* biotic and abiotic factors that promote and constrain the expression of traits (e.g. root colonisation; ecological fitness; and production of antibiotics/toxins, siderophores, biosurfactants, chitinases, lipases and proteases) that are often important to successful biocontrol? How rapidly are biocontrol metabolites like antibiotics and toxins produced and degraded in the rhizosphere, bulk soil and phyllosphere? In addition, some biocontrol traits are subject to phase variation, "a process of reversible high-frequency phenotypic switching that is mediated by mutation, reorgani[s]ation, or modification of DNA" (Lugtenberg and Kamilova, 2009). This process is well-described *in vitro* but the dynamics and frequency of its occurrence in the rhizosphere, bulk soil and phyllosphere is poorly described.

Without such fundamental information about biocontrol mechanisms *in situ*, it is difficult to predict where and under what conditions a BCA can be expected to perform. "Omics" research (e.g. genomics, proteomics, metabolomics, etc.) will be on the forefront in generating fundamental new information about the biocontrol process. It is notable that in the last several years, at least ten genomes of well-described *Pseudomonas* BCAs have been sequenced and each month more sequences of BCAs and related strains appear in the literature. Knowledge gained from analysis of these genomes is already helping to unravel the fundamental *in situ* interactions leading to biocontrol and also revealing new biocontrol genes.

One example of the benefit of genomics to biological control is seen in the analysis of the genome of *Pseudomonas protegens* Pf-5 (formerly *Pseudomonas fluorescens*) (Loper et al., 2012), the first BCA to be sequenced (Paulsen et al., 2005). In strain Pf-5 and the closely related strain *Pseudomonas protegens* CHA0 (formerly *Pseudomonas fluorescens*), surprisingly, a novel genomic locus encoding a large protein insect toxin termed Fit (for *Pseudomonas fluorescens* insecticidal toxin) was discovered. This toxin is related to the insect toxin Mcf (Makes caterpillars floppy) produced by the entomopathogen *Photorhabdus luminescens*, a mutualist of insect-invading nematodes. When injected into the haemocoel, strain Pf-5 or CHA0 killed larvae of the tobacco hornworm (*Manduca sexta*) and the wax moth (*Galleria mellonella*), whereas mutants of these two strains with deletions in the Fit toxin gene were significantly less virulent to these larvae (Péchy-Tarr et al., 2008).

Why transgenic biocontrol agents?

Genetic engineering offers an approach to enhance the consistency of performance, spectrum of activity and colonising ability of BCAs. All mechanisms of biocontrol (competition/pre-emptive exclusion, parasitism/predation, induction of systemic resistance and antibiosis/toxin production) have been targeted for improvement during the last 25 years. Selected examples of proof of concept studies are given below.

Competition/pre-emptive exclusion

Expression of the *Pseudomonas putida* WCS358 ferric siderophore receptor *pupA* in strain WCS374 increased the competitiveness of WCS374 against WCS358 when both strains were co-inoculated (Raaijmakers et al., 1995). Increasing the copy number of the *Pseudomonas fluorescens* WCS365 site-specific recombinase gene *sss* in F113 and WCS307 increased the competitive colonisation ability of the recombinant strains on tomato root tips (Dekkers et al., 2000). This gene plays a role in DNA rearrangements and is thought to help keep bacterial cells from becoming "locked in" a state unfavourable for competitive colonisation.

Parasitism/predation

Expression in *Pseudomonas putida* of *chiA* from *Serratia marcescens* gave improved protection of beans against *Sclerotium rolfsii* (Chet et al., 1993). Dunne et al. (2000) showed that overproduction of an extracellular serine protease by *Stenotrophomonas maltophilia* W81M3 or W81M4 resulted in improved control of Pythium damping-off of sugar beet by the recombinant strains as compared to the wild-type strain W81.

Induced resistance

Introduction of *pchCBA* from *Pseudomonas protegens* CHA0 (formerly *Pseudomonas fluorescens*) into strain P3 enabled salicylic acid production and improved the ability of P3 to induce systemic resistance in tobacco against tobacco necrosis virus (Maurhofer et al., 1998).

Antibiosis/toxin production

Transfer and expression of the HCN biosynthesis operon *hcnABC* from *Pseudomonas protegens* CHA0 into *Pseudomonas fluorescens* P3 resulted in improved control of black root rot of tobacco by the transgenic strain (Voisard et al., 1989).

Transfer of a recombinant plasmid *pCU203*, containing genes for the biosynthesis of 2,4-diacetylphloroglucinol (DAPG) cloned from *Pseudomonas* sp. F113, into *Pseudomonas* sp. strain M114 yielded M114(pCU203), which gained the ability to synthesise DAPG and control *Pythium ultimum* damping-off of sugar beet better than did M114 (Fenton et al., 1992).

Molecular genetic modifications to biocontrol agents

A very wide variety of genetic approaches have been used to genetically engineer BCAs with improved biocontrol or plant colonising ability, and these approaches can be grouped in three categories: *i)* deletion or mutation of existing genes; *ii)* alteration of gene regulation; *iii)* introduction of heterologous genes. Selected examples of these approaches are given below.

Deletion or mutation of existing genes

Agrobacterium radiobacter K84 is a well-described BCA of crown gall that is sold worldwide (Kerr, 1980). A transfer (Tra^-) mutant of A*grobacterium* K84 (designated K1026) was constructed to prevent the possible transfer of pAgK84 encoding agrocin 84 to *Agrobacterium tumefaciens*, which could result in the pathogen becoming resistant to the BCA (Jones et al., 1988). The recombinant strain K1026 is as effective as the wild type and is used commercially (Jones and Kerr, 1989).

Another excellent example of this type of genetic modification involves biocontrol of ice nucleating bacteria by an ice nucleating deficient *Pseudomonas syringae* (Hirano and Upper, 2000). An Ice strain of *Pseudomonas syringae* was constructed by deleting a fragment of the ice gene, followed by marker exchange of the mutated gene into the wild type. This engineered derivative was the first recombinant microbe deliberately released into the environment. Application of Ice^- mutants reduced populations of Ice^+ *Pseudomonas syringae* on potato and strawberry 50-fold by pre-emptive exclusion and reduced frost damage in the field (Lindow, 1995; Lindow and Panopoulos, 1988). The Ice^- strain faced a difficult path through regulatory, social and political obstacles prior to field release, which contrasted strikingly with the release of A*grobacterium* K1026, which faced little resistance.

Finally, Barahona et al. (2011) constructed a triple mutant of *Pseudomonas fluorescens* F113 in the genes *sadB*, *wspR* and *kinB*, resulting in hypermotility and better root colonisation. In addition, the mutant strain had improved biocontrol activity against *Fusarium oxysporum* f. sp. *Radicis-lycopersici* on tomato and *Phytophthora cactorum* on strawberry as compared to F113.

Alteration of gene regulation

Bacillus subtilis strain ATCC 6633 produces the lipopeptide mycosubtilin. Replacing the native promotor of the mycosubtilin operon in ATCC 6633 with a constitutive promotor yielded the recombinant strain BBG100. This recombinant produced up to 15-fold more mycosubtilin and suppressed *Pythium aphanidermatum* on tomato significantly better than the wild type did (Leclère et al., 2005).

The two-component regulatory system consisting of GacS (sensor kinase) and GacA (response regulator) is involved in the regulation of secondary metabolism. In a second example of altered gene regulation, Ligon et al. (2000) enhanced expression of the biosynthesis genes (*prnABCD)* for the antibiotic pyrrolnitrin in *P. fluorescens* BL915 by adding additional plasmid-borne copies of *gacA*, by changing the first base in the coding sequence of the *gacA* gene to a more efficient codon, or by replacing the native promoter of *gacA* with the stronger P_{tac} promoter. Each of these alterations resulted in a marked increase in both the amount of pyrrolnitrin produced by the various genetically modified strains and their level of control of *Rhizoctonia solani* on cucumber and impatiens. The level of antibiotic production was directly related to the level of control of *Rhizoctonia solani*.

Introduction of heterologous genes

Most research on engineered strains has focused on adding new biocontrol genes into known BCAs of pathogens, insects and weeds. For example, *Trichoderma atroviride* P1 suppresses a wide range of foliar and soilborne pathogens. Insertion of the *Aspergillus niger* glucose oxidase-encoding gene (*goxA*) under the control of the homologous chitinase (*nag1*) promotor into strain P1 yielded the transgenic strain SJ3-4 (containing 12-14 *goxA* copies) that induced systemic resistance against *Botrytis cinerea* and controlled *Pythium ultimum* and *Rhizoctonea solani* on bean better than did P1 (Brunner et al., 2005).

Bacillus thuringiensis cry genes have been introduced into a wide variety of bacteria (e.g. *Pseudomonas fluorescens*, *Agrobacterium radiobacter*, *Ancylobacter aquaticus*, *Clivibacter xyli* and *Herbaspirillum seropedicae*). These transgenic strains inhibited a variety of pests, including tobacco hornworm (*Manduca sexta*), malaria mosquito (*Anopheles stephensi*), leatherjacket (*Tipula oleraceae*) and European corn borer (*Ostrinia nubilalis*) (Downing et al., 2000; Obukowicz et al., 1986a, 1986b; Yap et al., 1994). *Bacillus* transformed with the mosquitocidal Cry and Cyt proteins of *Bacillus thuringiensis* and the binary toxin of *Bacillus sphaericus* showed 10-fold better efficacy against Culex spp. (Federici et al., 2003).

In another line of research, *Metarhizium anisopliae* ARSEF 549 was engineered to express the insect-specific neurotoxin AaIT from the scorpion (*Androctonus australis*). Toxicity of the transgenic strain increased 22-fold against tobacco hornworm (*Manduca sexta*) catepillars and nine-fold against adult yellow fever mosquitoes (*Aedes aegypti*) (Wang and St. Leger, 2007).

Most interesting was the report by Fang et al. (2011) who engineered *Metarhizium anisopliae* to produce and deliver molecules that selectively block the development of the causal agent of malaria (*Plasmodium falciparum*) in the mosquito.

A final example relates to biocontrol of weeds. Introduction of *NEP1* (encodes a phytotoxic protein from *Fusarium)* into *Colletotrichum coccodes* increased nine-fold the virulence of the fungus on the herbicide-resistant weed velvetleaf (*Abutilon theophrasti*).

The transgenic strain killed more rapidly and at a lower dose that the wild-type strain (Amsellem et al., 2002).

Case study: Introduction of phenazine genes into *Pseudomonas* spp.

Phenazines are colourful, redox-active antibiotics produced by members of some fluorescent *Pseudomonas* spp. and a few other bacterial genera (Mavrodi et al., 2006). Phenazines are produced in the rhizosphere (Mavrodi et al., 2012), where they are involved in the suppression of plant pathogens (Chin-A-Woeng et al., 2003; Mavrodi et al., 2006; Thomashow et al., 1990), can act as electron shuttles (Hernandez et al., 2004; Rabaey et al., 2005) and contribute to the ecology (Maddula et al., 2008; Mazzola et al., 1992), physiology and morphology (Dietrich et al., 2008; Price-Whelan, 2006) of the strains that produce them. Expression of the core seven-gene phenazine (*phz*) biosynthesis operon (*phzABCDEFG*) is controlled in pseudomonads by homoserine lactone (HSL)-mediated quorum sensing (Mavrodi et al., 2006). Phenazines and quorum sensing are required for the establishment and development of biofilms on surfaces, seeds and roots (Maddula et al., 2008; Mavrodi et al., 2006). In the rhizosphere, expression of *phz* genes can be induced by homoserine lactones produced by heterologous isolates (Pierson et al., 1998; Pierson and Pierson, 2007) or quenched by HSL-degrading rhizosphere inhabitants (Morello et al., 2004).

A disarmed Tn*5* vector (pUT: Ptac-*phz*ABCDEFG), originally constructed by L.S. Thomashow and colleagues, has been used extensively to stably introduce a single copy of the phenazine-1-carboxylic acid biosynthesis genes (isolated from *Pseudomonas fluorescens* 2-79) under the control of a P*tac* promotor into *Pseudomonas* spp. from sources worldwide to improve biocontrol activity. Strains transformed with the *phz* locus also serve as model organisms to determine the impact of transgenes on the ecological fitness and the impact of recombinant strains and on the indigenous rhizosphere microbial community (Ryan et al., 2009). For example, the *phz* operon was introduced into *Pseudomonas brassicacearum* (formerly *Pseudomonas fluorescens*) Q8r1-96 (Loper et al., 2012), a strain that naturally produces the antibiotic DAPG and suppresses Take-all disease of wheat. Several recombinants of Q8r1-96 were selected (Z30-97, Z32-97, Z33-97 and Z34-97) and all produced greater amounts of PCA than strain 2-79, the source of the *phz* operon, because the genes were under the control of a constitutive promotor. Surprisingly however, addition of the *phz* genes also caused elevated production of DAPG in all of the transgenic strains as compared to the wild type Q8r1-96. Although the transgenic strains were no more suppressive of Take-all and Pythium root rot than Q8r1-96, they showed remarkable suppression of Rhizoctonia root rot at a dose of only 100 CFU seed^{-1}, which was 100 to 1 000 times less than the dose required for similar disease control by the wild type Q8r1-96 (Huang et al., 2004).

In a similar study, *Pseudomonas fluorescens* SBW25 was transformed with the mini-Tn*5* vector carrying the *phz* genes and the transgenic strains gained enhanced ability to suppress *Pythium ultimum* damping-off disease of pea when compared to the wild-type strains SBW25 and 2-79 (source of the *phz* operon) (Timms-Wilson et al., 2000).

Some of the best studies of the population dynamics and non-target effects of transgenic BCAs in the field have been conducted with *Pseudomonas putida* strain WCS358r engineered to produce either PCA or DAPG by using the mini-Tn*5* vector system described above (Glandorf et al. 2001; Leeflang et al. 2002; Viebahn et al. 2003). PCA was shown to be produced in the rhizosphere by the transgenic strain, and both

cultivation-dependent and independent methods indicated that the wild-type and transgenic strains had transient effects on the composition of the rhizosphere fungal and bacterial microflora of wheat. The effects of the transgenic strains sometimes were longer lasting than those of WCS358r, and differed from year to year and study to study. These results were similar to those of others conducted under controlled or field conditions and were not surprising given that strain WCS358r and other BCAs often establish high population sizes soon after inoculation, and then the densities decline over time and distance from the inoculum source. In addition, introduced BCAs do not become uniformly dispersed throughout the rhizosphere or among roots of the same or different plants. Collectively, these and other studies of the non-target effects of wild-type and recombinant BCAs indicate that even though the introduced bacteria have definite impacts on non-target microbial communities, the effects vary from study to study and are transient (Ryan et al., 2009).

Conclusion

Microbial-based mechanisms of defense are especially important because plants lack genetic resistance to many common pathogens and insects, especially soilborne organisms. Suppressive soils are the best examples of indigenous micro-organisms protecting plants against pests. Natural instances of pathogen and insect suppression have been rich sources of micro-organisms for development into BCAs. Although the use of biocontrol technology remains only a small fraction of that of chemical pesticides, the number of new biocontrol agents and their performance continues to increase. However, inconsistent performance and narrow spectra of activity are issues that must be resolved before the use of biocontrol technology can reach its full potential as an integral part of sustainable agriculture in the 21st century. BCAs have been engineered to colonise better, tolerate stress better, perform more consistently and effectively, and have a broader spectrum of activity than their wild-type progenitors. All biocontrol mechanisms have been targeted for improvement: competition/pre-emptive exclusion, parasitism/predation, induction of systemic resistance and antibiosis/toxin production. A very wide variety of genetic approaches have been used to engineer BCAs and they can be grouped into three categories: deletion or mutation of existing genes, alteration of gene regulation and introduction of heterologous genes. When new genes are introduced into a BCA, they can influence the expression of biocontrol traits already present. Competitiveness of the transgenic BCA as compared to the parental strain can depend on the host crop. Current micro-organisms of choice for development as commercial BCAs (*Bacillus*, *Trichoderma*, *Beauveria* and *Metarhizium* spp.) will probably be the microbes of choice for future development as transgenic BCAs and *Pseudomonas* will continue to be an important research tool.

Understanding the biogeography of potential transgenes (i.e. those encoding antibiotics and toxins) and their role in nature should lessen concerns about the commercial use of recombinant BCAs. Future research should continue to focus on the development of novel engineered BCAs but broader field testing is needed for engineered agents that have been constructed during the last 25 years and are known to have enhanced activity. During the last 15 years, there has been much greater research emphasis on transgenic plants than transgenic microbes for pest control.

Note

1. www.epa.gov/pesticides/biopesticides

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Chapter 3

Lessons of the impact of genetically engineered micro-organisms on natural ecosystems like soil

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This chapter briefly describes some examples of past and present (next to potential novel) applications of genetically modified micro-organisms to soil, stressing the importance of analysing the putative impacts of such applications to the life support functions (LSF) of a living soil at three levels: i) *functioning for soil fertility;* ii) *functioning for pathogen suppressiveness;* iii) *functioning for the provision of clean drinking water. To understand the impact of such genetically modified micro-organism applications on the soil, it is important to deepen our understanding of the microbial communities that are responsible for the key LSF of that soil. Moreover, we need to understand how these might be affected mechanistically. It is, therefore, important to further develop databases that contain extensive data on the microbial communities in the soil systems under study. This chapter advocates the application of the currently available powerful methods, which enable the dissection of soil microbial systems into their individual components. Finally, the chapter proposes the definition of a normal operating range (NOR) to fit the dataset obtained into a framework which is quantifiable and may serve to support decision making.*

Introduction

Use of genetically modified micro-organisms

Over the last 20 years, several genetically modified micro-organisms (GMMs) have been and are still being developed for use in agricultural and other (e.g. waste treatment) settings. An early approach to application in agriculture, incited in several Chinese research institutes, consisted of the use of a genetically modified *Alcaligenes faecalis* strain A1501. This organism, now renamed *Pseudomonas stutzeri*, had been modified to constitutively fix atmospheric nitrogen in the rhizosphere of rice, even in the presence of reduced nitrogen. The reduced nitrogen (in the form of ammonium) would otherwise repress the nitrogen fixation system (Lin et al., 2000). Following its development, the organism was used, at a large scale, in the rice-growing areas of the People's Republic of China, and slightly consistent increases of crop yields (5-10%), as measured by plant biomass and grain yield, have been reported (Lin, personal communication). Moreover, the applications did not exert any observable deleterious effects on the (agricultural) environment. Parallel microcosm studies with the strain performed in the Netherlands did not provide any evidence for measurable effects, neither on the colony forming unit (CFU) counts of indigenous bacteria nor on the PCR-DGGE profiles representing the community structures of total bacteria present in the system (Lin et al., 2000). The only discernible effect found was the one resulting from the (ephemeral) presence of the inoculant strain, which was, for instance, selectable on the basis of its great capacity to quickly utilise lactate.

Another application includes the now famous long-standing application of the GMM *Agrobacterium radiobacter* strain K1026, which was modified from the biocontrol agent *A. radiobacter* strain K84 to combat the plant pathogen *A. tumefaciens* (causing crown gall disease) in soil (Ryder and Jones, 1991). The modification was intended to block the transfer of toxin-resistance genes to cells of *A. tumefaciens*, which would turn these insensitive to the control. *A. radiobacter* strain K84 contains a plasmid, termed pAgK84, which encodes the anti-pathogen (toxin) compound agrocin 84, next to the gene conferring intrinsic resistance to this toxin. It also contains another plasmid, pNOC, which codes for nopaline uptake and catabolism. Thus, strain K84 competes with the pathogen at gall sites for nutrients (opines), killing the pathogen by producing agrocin 84. Strain K84 turned out to be an efficient coloniser of plant roots and wound sites, providing protection after application. However, the possible transfer of the agrocin plasmid to cells of the pathogen, brought about as a result of transfer functions carried on the pNOC plasmid, might result in pathogenic strains becoming resistant to agrocin 84 and hence a breakdown of the control. In order to avoid this potential breakdown, the transfer (*Tra*) region of pNOC was deleted by genetic modification, to produce the transfer-minus derivative strain of K84 termed *A. radiobacter* K1026. With great success, strain K1026 is now commonly used in many *A. tumefaciens* control strategies. It represents the first GMM approved for release into the environment (originally under Australian regulation). It is regarded as being safe for humans, animals, plants and the environment, being, except for the deletion of part of its genome (including the plasmid genomes), identical to the naturally occurring counterpart.

Chapter 1 provides an account of other promising applications of GMMs. The future for such and other environmental applications of GMMs thus appears to be bright. In most cases, the respective GMMs are designed for specific tasks in the environment, and hence they will inevitably exert the effects related to their intended tasks whenever they enter the environment.

The potential (adverse) impact of genetically modified micro-organisms on ecosystems

Notwithstanding the ample possibilities for a successful use of GMMs in the environment, there are still those that question the potential impact of the unintended effects that may be caused by the release of GMMs. To exercise this issue, one could consider the following: GMMs may theoretically impact their recipient environment by:

1. chemical (abiotic) modification of the environment
2. outcompeting, antagonising or cross-feeding the local microbiota, thereby changing their population structure
3. exerting effects on local organisms, such as plants, and/or
4. spreading their (inserted) genes by horizontal gene transfer (HGT).

Several of the above impacts are well measurable, whereas others may be more difficult to discern. For instance, a range of methods has been developed that allows an optimised detection of indigenous microbial communities (Kowalchuk et al., 2004; see below). Thus, the putative effects of GMMs on such communities can be determined in an elaborate manner. In addition, chemical changes of the environment are often also well measurable. HGT from the GMMs into the indigenous microbial community is also measurable, up to a particular level of resolution, using standard molecular screening techniques (Kowalchuk et al., 2003). Thus, the advanced methods that are currently available would facilitate a thorough assessment of the potential impact of GMMs. However, against which background should systems that are potentially impacted by GMMs be tested or compared?

The need to define normality in target ecosystems like soil

It is common knowledge that the chemistry of the natural environment, and the natural microbial and other communities that inhabit it, are often prone to fluctuations in response to the natural or anthropogenic influences that impinge on it. Hence, it is important to establish, in any ecosystem, what is supposed to be "normal" and what goes beyond "normality" (defining, with respect to the aforementioned parameters, a baseline or normal operating range, NOR). This goes beyond the type of effect that is expected to be exerted by a GMM. Then, the magnitude and duration of any effect of a GMM should be weighed against the amplitude of variation offered by the NOR. This chapter will discuss how a soil NOR can be determined and to what extent it may be useful as a baseline to weigh the potential impacts of GMMs against. But first the chapter will examine the difficulties posed for analyses of living soil systems, the methods that have been developed to overcome these and the key microbial functions of soil.

The great plate count anomaly and methods that can overcome it

It is well known that a majority of the micro-organisms of soil does not easily grow on plates. This phenomenon has been coined the Great Plate Count Anomaly (GPCA; Staley and Konopka, 1985), and it can – for bacteria – amount to 99% of the total microbiota (Staley and Konopka, 1985; Ward et al., 1995). The GPCA impedes the easy and thorough understanding of the structure of soil microbial communities on the basis of traditional cultivation-based methods, simply because a majority of micro-organisms is unculturable and hence escapes functional analyses. The soil DNA- and RNA-based methods developed in the last two decades have provided a great thrust to man's

understanding of soil microbial communities, as these provide snapshot-type descriptive information sets. Indeed, a large suite of advanced methods is currently available for the assessment of the microbial community structure and diversity in soils (Kowalchuk et al., 2004; Van Elsas et al., 2007). Moreover, more recent strategies to: *i)* apply DNA micro-arrays containing suites of probes that can report on the phylogenetic and functional status of soil communities; and *ii)* apply direct pyrosequencing to soil DNA samples, offer great potential to foster our understanding of the composition and functioning of the microbial communities (DeSantis et al., 2007; Roesch et al., 2007; Van Elsas and Boersma, 2011). Other methods that focus on microbial functions (e.g. based on the Biolog system) enable a view of functional diversity, without specifically assessing the underlying micro-organisms.

In the light of such and other recent methodological developments, one can safely state that an era has commenced, in which, for the first time in history, a more or less complete inventory of the community structures and diversities of the microbiota of soils can be made. The following section discusses the importance of soils for life on planet Earth, then examines to what extent this enormous methodological capability can assist in the quest to define the "normality" of soil and its functioning, in the light of the current and future applications of GMMs.

The soil ecosystem – its natural (normal) status, functioning and resilience

As GMM applications in (agricultural) soil will undoubtedly be important, a special focus is placed here on the intricacies of the agricultural soil environment, in particular its quality and health status. This soil status is important as the following indispensable functions are supported:

1. the availability of plant nutrients (soil fertility)
2. the suppression of soil-borne plant diseases (see Chapter 2; Kennedy and Smith, 1995)
3. the cleaning function of soil, e.g. for the provision of clean drinking water (by filtering and biodegradation).

These three functions are known as the life support functions (LSF) of soil. They are very tightly linked to, and dependent on, crucial constituents of the soil microbial community. This section will briefly touch upon the first two functionalities.

Nutrient cycling function of soil

Soils are responsible for a large part of the nutrient cycling processes (i.e. the cycling of different forms of carbon, nitrogen, phosphorus and sulfur) that drive ecosystem functioning on Earth. As examples, key steps in the nitrogen cycle such as nitrogen fixation, ammonia oxidation and denitrification, are carried out by micro-organisms that inhabit soils. In particular, ammonia oxidation and denitrification in soil determine what chemical forms of nitrogen, ammonium, nitrite or nitrate, will be available in (ammonium), or are flushed out (nitrate) of soil. Both processes are driven by several microbial groups, with the connotation that ammonia oxidation (carried by a few groups mainly among the beta-Proteobacteria and archeae) is less broadly spread than denitrification (carried by many groups across the bacterial domain).

Suppressiveness of plant disease

The "health" of a soil can be defined in terms of its microbiological capacity to counteract (suppress) the activity of plant pathogens (see Chapter 2). This suppressiveness can conceptually be divided in "general" versus "specific" suppressiveness. General suppressiveness is defined as being caused by unspecified activities of a myriad of organisms (e.g. resultant from competition for essential nutrients with pathogens), whereas specific suppressiveness is related to a specific activitity, e.g. antagonism, exerted by defined organisms.

Specific suppressiveness is classically best illustrated by the causal relationship between the decline of Take-all disease in wheat by consecutive wheat monocropping and the concomitant increase of fluorescent pseudomonads that produce the antifungal antibiotic 2,4-diacetyl phloroglucinol (Raaijmakers and Weller, 1998). Here, the GPCA would seem to impede a thorough assessment of the specific or even general disease-suppressive properties of the system, although Mendes et al. (2011) have recently pointed to a molecularly-based assessment of suppressiveness. In particular, the ecology of the key microbial interactions that take place in the soil environment and strategies to direct these, need scrutiny. It has been hypothesized that the level of interactiveness in a soil system is related to the stability of function, in this case suppressiveness.

Resilience of soil

It has often been postulated that the quality or health status of a soil not only relates to the soil's functionality or disease suppression, but to its resilience in the face of stress as well, i.e. its capacity to return to the original status following such a stressful situation. In particular, disturbance of a soil microbial system may affect soil functioning to an extent that correlates inversely with the functional redundancy present in the system (Kennedy and Smith, 1995). This section argues that a greater microbial diversity, in particular in terms of function, may relate to a higher level of resilience, resulting in a better buffered system in the face of stressors that emerge. For both soils that perform nutrient cycling LSF and soils involved in pathogen suppression, this may mean that a greater diversity of nutrient-cycling, antagonistic or competitive functions correlates with higher degrees of resilience. It is of prime importance that methods are developed and applied that allow for a definition of the operational amplitude of healthy soil function, including "normal" responses to perturbances, versus what exceeds this NOR.

The normal operating range of soil function as the grand descriptor of normality

In the light of the plethora of functions exerted by a normal agricultural soil, normality can be defined as a status of the soil under which all relevant functions are within the limits set by the normal climatic and anthropogenic influences exerted on the soil. When different relevant parameters are measured, their combination into one overall parameter would establish an overall NOR of soil function (Pereira e Silva et al., 2013). Here, it can be strongly argued that the modern molecular techniques (Kowalchuk et al., 2004; Van Elsas and Boersma, 2011) are indispensable tools that allow an optimised definition of the soil NOR. However, even with the advent of these advanced soil monitoring methods, the nature of the soil microbiota, its dynamics, activities and interactions may remain enigmatic for a long time to come. This is because such an intricate understanding requires the application of the tools at scales which are at the moment not realistic. In order to truly understand the normal fluctuations in soil, including those that result from agronomical measures, it is important that large databases

are created that allow the storage of large data sets, including those obtained by molecular tools as well as the so-called metadata (data that describe the key parameters of soil, i.e. soil pH, organic matter content, chemical status and textural type). The resulting description of the dynamic soil status will then provide the background against which out-of-range situations are compared (Bruinsma et al., 2003; Kowalchuk et al., 2003). Furthermore, important and sensitive indicators of soil processes should be selected. Given the presence of multiple functions in soil ecosystems, such a framework will rely on a range of soil attributes that, when considered together, will provide an estimation of the quality of soil in terms of its biological function (Villamil et al., 2007; Romaniuk et al., 2011).

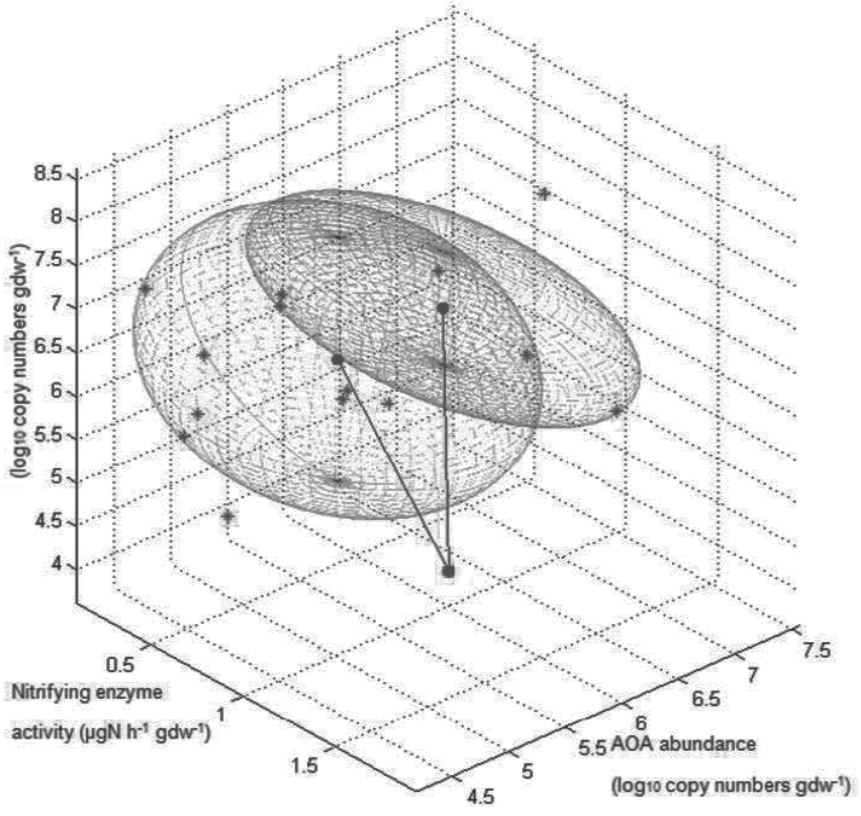
So, how can an NOR be established for soils? Looking to other parts of science where the NOR concept has been used, such as geochemical science (Wang et al., 2010), may help. In molecular ecology (De Boer et al., 2011), and recently in microbial ecology (Inceoglu et al., 2011; Pereira e Silva et al., 2011, 2012; Rutgers et al., 2009), the concept has been proposed, but so far no appropriate method or tool has been developed that satisfactorily defines the NOR of soils. To allow an assessment of the impact of practices such as the release of GMMs, the goal should be that key parameters quantify impacts for direct comparative purposes (Anderson, 2003).

A mathematics-based approach to defining the NOR is feasible. Let us consider the NOR as an ellipsoid in a space of *n* dimensions, where *n* is the number of parameters measured in a single system, and its borders represent the NOR. Such borders might be defined as the 95% confidence area of undisturbed states (Figure 3.1). They might also encompass all the data, so be defined by the extreme values that are still felt to be includable in normality. The distance between a particular state of the soil and the centre of the NOR will represent a quantitative measure that summarises the state of the soil, defined by Kersting (1984) as the "normalised ecosystem strain" (NES). Then, the strength of the "stress", or how much a soil is outside the NOR, can be determined by the distance between the "stressed" soil and the border of the ellipsoid. When the soil is in an undisturbed state, all combinations of the parameters fall within the NOR, giving an NES value that is smaller than one unit. Values exceeding 1 would indicate that the system is under "stress". The decision, however, whether a deviation of a soil from the NOR is "adverse" or not should be made by an educated guess with respect to the level of potential harm to the system. Ultimately, this would be a decision which is to be left to decision makers (Smit et al., 2012). The qualification whether a deviation represents harm or ecological hazard will depend on the use of the soil, e.g. for cultivation in agriculture or for nature development. It can only be done on the basis of the functions of that specific soil under evaluation (Rutgers et al., 2009).

Recently, an extensive study on particular soil parameters across Dutch soils was performed, over three consecutive years (Semenov et al., 2014). The study aimed to distinguish key soil parameters that could play an important role in the proper establishment of an NOR for soil function. In total, 22 measurable parameters were selected to define the NOR, including soil pH, organic matter, level of nitrate, abundance of bacteria, archaea, fungi, ammonium oxidizers, nitrogen fixers and denitrifiers. Moreover, nitrification and denitrification potentials were measured. In the work, the distance observed between a "stressed" soil and the NOR border, as based on nitrification-related parameters (activities, abundance and diversities), was much higher than the corresponding value between the NOR based on other relevant parameters (e.g. soil pH, OM, archaeal and fungal abundances and diversities; Pereira e Silva et al., 2013). The NES value was also higher when compared to the NOR based on more

redundant proxies (e.g. denitrification potential and abundance of total bacteria and denitrifiers). This supported the posit that by focusing on sensitive parameters such as those describing nitrification (taking the abundance, structure and function of ammonia oxidizers as parameters), a sound NOR of soil functioning is achieved. Consequently, the chances of distinguishing stressed soils (measurements outside the NOR) are likely higher when so-called sensitive parameters are considered than when randomly selected parameters are tested (Figure 3.1). Based on the above, a classification of potential biological parameters can be provided (Pereira e Silva et al., 2013), where nitrification-related measurements were top-ranked in relation to other (more redundant) measurements. However, it is noteworthy that these parameters were more sensitive in the sandy soils, supporting the idea that NORs should be built taking into consideration the type of soil under scrutiny.

Figure 3.1. **Representative example of a normal operating range of soils showing 3 of the 22 dimensions**



Notes: The ellipsoid 1 characterises the normal operating range for agricultural soil under tillage while ungrazed grassland is represented by the ellipsoid 2. The ellipsoids represent the borders of the NOR for three indicators (nitrifying enzyme activity and abundance of anmonia-oxidizing archaea (AOA) and bacteria (AOB)). Crosses (red) are observed values which characterise the normal operating range. The line is the distance between the centre of the normal operating range (dot (blue)) and the state of the selected soil (faint dot (green)). It is important to mention that the distance that reflects how much the selected soil (faint dot (green)) is outside the normal operating range is the distance between the faint dot (green) and the border of the ellipsoid, not the centre. Two ellipsoids are different in volume due to higher amplitudes of variation observed in the above-mentioned indicators for more disturbed soils (agricultural) compared to the grassland one.

Source: Pereira e Silva et al. (2013), "Microbe-mediated processes as indicators to establich the normal operating range of soil functioning", *Soil Biology & Biochemistry*, No. 57, pp. 995-1002.

Outlook

This chapter examined some key examples of the past and present (next to potential novel) applications of GMMs to soil and questioned the putative impacts of such applications to soil LSFs at three levels:

1. functioning for soil fertility
2. functioning for pathogen suppressiveness
3. functioning for drinking water provision.

To understand the impact of such GMM applications, it is important to deepen our understanding of the microbial communities that are responsible for the soil LSF and how these might be impacted. It is, therefore, important to focus on the further development of databases that contain extensive data on the microbial communities in the soil systems under study and are generated with the currently available powerful methods that enable the dissection of soil microbial systems into their individual components (Kowalchuk et al., 2004; Van Elsas and Boersma, 2011). Such databases should contain, next to the data on microbial communities, metadata that describe the local conditions. The microbiota-related dataset should be established at both the functional and phylogenetic levels, with an additional focus on the spatial and temporal relationships between the individual organisms and functions analysed.

This chapter presented a strategy that might define an NOR of soil function, which includes the ranges of variation incurred by the different parameters that are deemed important as descriptors of the soil NOR. In this perspective, nitrogen cycling was taken as a key asset that primarily might define the soil NOR. However, it is important to bear in mind that such a proposal to pinpoint a particular process as being more important than other processes is somewhat arbitrary, and is thus open to discussion. Another issue raised here is that soil NORs may be implemented per soil type, assuming that particular key soil processes run in similar ways per soil type, which is supported by some recent literature (Pereira e Silva et al., 2011; 2012). However, in this still-developing area, the novel datasets are expected to allow further fine-tuning the current, assumption-based inferences about how soil NORs can be best established. Finally, the datasets that will allow, for the first time in history, having a comprehensive overview of the complex soil microbiota, will need to be supported by powerful bioinformatics tools that enable a ready and fast ordination of the data. There is a problem (once denoted as the "informational or computational bubble"), as such tools are currently not available. Hence, investments in bioinformatics are dearly needed.

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Part II

The use of microalgae for production purposes

Chapter 4

The need and risks of using transgenic microalgae for the production of food, feed, chemicals and fuels

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This chapter provides a brief overview of the targets of algal genetic modification followed by a short description of the Netherlands legislation concerning genetically modified organisms, an overview of what is already known about the risks related to production systems of (GM-) algae, and the potential risks of GM-algae for human health and the environment.[1]

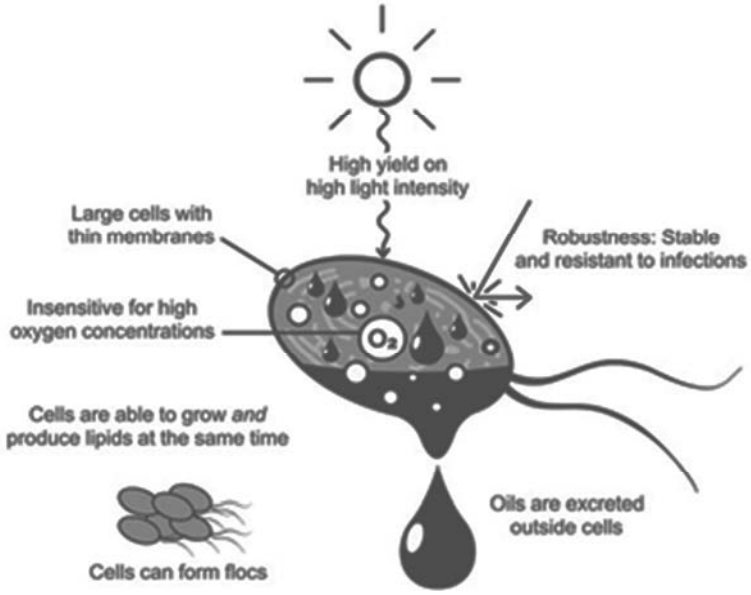
[1] This chapter is based on a study commissioned by the Netherlands Advisory Commission for Genetis Modification (COGEM), performed by Technopolis (2012).

Importance of transgenic microalgae

Microalgae may be used for the sustainable production of various commodities and products, such as feedstock and biofuels. Microalgae can be cultivated on seawater, using residual nutrients (carbon dioxide [CO_2], nitrogen [N], phosphorus [P]), and produce valuable co-products, e.g. lipids and proteins. Microalgae can be grown very efficiently. As an example, the total need for all transport fuels in Europe can be covered by microalgae cultivated on the surface area of Portugal. Four hundred million tons of protein would be produced as by-product, which is about 40 times the amount of soy protein imported into Europe. The EU FP7 programme[1] has funded a large number of research programmes aimed at further development of the use of (micro-) algae for various sustainable purposes.

There is a clear need for genetic improvement of the strains of microalgae that are currently being used, to create the "ideal micro-alga" (Figure 4.1). Features that could be improved include high biomass productivity, in particular of required molecules, such as proteins, saturated neutral lipids and unsaturated fatty acids, possibilities to grow under selective conditions, ease of harvesting and possibilities to use mild extraction conditions.

Figure 4.1. **Ideal microalga**



Genetic modification of algae

This section provides a short overview of the state of the art on transgenic research on algae, the algal strains that have been used as hosts for genetic modification and the DNA delivery methods. It then presents the targets of genetic modification of algae.

Genetically modified algal strains and their stability: DNA delivery methods

A first prerequisite transformation of the cyanobacterium *Synechocystis* was already reported in 1970 (Shestakov and Khyen, 1970). Successful transformation of the green alga *Chlamydomonas reinhardtii* was reported in 1989 (Harris, 2009). *C. reinhardtii* has

become the model species in molecular biology of (eukaryotic) algae and is therefore the best described one (Harris, 2009). Since then, successful genetic transformation of approximately 30 algal species has been demonstrated (Hallmann, 2007; Radakovits et al., 2010). Table 4.1 provides an overview of genetically transformed algal species.

