Genetically Modified Organisms for the Bioremediation of Organic and Inorganic Pollutants

Final Report

March 2002

WS Atkins Environment

Woodcote Grove, Ashley Road, Epsom, Surrey KT18 5BW
Tel:]01372] 726140@Fax:]01372] 740055

PREFACE

This study was carried out under DEFRA's *Genetically Modified Organisms (GMO) Research Programme,* which aims to underpin Government policy on the environmentally safe use of genetically modified organisms.

This report represents the findings of a desk study on the current and future uses of genetically modified organisms (GMOs) for the bioremediation of organic and inorganic pollutants, and an assessment of the risks of such uses to the environment and human health.

The report was compiled by Dr Colin Cartwright and Helen Folkard-Ward of WS Atkins Environment; Dr Ian Thompson, Dr Michelle Barclay and Prof Mark Bailey at the Centre for Ecology and Hydrology, Oxford; and Prof Andrew Smith from the Department of Plants Sciences, University of Oxford.

CONTENTS

List of Tables

List of Figures

EXECUTIVE SUMMARY

This report constitutes a review of the use of genetically modified organisms (GMOs) for the bioremediation of organic and inorganic pollutants. The report covers the current and future applications of GMOs in bioremediation, an assessment of the risks posed to the environment and human health, and the management strategies available to reduce the likelihood of any risks from being realised. The information presented in this report was compiled from details published in peer-reviewed journals, from information presented at a one-day workshop held as part of the project and the expertise of the project consortium.

Genetic modification technology is reported to offer a wide variety of current and potential applications for use in the bioremediation of pollutants. To date, three main types of GMOs have been developed and are currently undergoing field trials or already being used in commercial applications. These types are genetically modified microorganisms (GMMs) designed to degrade organic pollutants; genetically modified (GM) plants designed to hyperaccumulate or volatilise metal pollutants; and GMMs used as biosensors to detect the presence and toxicity of particular pollutants on site. No releases of GMOs into the environment have taken place in the UK for bioremediation applications, although contained applications of *lux* modified GMMs have been used at a commercial level to map the distribution of pollutants in contaminated sites.

All of the applications of GMOs for bioremediation have used bacteria or plants as the modified organism. Although fungi have the capability to degrade a wide range of often highly recalcitrant compounds, these organisms have not yet, to date, been genetically modified for use in bioremediation. Possible explanations for this include the relative complexity, at the genetic level of the degradation of organic pollutants by fungi compared to bacteria, and the identification of many fungi as plant pathogens, and therefore unsuitable for release into the environment.

In addition to their application in biosensors, the use of GMMs for bioremediation has focused on the degradation of organic pollutants, largely chlorinated compounds and hydrocarbons. In contrast, GM plants have been developed predominantly for the treatment of metal pollutants. Although GMMs have the potential to bioremediate metal pollutants, and GM plants are being developed to target organic contaminants such as nitroaromatic compounds, there is currently a distinction between applications for GMMs and GM plants.

This has arisen as a consequence of the existing levels of knowledge of the genes and metabolic systems involved, and as a result of the particular characteristics of the organisms themselves.

The genetic basis of the activity of microorganisms towards pollutants is less complicated than similar systems in plants. Consequently more information is available on the genes involved and the structures of the metabolic pathways. In bacteria, many of the processes involve only a few genes arranged on a single operon. This makes the identification and transfer of bacterial genes to other organisms relatively straightforward, certainly when compared to a similar approach with plants. However, although GM plants that incorporate bacterial genes have been developed, differences in guanine cytosine (GC) content and codon bias between microorganisms and plants mean that any bacterial genes used in plants may require some modification for them to function effectively.

The objective of the processes designed to bioremediate organic pollutants is the complete mineralisation of the compound. GMOs have been designed that are able to either mineralise the pollutant, or to perform one stage of the catabolic pathway that may otherwise prevent the biodegradation of the compound by the natural flora and fauna. Because metals cannot be biodegraded, their bioremediation requires the sequestration and/or accumulation of the metal by the organism. Whilst microorganisms are capable of sequestering metals, any bioremediation strategy using such organisms also requires systems to remove the metal/microbial complex from the environment. The advantage of using plants for the bioremediation of metals is that the metal pollutants can be accumulated in the plant, which can ultimately be removed from the contaminated site. GM plants have been developed to bioremediate metals, by the modification of plants with bacterial genes, for example the *merA* and *merB* bacterial genes used to bioremediate mercury contaminated sites; and also by the modification of faster growing high biomass plants with plant genes isolated from natural metal-accumulating plant species.