Table 4.1. **Overview of genetically transformed algal species**

Species	Stability of transformation[1]	Species	Stability of transformation[1]
Chlorophyta		***Heterokontophyta***	
Chlamydomonas reinhardtii	Stable	*Laminaria japonica*	Stable
Chlamydomonas reinhardtii	Stable (chloroplast)	*Undaria pinnatifida*	Stable
Volvox carteri	Stable	*Phaeodactylum tricornutum*	Stable
Dunaliella salina	Stable	*Navicula saprophila (Fistulifera saprophila)*	Stable
Dunaliella viridis	Stable	*Cylindrotheca fusiformis*	Stable
Haematococcus Pluvialis	Stable	*Cyclotella cryptic*	Stable
Chlorella sorokiniana;	Stable	*Thalassiosira weissflogii*	Transient
Chlorella kessleri (Parachlorella kessleri)	Stable	*Nannochloropsis sp.*	Stable
Chlorella ellipsoidea	Stable	**Dinoflagellates**	
Chlorella vulgaris	Transient	*Amphidinium sp.*	Stable
Ulva lactuca	Transient	*Symbiodinium microadriaticum*	Stable
Ostreococcus tauri	Stable		
Rhodophyta		**Cyanobacteria**	
Cyanidioschyzon Merolae	Stable	*Spirulina platensis (Arthrospira platensis)*	
Porphyra yezoensis	Stable/transient	*Anabaena sp*	
Porphyra miniata	Transient	*Synechocystis sp.*	
Kappaphycus alvarezii	Transient	*Synechococcus*	
Gracilaria changii	Transient	*Nosctoc muscorum*	
Porphyridium sp	Stable (chloroplast)		
Porphyridium sp	Stable	**Euglenids**	
Gracilaria	Stable	*Euglena gracilis*	Stable (chloroplast)

Note: 1. Nuclear transformation unless indicated otherwise.

Methods used for DNA delivery into eukaryotic algae are micro-particle bombardment (or biolistic), cell agitation with micro- or macroparticles (e.g. glass beads), protoplast transformation with polyethylene glycol or protoplast or whole cell transformation by means of electroporation, and finally *Agrobacterium* mediated transformation (Coll, 2006), i.e. methods that are also used for DNA delivery into plants.

Selectable traits used include resistance against antibiotics, chemical agents such as herbicides and genes that rescue mutations such as auxotrophies; marker genes allowing election of transformants include Gus and GFP genes (León-Bañares, 2004; Technopolis, 2012).

The promoters used to drive gene expression in transgenic algae are either homologous promoters, e.g. the Rubisco small subunit (RbcS2) or the ubiquitin (Ubi1) promoter or the heterologous promoters CaMV35S and SV40. CaMV35S, the cauliflower mosaic virus promoter, a typical promoter for strong expression in higher plants, works well in several algal strains while the SV40, the simian virus 40 promoter a polyomavirus promoter, has been shown to work in *H. pluvialis* and in *C. reinhardtii* (Coll, 2006).

Nuclear transformation of algae generally results in random integration of transgenes. In *C. reinhardtii* and *C. merolae* and *Ostreococcus* homologous recombination has been achieved but the frequency is low (Radakovits et al., 2010). Recently one alga, the oil-producing algae *Nannochloropsis* sp., was shown to have a high frequency of homologous recombination after transformation and selection (Kilian et al., 2011). In contrast, chloroplast transformation often results in homologous recombination (Lapidot, 2002; Purton, 2007).

Targets of algal genetic modification

Genetic modification as a tool to improve algal performance is more and more considered a necessity to achieve new and economical viable productions systems (Wijffels and Barbosa, 2010; Greenwell et al., 2010; Hannon et al., 2010; Scott et al., 2010; Schuhmann et al., 2012).

Three types of targets can be distinguished for genetic modification of algae: improvement of photosynthetic efficiency, improvement of productivity of selected products and new products.

Improvement of photosynthetic efficiency

Biofuel production efficiency with algae is directly dependent on the solar photon capture and conversion efficiency of the system. However, daylight intensity is most of the time above the maximum photosynthetic efficiency of algae and therefore growth is reduced, a phenomenon known as photo inhibition. Research in this area focuses on the light harvesting antenna complex (LHC) (Mussgnug et al., 2007; Anastasios, 2009).

Improvement of productivity of selected products

The rising market demand for pigments from natural sources has promoted large-scale cultivation of microalgae for synthesis of such compounds. Genes encoding enzymes that are directly involved in specific carotenoid syntheses have been investigated and further development of transformation techniques will permit considerable increase of carotenoid cellular contents, and accordingly, contribute to increase the volumetric productivities of the associated processes (Guedes et al., 2011). One example of such a gene (a phytoene desaturase) has already been published (Steinbrenner and Sandmann, 2006). Table 4.2 gives an overview of carotenoids produced by selected microalgae.

Table 4.2. **Carotenoids produced by selected microalgae**

Microalga source	Active compound
Dunaliella salina	B-carotene
Haematococcus pluvialis	Astaxanthin, cantaxanthin, lutein
Chlorella vulgaris	Cantaxanthin, astaxanthin
Coelastrella striolata var. multistriata	Canthaxanthin, astaxanthin, β-carotene
Scenedesmus almeriensis	Lutein, β-carotene

Research on lipid production has increased in the past decades due to interest in developing algal biofuels. Genetic modification is part of the strategy to increase lipid production with algae. Target genes are lipid biosynthetic genes, lipid storage genes and lipid degradation genes. Obviously, the first two categories have to be enhanced while the third category of genes should be reduced (Radakovits et al., 2010; Scott et al., 2010).

Another interesting aspect is the modification of the lipid characteristics. This could increase the quality of the lipids with regards to suitability as diesel fuel feedstock but could also make the lipids suitable for other applications, like industrial applications, food or feed (Radakovits et al., 2010). Genes for this purpose will originate from the group of fatty acid modifying enzymes, such as desaturases and thioesterases, which have been studied in genetically modified plants in detail for a long time (Napier, 2007).

New products

An emerging field in the biotechnology of algae is the introduction of genes or metabolic pathways in order to produce components of economic interest and which are not yet present in the wild type. Table 4.3 gives an overview of new products that have been made by algae through genetic modification. Two major groups of new products can be distinguished: energy products (like ethanol, hydrogen and fatty acids) and recombinant proteins.

Table 4.3. **New products that have been made by algae through genetic modification**

Product	Algae used	Reference
Hydrogen	*Chlamydomonas reinhardtii*	Melis and Happe (2001)
Hepatitis B antigen protein (HBsAg)	*Dunaliella salina*	Sun et al. (2003)
Human growth hormone (HGH)	*Chlorella vulgaris* *Chlorella sorokiniana*	Hawkins and Nakamura, (1999)
Poly-3-hydroxybutyrate (PHB)	*C. reinhardtii*	Chaogang et al. (2010)
Erythropoietin; Human fibronectin 10FN3 and 14FN3; Interferon β; Proinsulin; Human vascular endothelial growth factor (VEGF); High mobility group protein B1 (HMGB1)	*C. reinhardtii*	Rasala et al. (2010)
Bovine lactoferricin (LFB)	*C. reinhardtii*	Li and Tsai (2009)
Avian and human metallothionein type II; Antigenic peptide P57; Antigenic proteins VP19,24,26,28; Foot and mouth disease virus VP1 protein; Anti-glycoprotein D of herpes simplex virus; Anti-rabbit IgG; Human tumour necrosis factor; Bovine mammary-associated serum amyloid; Classical swine fever virus E2 viral protein; Human glutamic acid decarboxylase 65; Human erythroprotein; Antianthrax protective antigen 83 antibody; D2 fibronectin-binding domain	*C. reinhardtii*	Griesbeck and Kirchmayr (2012)
Flounder growth hormone (FGH)	*Synechocystis*	Liu et al. (2008)
Ethylene	*Synechocystis*	Sakai et al. (1997)
Ethanol	*Synechocystis*	Deng and Coleman (1999)
Fatty acid	*Synechocystis*	Xinyao et al. (2011)
Isobutyraldehyde	*Synechococcus elongatus*	Athumi et al. (2009)
Isoprene	*Synechocystis*	Lindberg and Milis (2010)
Poly-3-hydroxybutyrate (PHB)	*Phaeodactylum tricornutum*	Hempel et al. (2011)

None of the products from Table 4.3 are commercially available at the time. However, research on the application of algal systems for the production of these products is increasing (Angermayr et al., 2009; Beer et al., 2009; Specht et al., 2010; Griesbeck and Kirchmayr, 2012).

Examples of other research are the use of algae for CO_2 capture and wastewater treatment.

A review on recent research involving engineering cyanobacteria for the production of valuable compounds has been published by Ducat et al. (2011).

European regulations for working with genetically modified organisms

Working with genetically modified organisms (GMOs) in the Netherlands is governed by national regulations that implement the EC Directives 2009/41/EC (European Union, 2009) and 2001/18/EC (European Union, 2001) that deal with contained use of GMOs and with deliberate release into the environment of GMOs, respectively.

A risk assessment is the key element in both directives. Guidance notes to the EC directives, laid down in annexes to the directives, describe in detail the different aspects of such a risk assessment. Both Directive 2001/18/EC and 2009/41/EC state that the performance of an environmental risk assessment (ERA) is mandatory. In Directive 2001/18/EC an ERA is defined as "the evaluation of risks to human health and the environment, whether direct or indirect, immediate or delayed, which the deliberate release or the placing on the market of GMOs may pose". Under Directive 2001/18/EC "human health" is taken into consideration only as far as incidental exposure is concerned; food and feed safety are taken into consideration in the EU regulation 1829/2003 (European Union, 2003).

The EC directives on GMOs make a clear distinction between contained use and deliberate release into the environment:

- Contained use is defined as "any activity in which organisms are genetically modified or in which such organisms are cultured, stored, transported, destroyed, disposed of or used in any other way and for which specific containment and other protective measures are used to limit their contact with the general public and the environment".
- Deliberate release is defined as "any intentional introduction into the environment of a GMO or a combination of GMOs for which no specific containment measures are used to limit their contact with, and to provide a high level of safety for, the general population and the environment".

This chapter considers the environmental risks and the risk assessment of engineered algae in the context of these regulations.

Risks related to production systems of (GM-)algae

Three different production systems for large-scale production of algae can be distinguished: natural locations, open ponds (raceway ponds) and closed systems (photo bioreactors [PBRs]).

Releases in natural locations clearly are deliberate release into the environment since there are no effective protective measurements to prevent the algae from entering the surrounding environment.

Releases in open ponds can be regarded as deliberate release. Since ponds are not covered, there is contact with the environment through open air which could be considered intentional introduction into the environment. Contact with the environment

may also occur due to spillage which may occur due to, for example, large winds or floods, especially in very large-scale ponds.

Closed systems could be considered contained when placed inside a building. Cultivation of a GMO in a closed system which is placed in open air may be considered under the regulation of contained use when it meets the following criteria: "'contained use' means any activity in which micro-organisms are genetically modified (…) and for which specific containment measures are used to limit their contact with the general population and the environment" (European Union, 2009: Article 2c).

In the Netherlands, a safety level of Good Industrial Large Scale Practice may be applied to the use of micro-organisms in industrial settings. This safety level is based on the concept of Good Industrial Large Scale Practice (GILSP) that was originally developed in the OECD "Blue Book" (OECD, 1986). It implies that if a host organism has a long history of safe use in an industrial setting, the same industrial setting offers adequate containment for the use of a GMO derived from this host organism.

The rules of GILSP can be applied to the use of a GMO if:

- the host organism is non-pathogenic and has a long history of safe use under industrial conditions
- the GMO is derived from this host organism using a "safe" vector (if applicable) and a "safe" insert, and the resulting GMO has a reduced fitness in the environment compared to the host organism.

The concept of GILSP implies, *inter alia*, that living organisms of a culture grown under GILSP may be released in the environment inasmuch as that is usual also for the host organism.

Until now, there is still limited practice of algae production systems in Europe. In the Netherlands, local municipalities have granted environmental approval for growth facilities for non-modified algae, but have done so according to different regulations. For example, the algae production systems of AlgaePARC[2] needed to be contained, while for the production systems of Ingepro, no risk assessment was required.

Overview of potential risks of GM-algae for human health and the environment

The European Commission has developed guidance notes for risk assessment of the use of GMOs. Guidance Note 2000/608/EC (European Union, 2000) deals with risk assessment of contained use of genetically modified micro-organisms while Guidance Note 2002/623/EC (European Union, 2002) deals with the risk assessment of deliberate release into the environment of genetically modified organisms. This section discusses elements of the risk analysis methodology as developed in these guidance notes.

Safety of the algae, the insert, vector and the GM-algae

With respect to contained use, the risk assessment is aimed at identifying harmful properties of the algae due to the combined characteristics of the recipient organism, the insert, the vector and the resulting GM-algae with respect to human health and the environment.

There are only a few species of algae that are classified as pathogens in humans or animals. These algae belong to the *Prototheca* or *Chaetoceros* or are mentioned on the IOC-UNESCO list of harmful algae. However, quite a number of algal species, especially

belonging to the dinoflagellates and the diatoms, produce toxins that impact humans, animals and birds. In addition, some cyanobacteria also produce toxins that are harmful to humans and animals. For example, some genera that are industrially relevant contain species that are known to produce toxins, e.g. *Phormidium* (some strains do not produce toxins), *Anabaena circinalis*, *A. flos-aquae*, while *Synechococcus wickerhami* and *Prototheca cutis* are human or animal pathogens.

In the examples of GM-algae mentioned above, the DNA inserted in the recipient algae has been characterised. Although it is unlikely that GM-algae intended for use in outdoor cultivation systems contain inserts that have not been characterised, a differentiation between donor organisms in terms of toxin producer, pathogens or non-toxin producer non-pathogen will influence the risk assessment when uncharacterised genes have been used to produce the GM-algae, as uncharacterised genes may be involved in toxin production or pathogenicity.

When looking at the targets of genetic modification of algae, the following groups of genes used as inserts, can be distinguished:

- genes involved in photosynthesis
- genes involved in carotenoid biosynthesis
- genes involved in lipid biosynthesis
- genes encoding (pharmaceutical) proteins
- regulatory genes such as transcription factors or other metabolic regulators.

In general, the genetic modification of algae aimed at modifying either photosynthesis, carotenoid biosynthesis or lipid biosynthesis is not expected to generate harmful strains with respect to human health. None of the genes used encode for toxins or are suspected to lead to toxin production through enhanced metabolic steps or metabolic pathways, especially when they are expressed in "safe" algae hosts.

However, introducing genes in the host may have phenotypic effects and for that reason it is argued that these effects should be analysed. When expressing pharmaceutical proteins (e.g. antibodies), the potential effects of these proteins on humans have to be addressed in the risk assessment.

In eukaryotic algae, the donor DNA is integrated in the genomic or chloroplast DNA. Only *Chlamydomonas reinhardtii* has a history of stable genetic modifications and subsequent cultivation of the GM-strains. Stability of other GM-algae (which is mainly an issue in the production using these algae) still has to be confirmed, especially under non-selective conditions since stability will most likely be gene and integration dependent. As cyanobacteria are bacteria, vector DNA can be integrated into the genome, but vectors, which can replicate in the cytoplasm, are also used. The methodology of risk assessment used for GMOs can be applied to cyanobacteria without major modifications.

Transfer of genetic material to other organisms

An important aspect to be addressed in the ERA is the transfer of inserted genetic material to other organisms. Therefore, horizontal gene transfer (HGT) – the transfer of genetic material from one organism to another which is a natural mechanism and has played an important role in evolution – is a point of concern.

In cyanobacteria, where ~50% of extended gene families putatively have a history of HGT (either between cyanobacteria and other phyla, or within cyanobacteria, or both), HGT has played an important role in evolution (Zhaxybayeva et al., 2006; Monier et al., 2009). In these bacteria, HGT is a mechanism in real-time adaptation and for that reason it is part of the risk assessment of GM-bacteria.

In eukaryotic algae, HGT has been part of the evolutionary development; however, in these organisms, this is not a real-time event and poses no additional risk in GMOs.[3]

Vertical gene transfer uses reproduction as a means of gene transfer through generations and may be a risk with GM-algae when the species used has a sexual reproduction cycle and wild-type partners are present in the environment.

The transfer of antibiotic resistance or herbicide resistance is an issue in the debate on the safety of GMOs. Several governments in the European Union have recommended the phasing out of GM-crops containing any antibiotic resistance markers (European Federation of Biotechnology, 2001). Therefore, the use of GM-algae, without antibiotic resistance genes, for outdoor cultivation will almost certainly be more easily accepted by the public. However, as discussed above, in most of the genetic modification protocols for algae, antibiotic resistance is being used as the selection criterion. Some alternative selection systems have been used in algae (the nitrate reductase selection system, uracil selection), but more research on alternatives for antibiotic selection of algae GMOs is necessary. Genetic deletion of the antibiotic selection gene after generation of a stable transgenic line has also been achieved for some algae transgenic systems, so technology to avoid antibiotic genes in GM-algae is under development (Mayfield, personal communication).

Table 4.4. **Important data for environmental risk assessments of algae**

Data	Effect
Strain identity	Pathogenicity, toxin production
Growth conditions	Spreading into the environment
Algae production system	Open pond, closed tubes
Specific GMO properties	Enhanced or reduced growth, antibiotic resistance
Stability of the GMO	Horizontal gene transfer
Harvesting method	Chance of escape

Notes

1. http://ec.europa.eu/research/fp7/index_en.cfm.
2. www.algaeparc.com.
3. HGT from GM-plants to prokaryotes has been studied and was shown to pose negligible risks (Keese, 2008). Horizontal gene transfer from bacteria has also been studied in relation to mechanisms and barriers (Thomas and Nielsen, 2005) and to risk assessment of GMOs (Heuer and Smalla, 2007).

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Chapter 5

The benefits and advantages of commercial algal biomass harvesting

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This chapter outlines the concept of integrated bioremediation and co-product development using microalgae. It ties potential products with taxonomically governed biochemical profiles, which are essential criteria for product-driven strain selection. It closes by briefly describing the current challenges to commercial cultivation and biomass harvesting.

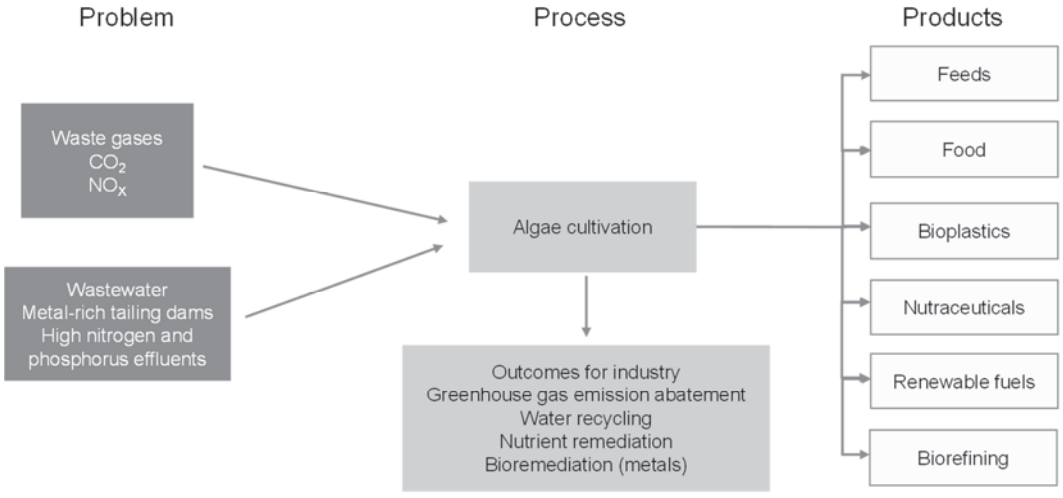
Concept of bioremediation using microalgae with value-adding co-product development

The unprecedented increase in greenhouse gas (GHG) emissions is predicted to lead to rapid environmental changes, such as, for example a general rise in global temperatures, more severe weather conditions and reduced freshwater availability, particularly in countries where freshwater is already a precious resource (Field et al., 2012). Global economies are under increasing pressure by governments and the general public to reduce their carbon emissions. For example, the global carbon dioxide equivalent (CO_{2e}) emissions for 2005 were 44.2 billion tonnes (Herzog, 2009). Many countries have introduced carbon taxes to force industries to rethink and actively work towards carbon reductions of their emissions (Ellis et al., 2010).

Global economies are not only pressured by GHG-induced proposed climate change scenarios, but are further challenged by the prediction of having reached or reaching peak oil and phosphorus in the foreseeable future (most likely in the next 15 years), which will negatively affect industries and agriculture (Cordell et al., 2009; Sorrell et al. 2009). It is possible that appreciable new fossil oil reserves exploration may be possible at greater depths; however, the quality of these so-called heavy oils is poorer, as the oil is more viscous, has a higher sulphur content and, hence, requires additional refining efforts. These efforts will be reflected in increased oil prices. Undeniably though and regardless, fossil oil reserves are not expected to be replenished within acceptable time frames to match the growing energy demands of the future world population (Owen et al., 2010). Peak oil also affects the agricultural sector, as farm machinery is oil driven and pesticides are oil-based products. The application of pesticides have led to sustained food supplies, which is directly linked to population growth (Pfeiffer, 2006). With regards to peak phosphorus, predicted population growth, limited arable land for food production, which is not predicted to increase substantially or in line with estimated population growth (United Nations, 2004), and scarcer freshwater resources as well as more unstable weather conditions and raised temperatures will challenge agriculture and aquaculture industries to meet future nutritional and food supply requirements.

Algae and the oxygenic photosynthetic cyanobacteria (chloroxybactria) offer ideal solutions to the above-mentioned imminent problems, because they can be cultivated year round on non-arable land in various wastewater streams or brackish to marine waters, alleviating the pressure on arable land and freshwater resources. As algae are naturally high in protein and ω-3 polyunsaturated fatty acids and vitamins, which are essential in a balanced diet, they may well become a promising food supplement or food source to ensure a healthy diet for the growing population (Cribb, 2011), most likely not achievable with traditional terrestrial crops. In addition, malnutrition or lack of essential amino acids, fatty acids, minerals, antioxidants and vitamins are linked to numerous diseases, such as nutritional anaemia (iron and B12 deficiency), xerophthalmia (vitamin A deficiency) and endemic goitre (iodine deficiency), which are, according to the World Health Organisation, of growing concern (Edwards, 2010). Many algal strains are also suitable for producing renewable fuels (biodiesel, bioethanol and kerosene), restoring the carbon balance and fertility in weathered soils (biochar) (Bird et al. 2011; 2012), for the bioremediation of carbon dioxide (CO_2) (1 DT of biomass remediate 1.83 T of CO_2 (McGinn et al., 2011) and nitric oxide containing flue gasses (Nagase et al., 1997) and metal- and nutrient-rich wastewaters (Perales-Vela et al., 2006) (Figure 5.1).

Figure 5.1. **Concept of bioremediation using microalgae with value-adding co-product development**



Taxonomic affiliations: Implications for potential end product use

Just like the rather diverse habitat algae colonise, they also show an incredible taxonomic diversity (Table 5.1) and were formerly classified as belonging to the kingdom Protista, which became a collection bag for seemingly unrelated organisms. To make sense of the classification mess, a new classification scheme was proposed for the eukaryotic protists (Adl et al., 1995), excluding the oxygenic photosynthetic chloroxybacteria (formerly cyanobacteria or blue-green algae), which is shown in Table 5.1 as far as it is relevant to the algae. The algae are now distributed amongst four supergroups and grouped in regards to their relatedness with non-photosynthetic protists (Table 5.1). For example, the Euglenophyta are more closely related to the obligate parasitic Trypanosomes and Leishmania, as are the dinoflagellates to the obligate parasitic Apicomplexa, which cause for example malaria (*Plasmodium* spp.), and the ciliates (Table 5.1). It is hence not surprising that 50% of the euglenoids and dinoflagellates are actually not photosynthetic (see explanation below) (Zhang et al., 2000). Indeed, the parasitic Apicomplexa have retained a rudimentary plastid, termed the apicoplasts (McFadden, 2011), which no longer has a photosynthetic function, but is still the location for *de novo* lipid synthesis (Huerlimann and Heimann, 2012). The largest change to the former protistan classification scheme is that the green algae (Chlorophyta) are now classified together with the Embryophyta in the kingdom Viridiplantae and the red algae (Rhodophyta) and the glaucocystophytes in the kingdom Rhodophytae, which now form the supergroup Planta (Table 5.1) and, strictly speaking are no longer regarded to be protists.

In order to understand algal diversity and classification schemes and their impact on end product suitability, it is necessary to understand the origin of the chloroplasts (plastids). The prokaryotic chloroxybacteria contain chlorophyll a only and water-soluble phycobilins as accessory pigments (Gould et al., 2008) and evolved oxygenic photosynthesis as a mechanism to convert solar energy into chemical energy and for carbon acquisition about 3.5 billion years ago. In essence, they created today's atmosphere, having evolved under essentially anaerobic conditions and high atmospheric CO_2 concentrations (Payne et al., 2011).

Table 5.1. **Taxonomic affiliation of algae**

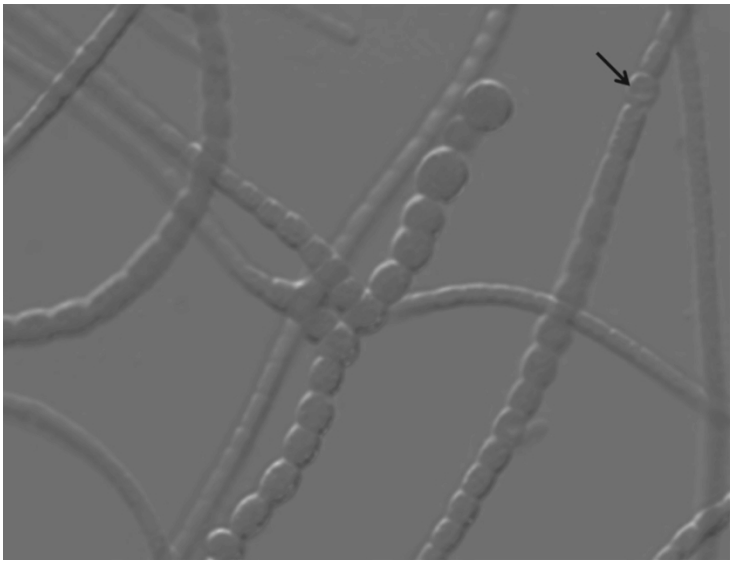
Supergroup	Kingdom	Taxa (phyla)	Classes
Excavata[1]	Discicristatae	Euglenophyta,[2] Trypanosomes, Leishmania	
Chromalveolata	Heterokontae	All heterokont algae also called *stramenopiles* or Ochrophyta	e.g. Bacillariophyceae, Phaeophyceae, Eustigmatophyceae
	Eukaryomonadae	Haptophyta, Cryptophyta	
	Alveolata[1]	Dinophyta,[2] Ciliata, Apicomplexa	
Rhizaria[1]	Cercozoae	Chlorachniophyta, Radiolarians, Eugliphids	
Planta	Viridiplantae	Chlorophyta, Embryophyta[3]	
	Rhodophytae	Rhodophyta, Glaucophyta (sometimes also called Glaucocysto-phyta)	e.g. the Glaucophyte, *Cyanophora paradoxa*

Notes: 1. Supergroup and kingdoms containing heterotrophic zooplankton or obligate parasites. 2. Microalgal phyla containing heterotrophic and non-plastidial genera. 3. Embryophyta are the plants and do not have any algal representatives in this phylum.

Source: Based on Adl, S.M., et al. (1995), "Diversity, nomenclature, and taxonomy of protists", *Systematic Biology*, No. 56, pp. 684-689.

Some chloroxybacteria are also capable of fixing atmospheric nitrogen. As the nitrogenase enzyme complex responsible for N_2 fixation is inhibited by oxygen, the process is either spatially separated into heterocysts (which only contain the non-water splitting and therefore not-oxygen evolving photosystem I) (Figure 5.2) or it is temporally segregated (occurring at night, when photosynthesis is not active and no oxygen is being produced) (Latysheva et al., 2012). This provides a growth advantage under nitrate-, nitrite-, ammonium- and/or urea-nitrogen-limiting conditions, which can be a large cost saver in commercial-scale production for various valuable end products.

Figure 5.2. **Micrograph of the branching filamentous cyanobacterium *Mastigocoleus* sp.**



Note: The arrow is pointing to the heterocyst.

These choroxybacteria were taken up by a heterotrophic host, providing it with photosynthates and energy. Over time, this endosymbiotic relationship transformed into the chloroplast (plastid surrounded by two membranes) through gene transfer from the endosymbiont to the host. This primary endosymbiotic relationship, which gave rise to

organisms with a primary plastids (chloroplast surrounded by two membranes) resulted in the evolution of the first photosynthetic eukaryotes; for the algae, these are the green algae, characterised by having chlorophyll a and b, the red algae and the glaucophytes (chlorophyll a only and phycobilins; they also still contain the bacterial peptidoglycan cell wall between the inner and outer chloroplast membrane, hence the organelle is known as the cyanelle instead of chloroplast) and for plants it is the embryophytes (aquatic and terrestrial plants characterised by having chlorophyll a and b) (Archibald, 2008; Gould et al., 2008).

Then in a secondary endosymbiontic event, a heterotrohpic eukaryote engulfed either a red or green lineage primary photosynthetic eukaryote (e.g. a red alga or perhaps glaucophyte for the red lineage and a green alga for the latter), giving rise to photosynthetic eukaryotes now containing plastids surrounded by three or four membranes (Archibald, 2008; Gould et al., 2008).

The origin of plastids, regarding red or green lineage, is still debated with one group hypothesising that the mechanisms required for the transformation of an endosymbiont into an organelle would be too complex to be derived from two separate events, hence claiming that all plastids were red lineage derived (Delwiche, 1999). Presumably, a green lineage endosymbiotic event gave rise to the Euglenoids and the Chlorachinophytes as both groups contain chlorophyll a and b. In contrast, it is assumed that generally all other genera in the supergroup Alveolata (Table 5.1) arose from a secondary endosymbiotic event with a red alga. There are exceptions to the latter in the case of the dinophyta, which are believed to have entered tertiary endosymbiotic events with various other algae (Chlorophyta, Haptophyta, etc.) (Delwiche, 1999).

The accumulation of membranes surrounding the plastid obviously made nuclear-encoded plastididal protein import quite complex and this together with the physiological and cell biological phyla/class characteristics would explain why the different classes of algae are characterised by different carbon storage products (Huerlimann and Heimann, 2012), which is important when considering commercial production for specific end-product development.

Taxonomic biochemical characteristics regarding carbon storage have the following implications for end-product development. The primary carbon and energy store for members of the Chloroxybacteria, the supergroup Planta and the Cryptophyta belonging to the supergroup Alveolata is starch (Graham et al., 2008), but the chloroxy bacteria and algae lack the structural complexity of terrestrial plant cell walls, which contain lignin. Starch and simple cellulosic materials are easily fermented to bio-ethanol (Hirano et al., 1997) and also extracted and transesterified to biodiesel (Sivakumar et al., 2010) as a renewable fuel. In addition, the main fatty acid composition of the green algae belonging to the class Chlorophyceae, particularly freshwater species, is very similar to terrestrial plants (vegetable oil) with α-linolenic acid as the main ω-3 polyunsaturated fatty acid. This would render this group of organisms ideal for replacing terrestrial vegetable oil crops, such as canola, etc. with a green algal-derived vegetable oil produced on non-arable land using non-potable freshwater or nutrient-rich wastewater. To date, the marine chlorophyte *Dunaliella salina* and the freshwater chlorophyte *Haematococcus pluvalis* are used for the commercial production of ß-carotene and astaxanthine, respectively, which are used for food colouring, as supplements in the aquaculture industry and as antioxidants in health foods, while biomass of the green microalga *Chlorella* spp and the chloroxybacterium *Arthrospira platensis* (formerly *Spirulina platensis*) are marketed unextracted as health food supplements based on the

content of Provitamin A and vitamins of the B-complex (Table 5.2). A 40-gramme supplement of *Arthospira platensis* per 34 kilogramme feed per dairy cow changed the fatty acid profile of the milk from predominantly saturated fatty acid to the dominance of mono- and polyunsaturated fatty acids (sampled on days 15, 30 and 45 of a 7-week feed trial period), which will help to market such milk products as health foods and potentially achieve higher prices for the product due to the offset of negative health impacts associated with saturated fatty acid diets (Christaki et al., 2012).

Many members of the supergroup Alveolata (the diatoms, eustigmatophytes, cryptophyes and haptophytes, but not the brown algae Phaeophyceae) primarily store photosynthetic carbon as storage lipids (triacylglycerides [TAG]) instead of starch but also produce storage sugars, such as chrysolaminarin, a ß-1,3-linked polysaccharide (Graham et al., 2008). Members of this supergroup are also characterised by having high content of ω-3 long chain polyunsaturated fatty acids, such as EPA (C20:5, eicosapentenoic acid) and DHA (C22:6, docosahexanoic acid), and the ω-6 long chain polyunsaturated fatty acid AA (C20:4, arachidonic acid), which are essential fatty acids in the diet of aquaculture organisms (Brown, 2002), but also humans (now usually provided as fish oil). In addition, it has been shown that a correct ω-6 to ω-3 ratio is critical for maintaining cardio vascular health (Simopoulos, 2002).

Given the biodiversity represented in this supergroup, there are very few species belonging to diverse genera (Bacillariophyceae: *Chaetoceros calcitrans* and *C. muelleri*, *Nitzschia* spp, *Phaeodactylum tricornutum*, *Skeletonema costatum*; Eustigmatophyceae: *Nannochloropsis oculata*; Haptophyta: *isochrysis* aff. *galbana*, *Pavlova salina* and *P. lutheri*; Cryptophyta: *Proteomonas sulcata*, *Rhodomonas salina*, Dinophyta: *Akashiwo sanguinea* (formerly *Gymnodinium sanguineum* [Heimann, 2012], *Crytpthecodinium cohnii*) that are currently primarily cultivated for aquaculture feed purposes, where they serve as a primary food source for crustaceans, filter-feeding molluscs and fish larvae, with the latter being fed either directly on the algae, or being fed micoalgae-reared *Artemia* and rotifers, if larger food particles are required (Brown, 2002; Harwood and Guschina, 2009; Heimann, 2012) (Table 5.2). Given the growing importance of aquaculture-reared seafood in maintaining a healthy diet for the growing population whilst protecting naturally oil-rich wild fish populations and crustaceans, the natural affiliation of aquaculture with microalgal cultivation and the nutrient-rich wastewaters this industry sector creates, it would make perfect sense to add algal commercial-scale cultivation and biomass-derived co-products to this industry's commodities, whilst bioremediation of the nutrient-rich wastewater would allow for efficient water recycling, reducing environmental impact and thereby allowing the industry to expand.

Even though the macroalgal food market is well established and lucrative, fetching USD 2 billion for Nori (*Porphyra* sp., Rhodophyta), USD 600 million for Wakame (*Undaria* pinnatifida, Phaeophyceae) and Kombu (*Laminaria* japonica, Phaeophyceae) and a global market potential ranging from 20 000 to 40 000 t (Jensen, 1993; Radmer, 1996), most of these materials are harvested from the wild, which might not be sustainablein the long term. The market potential for microalgae ranges from lucrative health food products (e.g. *Arthrospira* sp. sells at USD 100 kg^{-1}, (Radmer, 1996)

Table 5.2. **Use of algal products and current market prices**

Product	Use	Organism	Market price (USD)	Global market	References
Biodiesel	Renewable fuel		0.73 L^{-1}		Harun et al. (2010); Subhadra and Edwards (2011)
Bio-ethanol	Renewable fuel	*Arthospira* sp. (Chloroxybacteria), *Scenedesmus dimorphus* (Chlorophyta), *Porphyridium cruentum* (Rhodophyta)			Harun et al. (2010)
Bio-methane	Renewable fuel	*Ulva* sp. (Chlorophyta), *Gracilaria* sp. (Rhodophyta), *Laminaria* sp. and *Macrocystis* sp. (Phaeophyceae)			Harun et al. (2010)
Bio-butanol	Renewable fuel, fine chemical	*Dunaliella* sp. (Chlorophyta) or any of the above that are also suitable for bio-ethanol production			Harun et al. (2010)
Acetone	Fine chemical	*Dunaliella* sp. (Chlorophyta) or any of the above that are also suitable for bio-ethanol production			Harun et al. (2010)
Glycerine	Pharmaceuticals, paints, industry bulk chemical	By-product of bio-diesel production	320-500 t^{-1}		Subhadra and Edwards (2011)
ω-3 fatty acids	Neutraceuticals animal feed	*Nannochloropsis* sp.(Eustigmatophyceae), *Nitzschia* sp. and *Phaeodactylum tricornutum* (Bacillariophyceae), *Pavlova viridis* (Haptophyta)	50 000-135 000 t^{-1}	USD 1 billiion[1] USD 7 billion[2]	Subhadra and Edwards (2011)
EPA	Neutraceuticals, animal and aquaculture feeds	*Nannochloropsis* sp.(Eustigmatophyceae), *Nitzschia* sp. and *Phaeodactylum tricornutum* (Bacillariophyceae), *Pavlova viridis* (Haptophyta)	508 000 g^{-1}	954-1219 g^{-1}	Harun et al. (2010; Harwood and Guschina (2009); Spolaore et al. (2006)
DHA[3]	Neutraceuticals, animal and aquaculture feeds	*Crypthecodinium cohnii* (Dinophyta), *Ulkenia* sp., *Schizochytrium mangrovei* and *Thraustochytrium aureum* (Heterokontae, Thraustochytriaceae), *Amphidinium carterae* (Dinophyta), *Isochrysis galbana* (Haptophyta)		689-901 g^{-1}	Harun et al. (2010); Harwood and Guschina (2009); Spolaore et al. (2006)
AA (Arachidonic acid)	Neutraceuticals	*Porphyridium* sp. (Rhodophyta)		636-795 g^{-1}	Harwood and Guschina (2009); Spolaore et al. (2006)
γ-linolenic acid	Neutraceuticals	*Arthrospira* sp. (Chloroxybacteria)		445-636 g^{-1}	Harwood and Guschina (2009); Spolaore et al. (2006)
Chlorophylls	Food colouring, functional food, pharmaceuticals, cosmetics	*Chlorella* sp. (Chlorophyta)	184 000-268 000 g^{-1}		Harun et al. (2010)
Accessory non-polar pigments	Food colouring, functional food, pharmaceuticals, cosmetics				

Table 5.2. **Use of algal products and current market prices** (*cont.*)

Product	Use	Organism	Market price (USD)	Global market	References
Phycobiliproteins	Biomedical uses (fluorescent markers) Food colouring Pharmaceuticals Cosmetics	*Arthrospira* sp. (Chloroxybacteria), *Porphyra* sp. and *Rhodella* sp. (Rhodophyta), potentially also Cryptophyta and Glaucophyta	3 000-25 000 g^{-1}	USD 50 million	Milledge (2011); Radmer (1996)
Astaxanthin	Antioxidant Food colouring	*Haematococcus pluvalis* (Chlorophyta)	2 500 000 t^{-1}	USD 200 million	Milledge (2011)
ß-carotene[3]	Food colouring, functional food, pharmaceuticals, cosmetics	*Dunaliella salina* (Chlorophyta)	300 000-3 000 000 t^{-1}		Ben-Amotz (2007; Milledge (2011)
Vitamin B_{12}[3]	Neutraceuticals, health food	*Chlorella* sp. (Chlorophyta), *Arthrospira platensis*[4] (Chloroxybacteria)			Harun et al. (2010)
Provitamin A[3]	Neutraceuticals, health food	*Chlorella* sp. (Chlorophyta), *Arthrospira platensis*[4] (Chloroxybacteria)			Harun et al. (2010)
Algal meal	Animal feed	*Arthrospira* sp. (Chloroxybacteria), *Chlorella* sp. (Chlorophyta), *Laminaria* sp. (Phaeophyceae)	1 200-1 800 t^{-1}	635 million t[5] 5-10 million t[6]	Milledge (2011); Subhadra and Edwards (2011)
Stable isotopic amino acids			5 900 g^{-1}		Milledge (2011); Spolaore et al. (2006)
Stable isotopic nucleic acids			28 000 g^{-1}		Milledge (2011); Spolaore et al. (2006)
Agar[3]	Food, biomedical	*Gracilaria* sp., *Gelidium* sp., *Pterocladia* sp. (Rhodophyta)			Radmer (1996)
Agarose[3]	Biotechnological applications	Rhodophyta		Up to 25 000 kg^{-1}	Radmer (1996)
Alginates[3]	Food, paper, biomedical uses	*Ascophyllum* sp. *Laminaria* sp. and *Macrocystis* sp. (Phaeophyceae)			Radmer (1996)
Carageenans	Food products (gelling, thickening, stabilising)	*Eucheuma cottonii*, *Chondrus crispus* and *Eucheuma spinosum* (Rhodophyta)			
Fertilisers	Soil conditioners	All algae, but easier with macroalgae			

Notes: 1. 2008. 2. 2011. 3. Current commercial production.4. Formerly *Spirulina platensis*. 5. Animal feed. 6. Replacement with algae.

to relatively low-value products (e.g. biodiesel USD 0.73 L^{-1}; Subhadra and Edwards, 2011), but there is also an enormous potential to replace existing animal feeds with microalgal meal, as the former need to be raised in substantial quantities on arable land and in some countries (i.e. Australia) require irrigation and expensive import, which, considering carbon and energy budgets, may not be sustainable in the long run. For example, soy-, copra- and fish meal fetch USD 320-1 200 t^{-1}, when sold as animal feed (Subhadra and Edwards, 2011), yet microaglae contain a similar biochemical profile compared to soy (Table 5.3), but do not require arable land and can be cultivated in nutrient-rich wastewaters, thereby taking the ammunition out of the feed versus food debate. The same argument can be expanded to fish oil, currently produced from caught wild population, which is environmentally unsustainable in the long term and will not cover the growing needs of the future human population. The current market price of fish oil is USD 800-1 000 t^{-1} (Subhadra and Edwards, 2011), which is used in aquaculture and for supplementation of ω-3 long chain polyunsaturated fatty acids in human nutrition. Thus, the higher value markets for microalgal products (Table 5.2) exist, making economical production of a variety of microalgal products possible, if commercial production adopts a mixed product approach where production of high-value commodities offsets production costs for low-value market goods, such as biofuels. Ultimately, it will be necessary to guarantee food, feed and fuel security for future generations.

Table 5.3. **Microalgal biochemical profiles in comparison to soy**

Species	Lipid $100g^{-1}$	Protein $100g^{-1}$	Carbohydrate $100g^{-1}$
Nannochloropsis oculata (Eustigmatophyceae)	16.4-29.7[1]	29.7	38.2
Picochlorum atomus (Chlorophyta)[2]	9.7-40[1,3]	50	32
Soy meal	19.9	36.5	30.2

Notes: 1. Higher lipid content is achieved under nitrogen-limiting conditions. 2. Three times the growth rate of *Nannochloropsis oculata.* 3. Highest lipid yields under nitrogen starvation.

Considerations for strain selection for commercial-scale algal production

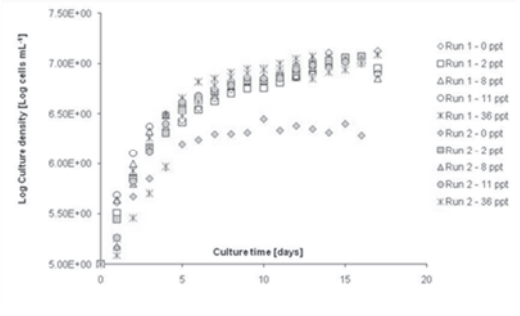
Strain selection must consider the quality of the water source, the environmental conditions, cultivation system, fertilisation regimes and integrated cultivation-harvest cycles, because they influence biomass productivity, product quality and hence marketability. Market potential and commercial viability in terms of required CAPEX (capital expenditure) and OPEX (operational expenditure) also need to be modelled to ensure successful commercial production. It is often best to invest in desk studies in order to create at least preliminary business plans. These should consist of a good knowledge of endemic strains to evaluate their use, particularly if water remediation (e.g. metal remediation from industrial tailing dams or secondary sewage) is the goal, as these organisms are likely already adapted to local conditions (water quality and environmental conditions) (Park et al., 2011).

Strain selection is intimately linked with product selection, especially when targeting fine chemicals or neutraceuticals, because biochemical composition is, as explained before, often class specific (e.g. the ω-3 long chain polyunsaturated fatty acids EPA, DHA, AA will only be produced in certain strains [Brown, 2002], while quantities of the desired product are often influenced by fertilisation regimes and environmental conditions [Huerlimann et al., 2010]). For example, growth of the green microalga

Picochlorum atomus was not affected by salinity levels ranging from 2 ppt to 36 ppt (Figure. 5.3A) (Alvensleben, 2010), neither was lipid content, while nitrogen limitation and starvation significantly increased total lipid content (Figure 5.3B). This makes *Picochlorum atomus* an ideal organism for wastewater remediation with vastly differing salinities, while the end product can range from health food supplements (e.g. like Chlorella tablets) and animal feeds (Table 5.3) to biodiesel (Table 5.4). Biodiesel quality parameters calculated from fatty acid methyl ester (FAME) profiles and compared to available standards and common plant oils used for biodiesel production showed that the green microalga *Tetraselmis* sp. and the eustigmatophyte *Nannochloropsis oculata* had the most suitable FAME profile for biodiesel production with regards to cetane number, iodine value and cold filter plugging point, followed by *Picochlorum atomus* with similar cetane and iodine values but less desirable cold filter plugging point and the haptophyte *Isochrysis* aff. *galbana*, which had the least suitable profile regarding cetane number and iodine value, but an exceptional low cold filter plugging point due to the high amounts of long chain polyunsaturated fatty acids (EPA and DHA) generally produced in haptophytes (Table 5.4). The large variation in quality parameters show that fatty acid profiles, and hence biodiesel quality, is strongly influenced by nutrient status (growth phase) and fertilisation regime.

Figure 5.3. **Salinity tolerance of *Picochlorum atomus***

A. The effect of salinity on growth



B. The effect of salinity and nitrogen limitation on total lipid content

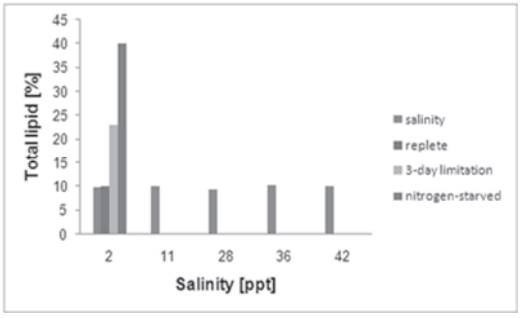


Table 5.4. **Biodiesel properties of select microalgae calculated from FAME profiles**

Standards	Cetane number	Iodine value	Long chain saturated fatty acids	Cold filter plugging point	Degree of unsaturation
ASTM D675	47 minutes	n.d.	n.d.	n.d.	n.d
EN 14214, AU 255	51 minutes	120	n.d.	n.d.	n.d
NPA Brazil	45 minutes	n.d.	n.d.	n.d.	n.d
Palm	55.1	73.9	3.6	-5.2	86
Soy	53.2	112.9	1.1	-13.0	131
Tetraselmis sp.	42.8-56.6 (51.5)	78.7-140.6	3.2-4.4	-2.8/-6.3	83.7-118.1
Nannochloropsis oculata[1]	30.9-54.8 (50.5)	83.4-163.2	3.0-6.5	-0.4/-8.5	74.1-118.5
Picochlorum atomus[1]	43.7-61.5 (42.2)	52.5-137.9	6.2-9.3	2.9/12.8	51.2-135.5
Isochrysis aff. *galbana*	28.1-40.7 (32.7)	149.1-205.5	1.7-7.0	-3.1/-11	104-128

Notes: 1. Strongly influenced by nutrient and growth status. Numbers in brackets denote average values of all FAME profiles obtained.

With regards to water remediation or raising aquaculture species with unknown dietary requirements, a green aquaculture approach may be beneficial, where a body of water is fertilised to allow the local microalgal flora to bloom (Neori, 2011; Park et al., 2011). However, there is little control over species composition, which can adversely affect product development, particularly when the microalgal community consists of taxa belonging to different phyla with markedly different biochemical profiles, as it will be difficult, if not impossible, to guarantee product quality. In addition, quite a number of microalgae, most notably cyanobacteria, which often contaminate microalgal cultures, can produce potent toxins which would render the biomass unsuitable for feed or food. A green aquaculture approach is nonetheless beneficial if the microalgal community to be cultivated belong to the same class or family, because this negates large biochemical profile changes due to dominance shifts or if a distinctive biochemical profile is less important, e.g. biochar applications (Atkinson et al., 2010; Bird et al., 2011). Experiments with chlorophyte consortia dominated by *Scenedesmus* spp showed that this group of organisms is capable of remediating up to 40-60 mg of nitrire L^{-1} day^{-1}, which is an expected conversion product of nitric oxide (NO_x) in water, and more than 350 mg of nitrate L^{-1} over seven days (data not shown), making them ideal for NO_x remediation from flue gas from coal-fired power stations and for nutrient-rich wastewater remediation (Park et al., 2011).

Tailing dams of coal-fired power stations also contain heavy metals, which is of concern when considering the biomass for use in animal feed applications. Experiments using the *Scenedesmus* spp-dominated consortia and supplemented with the average tailing dam concentrations of boron, molybdenium, vanadium and zinc, the metals identified as of concern with regards to animal feeds, and grown under low and high nitrogen and phosphorus, showed that green freshwater strains remediate 100% of these metals, with slightly higher uptake observed for boron and significantly increased uptake for zinc under high nutrient conditions (Table 5.5). These data suggest that *Scenedesmus* spp chlorophytic freshwater consortia are suitable for metal remediation from industrial tailing dams, but care must be taken when considering end product use, as biomass generated in such applications would need to be mixed with other uncontaminated feeds to avoid potential metal poisoning (Alvarez Roa, 2012). The same experiment also showed that metal treatment had no effect on growth or fatty acid composition, but the fertilisation regime had a significant impact on the amounts of polyunsaturated fatty acids (PUFA), which were nearly twice as high under high nutrient conditions compared to low nutrient conditions (Table 5.5). This has significant implications with regards to product choice for this group of organisms, suggesting that low nutrient conditions would render a biomass suitable for biodiesel production, while a high nutrient regime would allow use of the biomass in animal feed applications (Alvarez Roa, 2012).

For products where the biochemical composition is critical and must be guaranteed with regards to minimum and maximum content, e.g. animal feeds, health food supplements, etc., this is extremely important to know as is in which way fertilisation regimes and environmental conditions affect biochemical composition of the biomass. For example, photosynthetically acquired carbon can either be used for growth for incorporation into membrane lipids, DNA and RNA and proteins or diverted to storage as either storage oils (triacylglycerides, TAGs) or sugars, such as starch. Algal culture growth typically continues until carrying capacity of a particular cultivation system and maximum cell densities for a particular strain are reached, as long as none of the nutrients (nitrogen, phosphorus, minerals – such as iron) are limiting. This entails that rapidly

growing cultures deposit less carbon into storage, which is undesirable if products development relies on either high starch or TAG content such as bio-ethanol or biodiesel production, respectively. For example, it has been shown that nitrogen limitation (growth phase) and culture medium composition affect lipid content and fatty acid profiles of microalgae, but that the extent of the effect is strain dependent, affecting some species more than others (Huerlimann et al., 2010 and refrences therein). Thus, integration of fertilisation regimes with harvest cycles, e.g. allowing for a period of nutrient limitation prior to harvest to optimise lipid content, becomes an important consideration, which is, in an economical and environmental sense, at least as important as strain selection.

Table 5.5. **Metal bioaccumulation (µg L^{-1}) and effect of nutrient regime on fatty acid classes (%) in a chlorophyte community dominated by *Scenedesmus* spp**

Metal	Low nitrogen and phosphorus	High nitrogen and phosphorus
Boron	0.15 – 0.225	0.25 – 0.3
Molybenium	0.055 – 0.06	0.06 – 0.7
Vanadium	0.1 – 0.14	0.1 – 0.14
Zinc	0.19 – 0.28	0.35 – 0.45

Fatty acid class	Control, treatment	Control, treatment
Saturated fatty acid	30, 30	19, 22
Mono-unsaturated fatty acid	30, 32	19, 19
Polyunsaturated fatty acid	40, 38	38, 59

Source: Alvarez Roa, C. (2012), "Microalgae bioremediation of trace metals commonly found in ash-dam water from Tarong power station: A coal-fired power plant in Qld", School of Marine and Tropical Biology, Vol. Master of Applied Science, James Cook University, Townsville, Australia.

Cultivation considerations

Generally, three types of microalgal cultivation systems can be distinguished:

- open systems such as ponds, raceways and high rate algal ponds (HIRAPs) traditionally used in aquaculture and for the commercial production of microalgae
- closed systems: tube or plate photobioreactors, where the algal biomass is generally cycled through a solar compartment and a mixing compartment, which allows for degassing and nutrient addition
- hybrid systems: which are essentially open systems but operate under positive air pressure compared to the outside, making it less likely for contaminants to invade the system (da Rosa et al., 2011; Henrard et al., 2011; see also Chapter 4).

All cultivation systems have their advantages and disadvantages. Disadvantages of open systems are: prone to invasions, shallow, making mixing and gas solubilisation difficult, high water loss due to evaporation, large land requirements, low biomass productivities and often poor temperature control. Open systems also have significant advantages. The shallow depth allows for effective degassing of the photosynthetically produced oxygen, which can inhibit photosynthesis if it accumulates in the system, evaporative water loss provides a means of non-energy derived cooling, most microalgal species investigated can be grown in these systems and they are inexpensive in terms of CAPEX (Christenson and Sims, 2011; Weissman and Goebel, 1987). However, evaporative water loss and the large area requirement, particularly for biomass use for biofuel production, are of environmental concern considering future freshwater resources

(Murphy and Allen, 2011). To avoid these negative impacts, it would be mandatory that evaporative water loss is compensated for using non-potable wastewater and that system operation must occur on non-arable land. Currently, open systems are used for the commercial production of ß-carotene mainly using the chlorophyte *Dunaliella salina*, production of the chloroxybacterium *Arthrospira platensis* and the chlorophyte *Chlorella* sp. as a health food supplements (Table 5.2). Reported long-term operation averages for the eustigmatophyte *Nannochloropsis oculata* are 20 g dry weight m^{-2} day^{-1}, which still significantly exceeds productivities of even the most productive terrestrial oil crops (CSIRO, 2011), make such systems potentially useful to also secure high-quality aviation fuel, an area the aviation industry is actively pursuing. With reference to the sustainability of aviation fuel, it is noteworthy that the CSIRO considers bio-derived jet fuels the only sustainable replacement for fossil oil-derived aviation fuels, which will not interfere with arable land use for human food production and can be generated in sufficient quantities to make this a possibility (CSIRO, 2011).

Closed systems are believed to have significant advantages over open cultivation systems in that they are considered to be less prone to contamination, do not suffer from evaporative water loss, show higher productivities on a volume and area basis due to improved light penetration and biomass resuspension (Carvalho et al., 2006). Disadvantages of these systems are that current systems are relatively small scale, only very few organisms can be successfully cultivated, mixing and degassing (build up of photoinhibitory concentrations of photosynthesis-derived oxygen) is still problematic and energy-intensive, require extensive ground preparations for their set up and cooling due to the small volumes in tubular and thin plate solar compartments, are highly technical and very expensive requiring highly trained personnel, which almost prohibits operating them in less developed countries.

In general, improved productivities are typically not large enough to offset the higher costs of CAPEX and OPEX (energy requirements), making it energetically and economically unattractive to use them for the production of low-value end products, such as fuels (Xu et al., 2009). Volumetric daily productivities of closed photobioreactor systsms are being advertised as 4-6 g dry weight L^{-1} day^{-1}; however, long-term multi-year production records are lacking, which makes it unclear whether these productivities could be maintained year round. Regardless, as volumes in closed production systems are typically 10-20 times smaller than open systems, but costs are 10 times higher, it is questionable if this increased productivity would actually stand out compared to the reported long-term year-round productivities of open systems' 0.5-1 g dry weight L^{-1} day^{-1}, which for lower value products is most likely not the case. In terms of cost and volumes, closed photobioreactors are attractive for the cultivation of microalgal biomass for the high to very high value product market where much smaller biomass or compound quantities are required to strike economical success. As such, to date, commercial-scale cultivation is restricted to the freshwater chlorophyte *Haematococcus pluvialis* for the production of the antioxidant astaxanthin (Li et al., 2011).

Given the economical and energetic drawbacks of closed systems, current research also focuses on developing hybrid systems, which are essentially a semi-closed cultivation system where a positive air displacement between the system and the outside should restrict air-borne contamination. Another definition of hybrid system exists where the term describes a closed photobioreactor tasked with maintaining biomass for the inoculation of open systems for short-term cultivation in order to curb contamination (Singh and Dhar, 2011). Regardless of the definition used for hybrid systems, they are likely to be similarly expensive with regards to energy used for culture resuspension and

will also suffer from similar rates of evaporative water loss, displaying approximately twice the price tag of commercial-scale open systems. In essence, however, these systems have inherited the positive sides of the open cultivation systems and more of the advantages of the closed system. This makes these systems economically attractive for the mid-price range product market, as contamination is one of the major economic losses associated with open cultivation systems. Whether these systems display appropriate productivities remains to be shown, but initial results show that horizontal systems, which are comparable in depth and volumes to commercial raceways, show similar productivities and that these can be increased fivefold and more if cultivation occurs in vertically oriented systems (data not shown). The latter systems, however, are of much lower volume, thus it remains to be demonstrated whether vertical hybrid systems of similar volumes to horizontal ones and raceways would maintain this aerial productivity advantage.

It is also possible to grow many microalgal species (e.g. the chlorophytes *Chlamydomonas rheinhardtii* or *Chlorella protothecoides*) heterotrophically in fermenter-style cultivation systems on glucose or acetate in the absence of light, which increased lipid productivity around 24-fold (Xiong et al., 2008) compared to photosynthetically grown microalgae with high lipid productivities, such as the green alga *Tetraselmis* sp., a marine species belonging to the class Prasinophyceae (Huerlimann et al., 2010). While this approach shows immense promise for the production of low-value end products such as biodiesel, there are no ecological advantages to promote this to a commercial scale considering rising atmospheric CO_2 concentrations and the competition for arable land and irrigation-derived sugar, as heterotrophic growth generates CO_2 and the approach would enter the food versus fuel debate if conducted on a large enough scale to substantially contribute to renewable biofuels to meet growing future demands in industry and for general transport. In addition, the approach requires axenic (bacteria-free) cultures, which will be challenging to maintain on an industrial scale. Furthermore, the beneficial allelopathic interactions between the microalgae and their bacterial flora are lost in axenic cultivation, which leads to the cultivation of strains that are tolerant to this loss, thereby restricting strain choice. In addition, the demand for organic carbon would, at the required scales, negatively impact on sugar prices and arable land committed to carbohydrate production for fuel rather than human food, which has already been criticised with regards to the use of corn for bioethanol production (Liao et al., 2011). Even if life cycle and economic analysis were favourable, at this stage, the negative aspects outweigh the positive aspect of fuel security.

Harvest and process considerations

Following CAPEX and OPEX expenditures for commercial-scale cultivation systems, harvesting of microalgae grown in suspension cultures is the single largest CAPEX and energy expenditure often responsible for unfavourable economics and energy budgets for low-value commodities such as biofuels (Ghasemi et al., 2012). Harvest capacity is immediately important to closing the production cycle between biomass generation and biomass processing and the effectiveness of the harvesting system chosen will impact on cultivation regimes and will allow for the design of production cycles and biochemical optimisation of the biomass produced (e.g. incorporation of nutrient limitation phases prior to harvest and the ability to harvest on scale with biomass production). For example, different microalgal strains can show very different harvest requirements for growth phase and nutrient status in order to optimise biomass and lipid productivity (Table 5.6)

(Huerlimann et al., 2010). For example, scheduling of the harvest of the chlorophyte *Tetraselmis* sp. should be for the logarithmic phase, as total lipid content does not increase in the nitrogen-limited stationary phase (Table 5.6). In contrast, total lipid content of the eustigmatophyte *Nannochloropsis oculata* increases significantly during nitrogen limitation in the stationary phase and hence harvest for this species should be timed to coincide with this growth phase (Table 5.6) (Huerlimann et al., 2010). Implications of harvest integration with the culture growth phase are less critical for the haptophyte *Isochrysis* aff. *galbana* and the cryptophyte *Rhodomonas* sp.; however, as both biomass and lipid productivity are substantially reduced in the stationary phase without the offset of improved lipid content, harvest schedules should aim for harvests in logarithmic growth phase (Table 5.6). The harvesting methodology applied is also critically linked to cultivated microalgal strains, as differently sized and shaped cells will affect the harvesting process, which will necessitate optimising harvesting strategies for strain-dependent energy and economic efficiencies. Furthermore, different downstream biomass process technologies and end products will require different moisture levels of the biomass.

Table 5.6. **Growth phase-dependent total lipid content, biomass productivity and lipid productivity of four tropical microalgal species**

Species	Logarithmic phase			Stationary phase		
	Total lipid content	Biomass productivity	Lipid productivity	Total lipid content	Biomass productivity	Lipid productivity
	% of dm	g m^{-2} day^{-1}	g m^{-2} day^{-1}	% of dm	g m^{-2} day^{-1}	g m^{-2} day^{-1}
Nannochloropsis sp.	21.3	13.4	4.2	32.7	2.2	0.6
Isochrysis aff. *galbana*	23.5	18.8	4.4	28.6	3.2	1
Tetraselmis sp.	10.6	45.0	4.8	10.1	5.1	0.5
Rhodomonas sp.	9.5	13.4	1.3	12.5	4	0.5

Source: Huerlimann, R., R. de Nys and K. Heimann (2010), "Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production", *Biotechnology and Bioengineering*, No. 107, pp. 245-257.

Total suspended solid content (number of cells per unit volume) of commercial-scale cultivation systems normally does not exceed 1%. This means that 99% of the water-based cultivation medium needs to be separated from the 1% solids, which becomes harder and less economically and energetically feasible on scales required for sufficient microalgal biofuel production. The scaling aspect of harvesting of microalgal biomass is far less important for the production of lower volume and high-value end products and is therefore not often critically assessed, as centrifugation techniques (traditionally disc flow through centrifugation) are adequate in this context and initial CAPEX and energy costs are offset by the value generated by the end product (e.g. astaxanthine, ß-carotene, and *Chlorella* production).

The harvesting process is essentially a dewatering process that can draw on different strategies, dependent on the microalgal strain, biomass productivities, daily culture volumes that require processing, the size of the production facility (m^3) and the level of water content required for further processing of the biomass. The larger the facility with regards to culture volumes, the more important an integrated harvest-biomass production process becomes (Alabi et al., 2009). This makes the harvesting/dewatering process the biggest bottleneck to the commercial production of microalgae for sustainable renewable fuel generation.

Centrifugation is by far the most effective and most versatile harvesting technique; it is, unfortunately, also the least economical and energy efficient process with regards to large-scale applications (Alabi et al., 2009). Therefore, preconcentration of the dilute suspension culture is desirable. Current preconcentration techniques typically used are flocculation, either achieved through bio-flocculation (often self-aggregation) or chemical flocculation (using either inorganic or organic flocculants), a process that relies on neutralising the negative surface charges of microalgal biomass (Alabi et al., 2009). Chemical flocculation is not desirable as it can render the biomass unsuitable for neutraceutical products. Filtration is another dewatering process; however, costs at biofuel production scales are typically prohibitive. By far the cheapest way is gravity settling, which is achievable with appropriate microaglal strains, but time and land requirements still need modelling to scale with culture volumes required for renewable fuel production. Gravity settling is also not possible, with many of the small-sized microalgae that show promise for biofuel, bioplastic and higher value neutraceuticals or health food products (e.g. *Nannochloropsis occulata*, *Picochlorum atomus*, etc.). Dissolved air floatation, a dewatering process used in the paper industry, shows promise for dewatering, but consistent results would be highly strain and prior treatment (e.g. electro-coagulation) dependent. In short, with regards to harvest strategy, there will be no one size fits all, due to strain dependence and the amount of dewatering required for further processing of the biomass into desired end products.

Various process technologies exist for different end products. For biodiesel production, unless oil can be mechanically extracted from the biomass, hexane extraction followed by transesterification will be required, which potentially leaves the high protein and vitamin-rich microalgal meal unusable for animal feed production. This process technology also requires complete drying of the biomass, an energy expense that is hardly affordable given the energy requirements for cultivation and dewatering/harvesting. Several other process technologies show real promise, particularly for renewable fuel production such as subcritical hydrothermal liquefaction, as a certain amount of water is required, thereby avoiding the CAPEX and energy-intensive complete dewatering and drying requirements. This process has already been used successfully on dilute microalgal growth medium generated in HIRAPs for wastewater treatment, but complete lifecycle analyses will be required to assess economical and environmental sustainability of the process, which shows immense promise for biofuel production from microalgae, as the water content is beneficial rather than a hindrance in the conversion of the biomass (Lam and Lee, 2012).

Conclusion

Microalgal commercial-scale cultivation is achievable and superior with regards to biomass productivities to terrestrial crops, showing tremendous potential for the bioremediation of gaseous wastes and polluted waters, whilst affording cost recovery through value-adding co-product development. Simple commercially viable systems exist to produce sufficient biomass today, but a more integrated approach and complete lifecycle analyses still need to be conducted to evaluate large-scale potential environmental implications. The most promising approach to renewable energy and fuel production from microalgae lies in designing an integrated approach for cheap and environmentally/energetically cultivation, dewatering and applying new technologies for the conversion of the complete biomass, such as hydrothermal liquefaction, particularly for the generation of renewable aviation fuel.

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Chapter 6

Issues in the risk assessment of the use of microalgae for production purposes

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The use of microalgae for biotechnological purposes has increased rapidly in the past few years. In the United States, oversight of the development of the use of microalgae is included in the purviews of many laws and the regulations that implement those laws. Part of the responsibilities encompassed by these laws is a need to evaluate the risks as well as the benefits from the biotechnology industry. In the United States, efforts to co-ordinate the evaluation of research and the commercialisation of biotechnology, which includes the use of microalgae, have been ongoing since 1986. The recent development of a biofuels and bioproducts component of the biotechnology industry has resulted in new examinations of the roles government agencies play in the oversight of this industry sector. Risk and sustainability assessments for production of microalgae have recently been highlighted by private and government sponsored panels. This chapter discusses the progress of co-ordination and evaluation of such oversight in the United States.

Introduction

Over the past few years, the interest in microalgae for production purposes has grown vastly. In the United States, this is reflected in an increase in industrial activity, and many algae companies are headquartered in the United States. As algae are part of the alternative energy portfolio, their development for industrial use is supported by US government funds for alternative energy, made available by, for instance, the Departments of Agriculture, Energy and Defense. Moreover, algae are seen as industrially useful platforms because in addition to biofuel, they may be used to produce a variety of different products, including commodity chemicals, fine chemicals, food, feed, cosmetics and drugs. Algae are important in biotechnology because they can utilise light energy for growth, but some can also be cultured as heterotrophs, in conventional fermenters.

As regulatory oversight encompassing the algae industry is distributed among several laws in the United States, the harmonisation of risk assessment is part of the United States' interest in algae. Risk and sustainability reviews have been initiated due to mandates of laws requiring oversight or by needs of funding sources. An example of the latter includes a study by the US National Research Council entitled *Sustainable Development of Algal Biofuels in the United States* (Committee on the Sustainable Development of Algal Biofuels et al., 2012), that was supported by the Department of Energy.

Activities of the Algae Working Group of the Biomass Research and Development Board

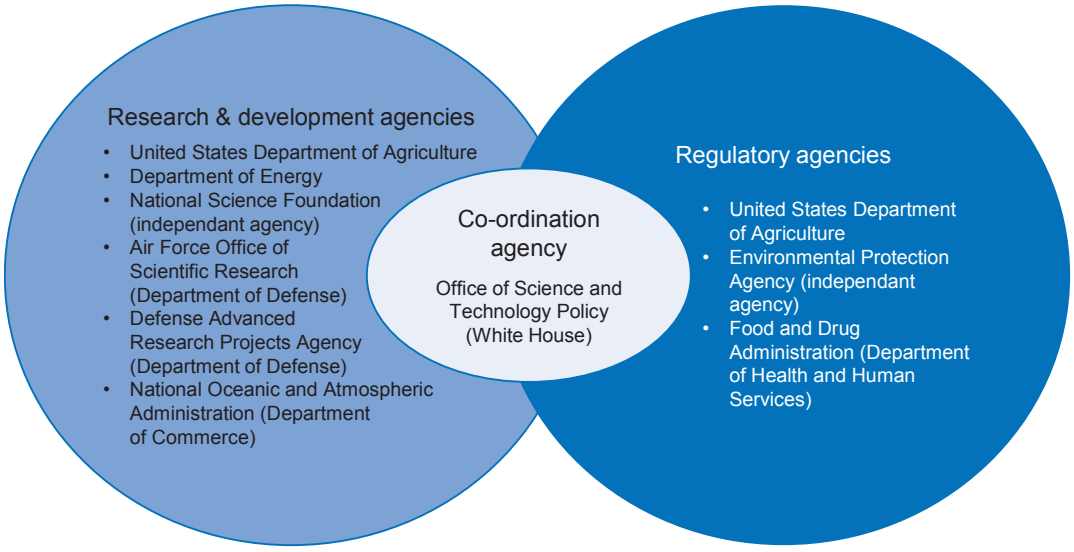
The Algae Working Group (AWG) is one of the support units of the Biomass Research and Development Board (BRDB), and is currently comprised of about 20 members from 8 departments or independent agencies (Figure 6.1). The scope of the AWG extends beyond the BRDB's needs, and includes topics such as research and regulation and other-than-energy interests, e.g. food, cosmetics, agriculture and the environment. The mission of the AWG, as described in 2012 was: advise, communicate and co-ordinate federal research, development, demonstration and deployment activities relating to the production and use of algae and their products/co-products in a sustainable manner within an appropriate regulatory framework.

Topic areas of the AWG in 2011 were: sustainability, algae biology and production, algae harvesting and extraction. Current topics include assessing the scope of oversight responsibilities within the US federal government and designing and developing an algae information resource on the aspects of algal technology, including information on the attributes of specific algae, descriptions of research and regulatory responsibilities, links to public resources of information, links to public information on research funding, and relevant event calendars. An overview of the AWG's activities and participation is presented in Table 6.1.

As noted, a variety of laws and regulations apply to the algae biotechnology industry in the United States. This results in a situation where regulatory oversight of algae is distributed among many statutes according to uses, such as foods, drugs and cosmetics, agriculture, or occurrences such as harmful algal blooms. Some examples of applicable regulatory legislation are the Food Safety Modernization Act (FSMA, 2011), the Toxic Substances Control Act (TSCA, 1976) and the Clean Water Act (CWA, 1972). To help resolve overlap of responsibilities, in 1986, most regulatory agencies involved with

biotechnology jointly described their oversight functions and agreed to lead responsibilities described in the Office of Science and Technology Policy's (OSTP) Coordinated Framework for Regulation of Biotechnology. However, some laws apply to all of the US federal government. This is the case for the National Environmental Policy Act (1970).

Figure 6.1. **The interagency Algae Working Group**



Source: The Interagency Algae Working Group/Biomass Research and Development Board (U.S.A.)

Table 6.1. **Overview of the Algae Working Group's activities (2012) and participation**

FY 2012 activities	Federal participants
– "Most/least wanted" algae list: genera of algae that are of particular interest as research models, production strains; also, algae strains that are problematic (invasive, toxic) – Scope of agency activities summarising agency mission areas and objectives related to algae – Working group report of activities: topical white papers resulting from meeting discussions, findings of knowledge gaps and descriptions of any other collaborative activities	Joyce Yang, Department of Energy/OBP Mark Segal, Environmental Protection Agency/OPPT Co-chairs Participating agencies and departments: – National Science Foundation – Food and Drug Administration (Department of Health and Human Services) – United States Department of Agriculture – Department of Energy – National Oceanic and Atmospheric Administration (Department of Commerce) – Department of Defense/Air Force Office of Scientific Research – Department of Defense/Defense Advanced Research Projects Agency – Environmental Protection Agency

Source: The Interagency Algae Working Group/Biomass Research and Development Board (U.S.A.)

Example: The Toxic Substances Control Act (TSCA)

Oversight of industrial and commercial chemicals production is provided by the Toxic Substances Control Act, with specific implementation for biotechnology micro-organisms provided by Microbial Products of Biotechnology; Final Regulation

under the Toxic Substances Control Act,[1] referred to simply as the TSCA, Biotechnology Rule (1997). This legislation applies to micro-organisms (including all types of algae) that are manufactured, imported or processed for commercial activities, including research and development (R&D) activities, that are considered "new", and describes the pre-manufacturing (MCAN) review requirements. "New" in this context means those that are not on the TSCA's Inventory of Chemical Substances. Examples of algae commercial applications covered by this regulation include biofuels, the production of specialty and fine chemicals and biofertilizers. New micro-organisms are defined as those comprised of genes from different genera and/or with chemically synthesised genes. Pre-manufacturing review of R&D (TERA) for the micro-organism is required if the micro-organism is not contained within a structure. Some chemical products of algae may be "new" chemicals, requiring pre-manufacturing (PMN) review of the chemicals.

Issues identified by the United States Environmental Protection Agency

The following issues were identified and presented in a 2009 EPA workshop:

- Environmental exposure from algae biofuel production under various levels of containment:
  - integrity of modern algae photobioreactors (e.g. plastic bags, other)
  - releases of algae for ponds (intentional and accidental)
  - environmental exposure under normal production:
    - dispersal by aerosols
    - dispersal by wildlife (birds, insects, reptiles, terrestrial animals
  - environmental exposure under catastrophic failure of containment systems.

Participants were asked to express why and when the listed scientific information is needed. The simple answer for "Why?" was that a scientifically credible risk assessment required that these kinds of data and information be available for evaluations, and that science-based risk hypotheses be taken into account, that are falsified based on high-quality scientific information that is useful for risk assessment. They also determined that there was an immediate need for this scientific information since, even in 2009, it was acknowledged that dozens of companies were currently operating with naturally occurring micro-organisms, and the use of genetically engineered strains by companies that were considering their commercialisation was on the horizon.

In another forum,[2] additional insight was provided by expanding on the topics identified in the 2009 workshop focusing on specific information needs as follows:

- Technology issues, e.g.: release potentials vary, depending on the design of the reactors used. Some design features may have positive or negative effects depending on the specific conditions:
  - open raceway ponds vs. closed photobioreactors vs. hybrid designs
  - inputs for production:
    - water use – freshwater, saline, brackish, wastewater, etc.
    - nitrogen and other nutrients
    - use for wastewater clean-up and CO_2 sequestration

- siting issues, e.g.:
  - the consequences of releases, when they occur, vary depending on the ecosystem in which the production facility is located, e.g. desert, coastal regions, surface freshwater, agricultural areas, urban regions.

In addition, other topics were noted:

- Human health and ecological effects, e.g.:
  - releases of algae into the environment:
    - ❖ phycotoxin production
    - ❖ propensity for blooming/anoxia
    - ❖ effects on food web by substitution of preferred food source (native algae) with dominant supply of alternate (escaped) algal species and/or different lipids produced by those algae)
    - ❖ stress-induced production of potentially bioactive biofuel molecules in the environment under commonly found nutrient-limited conditions
    - ❖ competition with indigenous species
    - ❖ dispersal in the environment
    - ❖ gene transfer from transgenic algae
  - release of wastewater and waste biomass, e.g.:
    - ❖ introduction of biological materials, chemicals, nutrients, additives (e.g. from flocculation) into the environment
    - ❖ bioaccumulation of heavy metals from industrial sources of CO_2.

Finally, other progress in identifying assessment issues for algae has taken a different track. A third workshop,[3] on assessing a new paradigm for risk assessment of micro-organisms designed using synthetic biology, was held in 2011. Participants were experts from multiple disciplines, who addressed how to perform risk assessments for micro-organisms produced by synthetic biology. The issues identified at this workshop are common to many algae biotechnology applications. To help identify the key issues, an example was used of a cyanobacterial species designed to produce a commodity chemical. Table 6.2 presents the results of the workshop-research needs, where the participants generated a summary of five main research categories for environmental risk assessments of synthetic biology applications.

Conclusion

Some items in the discussion on environmental risk assessment of genetically engineered algae may demand a special focus.

Familiarity with key algal species

While there is a some familiarity with a number of key species that have already been used extensively in actual production and that may serve as a baseline for assessment, for many species, little is known about their roles in the environment, and thus extrapolation from observations under culture to conditions expected if released from culture is difficult.

Table 6.2. **Summary of five main research categories for environmental risk assessments of synthetic biology applications**

Research category	Specific questions	Reasons given by participants
Rates of evolution and changes in functionality	– Investigate the rate of evolution for changes in functionality.	(Not given a high priority, and therefore no reason given)
Survival and persistence of the organism	– Is the organism compatible with the environment and other populations? – Can the organism survive in a dormant or resting state? – What is the "fitness cost" of the engineered gene and how much of a fitness cost would encourage rapid fall off or "extinction" of the organism in the wild? – How many survival competition tests are needed? Studies should include a whole community analysis, under a variety of environmental conditions. – Consider everyone (e.g. the grazers), not just the competitors.	– Encapsulates the genetic history of the organism and useful in understanding its evolution. – Companies are not expected to do a lot of work in this area; this information is difficult to come by, but important.
Fate and transport of functional genetic material	– Ability of DNA to persist after death? – Which (groups of) organisms may acquire the gene? – Does the target gene remain functional in other hosts? – In what ways can the target gene alter existing genomes? – Introduce fragments of the introduced cassette and measure what is picked up by other micro-organisms.	– As the general public would be very interested in this, a risk assessment would certainly need to cover this. – Fills in gaps, leads to useful information for both regulation and the development of organisms. – But it is also the subject that is least understood of what was talked about in the workshop, and therefore most interesting. – Most relevant from the policy perspective. – A risk we do not understand. – Limiting fate of genetic material.
Physiological differences and differences in functionality between the wild and novel organism	– What is the natural risk of these wild organisms (baseline considerations)? – How do we compare the additional risk due to novel genes? – Investigate secondary metabolites. How many should we look at and at what concentrations? – What are cells doing on a daily basis? Have they changed? Are they the same cells you started with? Are they behaving as desired? – Generate a profile of how the genome and the products of the cell are changed by the addition of engineered genes.	– Captures a broad understanding of the organism before it is modified and allows the modified organism to be compared with a baseline. – By focusing on this category, issues contained in research categories 1 and 2 would be addressed. – This is a "need to know" before it can be said whether the new organism will change ecosystems. – This category has the least amount of available data. – This represents the hazard part of the risk assessment which is important. – This will be the trigger of regulation. – This information is important for the first step for the risk assessment and will temper what questions to ask in other areas.
Probabilistic modelling of gene transfer	– Can modellers guide the parameters and data needed to predict gene uptake? – Would a model separate naturally occurring genes prevalent enough to assume that they have been thoroughly sampled throughout evolution from ones that are rare be useful? Can we create a threshold of exoticism for genes to guide us?	(Not given a high priority, and therefore no reason given)

Familiarity with a variety of existing production facility designs

Production facility design is undergoing rapid evolution. For some types of design, each manufacturer has developed an approach that may be unique for its needs. Much experience with these designs is proprietary. While experience with traditional open pond designs of the raceway approach is significant, those that involve advance photobioreactor or other non-traditional designs have little history of safe use. Thus, for both traditional and advanced facilities, an analysis should be made about their probability of failure, based on existing experience.

It may be expected that the production technology will develop rapidly with increasing success and needs. This includes both facility and organism design. It may be expected that new techniques will evolve for the genetic engineering of algae in order to make production more efficient. A thorough understanding of the effects of these technical advancements on potential risks associated with their use needs to be established concomitantly with the understanding of the effects of the advancements on improvements in production.

Notes

1. www.epa.gov/fedrgstr/EPA-TOX/1997/April/Day-11/t8669.htm.
2. Presented to the National Research Council Committee for Sustainable Development of Algal Biofuels, 17 March 2011.
3. "Comprehensive Environmental Assessment and Synthetic Biology Applications", held at the the Woodrow Wilson International Center for Scholars, Washington DC, in July 2011.

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Part III

The use of micro-organisms for bioremediation

Chapter 7

Designing bacteria for the environment: From trial and error to earnest engineering

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Since the mid-1970s, genetic engineering and the possibility of accidental or deliberate environmental release of modified micro-organisms has been the centre of debates concerning the consequences of altering the ordinary course of nature. For a sound discussion on risks, it is of essence to separate substantive scientific and technical issues from non-informed perceptions of the general public. This chapter advocates this question to be framed on the already extensive history and wealth of data on the design, performance and risk studies made since the early 1980s on genetically modified organisms and more specifically, on available records on genetically engineered micro-organisms (GEMs) designed for non-contained applications as in situ *bioremediation agents. Existing information provides a suitable background for tackling the uncertainties raised by newly engineered agents, including those that may stem from synthetic biology.*

Introduction

There are at least three ways in which genetically modified bacteria can help remove toxic waste. The first is, of course, by the use of environmentally friendly bio-processes and products which are designed *ab initio* precisely to avoid the production of noxious by-products (Schmid et al., 2001). The second case is the recycling or reuse of waste in source for either generation of added value products (e.g. conversion of lignocellulose into biofuels) or for mineralisation into CO_2 and H_2O (Keasling and Chou, 2008; Lee et al., 2008). Finally, there are frequent scenarios in which given chemicals have been released accidentally or chronically to soil or water ecosystems. This pollutes the area with concentrations of the compounds that are high enough to cause a detrimental effect on the biology of the site, but low enough not to warrant an intensive and costly, *ex situ* treatment. These cases are typical candidates for bioremediation interventions (Pieper and Reineke, 2000).