The types of risks posed by the use of GMOs for bioremediation are similar for both GMMs and GM plants, although the level of risk that might be realised differs between the two groups of organisms. The four hazards common to all GMOs (with the exception of GMMs used in contained systems for biosensor applications) are the transfer of genetic material, the accumulation of toxic compounds, the production of toxic metabolites and the disruption of other organisms and biological processes by the GMO.

The likelihood of each of these hazards is dependent on the characteristics of the individual GMO and the environment in which the organism is intended to be used. However, based on the types of GMOs that are reportedly being developed for bioremediation applications, the general consensus is that for GMMs the hazards most likely to be realised are the transfer of

genetic material and the disruption of other organisms or biological processes. For GM plants the transfer of genetic material and the accumulation of toxic compounds are identified as the most significant risks. However, in assessing the significance of the risks and the requirement of management strategies to minimise or reduce them, the unique characteristics of the contaminated site should be addressed.

Unlike the application of GMOs in agriculture, the contaminated sites that may potentially be bioremediated by GMOs usually have poor biodiversity and are often so contaminated that they do not support any vegetation. Therefore, the implications of the transfer of genetic material from GMOs to other organisms on the site, or the disruption on natural processes are likely to be low considering the limited number and diversity of other organisms present. Many of the modifications designed to bioremediate pollutants are also reported to offer the recipient organism no selective advantage outside the contaminated site, and may even offer a selective disadvantage to the organism in the absence of the specific pollutant.

Although the accumulation of toxic compounds has been identified as a hazard of using GMOs designed to sequester metal compounds for example, it should be noted that this is the intended purpose of the bioremediation strategy and the genetic modification. It should also be recognised that the use of plants to phytoaccumulate metals may be less hazardous to the environment and human health than alternative physical or chemical based remediation technologies.

Management systems are however available to minimise many of the risks identified. The insertion of the recombinant genes into the host organism by transposon mediated modification, rather than on a mobilisable plasmid, should reduce any subsequent transfer of those genes to other organisms. With GM plants, various horticultural techniques are available to prevent gene transfer, most of which are common to all GM plants. Examples of these include the use of sterile plants and species that reproduce vegetatively.

Restricting the GMO to the contaminated site by employing biological containment systems will reduce any impact the GMOs may have on the environment outside the contaminated site. Any bioremediation strategy (involving GM or non-GM organisms) will affect the organisms present in the contaminated site, as the strategy will, by design, alter the physical and chemical conditions present. The remediation of the site using physical or chemical processes will also have a significant impact on the organisms present, and this should be considered when assessing the risks posed by the use of GMOs.

Whilst a number of GMOs that have been designed for bioremediation are undergoing field trials, many of the developments are still at the laboratory or early field test stage. Improvements in the understanding of the genetic basis of the processes involved in the

biodegradation and bioaccumulation of pollutants, and as importantly, information on the basic mechanisms that determine how plants and microorganisms behave and interact in the environment, will lead to the development of a wide diversity of potentially very powerful applications to bioremediate pollutants. These have the potential to be able to target even the most recalcitrant pollutants in inhospitable environments at relatively low cost.

1. INTRODUCTION

BACKGROUND TO THE PROJECT

1.1 Anthropogenic activities such as heavy industry, mining and some agricultural practices have resulted in the contamination of the environment with a large number of organic and inorganic xenobiotic compounds. Such compounds are defined for the purposes of this report as compounds released through anthropogenic activities at concentrations that are higher than in the natural environment [1]. Xenobiotic compounds have the potential to harm environmental ecosystems directly, through their inherent toxicity [2], or through the disruption of important ecological processes [3]. Although improved practices have reduced the present level of chemical contamination of the environment, accidental releases of chemicals still occur, and a large number of contaminated sites still exist [4]. The total area of land in the UK contaminated by xenobiotic compounds has been estimated to range from 50,000 to 200,000 hectares (equivalent to almost 1 percent of total UK land area), and may include up to 100,000 contaminated sites [5].

1.2 Because of the potential for xenobiotic compounds to harm the environment and human health, and also the increasing demand for the re-use of contaminated (brownfield) sites for non-industrial purposes such as recreation or housing, there has been increased pressure to clean-up or remediate contaminated sites. The purpose of the remediation process is to prevent any subsequent exposure of the xenobiotic(s) at the contaminated site to the end-users of the site or to the flora and fauna present.