The conceptual frames behind such actions have evolved considerably since 1989, the time of the Exxon Valdez disaster (Harvey et al., 1990), as the deliberate addition of biodegrading bacteria (so-called bio-augmentation) has, in most cases, not been useful (Peterson et al., 2003). For the sake of enumerating biotechnological challenges related to microbial diversity, it should be mentioned that after a long period of stagnation, the field is experiencing a rebirth under the aegis of newly developed insights, for instance in systems and synthetic biology. New bioremediation approaches stem from the growing knowledge on the genomes of soil and marine bacteria and from the analyses of their whole transcriptomes, proteomes and metabolomes (Lovley, 2003; Watanabe and Hamamura, 2003; Pieper and Reineke, 2000; Katsivela et al., 2005; de Lorenzo, 2008). This wealth of data allows the construction of metabolic models that identify bottlenecks in biodegradation reactions. In some cases, these can be overcome through protein design and metabolic engineering aimed at fixing the problems found in natural bacteria. In other instances, the choice is the amendment of the afflicted site with given nutrients that may limit growth or catalysis of the indigenous micro-organisms otherwise (Wenderoth et al., 2003; El Fantroussi and Agathos, 2005). It is also feasible to associate degrading bacteria to plant roots (rhizoremediation), and even the expression of catabolic genes of bacterial origin in transgenic plants (Kuiper et al., 2004; Van Dillewijn et al., 2007).

These approaches are likely to produce successes in the degradation of otherwise recalcitrant pollutants *in situ*, such as chlorinated aliphatics and polychlorinated biphenyls as well as for binding heavy metals. However, bioremediation is not just the encounter of one bacterium with one chemical in a Petri dish. Real environmental cleanup involves various layers of multi-scale complexity involved in removal of toxic waste from polluted sites. Genetics and metabolism are the central, but not the only, aspects of bioremediation. A number of pre-catalysis processes upstream (diffusion in solid matrixes, bioavailability, weathering, abiotic catalysis of pollutants) and downstream post-catalysis (stress, production of toxic intermediates, predation, competition) constrain the outcome of the whole action (de Lorenzo, 2008). To this end, one needs to integrate multi-scale data from the all the biological, chemical and physical actors of the process – a challenging field of action for systems biology.

Genetically modified organisms for the environment: What went wrong?

The concept of using genetically engineered bacteria for environmental release as agents for *in situ* bioremediation of industrial pollution can be traced to the very beginning of the recombinant DNA technology. As early as 1972, Ananda Chakrabarty,

of the University of Illinois in Chicago, made global headlines in his attempt to patent a genetically modified *Pseudomonas* strain able to degrade a suite of petroleum components and thus holding a potential for dissipating oil spills (Cases and de Lorenzo, 2005). After ten years of litigation, the patent of the first man-manipulated live entity was granted, a seminal event that was to trigger a large number of consequences in many different realms e.g. scientific, legal, ethical, biosafey, biosecurity and social acceptance. In the meantime, the first usable tools for facilitating gene cloning were developed by Boyer and Cohen (Cohen et al., 1973) and the arch-famous Asilomar Conference took place (Berg et al., 1975a; 1975b). Although the patented Chakrabarty's strain did not really fulfill its promise, the entire case brought about considerable hype on the potential that genetic engineering could have to endow bacteria with a superior capacity to eliminate pollutants *in situ.* One distinct aspect of such an endeavour is that bacteria tailored for environmental release must be vigorously active rather than attenuated (as was recommended in Asilomar). This posed a fascinating challenge for the genetic engineers of the time, as strains had to be programmed to do their catalytic mission efficaciously while at the same time being safe. The approach proposed by that time was the design of genetic containment and biological containment systems to programme death of the engineered agents once the environmental purpose for which they had been created had been fulfilled (Diaz et al., 1994; Molin et al., 1993; Ramos et al., 1995; Ronchel and Ramos, 2001).

GEMs for *in situ* catalysis, for biological control and for plant protection have been for nearly 20 years the workhorses in which these early concepts have been tested and their success and failures examined (Cases and de Lorenzo, 2005). The balance is extremely good in having expanded the knowledge base on microbial ecology and biodegradation biochemistry – but clearly disappointing in terms of efficacious applications in the field. Despite some early successes in the engineering of sophisticated GEMs able to consume otherwise recalcitrant compounds (Rojo et al., 1987; Ramos et al., 1987) the reality is that bioaugmentation (i.e. increasing removal of pollutants by inoculating the target sites with catalytic bacteria) is not yet a reliable technology. Alas, this applies not only to GEMs, but to virtually all types of micro-organisms, natural or recombinant, the few exceptions being less than five. One is *Dehalococcoides*, an anaerobic bacterium able to cause reductive dechlorination of many chloro-organic compounds when inoculated in polluted aquifers (Lovley, 2003). A second one is *Geobacter* (Amos et al., 2007), which has shown its ability to remediate uranium-contaminated groundwater (Lovley, 2003). The best strains to do the job in both cases occur naturally. Furthermore, many of the toughest recalcitrant molecules (e.g. highly chlorinated aromatics) can be dealt with only by anaerobic bacteria, which are most often not amenable to genetic modification. To finish the less-than-rosy picture for transgenic bacteria, conditional killing circuits were far from achieving a certainty of containment which was hoped for.

On this basis, it is surprising to still see in environmental biotechnology numerous reports that propose engineering this or that bacteria for biodegradation of a target compound for potential use in bioremediation. There is a big gap between the potential and realisation and, for the sake of the field, it is better to accept that basically all early expectations of solving pollution and many other environmental problems through genetic engineering have conspicuously failed (Cases and de Lorenzo, 2005; de Lorenzo, 2009). In contrast, the field has yielded some dividends in the production and application of whole-cell biosensors (Ron, 2007; Vollmer and Van Dyk, 2004; Garmendia et al., 2008; de Las Heras et al., 2008) some of them for *in situ* application for detection of

underground chemicals, as well as bioadsorption an immobilization of heavy ions in engineered bacterial biomass (Valls et al., 2000). These are, however, minor victories in the midst of the debacle that has afflicted the pursuit of superbugs for combating pollution.

Think big: Global challenges

As the world becomes more global, we are becoming more aware that a large number of issues affect entire areas of the planet, with, next to climate change, the issue of global pollution by industrial waste and toxic chemicals. Pollutants produced at a given site are frequently mobilised to the upper layers of atmosphere and then deposited in remote areas, sometimes at high concentrations (Kallenborn, 2006; Daly and Wania, 2005). Unfortunately, it appears that nowhere in the world qualifies properly as a pristine, chemically virgin area. In this respect, it is worth noting that many antibiotics and other pharmaceuticals are eligible as authentic pollutants as well. In reality, there is not a sharp divide between synthetic molecules with antimicrobial activity and the many recalcitrant compounds produced or mobilised by the chemical industry (Alonso et al., 1999, 2001; Martinez et al., 2009). In other cases, xenobiotic compounds or their degradation intermediates become endocrine disrupters with devastating consequences for entire ecosystems. Finally, a set of convergent circumstances, i.e. changes in weather, global dissemination of microbial vectors through expanding transport networks and rapid evolution of antibiotic resistance, have led to the reappearance of epidemic diseases as well as the emergence of new ones. One daunting example of this regards the clear environmental origin of cholera outbreaks, which accounts for the sporadic and erratic occurrence of epidemics of this disease (Colwell, 1996; Colwell et al., 1998).

A better understanding of the connections between man-induced environmental changes and infectious diseases is desperately required. Such information is needed not only for explaining events in retrospect, but also for anticipating outbreaks and informing preventive measures. In summary, climatic change, pollution and infectious processes are at the top of the many issues that must be faced at a global scale. Is there any contribution of the genetic reservoir of microbial diversity for addressing these phenomenal problems?

The history of the planet Earth records a considerable number of changes in the composition of the atmosphere that can be traced to microbial action. One of them occurred 2 to 3 billion years ago, when primitive microbes acquired the ability to generate O_2 out of water using the energy from sunlight. This event altered altogether the ecology of Earth, as organisms were forced to cope with oxidative damage or else faced extinction. This change created new niches and heralded the emergence of the multi-cellular life forms during the Cambrian explosion (approximately 540 million years ago). Since then, the fossil record provides evidence of not less than five mass extinctions. Some of them have been attributed to a sudden change in the global composition of the atmosphere brought about by production of hydrogen sulphide by bacteria that lived in stagnant, deoxygenated water (Grice et al., 2005; Huey and Ward, 2005). Micro-organisms not only sense and reflect global environmental change, but they also contribute actively to bring it about. On this basis, only the global microbiota (which contributes the largest share of the Earth's biomass) has the high-scale catalytic power that would be required to decrease the ramping CO_2 levels, counteract the global warming and neutralise harmful emissions.

Our level of understanding of these processes is not enough yet as to be able to exploit them in our favour, so much more research is still required to this end. One ongoing (and timid) example of the use marine microbes for increasing CO_2 deposition involves the introduction of iron particles in the nutrient-rich, but iron-deficient, ocean waters in order to stimulate the growth of phytoplankton blooms (Pollard et al., 2009). A growing number of marine scientists (as well as businesses) are exploring such fertilisation as a way to foster the onset of plankton populations and sequester large amounts of CO_2 for reducing global warming and preventing ocean acidification. The approach is, however, not devoid of problems (Kintisch, 2008; Tollefson, 2008). When the organic material produced by a plankton bloom sinks to deeper waters, the resulting decomposition may use up oxygen in the medium and cause a destructive effect on marine life. Another concern is the effect of iron fertilisation on nutrients other than iron in the ocean, which may be depleted by phytoplankton growth. Yet, the iron fertilisation concept is not devoid of basis and will surely be applied intensively in the next few years, even at the risk of causing low-oxygen incidents and episodes of local anoxia (Kintisch, 2008; Tollefson, 2008). At the moment, little is known about how these procedures will affect marine food chains, which obviously know no borders. It is likely that the management and even deliberate stimulation of the catalytic capacity of marine microbes and soil bacteria at a planetary scale will be a serious matter of international politics in the not so distant future (Tollefson, 2008).

The onset of systems biology

The applications of systems biology to microbial ecology and environmental biotechnology were booming at the time of writing. The efforts embodied in this conceptual frame to address multi-scale microbiological complexity – from genes to whole communities – is the first step to comprehend more intricate setups where the microbiological constituent is just one of the players of a given system. Phenomena such as microbial pathogenesis, environmental catalysis, let alone climate change, involve a large number of biotic and abiotic components that interact dynamically. Yet, the various disciplines necessary to study these have traditionally been away from each other. Biofilm formation, which is at the core of a large number of microbial functions, is among many conspicuous examples of this sort.

Biofilms can be approached from at least two alternative conceptual frames, each of them using a distinctive descriptive language. Since the pioneering work of Bill Costerton (Costerton et al., 1995), many microbiologists see biofilm formation and evolution, in particular the generation of 3D structures, as the result of a genetically determined developmental programme (Monds and O'Toole, 2009), somewhat reminiscent of those found in animals. On the other hand, the very same phenomena can be described accurately with the only tools of physics and statistical mechanics, with no reference whatsoever to genetically programmed occurrences – a view advocated *inter alia* by Mark van Loosdrecht (van Loosdrecht et al., 2002; Nicolella et al., 2000). This is one of the cases where the divide between descriptive languages becomes more evident. Full understanding of the biofilm phenomena will surely requite the concourse of both approaches (Nadell et al., 2009). Another case involves the bioremediation scenarios mentioned above (de Lorenzo, 2008). The elements that influence the evolution of polluted sites include a combination of biotic and abiotic components, which are to be taken aboard for any useful understanding of each specific case.

Bioremediation could well be a privileged setting for the implementation of a systems science that merges and makes sense out of multi-scale data from all the biological, chemical and physical actors of the process. This endeavour is, however, plagued by the lack of a suitable format to compare and match results arising from different experimental systems and science fields. There is little consensus on the names of the genes, on the conditions of the experiments, on the definition of the parameters, on the activities of the various enzymes, etc. Researchers use *ad libitum* the International Union of Pure and Applied Chemistry's (IUPAC) nomenclature for compounds, together with vulgar names, thus an automated and interactive comparison of the data available is made very difficult to those not inside a given community. Maybe the key for degradation of polyaromatic hydrocarbons (PAHs) relies on a piece of data hidden in a publication on cancer that most microbiologists may never stumble across. Many relevant facts are surely documented, but in a cryptic form and we do not know how to access them and how to benefit from them (Cases and de Lorenzo, 2002). The literature already contains a great deal of information that cannot be properly extracted. The lack of tools to penetrate and process the abundant materials available in specialised publications prevents the translation of such information into useful general principles. Systems biology may provide a remedy to most of these problems because of its insistence on data standards, benchmarking experiments and expressing results in suitable quantitative formats. But we are not there yet. The concourse of computer scientists (including computational linguists) is a must to translate the soft narrative that is so typical of much of the (micro) biological literature into rigorous numerical descriptions of the systems under scrutiny.

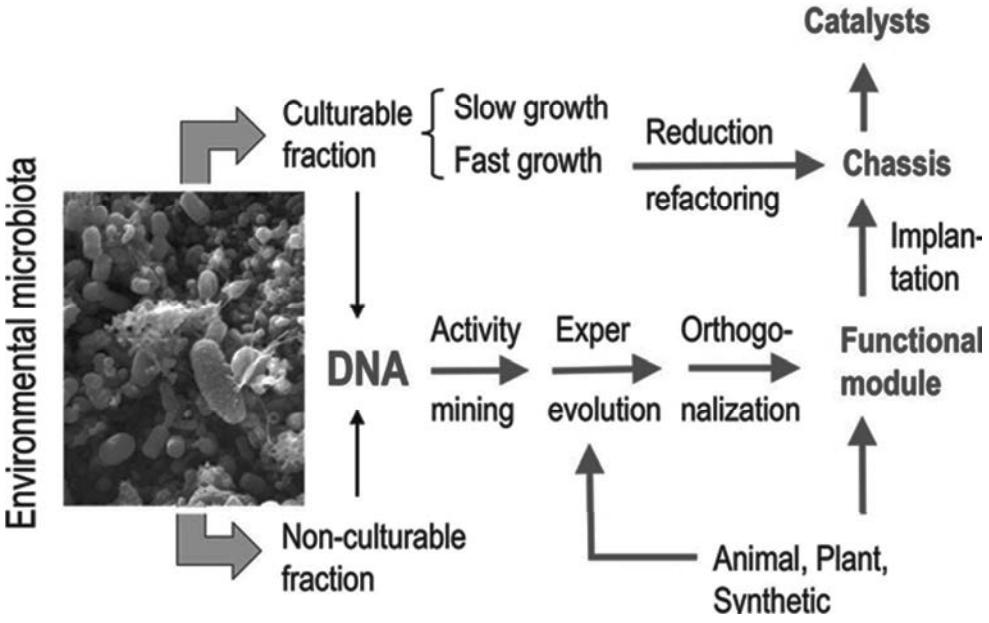
Synthetic biology: The next frontier

The early agenda of recombinant DNA technology in the late 1970s included the notion of genetic engineering as a metaphor of how the new methods would allow us to build new properties in biological systems. All of the activities under the umbrella of synthetic biology convert such an early engineering analogy into a veritable methodology. While traditional genetic engineering uses mostly trial and error approaches to produce new biological designs, synthetic biology attempts to reshape live systems on the basis of a rational blueprint (de Lorenzo and Danchin, 2008). To this end, biological objects are seen as wholes of stand-alone parts hierarchically assembled in modules, devices, subsystems and systems that can be abstracted and completely understood (Endy, 2005; Canton et al., 2008; Arkin, 2008). By the same token, the components of extant biological systems can be de-constructed and rationally re-constructed to build new biological objects with properties *à la carte*. This extreme engineering scene embodies the most extraordinary potential for both understanding the functioning of live systems and for constructing biological materials with a large variety of applications. Yet, implementation of this desirable scenario still needs to fill a large number of gaps in our knowledge of existing biological systems, including the definition of the biological building blocks that can be used for robust engineering; the adoption of a descriptive, quantitative language for biological transactions; and the identification and management of the physical, chemical and evolutionary constraints that frame the functioning of any autonomous biological system (de Lorenzo and Danchin, 2008).

The performance of virtually all biological objects – from proteins to communities – is context-dependent. Furthermore, live entities are perpetually changing under the inexorable laws of Darwinian evolution. Yet, existing biological systems are very robust so it should be possible to design them as well. To this end, a better conceptual frame is needed to understand what minimal biological building blocks are and how they can be

formatted and engineered. The nature of such biological parts is essentially different from e.g. components of electric circuits or mechanic engines. In addition, the nature and description of biological building blocks depends on the scale of the engineering objective. While genetic circuits may rely only on defined promoters and reporters, designing a whole cell will require complete functional modules as building blocks. Similarly, whole cells will be the parts for microbial community design and tissue engineering, and so on. There is a considerable list of research items associated with these issues. Fortunately, the growing ease of synthesising long DNA segments, even complete genomes, should make the field progress at a very fast rate.

Figure 7.1. **Flowchart for the generation of genetically engineered catalysts in the era of systems and synthetic biology**



Notes: The largest reservoir of biological activities is the non-culturable environmental microbiota, including the viral component. Various activity mining strategies employing wet or computational procedures can be used to identify pools of enzymatic activities of interest (pan-enzymes; de Lorenzo, 2008) in the corresponding metagenomic DNA. These can be evolved experimentally for an optimal performance and further orthogonalised (i.e. their functioning made autonomous from the final host). This gives rise to functional modules composed of one or more genes endowed with their cognate regulatory circuit – again, engineered for an optimal performance. On the other hand, the genomes of culturable fast-growing members of the microbial community can be minimised for deletion of undesirable features and optimised as the chassis for implantation of modules of either microbial origin or imported from other kingdoms, including non-natural biological objects (proteins, ribozymes, etc). The outcome of the flowchart is the production of robust and predictable whole-cell catalysts for *in situ* or *ex situ* environmental remediation. It is likely that the genomic chasses for these procedures will soon be altogether synthetic (Gibson et al., 2008).

Source: de Lorenzo, V. (2010), "Exploiting microbial diversity: The challenges and the means", in K. Timmis (ed.), *Handbook of Hydrocarbon and Lipid Microbiology*, Springer, Berlin-Heidelberg, pp. 2 438-2 458.

One possibility in this context is the creation of altogether artificial cells in which the whole genome is synthetic (Gibson et al., 2008) and can be programmed for a given application, an operation reminiscent of writing instructions in a computer programme (Danchin, 2009). Production of synthetic or semi-synthetic bacterial cells of this sort is now at hand, and the ultimate agenda of the genetic engineering that Cohen and Boyer started in the 1970s appears to be within reach. To avoid the re-enactment of the

controversy on GMOs that such synthetic cells could bring about, others see it more feasible to engineer DNA-free vesicles endowed with all basic features of live cells but without any ability to proliferate (Noireaux and Libchaber, 2004; Kuruma et al., 2008). Generation of synthetic cells is not only a biotechnological challenge, but also a serious scientific endeavour which touches upon very fundamental questions, e.g. the origin of life and the emergence of self-maintaining biological systems (Luisi, 2006).

New risks in sight?

The safety concerning accidental or deliberate release of semi-synthetic or entirely synthetic agents is the subject of much ongoing discussion (de Lorenzo, 2010a; de Lorenzo and Danchin, 2008; Schmidt and de Lorenzo, 2012). The large body of literature on GMOs and GEMs for environmental release shows that the more engineered one bacterium is, the less fit it is also to survive once released. However, even heavily engineered organisms function thus far on the basis of what one could call familiar biology, i.e. live systems based on DNA as information-bearing molecules, L-amino acids, D-sugars and a generally very conserved protein translation machinery. Despite the diversity of existing biological systems, they all share these basic building blocks and genetic software. Synthetic biology ultimately ambitions to emancipate biology from such constraints and create in the laboratory live objects based on other principles (Marliere et al., 2011; Marliere, 2009). While this is not yet at hand – and may not be in the near future – it is just a question of time that both organisms and properties new-to-nature (NTN) will be assembled. When the time comes, it will be necessary to anticipate new safety and risk scenarios associated to these new agents on the background of the benefits that they can bring about as well (Schmidt and de Lorenzo, 2012). But, as long as we remain in the realm of such a familiar biology, we may well handle virtually any possible scenario involving the release of GEMs for the next 10-15 years. The problem in most cases is that of its proper and efficacious performance of the engineered agents and not any risk of ecosystem takeover.

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Chapter 8

The intentional release of micro-organisms into the environment: Challenges to commercial use

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Bioremediation involves the application of micro-organisms for the removal of contaminants from the environment. Bioremediation competes effectively with other remediation approaches, such as thermal desorption and incineration. Further innovation of this technology involves the development of geneticically engineered strains with enhanced biodegradability capabilities. At present, however, there have been very few reported examples where genetically engineered micro-organisms have been released into commercial bioremediations. The main reasons for this include the lack of knowledge of the environmental risks and benefits of releasing genetically modified organisms into a contaminated area. In addition, non-specialist stakeholder support is often overlooked and remains a crucial area for improvement if sustainable remediation is to continue to develop. This chapter focuses on the application and risks associated with bioremediation.

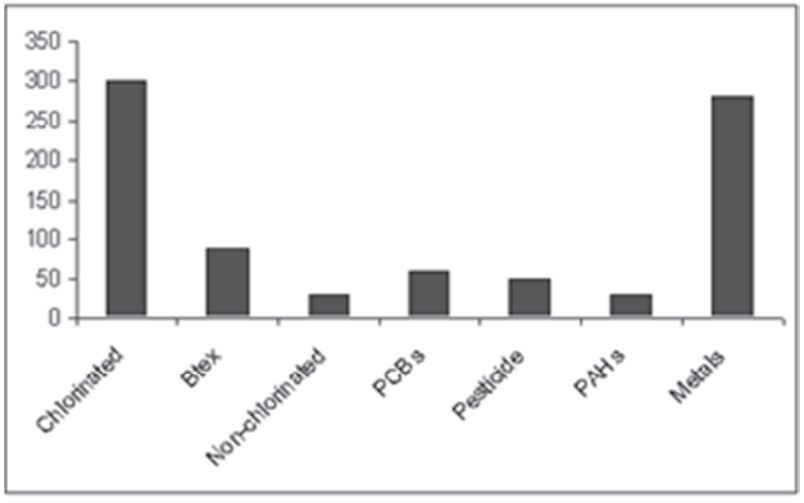
Introduction

The rapid expansion and increasing sophistication of the chemical industries in the past century, and particularly over the last 30 years, has meant that there has been an increasing amount and complexity of toxic waste effluents. It is estimated that there are around 60 000 chemicals in use in hospitals, households and in industry around the world; hundreds more are being introduced annually (Ball and Kadali, 2012). This has led to an unprecedented exposure of the environment to a vast array of chemicals. While most of the chemicals in use are used and subsequently disposed of correctly, it is inevitable that significant quantities of many of these chemicals will be released into the environment, becoming pollutants. This may occur in a number of ways, including (Ball, 2007):

- accidental release of chemicals during production and processing
- release of chemicals during use
- accidental release of chemicals during spillage
- deliberate release of the chemical into the environment.

At the same time, regulatory authorities have been paying more attention to problems of contamination of the environment. Industrial companies are therefore becoming increasingly aware of the political, social, environmental and regulatory pressures to prevent the escape of effluents into the environment. The occurrence of major incidents (such as the Union-Carbide (Dow) Bhopal disaster or the release of radioactive material in the Chernobyl accident, etc.) and the subsequent massive publicity due to the resulting environmental problems have highlighted the potential for imminent and long-term disasters in the public's conscience (Ball, 2007). Even though policies and environmental efforts should continue to be directed towards applying pressure on industry to reduce toxic waste production, bioremediation presents opportunities to detoxify a whole range of industrial effluents, as becomes clear from the example of an overview of toxic compounds and the number of sites where they occur in the United States (Figure 8.1).

Figure 8.1. **Number of sites in the United States that require treatment for pollution**



Note: Btex = benzene, toluene, ethylbenzene, xylene.

Once released into the environment, depending on the nature of the pollutant, the chemical can be found in air, soil and water. For example, if the pollutant (e.g. benzene) is in a gaseous state under atmospheric temperature and pressure, it will largely be found in the gaseous phase, while a solid contaminant (e.g. lead) will be found largely in soils or sediments (Ball, 2007). These pollutants can be found in air, water or soil and can be metals or organic compounds not normally found in nature. Once released into the environment, these pollutants may either be broken down or may persist until they are detected and quantified and their potential risk assessed. It may be that the pollutant(s) have to be removed and degraded, or degraded *in situ* (Figure 8.2) (Ball and Kadali, 2012).

Figure 8.2. **Sampling of groundwater for determination of hydrocarbon contamination**



Source: Andrew S. Ball, RMIT University, Australia

Remediation technologies

A number of options exist for the disposal (remediation) of pollutants found in the environment. These include (Ball, 2007):

- Incineration: the process of the destruction of a pollutant through conversion to carbon dioxide and water through combustion with the residue of incombustible material forming an ash residue.
- Burying: disposal of a pollutant by placing it in a sanitary landfill, which is engineered in a manner that protects the environment from the pollutant.
- Solidification: encapsulation of the pollutant in cement which after hardening can be disposed of safely in a landfill.
- Thermal desorption: this is an environmental remediation technology that utilises heat to increase the volatility of contaminants such that they can be removed from the soil. The volatilised pollutants are then collected or thermally destroyed.
- Bioremediation: the application of biological treatment to the cleanup of hazardous chemicals by metabolic conversion into non-toxic substances (Cookson, 1995).

This chapter focuses on the application and risks associated with bioremediation and consequently will focus on this sustainable remediation technology.

Bioremediation

The advantage of using bioremediation rather than digging up the contaminated soil and placing it elsewhere is that only moderate capital investment is required as the process is low in energy input. In addition, the processes are environmentally safe, do not generate waste and are self-sustaining. In many cases, bioremediation not only offers a permanent solution to the problem, but is also cost effective. Cleaning up existing terrestrial environmental contamination in the United States alone can cost as much as USD 1 trillion. Bioremediation can help reduce the costs of treatment as follows (Ball and Kadali, 2012):

- Treating contamination in place: most of the cost associated with traditional cleanup technologies is associated with physically removing and disposing of contaminated soils. Because engineered bioremediation can be carried out in place by delivering nutrients to contaminated soils, it does not incur removal-disposal costs.
- Harnessing natural processes: at some sites, natural microbial processes can remove or contain contaminants without human intervention. In these cases where intrinsic bioremediation (natural attenuation) is appropriate, substantial cost savings can be realised.
- Reducing environmental stress: because bioremediation methods minimise site disturbance compared with conventional cleanup technologies, post-cleanup costs can be substantially reduced.

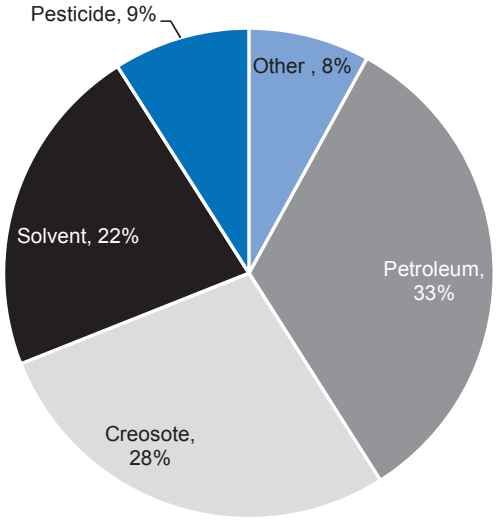
As a technology, bioremediation has a global application. In the United Kingdom alone it has been estimated that there are some 100 000 sites, which will take between GBP 10 000 million and GBP 20 000 million to clean up. In terms of the nature of the bioremediation process used, this depends greatly on the nature and quantity of the pollution. Nevertheless, bioremediation is an applicable technology for a range of pollutants. Figure 8.3 shows the range of industries that use bioremediation as a technology (Ball, 2007).

Technologies involved in bioremediation

In terms of technologies utilised within the wider remit of biotechnology, a number of specific terms are used to describe the activity of micro-organisms and the way they are used (Ball, 2007). This section discusses the main ones.

Monitored natural attenuation (intrinsic bioremediation) is one method of applying *in situ* bioremediation. One component of natural attenuation is the use of indigenous micro-organisms to degrade the contaminants of concern without human intervention (such as supplementing the available nutrients). Site characterisation and long-term monitoring comprise the activities required to implement natural attenuation. Long-term monitoring is used to assess the fate and transport of the contaminants compared against the predictions. The reactive transport model can then be refined to obtain better predictions. Natural attenuation processes typically occur at all sites, but to varying degrees of effectiveness depending on the types and concentrations of contaminants

Figure 8.3. **Range and weighting of industries that utilise bioremediation**



present and the physical, chemical and biological characteristics of the soil and groundwater. As they rely on naturally available micro-organisms in each site in combination with abiotic processes, natural attenuation processes may reduce the potential risk posed by site contaminants in three ways:

- the contaminant may be converted to a less toxic form through destructive processes, such as biodegradation or abiotic transformations
- potential exposure levels may be reduced by lowering of concentration levels (through destructive processes or by dilution or dispersion)
- contaminant mobility and bioavailability may be reduced by sorption to the soil or rock matrix.

In situ bioremediation (ISB) is the use of micro-organisms to degrade contaminants in place with the goal of obtaining harmless chemicals as end products. Most often, *in situ* bioremediation is applied to the degradation of contaminants in saturated soils, although bioremediation in the unsaturated zone can occur. ISB has the potential to provide advantages such as complete destruction of the contaminant(s), lower risk to site workers and lower equipment/operating costs. ISB can be categorised by metabolism or by the degree of human intervention. At a high level, the two categories of metabolism are aerobic and anaerobic. The target metabolism for an ISB system will depend on the contaminants of concern. Some contaminants (e.g. fuel hydrocarbons) are degraded via an aerobic pathway, some anaerobically (e.g. carbon tetrachloride) and some contaminants can be biodegraded under either aerobic or anaerobic conditions (e.g. trichloroethene).

Accelerated *in situ* bioremediation is where substrate or nutrients (termed biostimulation) are added to an aquifer to stimulate the growth of a target consortium of bacteria. Usually the target bacteria are indigenous; however, enriched cultures of bacteria (from other sites) that are highly efficient at degrading a particular contaminant can be introduced into the aquifer (termed bioaugmentation). Accelerated ISB is used where it is desired to increase the rate of contaminant biotransformation, which may be limited by lack of required nutrients, electron donor or electron acceptor. The type of

amendment required depends on the target metabolism for the contaminant of interest. Aerobic ISB may only require the addition of oxygen, while anaerobic ISB often requires the addition of both an electron donor (e.g. lactate, benzoate) as well as an electron acceptor (e.g. nitrate, sulfate). Chlorinated solvents, in particular, often require the addition of a carbon substrate to stimulate reductive dechlorination. The goal of accelerated ISB is to increase the biomass throughout the contaminated volume of aquifer, thereby achieving effective biodegradation of dissolved and sorbed contaminant.

The addition of either nutrients or micro-organisms generally bring about an increase in the rate of bioremediation, but the increased cost of utilising this approach ensures that their application is based around the particular requirements of the remediation. For example, if the site is to be built upon shortly, enhancing the natural rate of remediation through the addition of biostimulation and/or bioaugmentation may be necessary and cost effective. In contrast, if the site is to be left for some time (i.e. years) then monitored, natural attenuation will generally be employed as it is the most cost-effective bioremediation.

Environmental risks of bioremediation

In terms of deleterious effects of bioremediation on the environment, there are several potential problems which may arise:

- Firstly, there is the scenario that the bioremediation fails and the contaminant remains in the environment. This may be a result of the low bioavailability of the compound or perhaps pollutant toxicity.
- Secondly, there is the possibility that the bioremediation has resulted in only a partial breakdown of the pollutant. If the intermediate product is more toxic than the original compound, then this will lead to greater environmental damage. This has been observed during the degradation of polychlorinated ethene in groundwater where a more toxic intermediate, vinyl chloride, has been the main product of bioremediation rather than ethene.
- Thirdly, if biostimulation has been employed, then there is the possibility that the treatment itself (e.g. addition of nutrients such as nitrogen and phosphorus) may cause deleterious effects on the environment through increased nutrient availability, which in soils would mean the release of nutrients into surface water resulting in increased eutrophication leading to algal blooms.
- Finally, if bioaugmentation is employed, the addition of an organisms not native to that environment is added, there is an inherent risk that these organisms may significantly affect the functionality of the natural microbial community, causing deleterious effects on the environment.

In general, bioremediation utilises the natural ability of mixed populations of micro-organisms. The dynamics of such populations are complex and the potential for use of a released organism to enhance the bioremediation process therefore depends both on the environment and the nature of the pollutant (Aleer et al., 2011). However, with the release of any organism in the environment, the risk of utilising such a strategy must be fully considered. In some countries (e.g. Australia) the likelihood of being able to obtain permission from a body such as the Environmental Protection Agency to release a micro-organism is very small. However, in other countries (e.g. the United States), it is

more likely to be permitted if a case is made. Nonetheless, this obstacle remains a significant challenge to the commercial use of micro-organisms in many countries.

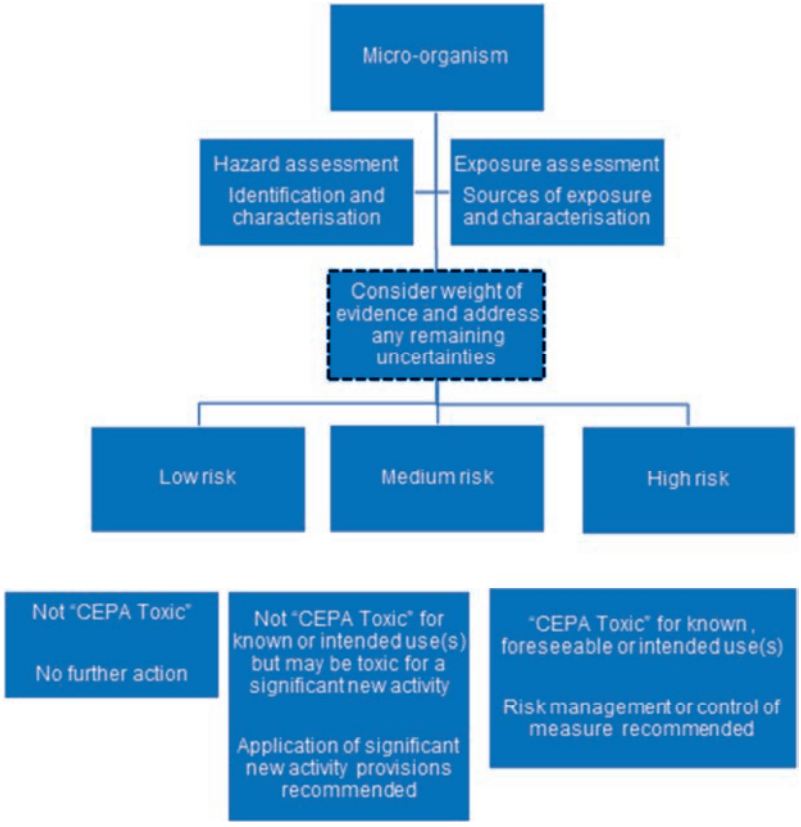
Nature protection and the introduction into the environment of micro-organisms

Given the potential risks associated with the release of micro-organisms into the environment, many countries have developed strategies and protocols for risk assessment. The approaches taken generally use the paradigm that risk is proportional to the product of hazard and exposure:

$$\text{Risk} \propto \text{Hazard} \times \text{Exposure}$$

For example, the Canadian EPA has established guidelines for risk assessment and a safety mechanism (Figure 8.4).

Figure 8.4. **Canadian draft guidelines for risk assessment for the release of micro-organisms into the environment**



Source: Department of Health and Ageing and enHealth Council (2002), "Environmental health risk assessment: Guidelines for assessing human health risks from environmental hazards", Commonwealth of Australia, Canberra, www.nphp.gov.au/enhealth/council/pubs/pdf/envhazards.pdf.

In terms of hazard assessment, this involves characterisation of the micro-organism and identifies the potential adverse effects on the environment and/or human health and predicts the extent and duration of these effects. This characterisation involves:

- taxonomic identification, for risk assessment purposes (OECD, 2003)

- assessment of strain history in terms of any known pathogenicity
- record of any genetic modifications related to the proposed strain
- the potential of the organism in terms of its potential for horizontal gene transfer
- consideration of the biological and ecological properties of the organism
- examination of any information relating to previous release.

As a result of the hazard assessment and together with the other assessments listed in Figure 8.3, the application is categorised into one of three risk estimates (Slovic, 1987; 1997):

- High risk: a determination of high risk implies that severe, enduring or widespread adverse effects are probable for exposure scenarios predicted from known, foreseeable or intended uses; control measures or risk management would be recommended.
- Medium risk: a determination of medium risk implies that adverse effects predicted for probable exposure scenarios may be moderate and self-resolving. In this case, use may be recommended with monitoring.
- Low risk: a determination of low risk implies that any adverse effects predicted for probable exposure scenarios are rare, or mild and self-resolving.

Examples of use of released micro-organisms in bioremediation

Over the last decade, a number of companies have been established to develop and commercialise biodegradation technologies. For example, one bioremediation company, Envirogen (New Jersey), has developed recombinant PCB (polychlorinated biphenyl) degrading micro-organisms with improved stability and survivability in mixed populations of soil organisms. The same company has also developed a naturally occurring bacterium that degrades trichloroethylene (TCE) in the presence of toluene, a toxic organic solvent killing many other micro-organisms. However, the use of microbes for bioremediation is not limited to detoxification of organic compounds. In many cases, selected microbes can also reduce the toxic cations of heavy metals (such as selenium) to the much less toxic and much less soluble elemental form. Other commercially available products include BioWorld Augmentation, which represents "a group of specific micro-organisms selected for each type of contaminant". Recently RemActiv™ has been introduced into the market; this is a liquid additive that contains selected micro-organisms and a specially formulated nutrient mix.

Feedback on the use of these organisms as a bioaugmentation treatment is mixed. This is not unexpected as the environmental conditions under which these additives operate effectively are limited and as every commercial bioremediation represents a unique set of pollutants and environmental conditions, it is not surprising that under some conditions, treatment is effective while under other conditions, treatment can be less effective or even ineffective. However, throughout the deployment of augmented organisms in the environment for remediation of a range of contaminants, to the best of the author's knowledge, there have been no reports of any detrimental environmental effects caused by the released micro-organism. This is an important observation, confirming that bioremediation represents a sustainable and environmentally safe

technology, provided that due testing and analysis in both the laboratory and the field have been completed prior to full-scale treatment.

Challenges to commercial use of bioremediation technologies

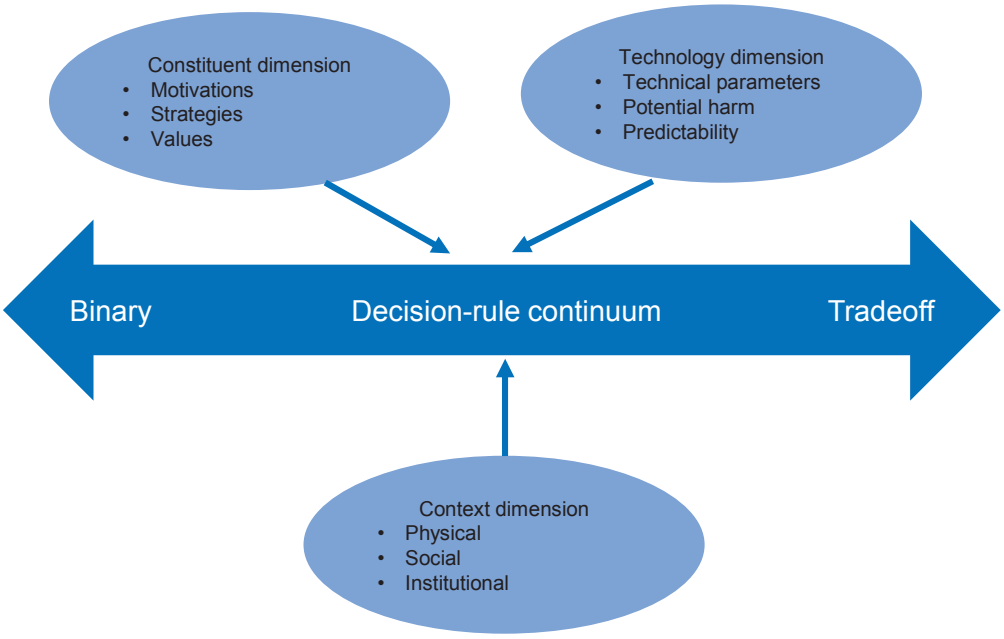
As this chapter has shown, bioremediation offers the possibility of technically effective and relatively less expensive remediation. Assuming that the promise of bioremediation strategies are realised, why would anyone object to using these natural treatments? A failure to anticipate issues that can derail plans to deploy any technology, including bioremediation, can be problematic (Axlerod, 1994). While some issues may revolve around the technical aspects of bioremediation, others may derive from non-technical, social concerns. Site-specific bioremediation decision making can be viewed as a social process that is informed by scientific and technical data, rather than as a physical process. While it is not asserted that bioremediation represents a controversial technology, the use of a simple clean-up option may become controversial (Priest, 1994). Bioremediation encompasses a suite of potential remediation options whose remediation targets, mechanisms and capabilities differ. Therefore, generic questions about the suitability of bioremediation have limited applicability to the particular situations in which it might be considered for deployment. Yet, neither is every possible permutation of contaminant, site, remediation mechanism and remediation goal likely to produce a unique social response. The approach probably lies somewhere in the middle – an exploration of the generic factors that may influence patterns of social responses to specific bioremediation applications (Hagedorn and Allender-Hagedorn, 1997).

To date, there have been relatively few systematic studies of social responses to bioremediation. However, a recent study (Conroy and Ball, unpublished data) suggests that a lack of education in terms of understanding the biological basis of the technology remains a barrier. Therefore, despite increasing applications of bioremediation, social issues related to its deployment have not been documented. While bioremediation may prove to be socially acceptable for cleaning up contamination, it may not be fully acceptable either across the suite of approaches it encompasses or across the range of sites at which it is proposed for deployment (Stern and Dietz, 1994; Davison et al., 1997). Further, the acceptability of this technology should be viewed as multidimensional instead of one-dimensional (e.g. as only as a matter of risk, or risk communication, or education). Acceptability evolves over time through interactions with individuals and organisations, and in response to new technical and non-technical information (Eagly and Kulsea, 1997). Without systematic data, complete analysis of the social dimensions of bioremediation cannot be undertaken. Instead, a systematic approach to identifying and analysing the social determinants of the acceptability of bioremediation can be made. This approach relies on a conceptual framework and draws from published literature to illustrate the attributes of bioremediation and its use.

Although the technology is based on natural processes and does not involve the use of genetically modified organisms, public concerns are centred on the apparent "lack of activity on site" which leads to a public perception that no real "effective treatment" is being applied to the site. To gain a better understanding of social acceptability issues and to improve the ability to predict outcomes in deliberations over the social acceptability of controversial technologies, Wolfe and Bjornstad (2002) developed a conceptual framework for organising what was perceived to be the most important issues. The resulting framework, PACT (Public Acceptability of Controversial Technologies), provides a common logic through which to view site-specific decision making about

remediation technologies (Figure 8.5). The PACT is built around dimensions that operate to influence decision-oriented dialogs over controversial remediation technologies in any location.

Figure 8.5. **Overview of public acceptability of controversial technologies (PACT), used to assist in site-specific decision making about remediation technologies**



Source: Wolfe, A.K. and D.J. Bjornstad (2002), "Why would anyone object? An exploration of social aspects of phytoremediation acceptability", *Critical Reviews in Plant Sciences*, Vol. 21, No. 5, pp. 429-438.

The factors relevant to specific decision settings and technologies varies from situation to situation. This PACT-based analysis focuses on an array of attributes that could strongly influence acceptability. In this context, acceptability refers to participants' willingness to consider the technology in question as a viable alternative, rather than to whether the technology ultimately is deployed. The PACT provides a framework through which to see how participants' position changes over time, from absolute positions of support or opposition at one extreme to completely negotiable positions at the other. Changes in positions may be related to any of the PACT's dimensions – from decisions about who should or should not participate in decision making to the kinds of technologies worth considering.

Conclusion

Bioremediation is now a successful environmental biotechnology, having a number of advantages (e.g. cost, environmentally friendly means of disposal) over any alternative treatment of contaminated land such as landfilling or incineration. There exist large areas of the world where contaminated land can be found, constituting an environmental and health hazard. Bioremediation offers the opportunity to utilise the natural microbial population to treat the contaminated site, returning the elements making up the contaminants to the natural nutrient cycling. However, each application varies with contaminants and environmental conditions and therefore there is no single "off the shelf"

solution for effective treatment. For petrogenic hydrocarbons, the natural microbial community often performs better than any introduced micro-organisms. For chlorinated hydrocarbons, the addition of non-genetically modified halorespiring organisms into an environment has proved successful in both North America and Europe. Whilst there exists a market for microbial inocula, the potential application and use of genetically modified organisms has yet to be realised. One of the main limitations to the use of this technology is social acceptance (Hoban et al., 1992). Applying the PACT to bioremediation reveals flaws in the typical one-dimensional method often used for gaining technology acceptance (e.g. educating the public about the technology and its benefits or communicating effectively the attributes of the technology in question).

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Part IV

The use of micro-organisms in cleaning products

Chapter 9

Microbial-based cleaning products in use and the potential role of transgenic micro-organisms

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This chapter provides a survey of the currently known uses of micro-organisms in different types of cleaning products based on searches conducted of publicly available information sources such as the scientific literature, patent databases and commercial websites. Examples of microbial species known to be used in different types of cleaning applications will also be given as well as potential human health and environmental issues associated with their use. A brief summary of Canadian regulatory experiences with these products, in particular those of the New Substances Program of Health Canada and Environment Canada, will be provided as well.

Introduction

Cleaning products are familiar to virtually everyone who lives or works in any kind of domestic residential setting, commercial place of business or institutional setting such as hospitals or daycare centres. Because of their widespread use, they are a large industry in many countries, including the United Kingdom (>GBP 3 billion in 2011) and the United States (USD 30 billion in 2010) (UK Cleaning Products Industry Association, 2011; American Cleaning Institute, 2012). Exact figures for sales of cleaning products in Canada could not be found, but it appears that a significant portion of the CAD 20 billion industry on consumer specialty products consists of soaps, detergents, disinfectants, sanitizers and air care products (i.e. deodorisers) (Canadian Consumer Specialty Products Association, 2012a; 2012b).

Cleaning products are mostly liquid formulations (although many come in powder form) used by consumers, typically in domestic settings, or by cleaning professionals in larger business or institutional settings. Any visit to a local supermarket, hardware or home renovation store indicates that the vast majority of cleaning products currently on the market in North America and Europe continue to contain chemical substances that tend to be reactive or corrosive in nature. Examples of these include solutions of sodium hypochlorite (household bleach), sodium hydroxide (found in many detergents and drain cleaners) and ammonium hydroxide (used in hard surface cleaners). Because of their reactive nature and their widespread use, these substances are very often a concern for human health effects as well as environmental impacts. In some cases, inappropriate mixing of some of these products have produced toxic chlorine and ammonia gases leading to acute poisoning and illness as well as more chronic effects (Nazaroff and Weschler, 2004).

In recent years, cleaning products containing various strains of micro-organisms as active ingredients have become increasingly prevalent in many countries as an alternative to chemically based cleaning products. These products appear to be increasingly sold for use in many of the domestic, commercial and institutional settings mentioned above, as well as for a variety of cleaning activities (hard surface cleaning, odour control, degreasing, septic tank treatments, etc.) where chemically based cleaning products have traditionally been used. Many of these products are very often advertised and described as "environmentally friendly", "biodegradable" and "non-toxic". These products are part of the larger category of "green cleaning products" that are available in supermarkets and hardware stores, and are very often advertised and sold online (an Internet search using a few relevant key words such as "bacteria" + "cleaning" + "green" + "enzyme", etc. produces many examples of these). Although microbial-based cleaning products are likely a relatively small portion of this market, it has been projected that the overall global market for green cleaning products may reach USD 9.32 billion by 2017 (PR Web, 2011).

The purpose of this chapter is to provide a survey of the currently known uses of micro-organisms in different types of cleaning products based on searches conducted of publicly available information sources such as the scientific literature, patent databases and commercial websites. Examples of microbial species known to be used in different types of cleaning applications will also be given as well as potential human health and environmental issues associated with their use. A brief summary of Canadian regulatory experiences with these products, in particular those of the New Substances Program of Health Canada and Environment Canada, will be provided as well as a proposal for a workshop to be hosted in Canada to further examine and discuss these and other issues.

Survey of microbes currently used in cleaning products

Known uses of these products

Table 9.1 provides a broad sample of what has been found through a search of publicly available information (scientific literature, patent databases, commercial websites, etc.) on current uses of microbial-based cleaning products and the types of micro-organisms they contain.

It thus appears that microbes (both as vegetative cells and as spores) are found in a wide variety of cleaning products and treatment applications where chemical agents have traditionally been applied for the same end uses. It should be noted that a large number of additional commercial websites were found advertising the sale of such products but without providing any specific details on the formulation of their products.

Although it is not within the scope of this chapter, there appears to be little publicly available information (aside from anecdotal evidence such as product testimonials) on the effectiveness of these products.

Microbial species used in these products

This section provides brief summaries of some of the microbial species that have been identified as being the active ingredients in these products.

Bacillus *spp.*

The most prevalent microbial species contained in these products appear to be those from the genus *Bacillus*. Most *Bacillus* species are commonly found soil micro-organisms which have the ability to form endospores in response to extreme environmental conditions. Of these, *B. subtilis* appears to be the one the most commonly identified. It is generally considered to be non-pathogenic and has been used as a probiotic and in the production of fermented foods (Hong et al., 2008) as well as a production organism for enzymes in detergents (Adisesh et al., 2011). *B. licheniformis* and *B. amyloliquefaciens* strains have also been used for this purpose (Adisesh et al., 2011). *B. polymyxa* strains have also been used as production organisms for topical antibiotics (Gelmetti, 2008).

Other bacterial genera

A variety of other bacterial genera appear to be represented in these products, many of which are not identified to the species level. These include *Achromobacter*, *Actinobacter*, *Alcaligenes*, *Arthrobacter*, *Rhodopseudomonas*, *Rhodobacter* and *Lactobacillus*. Of these, *Lactobacillus* is perhaps the best known, various species of which have been used as probiotics and in food production, and are generally considered non-pathogenic (Wassenaar and Klein, 2008). *Achromobacter* species are commonly found in fresh water and marine environments and are considered, among other things, as "beneficial bacteria" for use in aquaculture operations (Zhou et al., 2009). Various literature was found describing how species of some of these genera have been found to degrade various xenobiotic compounds (for example, see Perez-Pantoja et al., 2009). Other examples of this include various species of *Alcaligenes*, *Arthrobacter* and *Rhodopseudomonas* that have been found to degrade textile azo dyes (Xingzu et al. 2008; Pearce et al., 2003).

Table 9.1. **Micro-organisms used in cleaning products**

Product/process	Use	Micro-organisms in formulation	Number of micro-organisms in formulation	Information source/website
Liquid cleaner	Improve surface cleaning	Primarily *Bacillus subtilis*. Other potential organisms: *Pseudomonas*, *Arthrobacter*, *Enterobacter*, *Citrobacter* and *Corynebacter*	$1x10^6$ – $1x10^9$ /ml	Sybron Chemical Industries WIPO: EP0245560
Detergent	Soap	Lactic acid bacteria, yeast and photosynthetic bacteria	Yeast: $1×10^5$ /ml Lactic acid bacteria: $1.5×10^5$ / ml	EM Res Organization Inc. WIPO: EP1717301
Detackifying organic solvent	Degradation of water-borne paint containing high levels of organic solvent	Primarily *Bacillus subtilis, Pseudomonas fluorescens*. Other organisms used include: *Bacillus* sp., *Citrobarobacter* sp., *Aeromonas* sp., *Shewanella* sp., *Pseudomonas* sp., *Corynebacterium* sp., *Rhodococcus* sp. or a mixture thereof.	Not provided	Atotech Deutschland GMBHDEUTSCHLAND GMBH WIPO: WO2008094872
Cleaning solution	Removal of hydrocarbons	*Achromobacter*, *Actinobacter*, *Alcaligenes*, *Arthrobacter, Bacillus, Flavobacterium* and *Pseudomonas* species, and mixtures thereof. Preferred are those naturally occurring of the genus *Bacillus*, particularly *B. subtilis, B. licheniformis* and *B. polymyxa*	Not provided	Earth Alive Resources Inc. WIPO: WO2002033031
Cleaning agent	Enhance fermentation in soap production process	*Lactobacillus* group, yeast group and photosynthetic bacterium group	Not provided	EM Research Organization WIPO: KR1020070003956
Stable antimicrobial composition including spores, bacteria, fungi and/or enzymes	Various cleaning applications	Suitable spores (bacterial or fungal), vegetative bacteria, fungi or enzymes from *Bacillus*, *Pseudomonas*, *Arthrobacter, Enterobacter, Citrobacter, Corynebacter, Nitrobacter* species, mixtures thereof or the like; *Acinetobacter, Aspergillus, Azospirillum, Burkholderia, Ceriporiopsis, Escherichia, Lactobacillus, Paenebacillus, Paracoccus, Rhodococcus, Syphingomonas, Streptococcus, Thiobacillus, Trichoderma, Xanthomonas, Lactobacillus, Nitrosomonas, Alcaligenes, Klebsiella* species, mixtures thereof or the like.	$1x10^3$ – $1x10^9$ cfu/ml	Ecolab USA Inc. USPTO: 8211849 20110207649
Composition comprising organic matter and micro-organisms	Deodorizer, degreaser, mould inhibitor	Phototrophic bacteria belonging to: *Rhodopseudomonas, Rhodobacter, Rhodospirillum, Chromatium and Chlorobium*; lactic acid bacteria belonging to: *Lactobacillus, Propionibacterium, Pediococcus* and *Streptococcus* and yeasts: *Saccharomyces* and *Candida*	Not provided	USPTO: 20080085546
Probiotic micro-organism for the reduction of manure odour	Odour control	Micro-organisms belonging to the genus *Lactobacillus* or yeast belonging to the genera *Cryptococcus*, *Kluyveromyces*, *Candida* or *Metschnikowia*	Yeast content: 1.4×10^8 cfu *Lactobacillus* content: 5.4×10^9 cfu	OrganoBalance GmbH USPTO: 20110117068
Multi-action drain cleaning composition and method	Drain cleaning	*Bacillus subtilis* ATCC 6051, ATCC 14415 and ATCC 35946, *Bacillus licheniformis* ATCC 6598 and ATCC 1194, and *Bacillus polymyxa* ATCC 12060	Not provided	Organica Biotech USPTO: 20090263884

Table 9.1. **Micro-organisms used in cleaning products** (*cont.*)

Product/process	Use	Micro-organisms in formulation	Number of micro-organisms in formulation	Information source/website
Enz-Odor® 4, Enz-Odor® 4-2XFF , Nu-Bind 1* etc	Odour control agents	Liquid products containing exclusively spore-forming *Bacillus* species with preservatives, with or without additional ingredients	On average 2 x 10^9 cfu / g	Alken Murrary Corp: www.alken-murray.com/Odorindex.html; United States
EM-1 microbial inoculant	Aquarium/fish pond treatment	*Rhodopseudomonas palustris* and other unidentified phototropic and lactic acid bacteria, yeast	Not provided	Emerald Earth: www.emearth.com/NewFiles/Aquariums.html; United States
Clear-Flo (various products)	Surface cleaners	*Bacillus subtilis* strains	Various concs.; link provides extensive list of products	Alken Murrary Corp: www.alken-murray.com/CleaningIndex.html; United States
Drainbo	Drain cleaner	Several strains of *Bacillus*	Not provided	Drainbo The Natural Solution: www.drainbo.com/news.html; United States
Bio Source Zymo Super Concentrated Drain Cleaner, Green Bin Deodorizer	Drain cleaner and deodorizer	*Bacillus* cultures and substrains	Not provided	BioSource Solutions Inc.: www.biosourcesolutionsinc.com/site/products; Canada
Various cleaning products	Household drain cleaners and degreasers, septic tank additives and general cleaning products	*Pseudomonas aeruginosa* ATCC 31480, 700370 and 700371	Not provided	www.chemicalsubstanceschimiques.gc.ca/fact-fait/glance-bref/pseudomonas-eng.php; Canada
EcoWorks (various cleaning products)	Washroom cleaner, multi-surface cleaner, odour eliminator	*Bacillus* strains	5.25 x 10^7 /ml, equivalent to 200 billion/5 litres	www.eco-works.co.uk/probiotic-cleaning-liquids.html; United Kingdom
BioPure	Household cleaner	*Lactobacillus* cultures	Not provided	Pure Alternatives: www.purealternatives.net/biopure.html; Australia
EM-1 septic treatment	Septic tank additives/ treatment	Mixture of *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus delbrueckii, Bacillus subtilis, Saccharomyces cerevisiae, Rhodopseudomonas palustris*	1 million cfu/ml	Teraganix: www.teraganix.com/32oz-EM-1-Septic-Treatment-p/1022.htm; United States
Sporyzme BCC	Degrade organic waste and eliminate associate odours	Blend of *Bacillus* spores	5.0 x 10^{10} cfu/gm	NovoZymes: www.waterguru.net.au/pdfs/Sporzyme%20BCC%20PDS-1.pdf; United States

Sources: World Intellectual Property Organization (WIPO), www.wipo.int/portal/index.html.en; United States Patent and Trademark Office (USPO), www.uspto.gov.

Fungal species

Some of the cleaning products found in the literature were declared to contain *Saccharomyces* and *Candida* species. It is common knowledge that a number of *Saccharomyces* species (such as *S. cerevisiae*) have a long history of safe use in the baking, brewing and winemaking industries. In recent years it has also been recognised that a number of yeast species, including some belonging to *Saccharomyces* and *Candida*, have the potential to be effectively used in the biodegradation of a variety of hazardous chemicals (Xiuyan et al., 2011; Harms et al., 2011).

Potential targets of gene modification

No information was found indicating that any of the micro-organisms contained in the above-mentioned cleaning products were genetically modified in any way. However, there are indications in the literature that some of the genes involved in producing enzymes or biosurfactants and bioemulsifiers whose mode of action involves the increased solubilisation and breakdown of organic substances could be modified to enhance some of their properties. Thus, it is at least possible that genetically modified micro-organisms could find their way into cleaning products in the future, although it is questionable whether such products would continue to be regarded as "green".

Enzymes

Some of the main targets for gene modification have been those coding for the production of various amylases and proteases used in detergent products, mainly with the aim of improving their activity at lower water temperatures and more alkaline pH levels (Kirk et al., 2002). For example, *B. subtilis* strains have been engineered to express some of these modified genes (Ness et al., 1999). As well, a number of recombinant lipase enzymes have been produced using engineered *Bacillus* and *Aspergillus* species (Hasan et al., 2010).

Biosurfactants/bio-emulsifiers

Much research has been conducted recently towards engineering improved versions of various biosurfactant and bio-emulsifying substances (such as surfactin, rhamnolipids and emulsans) for use in detergent and other cleaning product applications. For the most part, the aim of the research has been to increase yields of these substances when expressed in various bacterial species (mostly *Bacillus*, but also in a number of *Acinetobacter*, *Pseudomonas* and *Serratia* species as well). A thorough review of this research is provided in Satpute et al. (2010).

Potential human health and environmental issues

A number of potential human health issues related to the use of microbial-based cleaning products have previously been described in a recent report on the use of such products, mainly in Europe (Spok and Klade, 2009). Environmental issues may also potentially exist because of the widespread use of such products and releases into the environment that may result. These issues can be categorised as issues: *i)* related to the micro-organism itself; and *ii)* related to formulation/use of the product.

Issues related to the micro-organism itself

Likely the single most important issue related to the micro-organisms themselves is the reliability of their taxonomic designation. Many of the micro-organisms found in these products were identified only to the genus level. For those identified to the species level, little to no information is provided as to what methods or tests were used to arrive at their identification. Some of the products do appear to have used micro-organisms from well-known culture collections (such as the ATCC), thus providing somewhat increased confidence in their taxonomic designation. From an overall risk assessment perspective, reliable taxonomic designation of a given micro-organism is the most important determinant of its potential hazard to human health and environment (Environment Canada and Health Canada, 2011a). A reliable taxonomic designation allows for the appropriate assessment of a micro-organism's infectivity, virulence and overall pathogenicity. This includes its ability to produce toxins, toxic metabolites and allergens as well as potential effects on sensitive populations (e.g. the immunocompromised, children/elderly, pregnant women, etc.) (Spok and Klade, 2009; Environment Canada and Health Canada, 2011a).

Based on the micro-organisms identified as being contained in the products listed in Table 9.1, even a cursory survey of the scientific literature reveals that it is possible that some of these products may contain pathogens. For example, some toxin-producing strains of *B. licheniformis* have been identified in outbreaks of food poisoning (Mikkola et al., 2000). Another example is *Acinetobacter baumanii*, which has recently emerged as a cause of healthcare associated infections (Fournier and Richet, 2006). A third example is several *Candida* species, including *C. albicans*, considered to be opportunistic pathogens for which a number of different virulence factors have been identified (Yang, 2003). In cases like these, proper taxonomic designation of a micro-organism to at least the species level (and in some cases, the sub-species or strain level) becomes very important, since it can help to distinguish between pathogenic and non-pathogenic strains.

Issues related to formulation/use of product

As far as the products themselves are concerned, a number of issues have become apparent. Somewhat related to the issue of reliable taxonomic designation mentioned above is the issue of consistency in quality control (QC) and quality assurance (QA) methods applied during the production of the micro-organisms and/or the end products. There are indications from previous studies (Spok and Klade, 2009), as well as from past experiences of the New Substances Program in Canada, that there is a wide variation in how QC/QA methods are applied in the production of these products. This includes procedures in place to monitor for potential contaminants. Currently, no broadly recognised standards for the QC and QA of cleaning products exist. However, in Canada, the EcoLogo Program, a voluntary third-party certification programme for environmentally preferable products, requires that all biologically based cleaning and degreasing products be manufactured in a facility that has a documented QC/QA system (EcoLogo, 2011).

As well, there are currently no regulatory requirements for specifically identifying microbial ingredients in these products in Canada. Since many of these types of products appear to be imported into Canada, and because the active ingredients are very often considered confidential business information, importers, distributors and end users very often do not know what micro-organisms are present in these products. There also do not

appear to be any specific labelling requirements for these products in the European Union or in the United States. However, as of April 2011, the United States Environmental Protection Agency's voluntary programme "Design for the Environment" requires that all non-trade secret ingredients be listed for all products that carry the Design for the Environment label, including cleaning products. Non-trade secret ingredients also need to be described as specifically as possible without revealing trade secret information (United States Environmental Protection Agency, 2011).

Considering the way in which microbial-based cleaning products would typically be used, human exposure to the micro-organisms contained within them is likely to some extent. Dermal exposure is the most obvious route; however, spray applications and powders can create aerosols leading to inhalational exposure as well. To a lesser extent, oral ingestion may also be possible, particularly if these products are applied anywhere near surfaces used for food preparation. Long-term exposures may also be possible since many of these products appear to contain spores that can remain viable for long periods of time. All of these exposures may also be enhanced by the fact that many of these products will be used in indoor settings where proper ventilation may not always be in place. There currently appears to be a significant lack of information in the scientific literature on the nature and magnitude of potential human exposures to micro-organisms through their use in these products, thus making any attempt to more precisely assess human health risks from such products somewhat difficult.

Regulatory experiences in Canada with these products

In terms of systematically assessing any potential risks to human health and the environment from the use of such micro-organisms in cleaning products in Canada, only one legislative authority currently exists: the Canadian Environmental Protection Act, 1999 (CEPA1999; Department of Justice Canada, 2012a). Information and data required from manufacturers or importers of new micro-organisms subject to CEPA1999 that are contained in cleaning products are outlined in the New Substance Notification Regulations (Organisms) (NSNR(Organisms); Department of Justice Canada, 2012b). Screening assessments are also currently being conducted on "existing" microbial strains found on the Canadian Domestic Substances List (DSL) (Environment Canada, 2012).

Assessments of "new" micro-organisms in cleaning products in Canada

Since 2000, four new (i.e. not on the DSL) micro-organisms intended for use in various types of cleaning applications were notified and assessed for potential risks to human health and the environment under CEPA1999. These applications included drain cleaning, carpet cleaning, in grease traps and in odour control. All four notified micro-organisms were *Bacillus* species, including strains of *B. subtilis, B. megaterium* and *B. pumilus*. None of these strains were genetically modified. Three of these strains were obtained from or have been deposited into well-known culture collections such as the American Type Culture Collection (ATCC) (American Type Culture Collection, 2012) or that of the United States' Department of Agriculture's Agricultural Research Service, also known as the NRRL collection (United States Department of Agriculture, 2011). The fourth was an environmental isolate.

Information substantiating the taxonomic designation of the notified micro-organism is the cornerstone of these assessments. A "polyphasic" approach is usually recommended, which typically involves any combination of information/data on cell and colony morphology, nutrient requirements, biochemical/metabolic testing (e.g. substrate

utilisation) and molecular and/or genotypic testing (e.g. fatty acid methyl ester – FAME, 16S rRNA, etc.). Typically, a taxonomic designation to the species level is expected. However, the primary goal of this approach for the purposes of conducting a CEPA1999 assessment would be to distinguish between potentially pathogenic and non-pathogenic strains. The assessment outcome in all four cases was "no suspicion of toxic" according to the definition of "toxic" found in Section 64 of CEPA1999.

DSL micro-organisms in cleaning products in Canada

The DSL is a list of all substances (chemicals, polymers and living organisms) that were: *i)* in Canadian commerce between 1 January 1984 and 31 December 1986; or *ii)* added to the list following notification and risk assessment, in accordance with CEPA1999. The list currently contains 67 microbial strains and 2 complex microbial cultures. Sixty-eight micro-organisms currently on the list were nominated based on the in commerce provisions described above. One complex microbial culture was added to the DSL following notification and risk assessment as a "new" substance, in accordance with the NSNR (Organisms). The current list of DSL micro-organisms can be viewed at Environment Canada (2011). All micro-organisms nominated to the DSL that have the potential to cause harm to human health or the environment must undergo a screening assessment as required under paragraph 74*(b)* of CEPA1999.

To establish whether micro-organisms on the DSL continue to be manufactured in or imported into Canada, a notice pursuant to paragraph 71(1)*(a)* of CEPA1999 was published in Part I of the *Canada Gazette* on 3 October 2009 for the 45 micro-organisms that were on the list in October 2009. Since then, 23 strains have been added to the DSL and these were not subject to this notice.

Based on information submitted by manufacturers and importers as part of the DSL nomination process as well as on the survey conducted as part of the CEPA1999 §71 notice mentioned above, 14 strains were found to be used in various types of cleaning products. These products included drain cleaners, degreasers, deodorizers/odour control, septic tank additives and aquarium/pond treatments. Several strains considered to be risk group 2 pathogens are among them. This information is based on activities that have occurred since 1984, so in almost all of these cases it is not clear whether these risk group 2 pathogens continue to be used in these products today. For example, there is no available information indicating that any of the three strains of *Pseudomonas aeruginosa* on the DSL are currently used in any type of cleaning products in Canada. However, a search of publically available information (Internet, patent databases) suggests that *P. aeruginosa* strains may possibly be found as active ingredients in commercial and household drain cleaners and degreasers, septic tank additives and general cleaning and odour-control products (Environment Canada and Health Canada, 2011b).

Knowledge gap in the use of Microbial-based cleaning products

Considering the current state of knowledge of the use of microbial-based cleaning products in Canada, the United States and Europe, it has become evident that there are significant gaps in terms of what is known about the extent of commercial and domestic use of these types of products as well as the specific strains of micro-organisms used as the active ingredients. These and other issues are to be the focus of a proposed international workshop on the subject of microbial-based cleaning products which will attempt to assemble stakeholders from government, industry, academia and public advocacy groups. Some of the more specific issues can include:

- Information gathering to fill in knowledge gaps: this includes information on the portion of the "green cleaners" market made up of microbial-based products, the specific microbial strains used in the different types of products, the extent of commercial and domestic uses of these products, their effectiveness compared to chemically based cleaners, etc.
- Industry stakeholder engagement: many of the information gaps identified above as well as other issues related to these types of products may not reliably be addressed unless there is engagement with industry stakeholders who could potentially benefit in the long-term by being more publically transparent about their products and thus gain greater public confidence in the safe use of these products.
- Human exposure scenarios: another significant knowledge gap which will need to be addressed in cases where more comprehensive risk assessments of the micro-organisms involved are deemed necessary.
- Environmental impacts: although environmental impacts are not expected as a direct result of their use, issues may arise should microbial-based cleaning products be manufactured, imported and/or used in exponentially greater quantities than what is currently known. These could result in significant environmental releases that may warrant greater scrutiny from a regulatory oversight perspective.
- Evaluation of current regulatory/policy frameworks: a re-evaluation of current regulatory and policy frameworks may be necessary once the above-mentioned issues are more thoroughly examined. This can include an evaluation of the most appropriate instruments (e.g. regulations, standards, codes of practice, etc.) to use for strengthening these frameworks to mitigate risks to human health and the environment without undue burden on the industry manufacturing and/or importing these products.

Conclusion

Based on the currently available scientific literature and information on microbial-based cleaning products, it appears that genetically modified micro-organisms could potentially play a significant role in the production of modified enzymes with enhanced properties for use as active ingredients in cleaning products for a variety of applications. However, currently known use patterns for these products may involve significant human exposure. As well, public perceptions regarding genetically modified organisms continue to be generally unfavourable. Thus, there is little indication at the present time that genetically modified micro-organisms themselves will find their way into commercially available microbial-based cleaning products as active ingredients in the foreseeable future.

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Chapter 10

Microbes in cleaning products: Regulatory experience and challenges for risk assessment

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This chapter: i) *provides an overview of the technology, products and applications of the use of micro-organisms in cleaning products;* ii) *discusses the application of existing legislation;* iii) *identifies and discusses possible environmental and health risks as well as environmental benefits; and* iv) *provides recommendations to regulators for further research and policy action.*

Introduction

Over recent years, consumer and environmental organisations have become increasingly aware of a novel type of cleaning products containing living micro-organisms as active ingredients (subsequently termed "microbial cleaners"). Given the lack of both general information on microbial cleaners in the public domain and product-specific information from developers, these organisations highlight difficulties in considering these products when providing recommendations to the public and private sector for green procurement. Information is considered to be particularly scarce on the environmental properties, health risks and efficacy of the cleaning products. Furthermore, it is not clear which legal regulations are governing the safety and marketing of these products.

Against this backdrop, this chapter: *i)* provides an overview on the technology, products and applications; *ii)* discusses the application of existing legislation; *iii)* identifies and discusses possible environmental and health risks as well as environmental benefits; and *iv)* provides recommendations to regulators for further research and policy action.

The analysis is based on a literature review (scientific literature, "grey literature", patents, company documents, regulatory and policy document, web-based information) and on interviews and consultations with representatives of manufacturers, blenders, professional cleaning service operators, governmental authorities, consumer and environmental organisations, and scientists. The overall focus is on the European Union context with a particular emphasis on Austria, though information on Canada and the United States was also considered.

A particular difficulty arose from the overall lack of information in the public domain, from the fact the manufacturers and blenders are not well represented in professional associations and, therefore, are difficult to identify, and from the reluctance of these business operators to share information which they consider as confidential business information. This was especially challenging as a wide range of applications and product designs was identified and because producers differ broadly in terms of production processes as well as quality and safety assurance.

Rationale of using micro-organisms in cleaning products

The overall rationale for using microbes is similar for all types of products. Living microbes are capable of enzymatically degrading substances associated with dirt, food residues, grease and other objectionable matter (known in cleaning terminology as "soil") and/or bad odours. Microbial action is aimed at controlling odour and to support the cleaning action of detergents. Producers of microbial cleaners frequently make environmental and efficacy claims.

Some micro-organisms produce a broad range of extracellular enzymes, including proteases, cellulases, amylases and ureases, which can degrade organic high molecular weight substances in soil. As opposed to cleaners with added enzymes, microbes can further metabolise (some of) these degradation products. Substances creating odour problems such as NH_3 can be metabolised, or the formation of H_2S may be avoided by transforming SO_3 into S_2. The microbes used in the cleaning products are also claimed to out-compete unwanted micro-organisms in colonising surfaces by using up the nutrients provided in the soil and from polluted surfaces. Other microbes can directly inhibit the

growth of unwanted microbes, for example, by lowering pH. Producers claim a long-term effect because micro-organisms will stay on the treated surface (as spores; many formulations contain spore forming bacteria, e.g. *Bacillus* spp.) and hinder re-colonisation by unwanted microbes.

Products and applications

Microbial cleaners are frequently marketed directly by manufacturers which are in almost all cases SMEs (small and medium enterprises). Most operators are blenders, i.e. they purchase the ingredients for their products from other specialised companies and blend them to yield the final products. Very few manufacturers seem to produce (all of) the microbes by themselves. We identified some 30 manufacturers in Australia Austria, Belgium, Canada, Germany, Japan, the Netherlands, Switzerland, the United Kingdom and the United States. Product data sheets of 20 companies were reviewed with more in-depth investigations of the information on products from 9 selected companies. Two companies provided detailed data including confidential business information (for details see Spök and Klade, 2009).

In commercial contexts, microbial cleaners are mainly applied for odour control in cases where conventional cleaners are considered less efficient: surface cleaning in sanitary facilities, but also more broadly as surface cleaners in buildings with a lot of visitors (e.g. public buildings, schools, restaurants, canteens, hotels, production facilities, nursing/retirement homes, animal shelters, veterinarian surgeries). Routine application by professional cleaning service companies was found, for instance, in train toilets in Austria, Germany and the Netherlands. A professional cleaning service company confirmed, in principle, the efficacy of these products, though there were considerable differences between products, but they highlighted the very high costs with some products. Products for hospitals are presently under evaluation. Here the rationale is that microbes sometimes causing problems in hospitals are outcompeted by the microbes used in the cleaner which would – according to the producer – in some areas render disinfection unnecessary. Besides hard surface cleaning, these products are also used for cleaning carpets and upholstery. Specialty products are used for cleaning drains, pipes and grease traps in order to remove deposits, and also in industrial production in the washing of machine parts, as well as for oil spills on masonry or concrete.

Products based on Effective Micro-organisms (EM®, EM Research Organization Inc.) represent a special type in terms of product design, producer, production process and marketing. An inoculum including a combination of bacteria and fungi is manufactured by licensed companies – mainly based in Japan – and marketed worldwide by specialised EM vendors and health food shops, partly via the Internet. The same and similar combinations of microbes are used for various outdoor and indoor purposes, including soil enhancement, composting, as feed additive and for cleaning. EM cleaners are not only applied in all the areas described above but recommended for a much broader range of indoor cleaning applications including tiling, stove, refrigerators, pots and pans, bio-waste containers, living spaces, wooden floors, closets, wardrobes, shoe cabinets, leather clothes, glass doors, washing machines, dishwashers, doormats, cars and even as laundry detergent. Although EM products are also being used in commercial contexts and by professional cleaning services, it appears that they are more often targeting consumers.

Manufacturers admit that microbial cleaners are still less efficient than conventional chemical products in terms of surface cleaning. In terms of odour control, however, these products are claimed to be superior. Unfortunately, with one possible exception

(Haslinger, 2006), no third-party evaluation of the efficacy of microbial cleaners could be found. The absence of generally agreed upon and standardised methods for comparing the efficacy of cleaning products might be one reason for this.

Microbial cleaners in the context of legislation

EU-harmonised legislation

Microbial cleaners clearly fall under the EU Directive on occupational health risks of biological agents. With respect to sectoral legislation, the picture is more unclear. It seems that the EU Detergent Regulation does not apply. The EU chemical legislation REACH is rather unlikely to apply, but that is not entirely clear. The EU biocide legislation might possibly apply to some, but not all, of these products. Thus, at present, no sectoral environmental legislation is clearly covering these products. If so, EU Directive 2001/95/EC (European Union, 2001) on general product safety would still apply and require a certain safety assessment and risk-related information to consumers by manufacturers and importers of these products. However, there is substantial leeway on how to interpret the requirements of this directive. Consequently, the only clear requirements established are for assessing certain risks for workers' health. There is no EU legislation regulating any environmental impacts of these products.

Occupational health

Microbial cleaners are covered by EU Directive 2000/54/EC (European Union, 2000) which regulates the minimum requirements for the protection of workers from risks related to biological agents. Employers (e.g. manufacturers and blenders of microbial products, professional cleaning service companies, other companies employing cleaning personnel) are required to conduct a risk assessment, including the classification of the micro-organisms used into one of four risk groups based on the pathogenic potential (European Union, 2000: Annex III). Potential allergenic or toxigenic effects (especially the former) are not reflected by the risk group scheme, but these effects also have to be considered (European Union, 2000: Articles 3, 3(d)). Only microbes which belong to risk group 1 are not considered to pose any hazards to human health. The use of microbes classified in risk group 2 or higher requires notification to the national competent authorities and preventive measures by the employer. The type of risk mitigation measures largely depends on the particular risk group and exposure scenario. Manufacturers claim that microbes classified into risk group 2 or higher are neither used nor considered for application in microbial cleaners and this was essentially confirmed in the product survey, with the exception of one product for special application in outdoor contexts.

Detergent legislation

Following a company request, the European Commission and EU member countries agreed that microbial cleaners – even if containing surfactants – do "not seem to have a cleaning action within the meaning of ISO definition (i.e. 'the process by which soil is dislodged from the substrate and brought into a state of solution or dispersion')" and are, therefore, out of the scope of the EU Regulation on Detergents (European Commission, 2009). However, this decision was based on an inquiry for one specific product where the cleaning action is claimed to result from bacteria feeding on the excrement of dust mites. It is not entirely clear if the rationale of this decision would also apply to all microbial products, e.g. to surface cleaner in sanitary facilities.

EU chemical legislation – REACH

All chemical compounds used in microbial cleaners are covered by the new EU chemical legislation REACH. Living micro-organisms and spores, however, do not meet the definition of "substance" as they can neither be understood as "well-defined substances" nor as UVCB substances (substances of unknown, variable composition, complex reaction products or biological materials) (European Chemical Agency, 2012). Manufacturers claim that this view has been confirmed by the Dutch and the Finnish national competent authorities. Still, some uncertainty remains. The *Manual of Decisions* of the EU chemical legislation prior to REACH explicitly excluded living (micro-) organisms from the scope of the legislation (European Chemicals Bureau, 2006; European Commission, 2008a) whereas the REACH guidance document does not (European Chemical Agency, 2012). It also remains unclear if the enzymes produced by the microbes and secreted outside the cells can be considered as UVCBs under REACH in analogy to enzyme (mixtures) added to cleaners. In fact, the very similar enzymes sometimes added to the microbial cleaner in addition to the microbes are covered by REACH, whereas those produced by the microbes are not. Despite the absence of a legal requirement, some manufacturers mention microbes in the Material Safety Data Sheets (MSDS), but not all manufacturers, and not in a consistent manner.

EU biocide legislation

Some microbial cleaners could potentially be considered as biocides, i.e. active substances, intended to destroy or otherwise exert a controlling effect on any harmful organism by chemical or biological means (European Union, 1998: Article 1), which would then be regulated under Directive 98/8/EC (European Union, 1998) for a number of reasons:

- Micro-organisms can, in principle, be considered as biocides, e.g. two *Bacillus* spp. including *B. subtilis* are listed as biocides in the annex to Regulation 1451/2007 (European Union, 2007). *B. subtilis* is frequently used in microbial cleaners.
- Drawing on analogies of other borderline cases, it appears possible that the outcompeting of unwanted micro-organisms by other micro-organisms via chemical or biological mechanisms could be considered a biocidal effect if it results from direct action (European Commission, 2003; 2008b). In contrast, a "physical" displacement of unwanted micro-organisms by overgrowing with beneficial micro-organisms or as a consequence of nutrient competition would presumably not be considered as biocidal activity. Manufacturers frequently highlight the latter effects. For many micro-organisms, however, including some species applied in microbial cleaners, it is described in the scientific literature that they can inhibit cell growth or even kill other microbes by producing and releasing bactericides or fungicides. Other microbes can inhibit growth by other means, e.g. lactic acid bacteria by lowering the pH. This type of mechanism could potentially be considered a biocidal activity. The question here is then whether these mechanisms would also apply to some of the strains used in microbial cleaners. Any clarification of this question would require a more comprehensive description of all the mechanisms of action for each micro-organism used.
- In certain cases, manufacturers are making claims which could be interpreted as claiming biocidal effects, in particular in the case of microbial cleaners used in

hospitals, but also for sanitary facilities, for cleaning carpets and upholstery when claiming deodorization or odour control.

According to two manufacturers, the national competent authorities in Belgium have confirmed that EU biocidal legislation does not apply to their products. A similar view was given by the Dutch Food and Consumer Product Safety Authority (VWA, 2004). No information was available on other types of products, from other competent authorities or from the EU level. Consequently, the applicability of the EU biocide legislation remains to be clarified, though, most likely restricted to specific applications and mechanisms of actions.

United States and Canada

In the United States, the use of naturally occurring microbes in microbial cleaners is not regulated. One exemption is the use of micro-organisms as pesticides (biocides). However, many microbial cleaners are not applicable to US pesticide regulation.