1.3 Remediation of contaminated sites involves treating the site in such a way that the contaminant is either removed from the site, converted into a less toxic form, or rendered immobile, so that subsequent exposure cannot occur. A wide variety of strategies have been proposed and/or demonstrated to remediate contaminated environments [6], with the type of pollutant to be removed and the characteristics of the site, including the location and distribution of pollutant, the principle determining factors. If the pollutant is present in discrete localised areas of a site, then those areas may be simply physically removed from the site and disposed of to landfill (so-called 'dig and dump' technology). This is a relatively inexpensive remediation strategy, but

does depend on a suitable receptor being available to receive the contaminated material. Where the contamination occurs over a wide area then removal of the contaminated material may not be feasible, and some form of on-site strategy is likely to be required. This may involve a physical based process such as air-sparging, or a biological/chemical based process to remove or degrade the pollutant.

1.4 The use of biologically-based processes to remediate environmental pollutants is described as bioremediation [7]. This type of strategy can offer a more suitable alternative to physical and chemical methods of dealing with contaminated sites. In particular, bioremediation can be less expensive [8] and importantly can be used to achieve the selective remediation of the target contaminant without incurring significant collateral damage to existing flora and fauna at the contaminated site [6]. Bioremediation strategies can be applied to the contaminated material *in-situ*, whereas removal of the polluted material to designated landfill sites, or extraction of the contaminants using physical processes simply concentrates the contaminated material in a different location [9].

1.5 Bioremediation is applicable particularly for the *in-situ* remediation of contaminants present at low concentrations, and for the selective removal of individual pollutants [4]. When applied to organic pollutants, bioremediation has the potential to achieve the complete destruction of the contaminant [10]. The bioremediation strategy that is employed may be as simple as the addition of nutrients to the contaminated site to improve the ability of the indigenous microbial community to degrade the pollutant (biostimulation) [11], or may be more complicated and involve the addition of particular organisms to the site (bioaugmentation) [6]. Such organisms are usually selected due to their capability to sequester, degrade or transform the pollutant and thereby bioremediate the contaminated site.

1.6 The purpose of this report is to review the current and potential future applications for the use of genetically modified organisms (GMOs) in bioremediation strategies. GMOs are defined as those organisms whose genetic profile could not have occurred naturally by mating and/or natural recombination. Although the capability to degrade many xenobiotic compounds is possessed by a range of non-GM (genetically modified) organisms, particularly microorganisms, the continuing persistence of some xenobiotics in the environment, including those that are known to be biodegradable, indicates that the biodegradative capacity of an environment's resident flora and fauna is not expressed completely or effectively [12]. Developments in molecular biology have the potential to produce organisms with new combinations of traits that can remediate previously unbiodegradable compounds [13], or degrade xenobiotics at a higher rate and/or to a greater extent than naturally occurring organisms [8].

1.7 The release of GMOs into the environment is regulated in the UK by DEFRA[1]. Applications to release GMOs are only approved on the basis that the release poses negligible risk to the environment and human health. Whilst no applications have been received by DEFRA to date in relation to the use of GMOs in the bioremediation of pollutants, the technology does exist to use GMOs in this way.

1.8 As a footnote to this review, it is recognised that the use of GMOs in the UK is the subject of intense public and political interest. Whilst this study has focused entirely on the scientific issues involved, it is recognised that the public can only benefit from access to current, objective and comprehensive reviews of both the use of GMOs and the likelihood of any risk to the environment and/or human health being realised.

AIMS AND STRUCTURE OF THE STUDY

1.9 The aims of this study were to review the use of GMOs for bioremediation and assess the risk of these applications to the environment and human health. These aims have been addressed through the completion of the following objectives:

(a) to identify and review current and potential future applications for the use of GMOs in the bioremediation of organic and inorganic pollutants;

(b) to assess the risks, in scientific terms, of the use of GMOs in the bioremediation of pollutants to the environment and human health; and

(c) to propose management strategies that could be employed to reduce any of the risks identified.

1.10 Whilst the report is intended as a review of the use of GMOs in bioremediation, and not the use of bioremediation strategies to treat contaminated sites, it is recognised that, to date, the use of GMOs in the field to bioremediate pollutants is very limited. Because applications of GMOs are likely to be developed to overcome the limitations of non-GM organisms, then this review will include some discussion of the use of bioremediation strategies involving non-GM organisms. This is particularly relevant to the future uses of GMOs in bioremediation.

1.11 The review of the use of GMOs in bioremediation has been presented in this report in four stages. These represent the principle types of GMOs that are, or have the

[1] Under Part VI of the Environmental Protection Act 1990 and Genetically Modified Organisms (Deliberate Release) Regulations 1992 (amended 1995 and 1997).

potential to be used in bioremediation applications, and cover the use of plants, bacteria and fungi. The fourth stage addresses the use of combinations of plants and microorganisms in bioremediation strategies.