Canada, in contrast, does regulate living organisms by extending the definition of substance in the Canadian Environment Protection Act (CEPA). Since 1999, a notification under the New Substance Notification Regulations (NSNR) is required if a micro-organism is not yet included in the Domestic Substance List (DSL) (see Environment Canada, 2000, 2012a; and Chapter 9). The DSL presently lists some 50 micro-organisms specified by strain and 2 combinations of microbes ("consortia") (see Environment Canada, 2012b). However, in all these cases, the producers could prove that these strains have already been used in Canada before and were, therefore, exempt from the NSNR. None of these micro-organisms has undergone the full-fledged assessment of health and environmental risks required for a New Substance Notification which has specific guidance (Environment Canada and Health Canada, 2010). Regulators also do not as yet have information on which of the listed micro-organisms are being used for microbial cleaners (Health Canada, personal communication).

Health and environmental risks

Micro-organisms in general can be harmless to human health and the environment and many micro-organisms have been used for decades and even thousands of years in the processing of food and feed. Other micro-organisms are pathogenic or toxic to humans, animals or plants. Also, allergenic properties have to be considered. Micro-organisms showing (a potential) for hazardous properties or having a long track record of safe use are usually described as such in the scientific literature and regulatory documents. For assessing the health or environmental hazards, it is therefore pivotal to know the identity of the micro-organisms contained in the cleaners.

Microbial cleaning products differ in the particular combination of micro-organisms used and the particular chemical ingredients, including enzymes (some cleaners also contain enzymes). The combination of micro-organisms and chemicals largely depends on the particular application, but there are also different product designs. In the present product survey, producers usually considered the precise identity (species, strain) as confidential business information. Only the taxonomic genus was declared, if such information was given at all. Very few producers provided more detailed information. The survey identified more than 30 different species, mostly bacteria and a few yeast and fungal species, though, in practice, the range of micro-organisms might be much broader as indicated in patent literature and other documents. The most frequently used microbes

are members of the genus group *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Rhodopseudomonas* and *Saccharomyces.* Some producers are specialised in combinations of different *Bacillus* spp. spores instead of using vegetative cells as spores to allow for a longer shelf life, up to one year (for details see Spök and Klade, 2009).

Producers claim that all of their microbes belong to risk group 1 and do not pose any health concerns. Moreover, some of the microbes used in cleaners are generally recognised as safe (GRAS) in food and other processing contexts or as QPS (qualified presumption of safety) in other contexts, indicating that they have a sufficient track record of safe use and handling may be exempted from certain risk assessment requirements. This is in accordance with information obtained in the product survey that all microbes identified on the species level can be classified in risk group 1. Exceptions only apply to one specialty purpose cleaner for outdoor purposes and to microbes suggested in patent literature. Some producers have also referred to additional safety reassurance from various OECD toxicity tests on rodents, although these test data are not in the public domain.

While all this suggests that there is no immediate threat for human health or the environment, this study has identified a number of issues which would need in-depth review, clarification and/or improvement.

The reliability of a key step in risk assessment – taxonomic identification – remains unclear

The classification in the risk group scheme, the assessment of potential hazardous properties and the existence of relevant experience in safe handling (history of safe use) based on scientific literature and regulatory documents is based on a reliable identification on the species (and frequently on the strain level). It is widely acknowledged that taxonomic identification can lead to erroneous results if not based on proper methods. This is important, as sometimes even taxonomically closely related species or strains can differ considerably in their hazardous properties. For instance, some strains within the same *Bacillus* species (including some species used in cleaners) can produce enterotoxins whereas other strains are not capable of doing so. Differentiation between such strains is also important for the QPS status; toxin-producing strains are explicitly excluded from the QPS status (European Food Safety Authority, 2008). Any erroneous identification could, thus, lead to entirely different results in the hazard assessment. Furthermore, microbial phylogeny and taxonomy have changed considerably over the recent 20 years, mainly due to insights from microbial genetics. These difficulties have also been recognised by the OECD which, in response, issued a guidance document for taxonomic identification of bacteria (OECD, 2003).

Little information was obtained on the taxonomic identification methods used by producers of microbial cleaners. The available information suggests different practices. Some of the organisms used came from widely acknowledged national microbial strain collections (e.g. American Type Culture Collection, ATCC).[1] Here, the source guarantees the application of proper methods for strain identification. Other microbes, however, were isolated from natural environments by the producers of microbial cleaners. Especially with the latter type of strains and in the absence of detailed information on the identification method, the reliability of the identification remains a potential concern. Sometimes the taxonomic identification is done by the producer; in other cases, it is done by an accredited microbiological laboratory. Also, the extent of in-house capability in microbiology seems to vary among producers. Moreover, identification is not only

conducted at the time when the strain is obtained once and for all – it remains to be an issue when maintaining an in-house strain collection from which inocula are being derived.

How to avoid unwanted microbes in cleaning products

The production of sufficient quantities of micro-organisms for a microbial cleaner is done by standard fermentation technology. Any fermentation process has the potential to result in unwanted micro-organisms present in addition to the desired microbes. Depending on the particular process conditions, these unwanted or contaminating microbes might include pathogens and/or might produce toxins. Moreover, they could also interfere with the intended microbial action. This is widely acknowledged (OECD, 2011), and operators of biotechnological processes have therefore established process controls and quality assurance systems aimed at both avoidance (too high levels) of and checking for contaminants.

Information from manufacturers indicated huge variations in process controls and quality assurance. In some cases, this raises doubts on hygiene, quality and consistency of the products. Such doubts are also reinforced by the findings of a study conducted by the Dutch Food and Consumer Product Safety Authority (VWA, 2004). The microbiological analysis of microbiological cleaning products identified huge variations in total viable counts, indicating problems with consistency and shelf life. They also found microbial contaminants including, in one case, a risk group 2 organism associated with human infections. These hygienic problems and the fact that some of the strains being used belong to microbial species known as either opportunistic pathogens or food contaminants, resulted in a VWA recommendation not to use microbial cleaner in areas of food processing and preparation and also not with particular risk groups (YOPI: young, old, pregnant, immune compromised). More recently, they also advised against the use in hospitals based on the same reasons (personal communication). Other applications, e.g. for sanitary purposes, are considered acceptable by the VWA.

Possible concerns in case of chronic respiratory exposure

The appropriate use of some microbial cleaner products leads to exposure scenarios which deserves particular attention. Spray application leads to aerosol formation, especially in closed rooms (e.g. toilets). Repeated application on carpets and upholstery can lead to an accumulation of spores and formation of dust-containing spores. Used in daily cleaning, chronic respiratory exposure therefore has to be considered in a health risk assessment. There is evidence in the scientific literature of sensitising properties and of hypersensitivity pneumonitis. In its microbial pesticide programme, the United States Environmental Protection Agency (US EPA) generally recognises that micro-organisms may be respiratory sensitisers. At the present time, in the course of its voluntary partnership environment label programme, Design for the Environment (DfE), the US EPA has generally excluded from consideration microbially based products intended for use on carpets, hard surfaces and other indoor environments until further information on their safety can be obtained (United States Environmental Protection Agency, 2009). Allergenic properties are also described for the mould species *Aspergillus oryzae* which is also being used in some cleaners.

It is not clear whether and to what extent these hazards are caused by the microbial enzymes and/or on other components of microbial cells and spores. Sensitising and allergenic properties of microbial enzymes, as well as some microbial cells, are well

documented. A difficulty is that there is no agreed upon test for respiratory sensitisation. In the European Union, microbial enzymes are therefore voluntarily considered by industry as respiratory sensitisers and labelled and handled accordingly (R42) (see Federal Environment Agency Austria and Inter-University Research Center for Technology Work and Culture, 2002). Further investigation of this question was, however, beyond the scope of this study.

In order to check to what extent and in what particular cases these concerns are also valid for microbiological cleaners, an in-depth scientific review needs to be conducted and quantitative data or robust estimates on the concentration of cells and spores in aerosols or dust, and the effects of those concentrations, would be required.

Environmental risks of the microbes

Little can be said on the environmental risks of the microbes used. While producers are generally keen to use safe microbes only, the risk group scheme for classifying microbes does not specifically consider plant or animal (in case there is no human) pathogenicity. The risk group scheme also does not consider toxicity to animals. Some companies referred to standard OECD oral toxicity tests on rodents as well as to eco-toxicity tests conducted with the *Bacillus* strains they are using and which did not – according to these producers – identify any environmental risks. This type of information does not seem to be available from all manufacturers or for all microbes.

Conclusion

Stakeholder and public information

There is little information about products, producers, applications to consumers, and in the public domain in general. Despite the fact that there are producers in many countries, there is no specific trade association for these producers, and producers and products are difficult to track. Whether microbes are being used or not is sometimes not clearly stated, or it is expressed in roundabout ways, such as “biological” cleaner, “biological”, “probiotic” cleaner, etc. More transparency to consumers and stakeholders would be a prerequisite for broader adoption by consumers. A product database should be established and the information collected in the course of this study should be expanded.

More science on the mechanism

The available information on the various mechanisms of action of the microbes is considered insufficient. This refers to a lack of transparency as well as to a lack of detailed knowledge on some products. Further scientific studies should be launched to investigate the physiological and biochemical basis of these mechanisms. Such information would also be important for clarifying a possible applicability of EU biocide and detergent legislation.

Health risks

Based on the available information, no clear immediate hazard could be identified. A qualification to this conclusion is that only a few producers decided to reveal the identity of their microbes to the project team. As a general pattern, risk-relevant information obtained from producers was fragmentary and lacking in technical detail.

As highlighted in the preceding section, some aspects deserve more attention, and presumably, regulatory oversight:

- the precise taxonomic identification of the microbes used as the basis of the entire risk assessment should be conducted according to OECD guidance
- the process control and quality assurance systems in place to avoid having unwanted microbes should be reviewed/included in the regulatory oversight
- the relevance of the risks associated with chronic exposure to dusts and aerosols containing vegetative cells and spores should be clarified
- the risks linked to the use of strains which belong to species known to include opportunistic pathogens and possible hazards for particular risk groups (e.g. YOPI) should be clarified; this is linked to possible restrictions in, e.g. hospitals, retirement homes, child care
- the risks associated with particular species, some strains of which are known from cases of food contamination and poisoning; should be clarified. This is linked to possible restrictions of the application in areas where food is being handled and processed.

Taking into consideration the different practices of producers in terms of risk assessment and quality assurance, a risk assessment protocol should be developed which also includes the requirements for taxonomic identification. In the course of establishing this protocol, the above issues could be clarified – even if uncertainties prevail – and the consequences for risk assessment and risk mitigation measures could be agreed upon. An internationally harmonised approach would thereby be in the interest of producers and users. Such an initiative, advocated by Canadian, Dutch and US authorities, could therefore be launched at the EU or international level, for instance at the OECD. A good starting point would be the existant guidance documents established for risk assessment in the context of the Canadian New Substance Notification and for the product review in the course of the US EPA DfE programme. Until these issues are properly addressed/clarified, a clear-cut recommendation in favour of using microbial cleaners as spray in closed environments or for cleaning carpets and upholstery cannot be provided.

Given the results of the VWA study and as long as there is no regulatory oversight, the occurrence of possible harmful contaminants should be checked by a third party. This could be done by conducting a microbial analysis of a microbial cleaner, e.g. at the beginning and the end of its shelf life. Very similar to the analysis of the VWA – which was conducted some ten years ago – such a study could verify the identity and quantity of the microbes intended to be present and identify possible (harmful) contaminants.

Legislation

It is recognised that microbial cleaners represent a novel type of product which does not smoothly fit into EU chemical, detergent or biocide legislation. The same may be true for other EU legislations, too. The applicability of either of these legislations might well depend on the particular product use and claims, thus, the adaptability of all three legislations should be further clarified. Alternatively, a specific regulation should be established tailored for these products to provide for regulatory oversight of environmental and health risks. In the absence of such a regulation, the observed differences in terms of quality assurance, hygiene and risk assessment might continue, which could potentially lead to products which differ markedly in terms of efficacy,

hygiene and even safety. Regulatory oversight would require developers to provide safety-relevant information in a harmonised and systematic way. Regulatory oversight would also be in the interest of producers, as approved products or notifications also represent a reassurance for new clients or users. It will be important to carefully balance the risk assessment requirements, otherwise this might be detrimental for the many SME-type developers.

Prospects for genetically modified micro-organisms in cleaning products

This survey revealed no indication that producers of microbial cleaners are developing genetically modified (GM) micro-organisms tailored for the use in cleaning products. Almost all producers of microbial cleaners are of SME type and it can be assumed that the development and market approval of genetically modified micro-organisms is too costly and the time to market – if successful at all – could easily take a decade. Moreover, the deliberate release of living GM micro-organisms is still lacking consumer/regulatory acceptance. In the related field of bioremediation, there is quite some research ongoing to enhance "cleaning" properties of micro-organisms by using GM techniques (oil spills, etc.) (see Chapter 8). A spill-over to microbes used in cleaning products can be expected once GM micro-organisms are considered more acceptable to be used in the environment.

Note

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Part V

Environmental applications of microbial symbionts of insects

Chapter 11

Use and release of mosquitoes for the control of dengue transmission: A world-first trial in Australia

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Mosquito-borne diseases such as malaria or dengue fever cause a huge health burden to people living in tropical and subtropical countries. Current control efforts are not always effective and many of these diseases have increased in prevalence, geographic distribution and severity. The transinfection of Aedes aegypti *mosquitoes with the endosymbiotic bacterium* Wolbachia pipientis *is a promising biocontrol approach for those diseases. Naturally occurring* Wolbachia *strains have been stably introduced from fruit flies into mosquitoes and shown that these strains can invade and sustain themselves in mosquito populations while blocking the replication of dengue viruses and other pathogens inside the insects. This chapter discusses the release of* Wolbachia*-infected* A. aegypti *mosquitoes in North Queensland, Australia. The regulatory process for this kind of release had no precedent in Australia and was authorised after a thorough community engagement process and an independent risk assessment. At the time of writing (April 2012), a second release trial was currently underway in Queensland and the technology will soon be deployed in dengue-endemic areas of Southeast Asia and in Brazil, once appropriate approvals are in place.*

Introduction

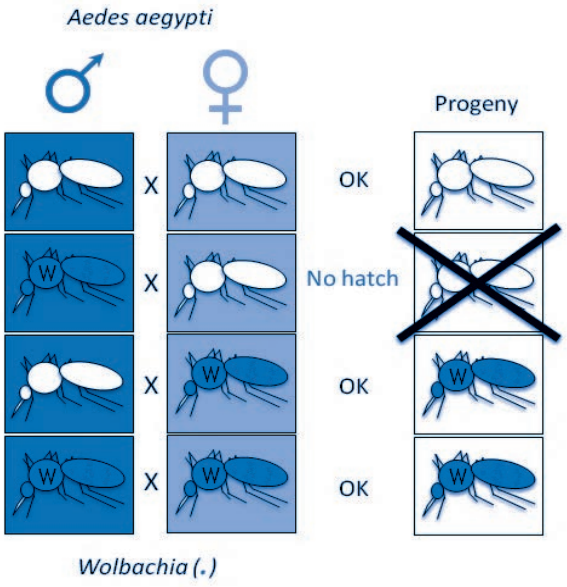
Mosquito-borne diseases are one of the major threats to human health. The malaria parasite transmitted by anopheline mosquitoes in particular causes an enormous health burden mainly among African children, and kills about 1 million people every year (World Health Organization, 2008). The second most deadly mosquito-borne disease, dengue fever, is caused by an RNA virus transmitted primarily by the bite of female *Aedes aegypti* (yellow fever mosquitoes). Causing about 50 000 deaths every year and affecting between 50-100 million people, this disease has increased in severity and distribution, and is now affecting more than 100 countries in tropical and subtropical regions of the world (Kyle and Harris, 2008; World Health Organization, 2009). *A. aegypti* mosquitoes are highly anthropophilic and breed in water containers around houses (old tyres, vases, fallen palm tree fronds, discarded items, etc.), therefore rapid urbanisation in developing countries has contributed to increasing mosquito populations and the concomitant spread of dengue. There are currently no effective vaccines or specific treatments for dengue fever nor the most severe form of the disease dengue haemorrhagic fever (Wilder-Smith et al., 2010), therefore disease monitoring and mosquito control programmes are the only preventive methods currently available. Traditional control approaches for dengue have targeted the mosquito by spraying insecticides, reducing breeding sites or using predatory copepods and fish to eliminate larvae (Kay and Vu, 2005), but these approaches can be very costly and they have not proven as effective as desired, in particular due to the rise of insecticide resistance (Kyle and Harris, 2008; Morrison et al., 2008). More recently, there has been a clear increase in activities related to the development and release of genetically modified (GM) mosquitoes, particularly to control the dengue and malarial vectors. The first generation of transgenic mosquitoes designed to suppress *A. aegypti* populations by effectively using a method similar to the sterile insect technique were released in the Cayman Islands in November 2009 (Reeves et al., 2012), while another release took place in Pahang, in Malaysia, between 2009 and 2012. These releases have been somewhat controversial and have not always been preceded by publication of the associated hazards and their regulatory approval processes (reviewed by Reeves et al., 2012).

The use of *Wolbachia* as a biocontrol agent

A new biocontrol strategy that does not involve genetic modification and does not have the environmental risks associated with the use of insecticides is currently being developed for the control of dengue. This approach uses *Wolbachia pipientis*, an intracellular alpha-Proteobacterium that is a very common endosymbiont of insects and other arthropods, but does not infect vertebrates and is harmless to humans. It is estimated that up to 76% of all insect species harbour *Wolbachia* infections, making this probably the most prevalent microbial symbiont in the biosphere (Hilgenboecker et al., 2008; Jeyaprakash and Hoy, 2000). These bacteria, discovered in the 1920s in the ovaries of *Culex* mosquitoes (Hertig and Wolbach, 1924), frequently induce a series of reproductive distortions in their insect hosts (Werren et al., 2008), the most common being cytoplasmic incompatibility (CI), a form of embryonic lethality that occurs when *Wolbachia*-infected males mate with uninfected females (Figure 11.1). The CI gives *Wolbachia*-infected females a reproductive advantage over uninfected ones, allowing *Wolbachia* to spread into populations (Hoffmann and Turelli, 1997), since these bacteria are maternally (vertically) transmitted through the egg cytoplasm. *Wolbachia*'s invasion ability has tremendous potential for the control of mosquito-borne diseases as they could be used to

spread antiparasitic traits into insect populations, with the intention of making them refractory to disease. Alternatively, *Wolbachia*'s CI phenotypes could be used to render mosquito populations incompatible and induce population suppression. The use of *Wolbachia* for the control of mosquitoes was postulated as early as the 1960s (Laven, 1967), and some preliminary field trials were done temporarily in Burma and India to control *Culex* mosquitoes (Curtis and Adak, 1974).

Figure 11.1. **Schematic representation of the cytoplasmic incompatibility phenotype induced by *Wolbachia* in *Aedes aegypti* mosquitoes**

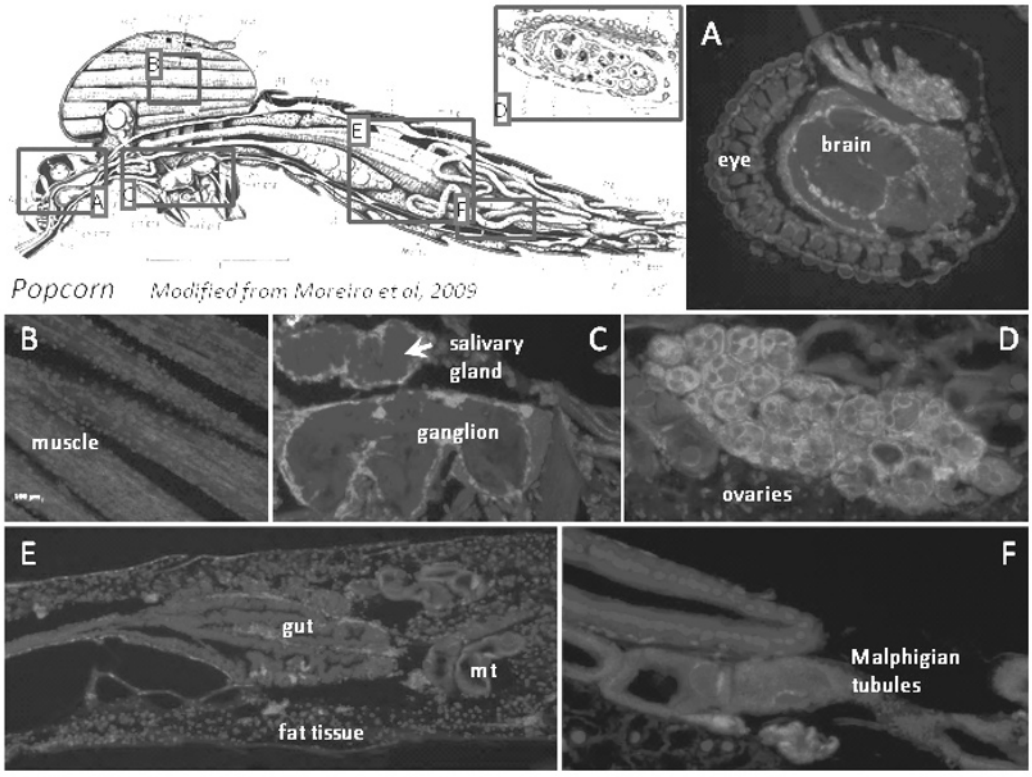


Out of the hundreds of different *Wolbachia* strains present in insects, a strain named *popcorn* (*w*MelPop) appeared to be particularly promising for the control of mosquito-borne disease. This strain, originally discovered in *Drosophila melanogaster* fruit flies in 1997 (Min and Benzer, 1997) over-replicates to high densities in fly tissues and induces CI in infected hosts, while reducing lifespan by about 50%. This is important because the longevity of insect vectors is a key factor affecting disease transmission. Insect-transmitted pathogens, such as dengue viruses or malaria parasites, require a period of replication within the mosquito body before they can be transmitted to another person bitten by the vector. This time, termed the extrinsic incubation period, usually takes about two weeks, a large proportion of the insect's lifespan. Therefore, only the older insects in a population are capable of transmitting dengue (Salazar et al., 2007). The idea behind the use of *Wolbachia* for dengue biocontrol was relatively simple; *popcorn Wolbachia* could be stably introduced into *A. aegypti* mosquitoes, which contain no *Wolbachia* infections in the wild, and CI would allow the bacterial infection to spread within the mosquito population, while eliminating the older (disease transmitting) individuals (Sinkins and O'Neill, 2000; McMeniman et al., 2009).

Despite *Wolbachia* being extremely common symbionts of insects and other arthropods, including some mosquito species, *A. aegypti* mosquitoes are not naturally infected with this bacterium. Therefore, for this approach to work, the *Wolbachia* infection must be transferred to mosquitoes in the laboratory using technically challenging methods such as embryonic microinjection. In 2006, two stably transinfected mosquito lines containing *popcorn Wolbachia* were generated following thousands of

embryo injections (McMeniman et al., 2009). Initial efforts using *Wolbachia* isolated from *popcorn*-infected *D. melanogaster* flies were unsuccessful. Infected mosquitoes were finally obtained after using *Wolbachia* that had been maintained in *A. albopictus* cell lines *in vitro* for several years with continuous serial passage (McMeniman et al., 2008). It is believed that this period of adaptation to a similar host intracellular environment was a key factor for the success of the microinjection, and cell adaptation approaches are being used for the generation of additional infections in other mosquito species. *Popcorn*-infected *A. aegypti* mosquitoes contain very high *Wolbachia* densities and they are widely distributed in most tissues including fat bodies, muscle, nervous tissue, salivary glands, Malphigian tubules, and in particular, ovaries (Figure 11.2) (Moreira et al., 2009). Strong ovarian infection is important for the stability of the transinfected lines, as it allows the bacteria to spread to the female progeny at extremely high rates and be maintained in the population once the initial infection has been created.

Figure 11.2. **Fluorescence *in situ* hybridisation of paraffin sections**



Note: This figure shows the localisation of *Wolbachia* (in red) in different tissues of *A. aegypti*. 8 µm sections were hybridised with two *Wolbachia* specific probes labelled with rhodamine (Moreira et al., 2009). DNA is stained with DAPI (blue). The top diagram has been adapted from Jobling (1987). (A) Head section showing *popcorn Wolbachia* in the brain and ommatidia. (B) *Wolbachia* in the thoracic muscle. (C) Salivary gland and thoracic ganglion. (D) Ovaries. (E) Midgut, fat tissue and Malphigian tubules (mt). (F) Malpighian tubules.

The presence of *popcorn Wolbachia* in mosquitoes reduces their adult lifespan by about 50% (McMeniman et al., 2009; Yeap et al., 2011), similar to the original infected fly hosts (Min and Benzer, 1997). *Wolbachia* also induce strong CI in *A. aegypti*, which allows the infection frequency to increase in the population. However, the most interesting effect from the *popcorn* infection in *A. aegypti* was discovered in 2009, when

Moreira et al. (2009) found that the bacteria have a strong inhibitory effect on dengue virus replication within the mosquito body. *Wolbachia*-infected mosquitoes have dramatically reduced dengue levels compared to uninfected counterparts after being fed on dengue-infected blood or being injected in the thorax with dengue viruses. These decreased dengue titers were confirmed by RT-PCR and also in immunostaining studies that showed the absence of dengue in the presence of *Wolbachia* (Moreira et al., 2009). Numerous recent studies have found similar inhibitory effects against a variety of insect-borne pathogens and insect viruses, including the Chikungunya virus, *Plasmodium*, *Drosophila* C virus, cricket paralysis virus, filarial nematodes, West Nile virus, etc. (Moreira et al., 2009; Panteleev et al., 2007; Hedges et al., 2008; Teixeira et al., 2008; Osborne et al., 2009; Kambris et al., 2010; Bian et al., 2010; Glaser and Meola, 2010; Hughes, G.L. et al. 2011). The molecular basis for the interference between *Wolbachia* and dengue remains unknown, although the two main hypotheses to explain it are based on the upregulation and priming of the mosquito immune system by the novel *Wolbachia* infection (Moreira et al., 2009; Kambris et al., 2009; Rances et al., 2012), and the direct competition for resources between *Wolbachia* and dengue viruses (Moreira et al., 2009; Iturbe-Ormaetxe et al., 2011).

A second *Wolbachia* strain (*w*Mel) from *D. melanogaster* flies was introduced into *A. aegypti* in 2009 by embryo injection (Walker et al., 2011). This strain is very closely related to *popcorn*, and is globally distributed in wild *Drosophila* populations (Riegler et al., 2005) and does not significant induce life-shortening in their native fly host or in transinfected *A. aegypti* (Walker et al., 2011). *w*Mel induces complete CI in mosquitoes and is also less abundant in *Aedes* tissues and as a result has lower fitness costs to the mosquitoes than *popcorn*, and as such, has stronger potential to spread into uninfected populations (Yeap et al., 2011; Turelli, 2010). Interestingly, *w*Mel also blocks DENV replication, although at slightly lower levels than *popcorn* (Walker et al., 2011), which makes it a very good candidate for a release trial. The potential of *w*Mel to spread and invade insect populations is further demonstrated by the global invasion of this strain in *D. melanogaster* during the past 80 years (Riegler et al., 2005), where it replaced a strain more closely related to *popcorn*.

Field releases of *Wolbachia*-mosquitoes in Australia: The regulatory process

The Eliminate Dengue Program[1] is a multinational project primarily funded by the Foundation for the National Institutes of Health through the Bill and Melinda Gates Grand Challenges in Global Health Initiative, and is aimed at using *Wolbachia*-infected *A. aegypti* as a novel strategy for the control of dengue. This programme is led by Australian scientists but includes international collaborators from Brazil, Indonesia, the People's Republic of China, Thailand, the United States and Viet Nam.

Subsequent to the encouraging scientific data, and in preparation for a pilot release of *Wolbachia*-infected mosquitoes in Australia, contained semi-field cages were constructed at James Cook University in Cairns, north Queensland, Australia (Ritchie et al., 2011). The environment in these greenhouse-like cages mimicked the typical Cairns backyard garden and contained potted plants surrounded by mulch, as well as a structure simulating the understory of a traditional north Queensland home, a classic spot where *A. aegypti* usually rest in this area. Cohorts of *Wolbachia*-infected mosquitoes were released into a wild-type population and the experiments demonstrated that both *w*Mel and *popcorn*-infected *A. aegypti* were able to invade and successfully replace uninfected

populations of mosquitoes, reaching fixation in the cages within one to three months (Walker et al., 2011).

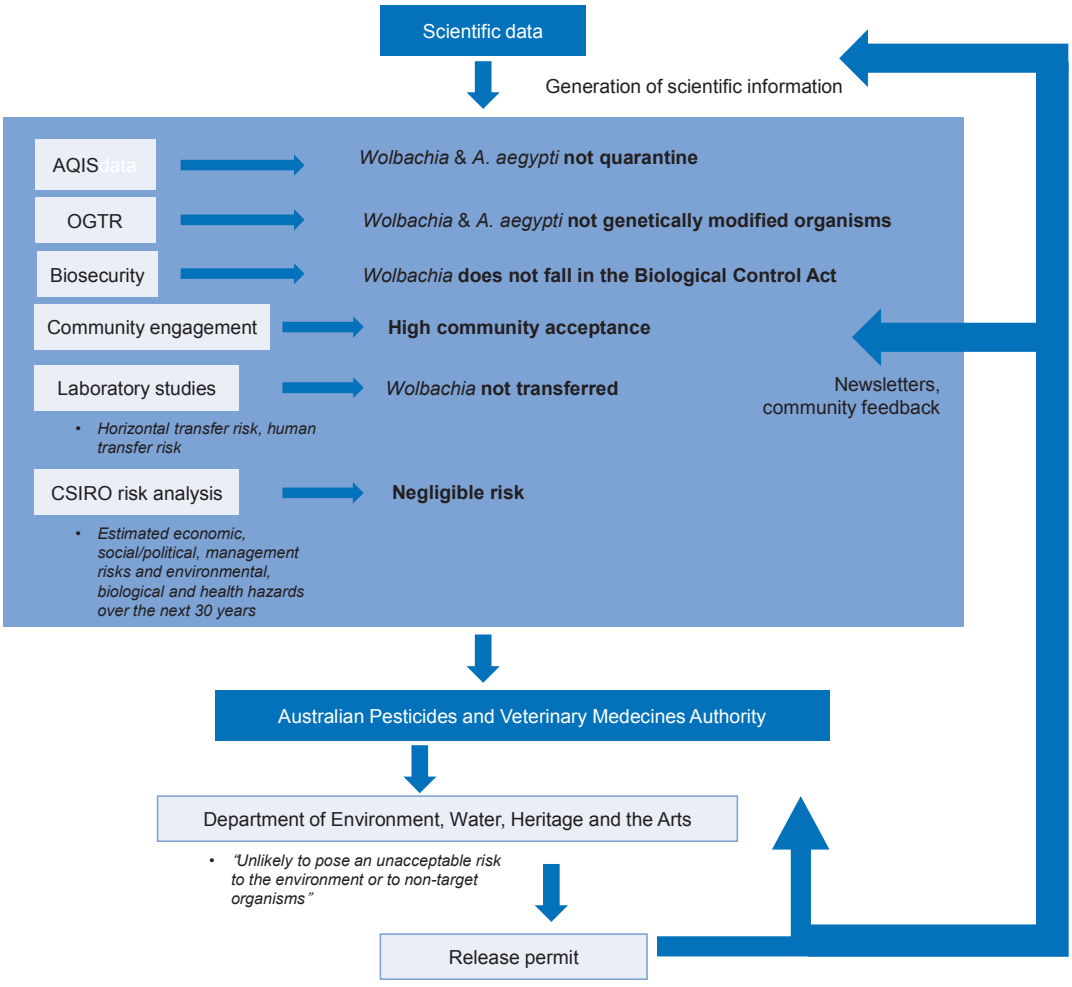
Following the promising results from the laboratory and field-cage studies, a research trial involving the open release of mosquitoes into dengue-prone areas of northern Queensland, Australia was planned. The release of *Wolbachia*-infected mosquitoes for biocontrol purposes had no precedent in Australia, therefore the regulatory pathway for this trial had to be mapped out. Australia has a very strict approach to the importation and release of exotic organisms into the environment and there are four major pieces of legislation that regulate it: the Quarantine Act 1908, the Biological Control Act 1984, the Environment Protection and Biodiversity Conservation Act 1999 and the Gene Technology Act 2000 (De Barro et al., 2011).

Figure 11.3 illustrates the process that took place before the release permit was granted. After initial consultation, the Australian Quarantine and Inspection Service (AQIS, now DAFF) ruled out that *Wolbachia* are not subject to quarantine as they naturally occur into the Australian environment, and as such are not regulated under the Quarantine Act. In fact, studies have revealed that *Wolbachia* are quite prevalent in Australian insects and arthropods, including some iconic species that are common in the release areas, such as the Cairns birdwing butterfly, or very well-known arthropods such as huntsman spiders or fruit flies.[2] Humans have constantly been exposed to *Wolbachia*-infected insects, either by sharing their environment, being bitten by them or by consuming plant products that are infected or contain residues from these insects – even by directly eating *Wolbachia*-infected insects as part of some diets or culinary traditions. Moreover, as up to 76% of all insect species are naturally infected with *Wolbachia* (Hilgenboecker et al., 2008; Jeyaprakash and Hoy, 2000), probably many of the insects deliberately released into the environment for other biocontrol purposes have been inadvertently infected with these bacteria.

Following the assessment by AQIS, the Chief Biosecurity Officer in Queensland determined that *Wolbachia* was not a foreign biological organism, and as such did not fall within the Biological Control Act. Similarly, the Office of the Gene Technology Regulator (OGTR) in Australia, who decides on licence applications to release genetically modified organisms, concluded that *Wolbachia*-infected mosquitoes were not within its remit, because neither the mosquito nor the bacteria have been genetically modified and they can be considered a biological control agent, but not a GMO. In fact, no genetic transformation technologies have yet been developed for *Wolbachia* despite extensive attempts by various laboratories, so all biocontrol efforts are focused on using the traits found in wild type strains. The fact that neither organism in the *Wolbachia-Aedes* association is genetically modified has been a key contributing factor to the relatively fast deployment of this strategy in the field, given the current public and regulatory hurdles to the release of genetically modified organisms in Australia and many other countries.

Regulatory approval for the release was finally granted by the Australian Pesticides and Veterinary Medicines Authority (APVMA), which decided to regulate *Wolbachia* as a "veterinary chemical product" (Figure 12.3). This was based on § 5(2) of the Agriculture and Veterinary Act 1994, that defines a veterinary chemical product as "a substance that is used for application to an animal by any means, as a way of directly or indirectly modifying the physiology of the animal so as to alter its natural development or reproductive capacity" (De Barro et al., 2011).

Figure 11.3. **Regulatory pathway followed in Australia for the release of *Wolbachia*-infected *Aedes aegypti* mosquitoes for the control of dengue**



Note: The release permit granted by the APVMA requires the generation of reports on the spread of *Wolbachia*. The affected communities are informed about the results. These releases have generated a large amount of scientific data that will facilitate further releases.

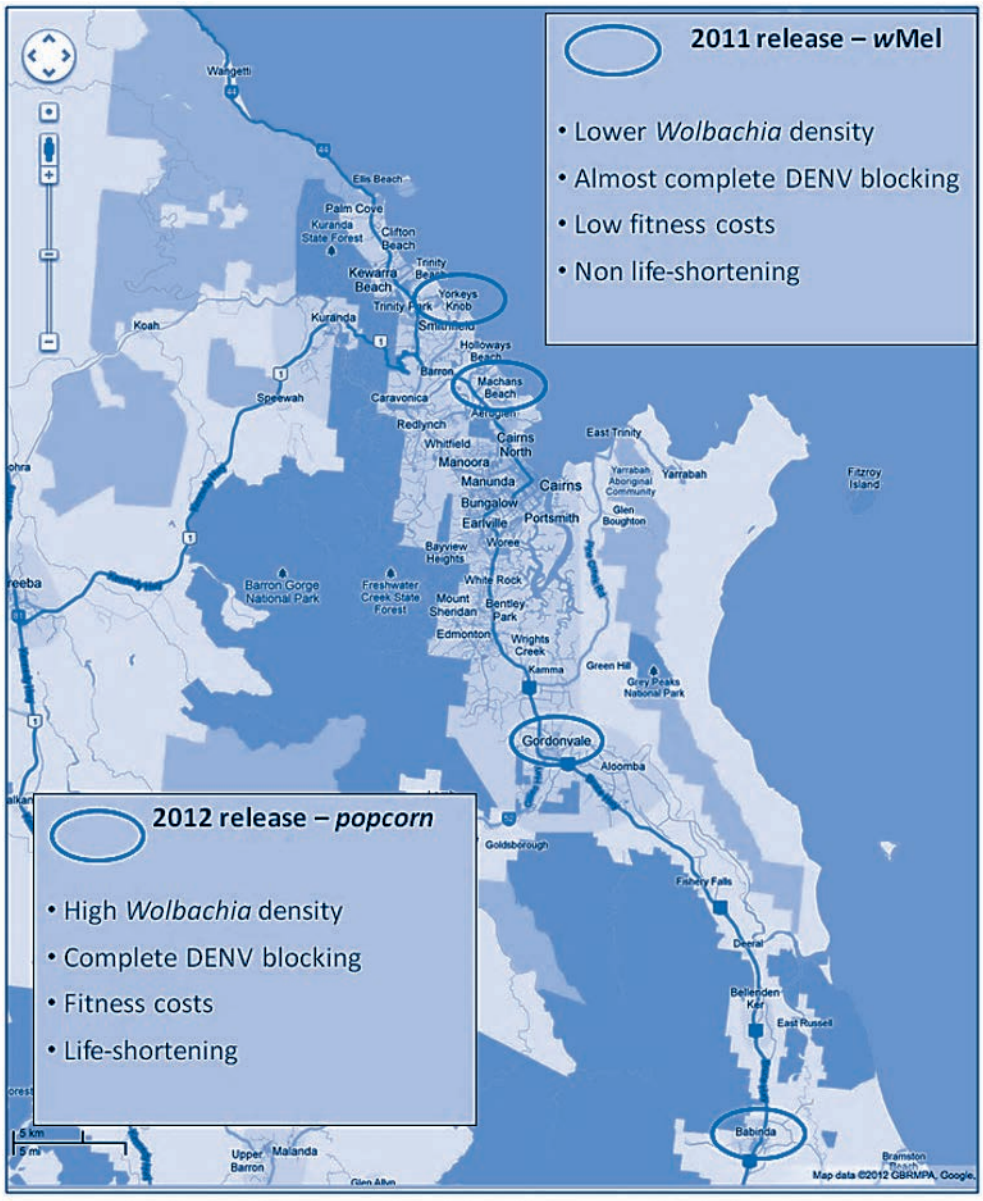
Key for the approval of the release by the APVMA was the risk analysis study conducted by the Commonwealth Scientific and Industrial Research Organisation (*CSIRO*). During an eight-month period, an independent panel of experts estimated the economic, socio-political, management, environmental, biological and health hazards over the next 30 years, determining the likelihood and consequences of these. Fifty hazards were initially considered and later grouped into 30 main hazards (Murphy et al., 2010), which included harm to the environment, the local economy, the tourism industry, human health, even the risks of people perceiving that if this strategy was successful there was no further need to be vigilant against mosquitoes. This study concluded that there was a "negligible risk (lowest possible rating) that the release of *Wolbachia-A. aegypti* will result in more harm than currently caused by naturally occurring *A. aegypti* mosquitoes over a 30-year period".

The APVMA also undertook a further risk assessment with the support from the Federal Commonwealth's Government Department of Sustainability, Environment, Water, Population and Communities, which supported the release. As part of the environmental risk assessment by the APVMA and the CSIRO, as well as community concerns identified during the social studies that took place in the release sites before release, laboratory studies were conducted to demonstrate that *Wolbachia* is not transmitted to humans during mosquito biting (Popovici et al., 2010). The sera from human volunteers that have blood fed thousands of *Wolbachia*-mosquitoes during the course of the project was compared to sera from control individuals that never fed these mosquitoes, and no evidence of *Wolbachia* antibodies in the sera of blood feeders was found. This is likely due to the fact that *Wolbachia* bacteria are too large (0.5-1µm) to pass through the mosquito salivary duct during feeding. These studies also showed that *Wolbachia* are not stably transferred to non-target species that feed on mosquito larvae (spiders, fish or crustacean predators) or share the environment where the mosquitoes live, and they cannot survive in the environment (plants, soil) where mosquitoes are kept (Popovici et al., 2010). Despite the fact that *Wolbachia* are extremely common in many arthropod species, natural horizontal transfer events are extremely rare, and the wide distribution of *Wolbachia* among insects is explained by the many millions of years that *Wolbachia* is believed to be associated with insects.

Wolbachia establishment in north Queensland mosquito populations

Between January and April 2011, up to 300 000 *A. aegypti* mosquitoes infected with the *w*Mel *Wolbachia* strain were released in the localities of Gordonvale and Yorkeys Knob, near Cairns, north Queensland (Figure 11.4) (Hoffmann et al., 2011). Adult (male and female) mosquitoes bred at the Mosquito Research Facility at James Cook University were placed in plastic cups and released weekly on ten occasions at every fourth house. The release was preceded by the removal of water from breeding containers in these sites one month earlier, to reduce the local *A. aegypti* population and maximise the proportion of *w*Mel mosquitoes. Only households that agreed on the release were targeted. The thorough community engagement process and the information campaign that preceded the release, together with the desire of people to participate in a novel dengue control strategy, generated extremely high community support. In order to monitor the spread and invasion of *Wolbachia*-infected mosquitoes in the release sites, a grid of up to 320 mosquito ovitraps were deployed in houses within and around the release areas. Collected eggs were hatched, reared into 2nd-3rd instar larvae, and then sent to a molecular lab in order to test for the presence of *Wolbachia*, as well as to determine whether the larvae were *A. aegypti* or not, by PCR. These studies demonstrated that the *Wolbachia* infection was able to spread and invade the release areas within four months, with percentages of *Wolbachia*-infected mosquitoes rising from 0% to above 80-90% in Gordonvale and Yorkeys Knob just before the dry season (Figure 11.5) (Hoffmann et al., 2011). These percentages reached 100% when the mosquito population was tested again at the beginning of the next wet season (unpublished data), showing that the *Wolbachia* infection has become fixed in these sites. None of the thousands of non-*A. aegypti* eggs collected during this period in the traps and tested by PCR were found to be infected with *w*Mel *Wolbachia*, which highlights the lack of horizontal transfer among mosquito species co-habiting in the same environment.

Figure 11.4. **Location of the 2011 and 2012 *Aedes aegypti* release sites in north Queensland, Australia**

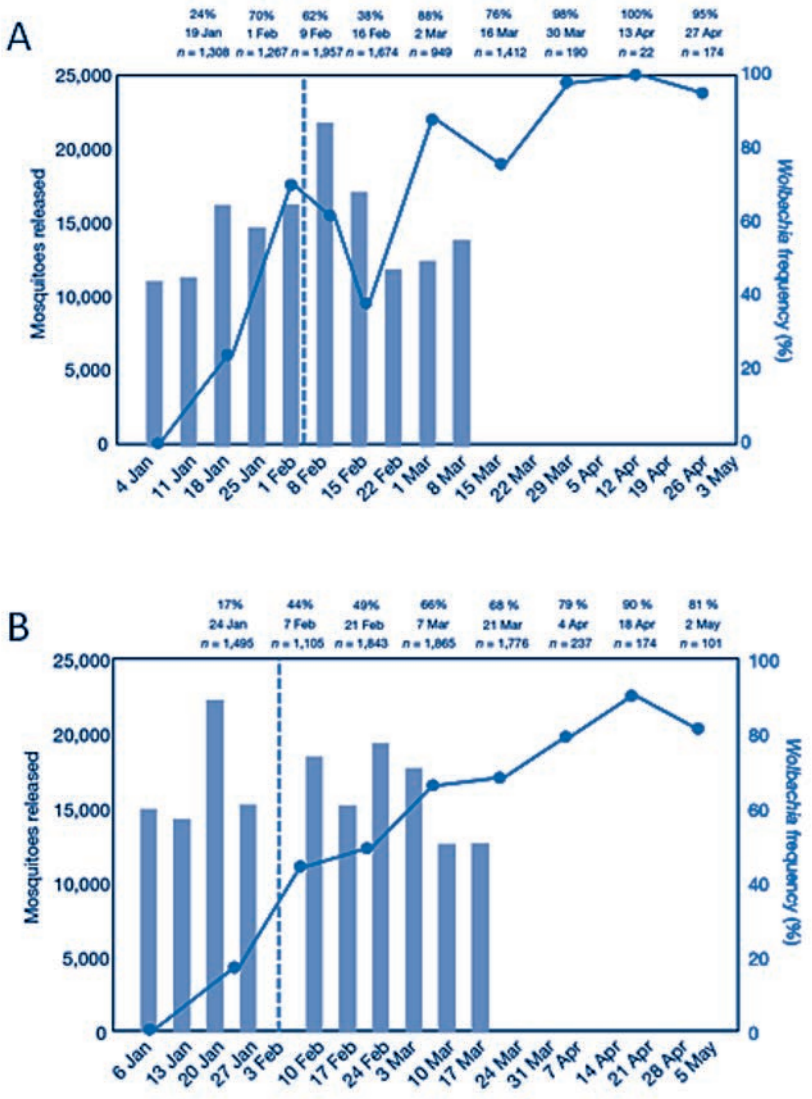


Note: The main phenotypes induced by the *w*Mel and *popcorn Wolbachia* strains in transinfected mosquitoes are described. This document and any map included herein are without prejudice to the status of or sovereignty over any territory, to the delimitation of international frontiers and boundaries and to the name of any territory, city or area.

During the 2012 wet season (January-April), a second release trial took place in the localities of Machans Beach and Babinda, near Cairns, following further support from the local communities. This release was supported by an amended permit from the APVMA, based on the submission of reports from the first release. This time, *A. aegypti* mosquitoes infected with the *popcorn* strain were used. This *Wolbachia* strain, although conferring more fitness costs to the mosquitoes, has much stronger dengue-blocking abilities than *w*Mel, and as such might represent a better alternative in dengue-endemic countries. Of particular interest will be to determine whether these mosquitoes are able to spread and

then survive the dry season, since the presence of *popcorn Wolbachia* has been shown to affect female fecundity and the survival of desiccated eggs (McMeniman and O'Neill, 2010). So far, the *popcorn* infection has spread in Machans Beach and Babinda, and at the time this chapter was written in April 2012, almost 80% of the *A. aegypti* mosquitoes in these areas were infected with this strain.

Figure 11.5. **Increase in the frequency of *Wolbachia*-infected mosquitoes in Gordonvale and Yorkeys Knob during the 2011 release**



Notes: In grey (bar graph), the number of mosquitoes released; in green (line graph), *Wolbachia* frequency. The dotted line indicates the time when tropical storm Yasi landed near Cairns, disrupting some of the monitoring collections.

Source: Hughes, G.L., et al. (2011), "*Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*", *PLoS Pathogens*, No. 7, e1002043.

In order to minimise the spread of *Wolbachia*-infected mosquitoes to non-target areas during the trials, only release sites that were isolated from neighbouring localities by physical barriers to *Aedes* dispersal (highways, sugar cane fields, forests, the ocean) were chosen (Hemme et al., 2010). A key safety consideration addressed by the APVMA is the monitoring of *Wolbachia* in neighbouring areas, therefore a grid of ovitraps was also deployed in various localities adjacent to the release sites (Hoffmann et al., 2011). Only small numbers of *Wolbachia*-infected *A. aegypti* were detected occasionally in some areas near the release sites, probably due to movement through vehicles or adult dispersal. Modelling studies have shown that the proportion of *Wolbachia*-infected mosquitoes must be above a threshold before a successful invasion takes place, so even if a small number of mosquitoes were to be dispersed to new sites, they would find it very difficult to establish a persistent local infection and would be easily swamped by wild-type mosquitoes (Barton and Turelli, 2011). Currently, there is no evidence to suggest that *w*Mel has been able to establish in neighbouring areas.

Future directions for *Wolbachia*

This novel strategy for dengue control has clearly demonstrated that, at least in the Australian environment, *Wolbachia*-infected mosquitoes can successfully invade and replace native uninfected populations when released in sufficient numbers. The establishment of *Wolbachia*-infected mosquitoes in the field should facilitate the future deployment of this strategy to other countries. Additional releases would no longer require the labourious rearing of thousands of adult mosquitoes in the laboratory but could instead be implemented by relocating field-collected mosquito eggs from infected sites to naive locations.

Determining whether these mosquitoes will have an actual effect on dengue transmission cannot be easily resolved in Australia, since dengue is not endemic in the country and the number of cases can vary enormously from year to year, depending on reintroductions from infected travellers (Gould and Solomon, 2008). Such a large epidemiological study is only feasible in dengue-endemic areas and this is now being proposed for countries such as Brazil, Indonesia or Viet Nam, where future deployments of *Wolbachia*-infected mosquitoes are currently being prepared. The Australian trial is being used as a template to develop community engagement strategies and risk assessment analyses for these settings, as well as for paving the pathway for regulatory approval in these countries.

Wolbachia-based strategies are well advanced in *A. aegypti,* where other strains have also been introduced, such as the *w*AlbB *Wolbachia* strain from *A. albopictus* (Xi et al., 2005), but they are not limited to this mosquito species (Iturbe-Ormaetxe et al., 2011). *A. albopictus*, an invasive species that has spread from Asia to the United States, Africa and southern Europe (Gratz, 2004) and is a secondary vector for dengue and Chikungunya, was very recently stably transinfected with *w*Mel *Wolbachia*, which also induces CI and blocks dengue transmission in this species (Blagrove et al., 2012). *A. albopictus* are dengue vectors despite being naturally infected with two *Wolbachia* strains, *w*AlbA and *w*AlbB (Sinkins et al., 1995). Other mosquitoes, such as *Armigeres subaltatus* or *A. fluviatilis*, are also naturally infected with *Wolbachia* strains, and are vectors for Japanese encephalitis virus (Tsai et al., 2006) and *Plasmodium gallinaceum* (Moreira et al., 2009), respectively. The work by Blagrove et al. and previous studies (Hedges et al., 2008; Osborne et al., 2009) have shown that not all

Wolbachia strains have the same pathogen interference phenotypes, and choosing the right genotype is essential for the approach to work.

Alternative technological strategies for disease control

The use of *Wolbachia* symbionts for the control of mosquito-borne disease is compatible with the use of alternative strategies currently being developed, such as vaccines, as well as traditional approaches such as the use of insecticides. *Wolbachia* mosquitoes add to the arsenal of disease control weapons being considered, such as the development of genetically modified mosquitoes expressing anti-parasitic molecules or the creation of paratransgenic approaches that uses symbiotic or gut-associated recombinant bacteria that express this molecules (reviewed by Caragata and Walker [2012], and see Chapter 12). The main scientific challenge with these approaches are the identification of pathogen or mosquito targets that can be engineered to reduce disease, as well as the development of mechanisms that allow the maintenance and spread of these genes in the populations. Obtaining the regulatory and the community consent to release these organisms into the environment may be the more difficult hurdle to overcome. The emphasis from the Eliminate Dengue team on communication with the local community before, during and after the releases was crucial for the acceptance and success of the strategy.

Although the release of *Wolbachia* mosquitoes in Australia was obviously not regulated as a genetically modified organism, the social, scientific and risk studies that preceded it, together with the success of the deployment strategy, can serve as a very interesting model of regulation of mosquito releases. The Australian regulatory experience also revealed that despite the approach being beyond the regulatory process for GMOs, the level of scrutiny with regards to biosafety was very rigorous (De Barro et al., 2011). This strategy is planned to be further tested in the future, when additional releases are carried out in South East Asian countries.

A comprehensive list of *Wolbachia* literature and resources can be found at the *Wolbachia* website[3] and full information about the field release of *Wolbachia*-infected mosquitoes for dengue control is also available online.[4]

Notes

1. www.eliminatedengue.com.
2. www.eliminatedengue.com.
3. www.wolbachiawebsite.org/index.html.
4. www.eliminatedengue.com.

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Chapter 12

Fighting malaria with engineered mosquito symbiotic bacteria

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Insecticides that kill the mosquito and drugs that kill the parasite are the only weapons presently available to fight the unbearably high human malaria toll. As mosquito and parasite resistance to these agents limits their effectiveness and there is currently no effective malaria vaccine available, clearly new means to fight the disease must be developed. This chapter explores the feasibility of an alternative strategy: rather than kill the vector mosquito, modify it to render it incapable of sustaining parasite development. This chapter investigates genetically modifying the symbiotic bacteria that naturally occur in the mosquito's midgut, by producing bacteria that carry the same anti-parasite genes. Major remaining challenges are to devise means to introduce the modified bacteria into mosquitoes in the field and to resolve regulatory and ethical issues related to the release of genetically modified organisms in nature.

Introduction

Malaria remains one of the world's most devastating diseases, afflicting close to 500 million people (nearly 1 in 12 humans) and causing over 1 million deaths every year. The available means to fight the disease are clearly insufficient and the development of new strategies is urgently needed.

Unlike AIDS and tuberculosis (the two other major infectious disease killers), which are transmitted directly from person to person, malaria is different in that the pathogen has to transit through a vector mosquito before it can be transmitted to another human. Consequently, eliminating the mosquito will stop transmission. As such, insecticides have been, and continue to be, the principal weapon to fight malaria. A major limitation in the use of insecticides is the rapid evolution of mosquito resistance to these agents (Maxmen, 2012) and another equally important, but rarely considered, limitation is that insecticides leave intact the biological niche where mosquitoes reproduce. In other words, mosquito breeding sites are unaffected by insecticide applications that occur mostly indoors, leading to two deleterious consequences: *i)* the cycle of continuous breeding (outdoors) and insecticide killing (indoors) constitutes a strong selection pressure for insecticide-resistant mosquitoes; *ii)* mosquito numbers that decline after insecticide use quickly revert to pre-treatment levels as soon as use is interrupted. Therefore, insecticide use can never stop, it would have to be used forever. In summary, insecticides are a very important weapon to fight malaria, but have significant limitations.

The other major weapon to fight malaria is drugs that kill the parasite in the infected human. However, in areas where the disease is endemic, drugs are used almost exclusively to treat, not to prevent, disease. A major limitation of this approach is the rapid evolution of parasite resistance, not unlike bacteria development of resistance to antibiotics. In the case of the malaria parasite, its exceptionally malleable genome exacerbates the problem, as drug resistance evolves quite rapidly, usually in the span of a few years. Another important limitation to the use of drugs is that the logistics needed to distribute the drugs to the people in need, largely in rural areas, is not easy to implement, as the countries with the highest malaria prevalence do not usually have adequate resources. The cost of the drugs and their distribution is also a limiting factor.

The third weapon under development is vaccines that either protect the vaccinated individual or prevent transmission (transmission-blocking vaccines). While extensive efforts have been invested in the last few decades into the development of vaccines, none is yet available. However, a partially effective vaccine is presently under phase III trial and will hopefully be added to the anti-malaria arsenal in a not too distant future (Agnandji et al., 2011).

Transgenic mosquitoes

Recent advances in mosquito molecular genetics and vector-parasite interactions suggest a new strategy to combat malaria, namely, rather than killing the mosquito, rendering it incapable of sustaining parasite development. Since the mosquito is essential for parasite transmission, hindering the mosquito's ability to sustain parasite development can be used to reduce or eliminate transmission. Considerable progress has been made toward this goal (Riehle et al., 2003). Indeed, mosquitoes can be genetically modified to substantially reduce their vectorial capacity (Ito et al., 2002). Despite this and other major advances made toward the generation of *Plasmodium*-resistant mosquitoes, important challenges still remain.

One crucial unresolved question is how to introduce effector genes (whose products interfere with parasite development in the mosquito) into wild mosquito populations. Several possible approaches have been proposed, such as the use of transposable elements or the bacterium *Wolbachia*, but each has serious limitations. In a recent major technological advance, cage experiments have shown that the MEDEA drive system can be used to introduce transgenes into *Drosophila* populations (Chen et al., 2007). While promising, this approach will take time to implement because the necessary tools (e.g. anopheline maternal effect genes, anopheline embryonic promoters) are not yet available.

Another limitation of this approach is that at least in the published cage experiments, a very high initial introduction rate (~25%) was necessary. Finally, this approach cannot overcome the reproductive barriers posed by reproductively isolated anopheline populations (cryptic species) which are common in malaria endemic areas (Powell et al., 1999). Another approach being explored for the spread of genes is the use of homing endonuclease genes originally derived from micro-organisms, but also synthetically assembled (Deredec et al., 2011). While this approach has promising features, there are technical obstacles to be solved, including the problem common to all genetic drive strategies of overcoming the barrier of reproductively isolated populations. It is not clear in what time frame these obstacles will be overcome.

Paratransgenesis

This section explores the use of an alternative strategy to render mosquitoes resistant to the parasite. It takes advantage of the fact that like the majority of higher organisms, including mammals and humans, the mosquito carries a significant microbiome (symbiotic bacteria) in its gut (Pumpuni et al., 1996; Straif et al., 1998). The idea is then to engineer these symbiotic bacteria to produce interfering products (effector molecules) that arrest parasite development. This approach is also referred to as paratransgenesis. An important strategic consideration is that the bacteria occur in the same compartment (the mosquito midgut), where the most vulnerable stages of the parasite cycle occur (Drexler et al., 2008). It is also important that midgut bacteria numbers increase dramatically (two to three orders of magnitude) after ingestion of a blood meal (Pumpuni et al., 1996), and therefore production of effector molecules can be expected to increase accordingly.

Initial experiments used a laboratory strain of *Escherichia coli* to produce a dimer of the salivary gland and midgut peptide 1 $(SM1)_2$ that interferes with ookinete invasion of the midgut (Ghosh et al., 2001) or a modified phospholipase A2 (Moreira et al., 2002). These experiments were promising as mosquitoes carrying these bacteria had a significantly decreased competence to sustain parasite development (Riehle et al., 2007). However, inhibition of parasite development was not robust for two main reasons: *i)* the *E. coli* used for these studies was an attenuated laboratory strain that did not survive well in the mosquito midgut; *ii)* the bacteria were engineered to display the recombinant proteins on their surface, therefore not allowing their diffusion to their intended targets on the parasite or the mosquito midgut.

In view of the promising results of the initial experiments (Riehle et al., 2007), the strategy was improved by focusing on four issues. First, a bacterial strain isolated from the mosquito gut was used instead of an attenuated laboratory bacterium. After isolation, this bacterium – *Pantoea agglomerans* – was further adapted to the mosquito midgut conditions by repeated passages through mosquitoes (Riehle et al., 2007). *P. agglomerans*

is commonly found in field anopheline mosquitoes, as well as in Africa (Pumpuni et al., 1996; Straif et al., 1998). Second, it was important to engineer the bacteria to secrete the effector proteins. While producing recombinant proteins in bacteria is straightforward, engineering Gram-negative bacteria to secrete recombinant proteins can be challenging. An efficient secretion of effector proteins by *P. agglomerans* was engineered making use of the *E. coli* hemolysin A (HlyA) secretion system (Tzschaschel et al., 1996). Third, to improve protein production by the bacteria, the genes encoding anti-malarial effectors were engineered by synthesising them with codon usage optimised for *P. agglomerans* (codon harmonisation). Fourth, several new effector peptides/proteins were developed and existing ones were adapted as follows:

- mPLA2: a mutant phospholipase A2 that inhibits ookinete invasion, possibly by modifying the properties of the midgut epithelial membrane (Moreira et al., 2002).
- Pro: a chitinase propeptide that inhibits chitinase activity, thus hindering ookinete traversal of the mosquito peritrophic matrix (PM; Bhatnagar et al., 2003). The PM is a chitin-based extracellular structure that surrounds the entire blood meal.
- Shiva1: a synthetic anti-parasitic lytic peptide (Jaynes et al., 1988).
- Scorpine: a scorpion (*Pandinus imperator*) anti-malaria lytic peptide, which has hybrid properties of the lytic peptides cecropin and defensin (Conde et al., 2000).
- $EPIP_4$: four copies of *Plasmodium* Enolase-Plasminogen Interaction Peptide (tetra-peptide), that inhibits mosquito midgut invasion by preventing plasminogen binding to the ookinete surface (Ghosh et al., 2011).
- Pro:EPIP: a fusion peptide composed of Pro and EPIP.

Bacteria that secrete these effector molecules were administered to mosquitoes followed one day later, by a *Plasmodium*-infected blood meal. Control mosquitoes were fed bacteria transformed with the HlyA parental plasmid and did not produce an effector protein. The recombinant bacteria strongly inhibited *Plasmodium* development in mosquitoes. Inhibition varied from 85% for mPLA2 to 98% for scorpine and $(EPIP)_4$ (Wang et al., 2012). Perhaps more importantly, the percentage of mosquitoes that had at least one parasite dropped from 90% in controls to 14~18% in mosquitoes carrying scorpine- or $(EPIP)_4$-expressing bacteria. This strong decrease in the proportion of infected mosquitoes should translate into an important reduction of transmission in the field. The use of multiple effector molecules, each acting by a different mechanism, should greatly reduce the probability of selecting resistant parasites. The inhibition of parasite development was equivalent when using an African mosquito (*Anopheles gambiae*) and an Asian mosquito (*An. stephensi*). Also, inhibition of *P. berghei* (a rodent parasite) and *P. falciparum* (a human parasite) was equivalent, suggesting that this approach may also work for other human parasites, such as *P. vivax*. Thus, the paratransgenesis strategy may well turn out to be "universal", being effective for multiple mosquito and parasite species.

Additional considerations in favour of transgenesis are that: *i)* genetic modification of bacteria is much easier to achieve than genetic modification of mosquitoes; *ii)* bacteria are easier to introduce into mosquito populations than transgenes and are unaffected by known genetic and reproductive barriers in wild mosquito populations; *iii)* bacteria can be produced easily and cheaply, also in disease endemic countries; *iv)* the paratransgenesis approach is compatible with, and could complement, other control strategies, such as insecticides, population suppression including transgenic mosquitoes.

Challenges ahead

It is important to emphasise that while many technical aspects have been successfully addressed, several major issues need to be considered before paratransgenesis can be implemented. One key issue is to devise means to effectively introduce the engineered bacteria into mosquitoes in the field. One possible approach that is beginning to be explored is to place baiting stations (cotton balls soaked with sugar and bacteria and placed in clay jars) around villages where malaria is prevalent. Other major topics that need to be addressed are the resolution of regulatory, ethical and social issues related to the release of genetically modified bacteria in nature.

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Part VI

Environmental risk assessment of the deliberate release of engineered micro-organisms

Chapter 13

Next generation sequencing-based metagenomics for monitoring soil microbiota

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DNA sequencing is a powerful method to unravel the genetic diversity of micro-organisms in nature. In recent years, revolutionary next-generation sequencing technologies have become widely used in various microbiological disciplines, including microbial taxonomy and ecology. This chapter reviews the species concept of prokaryotes, including bacteria and Archaea, and presents the development of a comprehensive methodology for monitoring microbes in soil. Next-generation sequencing-enabled metagenomics should be useful and can be widely applied to modern microbiology and biotechnology.

Next-generation sequencing

In 1977, the chain-termination based DNA sequencing method was developed by Frederick Sanger (Sanger et al., 1977). The principle of this chain-termination method (or Sanger method) was the incorporation of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators during the synthesis of complementary strand of template single-stranded DNA. As the ddNTPs are radioactively labelled, DNA fragments that are the result of chain termination after incorporation of ddNTPs can be detected based on one-dimensional polyacrylamide gel electrophoresis and autoradiography. The dramatic improvement of the original Sanger method was achieved by using fluorescently labelled ddNTPs and capillary electrophoresis (Smith et al., 1985; 1986). By the development of this automated Sanger sequencing method, DNA sequencing has become easier and orders of magnitude faster. The partially automated Sanger DNA sequencing method has dominated the fields of molecular biology for almost two decades and led to numerous scientific accomplishments, including the completion of the only finished-grade human genome sequence (Consortium, 2004). Despite substantial technical improvements during this period of time, the limitations of automated Sanger sequencing arose and presented a strong need for new and improved technologies for DNA sequencing with much higher throughput, such as required for sequencing large numbers of human genomes. Recent efforts have been directed towards the development of methods with a completely new basis, leaving Sanger sequencing with fewer reported incremental advances (Metzker, 2010).

Very recently, several types of high-throughput and low-cost platform for DNA sequencing methods have been developed and have made important progress in DNA sequencing (Mardis, 2008; Margulies et al., 2005; Valouev et al., 2008). The automated Sanger method is considered as a "first-generation" technology, and these newer methods are referred to as next-generation sequencing (NGS) (Pettersson et al., 2009). Currently, several NGS technologies are commercially available or about to become available, including Roche/454 (Margulies et al., 2005), Illumina/Solexa (Bentley et al., 2008), Life Technologies/APG (Valouev et al., 2008), Helicos BioSciences (Harris et al., 2008), Polonator (Shendure et al., 2005), Pacific Biosciences (Eid et al., 2009), Oxford Nanopore Technologies (Clarke et al., 2009) and Life Technologies/Ion Torrent (Rothberg et al., 2011). These new technologies employ various strategies applying multiple technological disciplines and rely on a combination of template preparation, sequencing and imaging, and genome alignment and assembly methods. One of the major advances offered by NGS is the ability to generate an enormous volume of data cheaply – in some cases in excess of 1 billion short reads per instrument run. This feature puts NGS into the new realm of experimentation such as transcriptomics, beyond just determining the order of bases (Metzker, 2010).

454 pyroseqencing

Currently, the Roche/454 pyrosequencing method dominates the NGS market together with Illumina/Solexa Genome analyzer (GA). The pyrosequencing of Roche/454 is a technology to be first introduced commercially among the next-generation sequencing methods. The pyrosequencing is a massively parallel sequencing technique based on enzymatic detection of inorganic pyrophosphate release on nucleotide incorporation (Leamon et al., 2003; Ronaghi et al., 1998). This technology employed emulsion PCR for amplification of template DNA where a single DNA template is attached to a single primer-coated bead that is then amplified to form a clonal colony

inside water droplets in an oil solution. The sequencing takes place in many picolitre-volume wells each containing a single bead and sequencing enzymes. Pyrosequencing uses luciferase to generate light for detection of the incorporation of individual nucleotides added to the nascent DNA, and the combined data are used to generate sequence read-outs (Margulies et al., 2005).

This technology provides intermediate read length and price per base compared to the conventional Sanger sequencing on one end and Illumina GA and Life Technologies SOLiD on the other (Schuster, 2008). The first version of pyrosequencing machine, called 454 Genome Sequencer (GS) 20, was released in 2004. It has been improved in the second version, 454 GS FLX, with great enhancements in terms of single-read accuracy and read length (average read length of 250 bp). The latest version of FLX series, called 454 GS FLX Titanium, generates more than 1 000 000 individual reads with improved quality of 400-500 bp in length per 10-hour instrument run (Droege and Hill, 2008; Metzker, 2010). It is currently applied to a wide variety of biological studies, such as human genetics, RNA analysis, metagenomics and ancient DNA sequencing.

Bacterial species concept and its use of genome sequence in taxonomy and metagenomics

One of the primary goals of metagenomics of the environment is to characterise the micro-organisms present in a given environmental sample as understanding the taxonomic composition of microbial communities can lead to an understanding of their ecology and function. A prokaryotic species concept is a fundamental basis of such an analysis.

A prokaryotic species is defined as a group of genetically related strains with the type strain as a centroid. A species boundary is defined by either DNA-DNA hybridisation (DDH) or 16S rRNA gene sequence similarity values. A 70% similarity level over the genome by whole genome DDH is the golden standard for species delineation (Wayne et al., 1987). The general principle of DDH requires: *i)* shearing the genomic DNA(gDNA) of the target strain and reference strains into small fragments of 1 Kb; *ii)* dissociating the double-strand gDNAs into single-strands by heating the mixture of DNA from both strains; *iii)* reannealing the fragments by subsequently decreasing the temperature. The hybrid DDH value is usually specified relative to the DDH value obtained by hybridising a reference genome with itself (Auch et al., 2010). However, the complex and time-consuming experimental procedure of this technique and the impossibility of building cumulative databases based on DDH results are the major drawbacks of this method. Thus, 16S rRNA gene has served as the primary key for phylogeny-based identification among the several thousand genes within a bacterial genome, because the amount of evolution or dissimilarity between the – highly conserved – rRNA sequences represents the variation shown by the corresponding genomes (Woese and Fox, 1977). A cutoff of 3% divergence in 16S rRNA has been used as a conservative criterion for species demarcation (Stackebrandt and Goebel, 1994; Tindall et al., 2009; Wayne et al., 1987).

In microbial molecular ecology, an operational taxonomic unit (OTU) or phylotype often corresponds to a prokaryotic species, which is defined as a group of organisms with high (≥97%) 16S rRNA gene sequence homology. The identification of new bacterial isolates also widely relies on the 16S rRNA gene sequence homology analysis by comparison with existing sequences in the reference databases. Because of the experimental simplicity and the availability of public databases of 16S rRNA gene

sequences, the use of this gene as a single marker for species circumscription has been well received, and it will be argued below that useful metragenomics data can be based on the study of 16S RNA. However, being a highly conserved molecule, the 16S rRNA gene does not always provide sufficient resolution at species and strain level (Konstantinidis et al., 2006). Moreover, single gene-based phylogeny may cause problems because of the possibility of horizontal gene transfer and intra-genomic heterogeneity of multiple copies of the genes (Rajendhran and Gunasekaran, 2011). The experimental difficulty of DDH and the lack of resolution of 16S rRNA gene sequence within species have raised the demand for a better method for species delineation (Stackebrandt et al., 2002).

Now, in the NGS era, in which high-quality genome sequence can be analysed easily and can be compared with other genomes in the public databases, average nucleotide identity (ANI) value between a given pair of genomes has been recognised as a simple and effective way to reconcile the genomic information with the current prokaryotic species concept (Goris et al., 2007; Konstantinidis and Tiedje, 2005). The inter-genomic distances are calculated from fully or partially sequenced genomes after cutting them into small pieces *in silico* (e.g. 1020 bp-long). Then, high-scoring segment pairs (HSPs) between two genome sequences are determined using BLAST algorithm (Altschul et al., 1997; Goris et al., 2007), or maximally unique matches (MUMs) between genome sequences are determined using MUMmer, an ultra-rapid aligning tool (Kurtz et al., 2004; Richter and Rossello-Mora, 2009). The ANI is then calculated from the sets of HSPs or MUMs. The comparative efforts undertaken to evaluate the ANI led to ascertain that the ANI reflects the degree of evolutionary distance between the compared genomes, and a value of 94-96% identity represents the DDH boundary of 70% (Auch et al., 2010; Goris et al., 2007; Konstantinidis and Tiedje, 2005; Richter and Rossello-Mora, 2009). The cases of using the ANI as a substitution of the DDH are beginning to increase in taxonomic studies (Vanlaere et al., 2009; Yi et al., 2012).

Microbial community analysis: Conventional methods

It is generally known among microbiologists that there is a huge potential of prokaryotic diversity made up of hitherto uncultured micro-organisms (Pace, 1997; Ward et al., 1990). Molecular techniques directed toward analysing the community composition of environmental samples indicate that hitherto classified prokaryotic species account for only the tip of the iceberg, considering the huge number (estimated as 4-6 x 10^{30}) of undiscovered prokaryotes present on Earth (Whitman et al., 1998). Usually, profiles of microbial communities in environments have been surveyed using genetic fingerprinting methods. Genetic fingerprinting is a DNA-based technique which generates a fingerprint, the barcode-like DNA fragment pattern. This is a direct analysis of whole genomes extracted from environments or PCR products of selected genes amplified from environmental DNA, based on either sequence polymorphism or length polymorphism. These techniques include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism analysis (T-RFLP), single-strand conformation polymorphism (SSCP), random amplified polymorphic DNA (RAPD), ribosomal intergenic spacer analysis (RISA), length heterogeneity PCR (LH-PCR), amplified ribosomal DNA restriction analysis (ARDRA) and DNA microarrays. In general, genetic fingerprinting techniques are simple and rapid, and allow simultaneous analyses of a large number of multiple samples. The "fingerprints" from different samples are then compared using computer-assisted cluster analysis and community relationships or differences between

microbial communities are inferred (Rastogi and Sani, 2011). However, fingerprinting approaches do not provide direct taxonomic identities of the members comprising the microbial community. Building up a comparable database is also impossible for fingerprinting-based methodology due to the variability of fingerprinting patterns depending on the gel-electrophoresis conditions.

Microbial community analysis: Metagenomics

Thanks to recent technological advancements, methods for the elucidation of microbial community structures have shifted from indirect methods, such as DGGE, T-RFLP and DNA microarrays, to direct methods called metagenomics (Rondon et al., 2000; Schmidt et al., 1991). Metagenomics is a study of collective set of genetic materials extracted directly from environmental samples, and does not rely on cultivation or prior knowledge of the microbial communities (Riesenfeld et al., 2004). Thus, it is a powerful tool to unravel environmental genetic diversity without potential biases resulting from culturing or isolation. Metagenomics is also known by other names, such as environmental genomics or community genomics, or microbial ecogenomics (Rastogi and Sani, 2011). The two major interests of metagenomics are which organisms are present and what metabolic processes are possible in the community (Allen and Banfield, 2005). The former is surveyed mainly based on 16S rRNA gene profiling, the prevalent marker gene for identification of prokaryotic species (Weisburg et al., 1991). Metagenomic investigations have been conducted in several environments, ranging from the oceans to soil, the phyllosphere and acid mine drainage, and have provided access to phylogenetic and functional diversity of uncultured micro-organisms (Handelsman, 2004).

Several major technical limitations have long been in existence with respect to metagenomics. PCR was usually used in metagenomics to selectively amplify target genes and then cloned into vectors for sequencing (Lane et al., 1985). This approach could amplify a minute amount of target genes from the bulk DNA to a reasonable quantity for analysis, but this analysis is subject to PCR-inherent bias (Polz and Cavanaugh, 1998) and thus may not reflect actual microbial community structure. By the advances of meta-strategies in biotechnology and bioinformatics, the need for PCR can be avoided by adopting shotgun sequencing into metagenomics (Breitbart et al., 2002; Tyson et al., 2004). This was feasible by using randomly sheared environmental DNA as it is for insert to be sequences, but still the potential bias imposed by cloning remained as a significant concern in shotgun metagenomics (Handelsman, 2004).

As described above, NGS methods such as Roche 454 pyrosequencing have brought a revolution in metagenomics not only by producing a large amount of data at a low cost, but also by excluding time-consuming and bias-imposing step such as clone library construction.

For the purpose of collecting metagenomics data, DNA is extracted from an entire microbial community, and a target region flanked by highly conserved primers is amplified by PCR before sequencing. This generates a mixture of amplicons, in which every read stems from a homologous region, and the sequence variation between the reads reflects the phylogenetic diversity in the community (Quince et al., 2009). Usually, the hypervarialble regions of 16S rRNA gene sequences are used for the target of pyrosequencing. The produced sequences are short (400~500 bp), but provide useful phylogenetic information. For example, investigation on the spatial changes in soil bacterial communities was explored using 88 soil samples and a massive bar-coded pyrosequencing technique (Lauber et al., 2009). The V1 and V2 hypervariable region of

16S rRNA genes was the target of sequencing. The results demonstrated that soil bacterial communities contain a large number of microbial species, implying extreme diversity; at least 1 000 species per soil sample. A large "rare biosphere" represented by an enormous number of low-abundance unique taxa also supports this finding. Such studies highlight the importance of large-scale sequencing techniques in investigating the highly diverse soil microbial communities (Rastogi and Sani, 2011). Now, this kind of microbial metagenomic sequencing data itself have become generally affordable and researchers are flooded by an unprecedented amount of DNA sequence data from various environments (Huber et al., 2007; Jones et al., 2009; Warnecke et al., 2007; Wegley et al., 2007).

Soil metagenomics: Practical applications

Phytoremediation, which is the use of plants to clean up environmental pollution, has received much attention as a promising method for the removal of metal pollutants in soils (Cherian and Oliveira, 2005; Van Aken, 2008). Phytoremediation is a cost-effective and environmentally friendly approach compared to other environmentally invasive, expensive and inefficient clean-up technologies (Van Aken, 2008). A number of plant species are capable of high-level organic compound degradation or heavy metal hyperaccumulation. However, slow rates of removal and incomplete metabolism have restricted the application of phytoremediation in the field (Van Aken, 2008). Thus, genetically engineered plants that exhibit enhanced performance with respect to the metabolism of toxic compounds have been developed by the over-expression and/or introduction of genes from other organisms (Doty et al., 2007; French et al., 1999). Engineered poplars have greatly increased the possibility of the practical application of phytoremediation. However, this technology is still in the developmental stage, with the field testing of transgenic plants for phytoremediation being very limited. The major obstacle is biosafety concerns, because the potential unwanted effects of genetically modified organisms are not fully understood.

One of the most postulated potential unwanted effects of genetically modified (GM) plants is alteration to the structure of indigenous microbial communities. Micro-organisms have an important role in regulating soil conditions (Wolfenbarger and Phifer, 2000). Soil micro-organisms are in charge of the global cycling of organic and inorganic matter. A number of microbes decompose organic matter into forms useful to the rest of the organisms in the soil food web, and can break down pesticides and pollutants in soil. Soil microbes perform important services related to water dynamics, nutrient cycling and disease suppression. They also produce substances that constitute the soil structure (Conrad, 1996). Thus, alteration in the diversity or activity of microbial communities may have adverse effects on soil ecology (Kennedy and Smith, 1995), and understanding how GM plants, and plants in general, might alter the soil microbial community is of great interest.

The effect of GM plants on soil microbial communities remains highly controversial. Several studies have reported that microbial communities are clearly altered by engineered plants (Bruce et al., 2007; Donegan et al., 1999; Gyamfi et al., 2002; LeBlanc et al., 2007; Lee et al., 2011; Siciliano and Germida, 1999; Smalla et al., 2001). In contrast, other studies have shown that the associated changes in microbial communities with engineered plants are statistically insignificant (Dunfield and Germida, 2004; Heuer et al., 2002; Kim et al., 2008; Lottmann et al., 2000) or very minor (Di Giovanni et al., 1999; Donegan et al., 1995, 1999; Dunfield and Germida, 2003;

Griffiths et al., 2000; Gyamfi et al., 2002; Jain et al., 2010; Lukow et al., 2000; Schmalenberger and Tebbe, 2002). Most of these studies have used non-sequencing based methods, such as community-level physiological profiles (CLPPs), fatty acid methyl ester (FAME), DGGE and T-RFLP. These techniques are useful for evaluating differences in overall community structure, but these fingerprinting methods are limited in their capacity to detect minor changes and the components of these changes. In addition, the number of clone sequences (≤100 sequences per sample) surveyed in a few studies (Kim et al., 2008; LeBlanc et al., 2007; Lee et al., 2011) is insufficient to determine overall community profiles.

Thus, to evaluate the effect of GM plant use on soil microbial communities, extensive sequencing-based community analysis was conducted, while controlling the influence of plant clonality, plant age, soil condition and harvesting season (Hur et al., 2011). The rhizosphere soils of GM and wild type (WT) poplars at a range of growth stages (i.e. rhizosphere of 1.5-, 2.5- and 3-year-old poplars) were sampled together with non-planted contaminated soil, and the microbial community structure was investigated by pyrosequencing the V3 region of prokaryotic 16S rRNA gene. Based on the results of DNA pyrosequencing, poplar type and growth stages were associated with directional changes in the structure of the microbial community. In detail, for both GM and WT poplars, the microbial community of poplars started separating from that of the control soil in the early stage of poplar cultivation (1.5 years), advanced to the middle-stage group (2.5 years), and finally reached the late-stage group (3 years), the composition of which was very different from that of the contaminated soil community. However, the rate of microbial community change was slower in WT poplars than in GM poplars. This phenomenon possibly occurs because of the more active metal uptake ability of GM poplars compared to WT poplars, which resulted in faster changes in the soil environment, and hence the microbial habitat. In conclusion, the shift in the microbial community structure to the late stage was driven faster by the effect of GM phytoremediation than WT phytoremediation. The results of the study demonstrated the superiority of NGS-based technique over traditional risk assessment approaches in the aspect of capacity to detect minor changes and the components of these changes. The next-generation sequencing-enabled metagenomics should be useful and can be widely applied to modern microbiology and bio-technology.

Conclusion

The NGS techniques, coupled with metagenomic analysis, has opened up a new era in the study of microbial diversity with direct access to the indigenous microbial communities in the environments. The superiority of NGS-metagenomics over conventional DNA fingerprinting or Sanger-metagenomics is evident from numerous microbial diversity studies. This NGS-metagenomics also provides further research strategies at the molecular level, such as gene-level functional analysis and gene expression analysis. In a near feature, this NGS-metagenomics will be able to be used as a universal diagnostic tool also in clinical bacterial or viral samples. The new NGS-enabled diagnosis requires no prior knowledge of the host or pathogen, and thus will expedite the entire process of novel pathogen discovery, identification, pathogen genome sequencing and the development of more routine assays.

Because the NGS techniques are still rapidly evolving, researchers continue to meet challenges in fully optimising NGS platforms as well as in analysing and managing data. Many technological developments are focusing on the sample-preparation protocols,

sequencing-library construction protocol, the quality and quantity of sequencing reads, and the analysis of massive data. One of the most challenging parts of those is developing novel algorithms and bioinformatic tools that scale with the tremendous amount of short reads generated through NGS-metagenomics. As the NGS technologies are producing a tsunami of data, the bioinformatics community needs to act quickly to keep up to pace with it. Particularly for NGS-metagenomics, efforts should be made to prepare tools for error-free estimation of species diversity and gene family frequency, tools for comparative metagenomics and tools for removing 16S rRNA chimeras.

NGS-metagenomics is useful and can be widely applied to modern microbiology and biotechnology. It has the potential to answer fundamental biological questions. The current progress toward understanding the uncultured bacteria, archaea and viruses through NGS-metagenomic analyses will lead to the comprehension of the genetic diversity, population structure and ecological function of complex microbial assemblages in the environments.

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Chapter 14

Reflection on environmental risk assessment of micro-organisms

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Due to the inter-dependent network of organisms, a microbe's interaction with the physical environment and its continuous evolution through mutation and horizontal gene transfer, microbial diversity is never static, which makes an analytical approach almost impossible for assessing the risk of the environmental use of microbes. One possible alternative approach could build on concepts developed by the OECD in the early 1990s: familiarity and substantial equivalence.

According to the Cartagena Protocol on Biosafety, *"*t*he objective of* a *risk assessment is to identify and evaluate the potential adverse effects of living modified organisms on the conservation and sustainable use of biological diversity in the likely potential receiving environment, taking also into account risks to human health"*. The "*p*otential adverse effects" are not always easy to identify *and i*nterpreting them in different circumstances has been a long-standing question. *Ambiguity surrounding this key word appears to have caused regulatory uncertainty.*

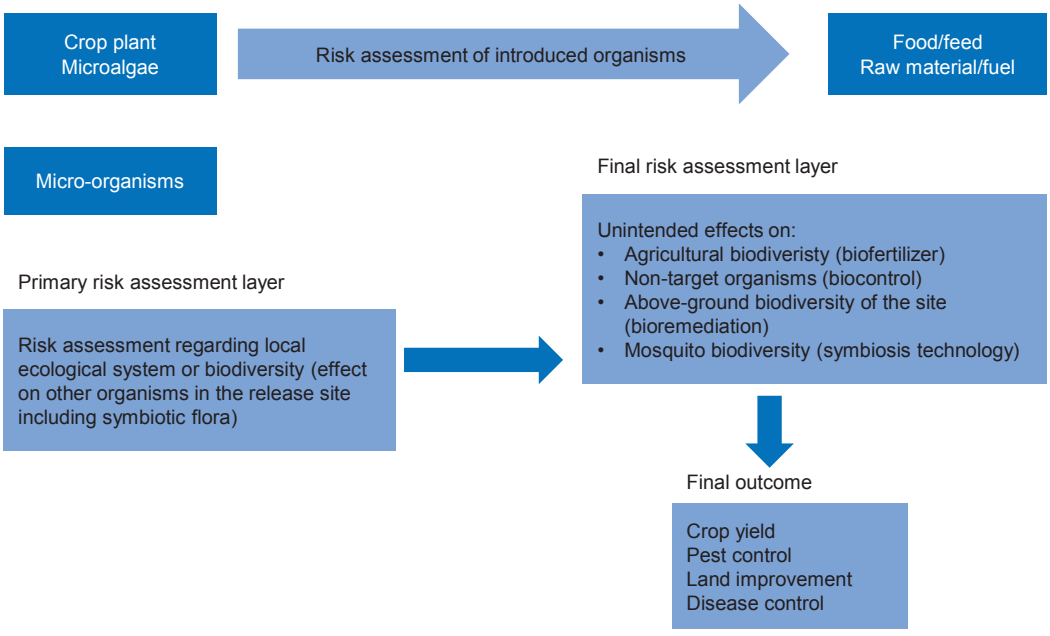
This chapter *addresses the t*arget of risk assessment of environmental application of microbes *and the difficulty of using an analytical approach in assessing the risk of micro-organisms used in the environment.*

Target of risk assessment

For crop plants or microalgae for food, feed, fuel or feedstock, the plants or microalgae introduced into the environment are clearly the target of risk assessment. However, for biofertilizer, biocontrol, bioremediation or mosquito control through paratransgenesis, this is not necessarily the case. For example, for paratransgenesis for mosquito control, while the biodiversity of living organisms impacted by the presence of mosquitoes is the main target, the biodiversity primarily affected is that of the symbiotic microbial flora of mosquitoes. For biocontrol or biofertilizer, while the non-target effect on the above-ground organisms could be the main target, the biodiversity primarily affected is the underground microbial flora (bacteria, fungi, nematodes, insects, plant roots, etc.). Thus, in these cases, the risk assessment consists of two layers. Such a bi-layered structure inherent in the risk assessment of microbes will be an important consideration in structuring the risk assessment of the environmental use of microbes (Figure 14.1). To which layer should we focus more in the risk assessment?

It should be noted that very often where risk assessment cannot identify the main target, such as in case of biofertilizer or bioremediation, risk assessment tends to focus excessively on the effect on microbial ecology.

Figure 14.1. **Bilayered structure inherent in risk assessment related to micro-organisms**



Difficulty of using an analytical approach in assessing risk of micro-organisms used in the environment

Problems encountered in assessing the "microbial diversity" is seen in Baas Becking and Beijerinck's statement, "Everything is everywhere, but the environment selects" (De Wit and Bouvier, 2006).

"Environment selects" refers to the fact that microbes are always under the influence of environmental factors, such as light, temperature, humidity, water, carbon, nitrogen, phosphate, sulphate, minerals, organic matters or organisms with which the microbes interact. Effective use of microbes in the environment requires the appropriate

environmental conditions. The introduced microbes alone do not determine the environmental consequences of releasing such microbes into the environment, but the combination of the microbes and the environment do. This consideration is particularly important in view of the changing climate and increasing human population which enhances the anthropogenic consequences (Smol, 2012).

"Everything [in terms of microbes] is everywhere" relates to the inherent difficulty of assessing microbial ecology. For example; "a pond 1 ha in area and 10 m deep will host 10^{18} bacteria, 10^{16} protists, 10^{11} small animals; species with 10^{7} individuals or less in the pond are unlikely ever to be detected" (Fenchel and Finlay, 2004). There will be ten-fold more viruses in addition to the ones that are inventoried. Use of metagenomics may reveal further biodiversity of microbes in the environment, but its capacity will be far short of what is required for its full understanding. Every microbe may be everywhere but it may not be noticed because its population remains small.

Microbes, i.e. bacteria (*Archaea* and *Eubacteria*), viruses (bacterial, animal and plant viruses), fungi, nematodes, arthropods and underground animals, make a complex interacting community. They constitute a metabolic and genetic consortium. Such a consortium is under the influence of the physical environment, such as temperature, water supply, nutritional content of the soil or water, etc, which are, in their turn, affected by microbial activities through cycling of carbon, nitrogen, phosphorus, sulphur and other molecules. Human activities such as agriculture, building cities and industries, strongly change the soil and water environments through pollution and land/water use.

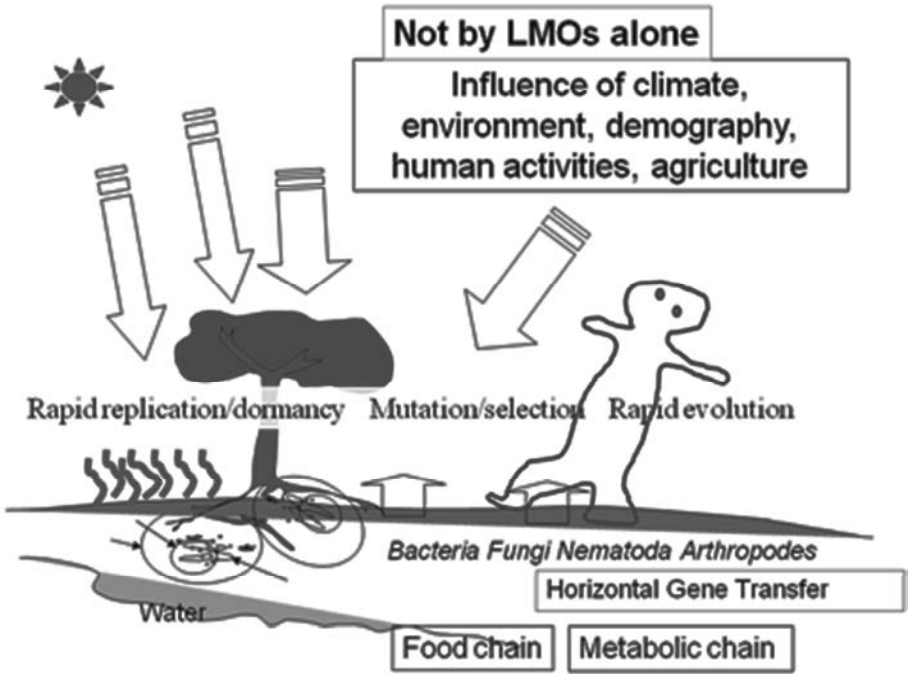
Microbes, owing to their rapid growth and capacity of clonal expansion and through mutation and selection, are undergoing a constant evolutionary process, which is enhanced by horizontal gene transfer, which occurs every moment even between different species (Figure 14.2). In fact, "horizontal gene transfer is essential for the evolution of prokaryotes and can be legitimately viewed as a necessary condition of the long-term survival of archaea and bacteria. Any asexual population is headed for eventual extinction because it does not possess effective means to eliminate the inevitably accumulating deleterious mutations" (Koonin, 2012).

In short, microbes do not exist independently; they are part of an inter-dependent network of primary production, degradation of organic material, prey-predator relation, etc. Microbes are under continuous evolution through mutation, horizontal gene transfer and selection in response to environmental change. Microbial diversity is never static.

All these situations make the environmental risk assessment of micro-organisms difficult, which is already addressed in the consensus documents (Table 14.1), i.e.:

- Incomplete information on the number of existing microbial species (OECD, 2003).
- Viable but non-culturable microbes (OECD, 2004).
- Frequent inter-species horizontal gene transfer including that of pathogenicity-related genes (OECD, 2010). Species name is an inexact marker of risk (OECD, 2011).
- The new knowledge has not always brought us closer to understanding of speciation in bacteria. There is no best method for taxonomy, not even if we restrict ourselves to aspects of taxonomy that are meaningful in risk assessment (OECD, 2003).

Figure 14.2. **Many factors other than LMOs influencing on biodiversity**



As a consequence, we may get into a situation where: "Because most GM micro-organisms cannot reveal their potential until release, and because some testing relevant to risk assessment cannot be done until release, one can't test without release, but one can't get permission to release without testing" (OECD, 2003). There is surely a limitation to the analytical approach in assessing the risk of environmental use of microbes.

Table 14.1. **Past OECD work on the environmental risk assessment of micro-organisms**

Regulatory oversight series
Guidance documents on:
– The Use of Taxonomy in the Risk Assessment of Micro-Organisms: Bacteria (No. 29)
– Horizontal Gene Transfer Between Bacteria (No. 50)
– Detection of Micro-Organisms Introduced into the Environment: Bacteria (No. 30)
– Pathogenicity Factors in Assessing the Potential Adverse Health Effects of Micro-Organisms (No. 52)
– Information used in the assessment of environmental applications involving:
– *Acinetobacter* (No. 46)
– *Acidithiobacillus* (No. 37)
– *Pseudomonas* (No. 6)
– *Baculovirus* (No. 20)
Important concepts:
Substantial Equivalence or Comparative Safety Assessment: Safety Evaluation of Foods Derived by Modern Biotechnology: Concepts and Principles (1993)
Familiarity and Stepwise Scale-Up: Safety Considerations for Biotechnology: Scale-Up of Crop Plants (1993)

An alternative approach is needed. One approach could be going back to the two complementary concepts developed by OECD in early 1990s: familiarity and substantial equivalence.

The concept of "familiarity" was proposed in combination with a scale-up process to gain "familiarity" (OECD, 1993a). It could be a process "from trial-and-error to earnest engineering" as expressed by Prof. Victor de Lorenzo.

"Substantial equivalence" or "comparative safety assessment" uses, as a reference, a "conventional counterpart with history of safe use" (OECD, 1993b). The principle is appropriate use of experience; it was first developed in relation to food safety assessment, but may be adjusted conceptually to an environmental risk assessment. Uncertainty is removed or reduced only through experience. The concept was successfully used in the Codex Alimentarius Commission that agreed on a series of texts on foods derived from modern biotechnology (Codex Alimentarius Commission, 2009).

The OECD's Working Group on Harmonization of Regulatory Oversight in Biotechnology has developed consensus documents since its first session in 1995 (Table 14.1). Examples in such documents shown in Table 14.1 will be an important information source on the history of the safe use of microbes (Table 14.2).

Table 14.2. **Environmental use of microbes in the past**

Acinetobacter spp.
– removal of phosphates
– bioremediation of sites contaminated with hydrocarbons, heavy metal, pesticides
– plant growth promoters and biocontrol agents against bacteria and fungi
– biosensors for pesticides metaphos, sumithion, etc.
Acidithiobacillus
– removal of sulphides from industrial waters, heavy metals from sludge and mine waters
– bioleaching of copper, uranium, etc.
– desulphurisation (remove sulphur from coal); bioleaching of pyrite from oil shale
– agricultural fertilisation (through involvement in sulphur cycle)
Pseudomonas
– *P. aeruginsa*: washing hydrocarbons from soil (biosurfactant)
– *P. fluorescens*: ice-minus; plant and fish disease control by inhibiting growth of fungi; degradation of chlorinatedaliphatic hydrocarbons, etc.
– *P. putida*: degradation of PCB, etc.
Baculoviruses
– biological control as insecticides (moth, cotton bollworm, etc.; no negative or unintended effects)
– registered insecticides: Adoxophyes orana (GV); Agrotis segmentum (GV); Anticarsia gemmatalis (MNPV)

If microbial biotechnology is to be used for the improvement or conservation of the environment, we should not miss timing. The underground microbial community is interacting with the above-ground community. Once the land becomes barren, the introduction of any microbes requiring above-ground plants will not work.

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Annex 14.A1

Text of paragraphs 8 and 9 of the Annex III to the Cartagena Protocol on Biosafety

8. To fulfill its objective, risk assessment entails, as appropriate, the following steps:

- (a) An identification of any novel genotypic and phenotypic characteristics associated with the living modified organism that may have adverse effects on biological diversity in the likely potential receiving environment, taking also into account risks to human health.
- (b) An evaluation of the likelihood of these adverse effects being reali[s]ed, taking into account the level and kind of exposure of the likely potential receiving environment to the living modified organism.
- (c) An evaluation of the consequences should these adverse effects be reali[s]ed.
- (d) An estimation of the overall risk posed by the living modified organism based on the evaluation of the likelihood and consequences of the identified adverse effects being reali[s]ed.
- (e) A recommendation as to whether or not the risks are acceptable or manageable, including, where necessary, identification of strategies to manage these risks.
- (f) Where there is uncertainty regarding the level of risk, it may be addressed by requesting further information on the specific issues of concern or by implementing appropriate risk management strategies and/or monitoring the living modified organism in the receiving environment.

Points to consider

9. Depending on the case, risk assessment takes into account the relevant technical and scientific details regarding the characteristics of the following subjects:

- (a) Recipient organism or parental organisms. The biological characteristics of the recipient organism or parental organisms, including information on taxonomic status, common name, origin, centres of origin and centres of genetic diversity, if known, and a description of the habitat where the organisms may persist or proliferate.
- (b) Donor organism or organisms. Taxonomic status and common name, source and the relevant biological characteristics of the donor organisms.
- (c) Vector. Characteristics of the vector, including its identity, if any, and its source or origin, and its host range.
- (d) Insert or inserts and/or characteristics of modification. Genetic characteristics of the inserted nucleic acid and the function it specifies, and/or characteristics of the modification introduced.

- (e) Living modified organism. Identity of the living modified organism, and the differences between the biological characteristics of the living modified organism and those of the recipient organism or parental organisms.
- (f) Detection and identification of the living modified organism. Suggested detection and identification methods and their specificity, sensitivity and reliability.
- (g) Information relating to the intended use. Information relating to the intended use of the living modified organism, including new or changed use compared to the recipient organism or parental organisms.
- (h) Receiving environment. Information on the location, geographical, climatic and ecological characteristics, including relevant information on biological diversity and centres of origin of the likely potential receiving environment.

Chapter 15

Risk assessment considerations of genetically modified micro-organisms for releases

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The environmental risk assessment of a genetically modified micro-organism (GMM) needs to consider its potential interactions with indigenous microbial communities in a given habitat. Interactions can relate to the survival of the GMM and/or the transfer of recombinant genes to indigenous community members. While there is already considerable knowledge about the biology and ecology of some species used as hosts for genetic modifications to inform their environmental risk assessments, in-depth studies on the biology, genetics and eco-physiology of other GM species may still be required before considering their use in not-strictly contained systems, for example for biofuel production or as biocontrol agents. Containment can be achieved when using GMM symbionts which are non-viable outside of their hosts, as demonstrated with Wolbachia *sp. and insects. Given the potential of non-symbiotic micro-organisms to spread in the environment, it appears desirable that a GM should not persist after its intended purpose of application has been achieved, even if it's presence does not necessarily translate to a risk, as it may have no adverse properties. In summary, in addition to a detailed characterisation of the genetic and biological properties of a GMM, in-depth knowledge about its interactions with its target and non-target environments is not only crucial to improve its efficiency, but also important to assess their environmental risks.*

Microbial community networks and resilience

The metabolic activity of micro-organisms is crucial for life on Earth. The cycling of atoms and molecules, which provide the basis for life, is only possible due to the metabolic versatility, niche colonisation, environmental persistence and overall abundance of microbial cells. As much as micro-organisms modify their immediate environment by transforming nutrients and excreting metabolites, the surrounding environmental conditions select for specifically structured microbial communities. While a huge part of the diversity of micro-organisms on Earth is still unknown in terms of species identity and particular physiological properties and potentials, the increasing speed of new nucleic-acid sequencing technologies and their high-throughput bioinformatic analyses opens access to many of them. These technologies increasingly allow viewing and appreciation of the complexity of microbial communities as they occur in ecosystems, e.g. soils, gastrointestinal tracts or intercellular niches provided by plants (Barriuso et al., 2011; Shokralla, 2012). Due to their long evolutionary history of millions or billions of years, the complexity of microbial communities is not random, but extremely stabilised in networks of interactions among their individual members. This microbial networking not only provides ecosystem services such as the biogeochemical cycling of elements, but is also required to directly protect humans, animals or plants against pathogenic micro-organisms (for an example for plants, see Van Bruggen et al., 2006).

While microbiologists do not doubt that microbial communities and their networking lay the foundation for life on Earth, it is still controversial whether or not they require protection and how this would be done, or whether they are self-regulated and highly stable in the first place. In this context, the characterisation and evaluation of resilience and robustness of such communities is a crucial factor (Allison and Martiny, 2008; Silva-Roche and de Lorenzo, 2010). This resilience of microbial community structure and function to disturbance can, in fact, be highly variable depending on the type of community and its environment, e.g. the buffering capacity of a highly diverse microbial community in a clay soil with organic matter is much higher than a community in a low pH sandy soil (Griffiths et al., 2008). Resilience will be different in systems with natural perturbation, e.g. variable amounts and qualities of inflowing nutrients, e.g. the human gut (De La Cochetiere et al., 2005; Dethlefsen et al., 2008), than in systems which are stratified and mainly undisturbed, i.e. biological soil crusts in desert ecosystems (Berard et al., 2011; Kuske et al., 2012).

The issue of resilience and buffering capacity of indigenous microbial communities translates into the problem of assessing the risks which would be associated with a release of genetically modified micro-organisms (GMM). There are several peculiarities about GMM as compared to genetically modified plants – for instance, their release is irreversible. While plants may be removed from a site with appropriate soil management strategies, the elimination of GMM from larger areas, e.g. agricultural fields or contaminated industrial soils, is hard to achieve. A practical example of the environmental persistence of a GMM was given by the first deliberate field release of two strains of the GM soil bacterium *Sinorhizobium meliloti* conducted in Germany in the early 1990s. These strains were tagged with a chromosomally inserted luciferase marker gene derived from a firefly. *S. meliloti* is capable of colonising the roots of certain legumes causing nodulation, i.e. lucerne (alfalfa) in which they mediate biological fixation of atmospheric nitrogen. But the species can also survive and grow in soil independent of such symbiotic partners. The strains, released in conjunction with seeding

of lucerne, were detectable for several years after their soil inoculation. Populations could be stabilised in the presence of lucerne in soil, but they were also maintained at a small, but rather stable, size independent of lucerne (Selbitschka et al., 2006) over several years. There were no indications that the inoculation affected the overall abundance and diversity of the dominant indigenous soil microbial community or the microbiological soil functions (Schwieger and Tebbe, 2000; Tebbe and Miethling-Graff, 2006). In environments with low resilience microbial communities, the presence of a GMM may already cause a structural or functional shift, even at lower population sizes, but in highly robust communities, huge amounts of GMMs would have no effect if they fail to occupy a niche within such systems, as demonstrated with the above-mentioned field release.

Risk assessments based on information on recipients

Chapters in the present volume give a good impression of how diverse the properties of GMM and their targeted environments can be. It is evident that the resilience of natural microbial communities may, in fact, limit the efficiency of a GMM to survive and perform its task, e.g. to promote plant growth in the rhizosphere or to degrade a pollutant in soil. A major challenge for the safe environmental application of a GMM is to construct on one side a competitive GMM but on the other side to limit its capacity to interfere with ecosystem services provided by the natural microbial communities. An environmental risk assessment should therefore consider how resilient or vulnerable an existing microbial community would be. Depending on the expected resilience of the natural microbial community, the level of scrutiny required in the risk assessment procedure could be different.

The severity of challenging the resilience of natural microbial communities also depends on the particular physiological and genetic properties and potentials of the GMMs themselves. Consideration of the environmental impact of the release of GMMs in general began with the advent of the possibility to generate them, as already described in the OECD "Blue Book" published more than 25 years ago (OECD, 1986). To date, there are a number of well-elaborated national and international consensus documents and guidelines for the environmental risk assessments of GMMs, including several documents issued by the OECD. For risk assessment of GMMs associated with the food/feed sector, a new guideline by the European Food Safety Authorities (EFSA) has been published (EFSA Panel on Genetically Modified Organisms, 2011b). All types of risk assessment consider the following aspects: molecular characterisation, hazard identification, exposure, direct and indirect effects of a GMM, intended and unintended effects on target and non-target organisms, comparisons with closely related non-GM organisms, and the availability of monitoring tools. As a basic requirement, the risk assessment and the derived risk management should be clear about protection goals on which they are based. Finally, risk assessments are not finished in advance of a field application or commercialisation, but continue to monitor effects, anticipated or not, once the GM organism or product is used. For GM plants, guidelines for such a post-market environmental monitoring have been developed (EFSA Panel on Genetically Modified Organisms, 2011a) and the principles would equally apply to GMMs for environmental use.

The molecular characterisation of the GMM and comparison to its non-modified counterpart, e.g. the non-modified parental strain, is an important starting point in risk assessment. Knowledge about the biology of a GMM can be gained from familiarity with the counterpart. Due to the fact that genetic modifications are preferentially done on

genetically very well-studied micro-organisms with a history of safe use, information on their biology and ecology is often available, e.g. in OECD consensus documents on certain micro-organisms, i.e. *Pseudomonas*, *Acinetobacter* or *Acidithiobacillus* (OECD, 1997; 2006; 2008). More recently, however, insights from cultivation-independent community analyses combined with novel techniques of cultivation of micro-organisms (Janssen et al., 2002; Stevenson et al., 2004), result in an increasing access to a novel diversity of environmental micro-organisms with the potential for biotechnological use. In such cases, additional in-depth studies on the biology, genetics and eco-physiological properties of a novel microbial species appear to be fundamental in the context of predicting their performance and assessing their environmental risks. GM cyanobacteria, as well as eukaryotic algae for the production of food, feed, chemicals or biofuels, were discussed at the OECD conference and it was indicated that due to a lack of familiarity with specific species, additional knowledge would be desirable in regard to their potential to colonise niches outside of their immediate application (contained use) in order to gain information for environmental risk assessments. This could also apply to GMMs used as biocontrol agents, exhibiting a capacity to colonise a target environment, e.g. a plant or the gut of an insect.

Environmental performance and containment

While knowledge of the biology and environmental behaviour, including pathogenicity, of a GMM is of crucial importance in a risk assessment, the recombinant genes also need to be assessed, since they may dramatically change the potential of an organism to survive outside of the laboratory. However, increasing the capacity of a GMM to survive in the environment is not a risk per se, as it may be an intended effect of the modification, e.g. to persist in a contaminated soil and degrade organic pollutants. Results with current bacterial inoculants, in fact, indicate that the risk of failure of a GMM to perform its desired activity in such soils is much higher than the risk it would impose on natural microbial communities (de Lorenzo, 2009). Similar constraints are likely to limit the success of bacterial inoculants in agriculture, e.g. to replace chemical fertilisation by biological nitrogen-fixation or phosphate mobilisation. A potential approach to enhance the viability and desired biological activities of bacterial inoculants could be to alter the expression of their natural genes by engineering their own promoters (Ryan et al., 2009). The huge gain of knowledge due to high throughput DNA-sequencing and bioinformatics delivers the tools which will probably allow progress from "spray and prey" to the successful design of GMM for more effective and reliable environmental applications (de Lorenzo, 2008). Should their survival and environmental exposure be enhanced through these practices, then the environmental risk assessment could differ in its level of required scrutiny from those applied before.

Ideally, GMM, once they have finished the job (for which they were designed), should disappear from the environment. A number of such concepts for containment, including bacteria with decreased fitness to repair mutations or substrate-inducible suicide-systems, have been developed and tested in the field and this principle of biological containment may become important for future applications (Molin et al., 1993; Schwieger et al., 2000; Torres et al., 2000). Due to the potential for mutational changes or other factors, such containment systems may not be 100% secure. On the other hand, bacterial symbionts, i.e. *Wolbachia* appear to be highly efficient containments systems, suggesting that for the control of insect-borne diseases the environmental spread of a GMM would be negligible (Alphey et al., 2002; Moreira et al., 2009).

An unintended environmental persistence of a GMM does not immediately and necessarily present a risk, since micro-organisms may be in resting cell stages, thus, metabolically inactive outside of their natural niche, or their metabolic activity may not interfere with the ecosystem functions provided by the existing microbial communities (see above the example of *S. meliloti*). The environmental persistence of a GMM may, however, correlate with its potential to travel beyond the immediate areas of application and thus enter non-target environments and ecosystems, which consequently would require an extended risk assessment of non-target effects. In this respect, GMMs with a tight symbiotic relationship, i.e. *Wolbachia* with insects or *S. meliloti* with certain legumes, could be preferable species for environmental applications.

Horizontal gene transfer

Horizontal gene transfer (HGT) is the stable transfer of genetic material between organisms without reproduction (OECD, 2010). The risk assessment of recombinant genes of a GMM must therefore not exclusively be linked to the GM host but require consideration of what those genes could do in another biological and ecological context (Davison, 1999; Snow et al., 2005). The environmental persistence of a recombinant gene may totally change once it has been transferred from one organism, e.g. a bacterium optimised to grow and survive in the gut, to another bacterium, capable of growing under nutrient limitation in soils or surface waters or a surface-colonizer with resistance to sunlight. Thus, because of the spread of microbial cells and because of HGT, an environmental risk assessment of a GMM should not only look at target environments but also at relevant non-target environments.

The likelihood of HGT depends on temporal and spatial aspects, densities of donor and recipient cells and their *in situ* physiological status, but also on molecular characteristics of the recombinant genes and their genetic context, e.g. the presence of homologous DNA stretches which may serve as sites for recombination, and whether the genes are located on mobile genetic elements and what the host range of such elements would be (Brigulla and Wackernagel, 2010; Thomas and Nielsen, 2005). Even though these factors have a dramatic influence on the likelihood of HGT in the range of ten or more orders of magnitude, the crucial question to be answered in risk assessing a GMM is what a hazard of the recombinant gene could be in any imaginable host, including potential pathogens, thus following a worst-case scenario approach. It should be noted that the HGT event itself has, in principle, no immediate consequences as it normally would occur between single cells within a background of billions. To become detectable and environmentally significant, growth in competition with indigenous micro-organisms would be required. Thus, the consideration of whether a selective advantage would be provided by the genetic modification is a crucial component in considering the environmental risks of horizontal gene transfer.

Conclusion

In addition to direct biological effects of a GMM, including their potential for HGT, hazards may also be caused by indirect effects. Indirect effects may include consequences of the replacement of an existing technology by utilising one linked to the use of a GMM. The assessment of indirect effects can be complex and may require interdisciplinary approaches, including modelling to making predictions from small-scale experiences and contained uses to broader non-contained applications. This effort incorporated into a risk assessment of a GMM, as described above, is clearly rewarding if GMM technologies, as

presented in this volume, are developed to improve the efficiency and environmental friendliness compared to current applications.

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Chapter 16

Overarching issues in the environmental risk assessment of deliberate release of transgenic micro-organisms

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A lot of work has been done on a large number of bacterial species that we know are present in the environment. This work has yielded important results for fundamental science as well as for biotechnological applications. But the environment has much more to offer in terms of bacterial variety, genomic variety and useful genes that remain to be discovered. One way to exploit these possibilities is the study of the soil metagenome, DNA sequences directly isolated from soil samples.

The genes that are isolated by the various techniques can be used in genetic engineering to improve bacterial strains that are available and that can be handled. This raises questions about risks, for instance the risk of horizontal transfer of these transgenes between organisms, i.e. between higher organisms and bacteria, as well as between different bacteria. One way to minimise the chances of such horizontal gene transfer (HGT) is to reduce the homology between transgenic DNA in donor organisms and the DNA in recipient organisms. With all the enthusiasm about the environment as a source of biodiversity, it should be recognised that the environment is very promising, but also extremely difficult to investigate, and difficult to control.

Exploitation of bacterial diversity in the environment

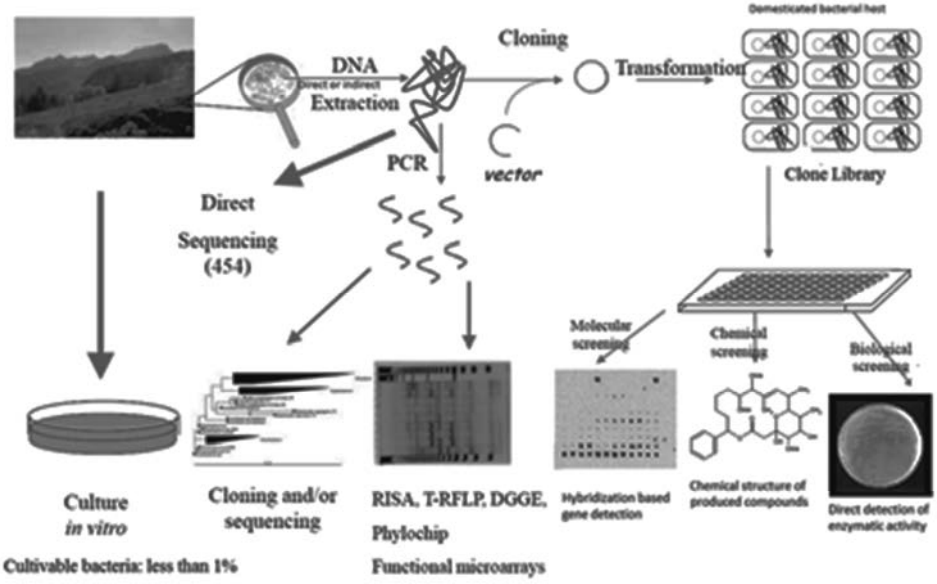
This volume has presented much fascinating information about prokaryotes in the environment and their (potential) roles in environmental processes. Still, it should be recognised that we have seen only a small part of what the environment has to offer in terms of useful micro-organisms and their functions. The list of organisms that are routinely used is already quite long, and the use of biotechnology and genetic modification may add organisms to the list, for instance attenuated strains of pathogens.

But there is a huge reservoir of microbial functions that has as yet been hardly tapped. But this is within reach: the functions that can be identified through metagenomics and exploited through genetic engineering are increasing rapidly, which together brings us the potential of "synthetic biology".

Metagenomics is the study of genes isolated directly from environmental samples, not from organisms cultured from environmental samples.

Figure 16.1 shows different ways to exploit the metagenome. Traditional experiments that look for cultivable bacteria will yield less than 1% of the existing diversity. By DNA extraction, the full genomic diversity can, in principle, be accessed (that is, if all DNA is extracted, see below). The DNA sequences can be analysed directly by various sequencing and hybridisation techniques or they can be cloned as a library, and the clones can be characterised by hybridisation-based gene detection, by analysing the chemical structure of produced compounds, or by direct detection of enzymatic activity.

Figure 16.1. **Metagenome exploitation**



Source: Lombard, N., et al. (2006) "La métagénomique des communautés microbiennes : Écologie microbienne des sols", *Biofutur (Puteaux)*, No. 268, pp. 24-27.

The method of hybridisation-based gene detection can be used to explore an environmental DNA sample for genes encoding a certain type of enzyme; for example the industrially interesting Type I polyketide synthases (PKSI). Using the ketosynthase domain of one of the enzymes, 140 of 60 000 clones showed up positive in hybridisation,

and 40 genes that were sequenced were all new PKSI type enzymes showing no redundancy (Ginolhac et al., 2004).

The concepts developed in metagenomics and the results obtained in practice are important for fundamental as well as for applied science. As we learn more about the genomes of a bacterial species, the species concept in bacteria becomes even more challenged than it was already. This has led to the development of the concept of the pangenome: the total genetic information that is found in all different strains that belong to a species. Thus, the isolation of new genes and new pathways is important for fundamental science, trying to explain the taxonomy of bacteria, as well as for biotechnology, where these new genes and pathways can be used and "new" organisms can be constructed using the newly characterised genes.

There is also an important issue for risk assessment. The fate of the new organisms with new genes after their inoculation into the environment should be known, as well as the fate of their genes that can be transferred from one organism to another by HGT.

Horizontal gene transfer of transgenic DNA

The HGT of transgenic DNA, if and when it occurs, could be seen as biological pollution, and, in contrast to chemical pollution that is diluted over time, this biological pollution could be augmented in the environment if the new genes increase the fitness of the organisms. It should be kept in mind that a genetically engineered micro-organism, once it is released into the environment, cannot be called back.

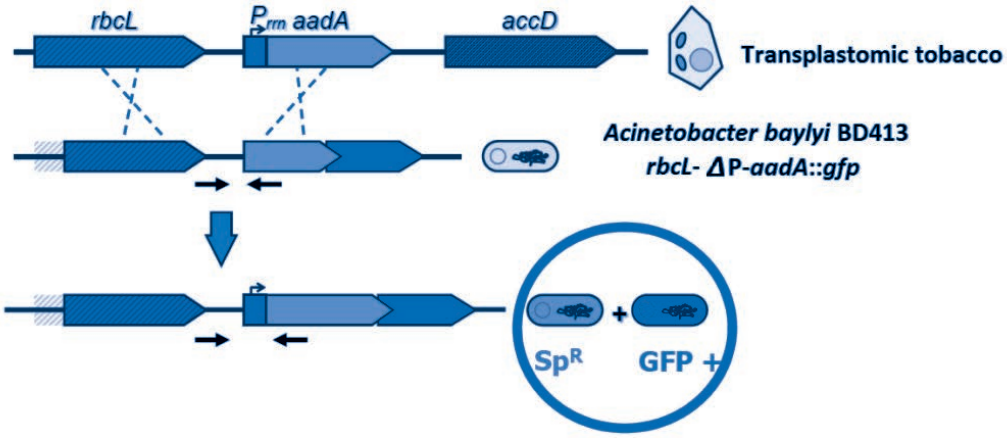
HGT is a very fundamental adaptive mechanism for prokaryotes. This is also evident from the sequences of bacterial genomes that are now known, and that all show evidence of being mosaic, i.e. made up of parts of genomes of other organisms. The question then becomes: what circumstances optimise HGT, and are there hot spots for HGT? A lot is known about the HGT of transgenic DNA from plant genomes to bacterial genomes, and from that we can learn some general facts.

First, Gebhard and Smalla (1998) showed that plant DNA carrying suitably selectable genes such as antibiotic resistance can be acquired by HGT *in vitro*. Transformation of bacteria also occurs *in situ*, as has been shown by Kay et al. (2002), who showed that in *Ralstonia*-infected plants there can be HGT of plant DNA to the *Acinetobacter baylyi* strain BD413. This gene transfer requires homology between the plant DNA and the bacterial DNA, but then the rate of gene transfer can be much higher in the plant than it is *in vitro*. HGT can now be visualised *in planta* (Figure 16.2; Pontiroli et al., 2009). Again, HGT is dependent on homology between the transforming DNA and resident DNA in the recipient.

Figure 16.3 shows transformants that result from HGT *in planta*, using this system.

The results show that sequence homology is the only barrier for HGT: when the experiment is set up in a way that there is sufficient sequence homology between the plant DNA and the DNA of the recipient micro-organism, HGT may occur at frequencies that can easily be observed, especially in decaying plant material. In conclusion: the potential for HGT in plant-bacteria interactions, and consequently also in interactions between bacteria, exists, and is an issue that has to be taken into account in risk assessment, but it can be mitigated by minimising sequence homology between transgenic DNA sequences and DNA sequences in potential recipients.

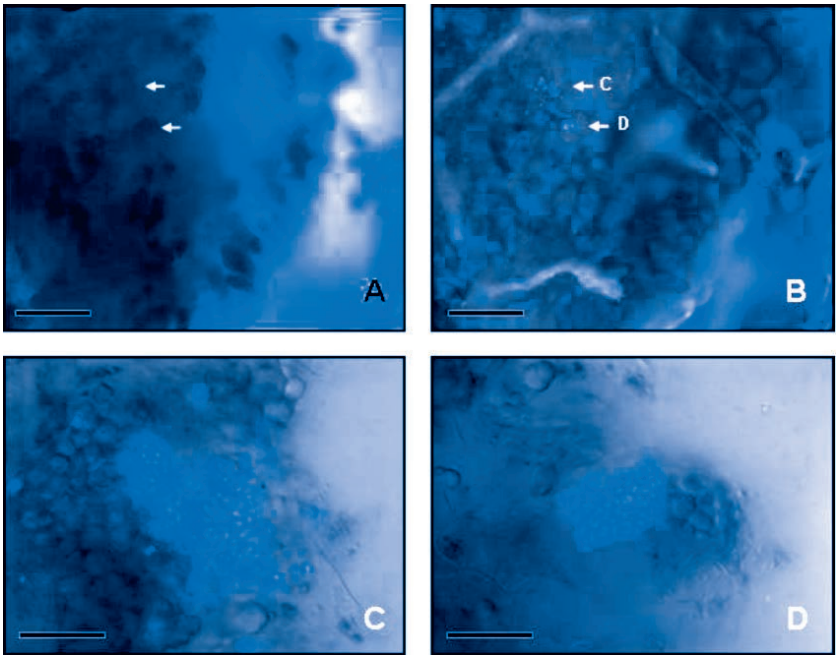
Figure 16.2. **Visualisation of horizontal gene transfer *in planta*: Genetic approach**



Notes: Transgenic chloroplast DNA carrying a bacterial *aadA* gene next to the chloroplast *sbcL* gene in the plant may transform *Acinetobacter baylyi* carrying the same genes, the *aadA* gene being deleted for the promoter and fused to a functional GFP gene. Recombination between the two gene sequences will result in *Acinetobacter baylyi* that has become spectinomycin resistant by expression of the *aadA* gene, and fluorescent by expression of the GFP gene.

Source: Pontiroli, A., et al. (2009), "Visual evidence of horizontal gene transfer between plants and bacteria in the phytosphere of transplastomic tobacco", *Applied and Environmental Microbiology*, No. 75, pp. 3 314-3 322.

Figure 16.3. **Visualisation of horizontal gene transfer *in planta***



Notes: A) Bright-field image, arrows point at the localisation of transformants. B) Epifluorescens micrograph showing transformants (green), chloroplasts (red) and veins (cyan. C and D) Details showing cell clusters of *A. baylyi* transformants expressing the GFP after restoration of the promoter activity through horizontal gene transfer between the plant and the bacteria. Bars: A, B: 50 µm; C, D: 20 µm.

Source: Pontiroli, A., et al. (2009), "Visual evidence of horizontal gene transfer between plants and bacteria in the phytosphere of transplastomic tobacco", *Applied and Environmental Microbiology*, No. 75, pp. 3 314-3 322.

Soil as a heterogeneous and complex environment

Ever since we have known about the vast amount of non-cultivatable bacteria in the soil, it is clear that soil systems are extremely heterogeneous and complex. It is a reservoir of genetic diversity but also a reservoir of problems. The Metasoil project,[1] which aims to establish the complete genome sequence of a soil sample, has shown a number of problems that have to be overcome. One problem and one of the impediments for establishing the metagenome of a soil sample is the difficulty to ensure that "all" DNA is extracted from the soil. There are quite a number of DNA extraction methods available, and it was found that the identity and the diversity of the DNA sequences isolated is very much dependent on the number of different extraction methods employed. As an example, using only one extraction method yielded only about 40% of all the sequences present on the Rothamsted soil phylochip, while by the use of 15 different methods on the same soil sample, 99% of the sequences were found. Thus, using two different methods on the same soil sample will yield two different populations of DNA sequences, and the degree of difference is similar to when DNA sequences derived from two different soils are compared.

This means that the metagenome approach is very promising, but also fraught with limitations as long as there is not better control over the DNA sequences that are extracted from the soil.

The take-home lesson of these considerations is: the soil is a most interesting environment, but it is extremely difficult to investigate, and difficult to control.

Note

1. www.genomenviron.org/Projects/METASOIL.html.

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