1.12 The information presented in the desk study was supported by the findings of a workshop, held at Magdalen College, Oxford. The one-day workshop brought together people with expertise in bioremediation and plant and microbial disciplines, to discuss the use of GMOs in this field. The workshop provided the opportunity to review specific bioremediation applications of GMOs in more detail, and also to discuss the relative risks and benefits of the use of GMOs in bioremediation and the potential management strategies available. The specific areas that were addressed in detail during the workshop were:

- the underlying constraints affecting the application of microorganisms and plants in bioremediation;
- the use of reporter gene based biosensors for diagnostic bioremediation;
- the use of GMMs for the bioremediation of organic pollutants and as an online process monitoring tool during a long-term field release;
- the application of plants for the accumulation of metals;
- the use of GM plants for the phytoremediation of pollutants, particularly mercury; and
- the development of GM plants for the degradation of explosives.

1.13 A full report of the presentations and discussion that took place at the workshop is presented in Chapter 5 of this report.

2. REVIEW OF THE CURRENT AND POTENTIAL FUTURE USES OF GMOs IN BIOREMEDIATION

2.1 The purpose of this chapter is to review the current and potential future uses of GMOs in bioremediation strategies. The chapter is divided into sections addressing the use of bacteria, fungi and plants, and also strategies that employ a combination of organisms.

2.2 Applications to use GMOs for the bioremediation of pollutants encompass a range of strategies, for example, the development of organisms with the capability to degrade a specific contaminant. Although the approach used is often tailored to the individual characteristics of the xenobiotic and the contaminated site, most approaches can be assigned to a particular type of bioremediation strategy.

2.3 The most direct application of GMOs in bioremediation is the development of a GMO that can be added to the contaminated site and will degrade the xenobiotic *in situ*. This approach however requires that the GMO is able to survive in the contaminated environment and still function as required, and that the contaminant is available in a state in which it can be biodegraded (GMOs can be designed to improve the bioavailability of the target contaminant as well as degrade it, if bioavailability is identified as a significant inhibitor of bioremediation) [14]. Many contaminated sites, particularly those that are historically polluted by industrial activities are polluted with a cocktail of both organic and inorganic compounds [10].

2.4 The presence of other contaminants, in addition to the target pollutant, may prevent degradation of the target compound through inhibition of the catabolic process or as a consequence of their toxicity to the introduced GMO [10]. For example, where a site is contaminated with a mixture of chlorinated solvents and radionuclides, any GMO that is introduced to degrade the chlorinated solvents must also be resistant to radiation. Strains of the microorganism *Deinococcus radiodurans* are reported to be applicable for use in such environments, and GM *D. radiodurans* strains are reported to be capable of degrading 125 n mole ml^{-1} chlorobenzene in environments with radioactivity levels of up to 60 Gy h^{-1} [15].

2.5 The bioremediation of multi-contaminated sites must consider all the xenobiotics present before the addition of possible bioremediating organisms. No single organism or community is likely to degrade all the organic pollutants present [10]. Survival of the inoculant in the contaminated environment may also depend on the concentration of target compound present. This is particularly relevant to microbial degraders, many of which use the target compound as a source of carbon, energy and/or nitrogen. If the concentration of target pollutant is too low, then the degrader will not obtain sufficient biochemical or metabolic benefit from utilising that compound, and degradation will not occur. If the concentration of the target contaminant is too high, then any toxic effect of the compound may inhibit the activity of the inoculant and degradation may be retarded [10]. If necessary, a consortium of inoculants or a combined strategy may be required where one or more of the inoculants present is designed to support the degrader with alternative carbon and/or nitrogen sources. Combined strategies using plants and rhizosphere competent microbial degraders (fungi or bacteria) are particularly suitable in these situations.

2.6 The low solubility and high hydrophobicity of many organic pollutants means that they are often not available for biodegradation. Biological reactions occur in or at the interface of the aqueous phase [4], and the rate and extent of the biodegradation of a pollutant is often limited if the contaminant partitions towards the particulate phase in an environment [16]. Strategies to bioremediate a poorly bioavailable compound, may require the addition of an organism able to improve the bioavailability of the compound, rather than degrade it (the capability to degrade the xenobiotic may already exist in the contaminated site, but may only operate at a low level). Such organisms include those that produce biosurfactants, and may be inoculated directly into the contaminated environment, or grown *in vitro* and the biosurfactant harvested and added to the environment on its own. The high cost of producing biosurfactants has, however, limited their application, and current efforts are reported to be directed towards designing inoculants that have both the desired catabolic trait and the capability to produce the required biosurfactant [14].

2.7 The ability of the natural flora and fauna to biodegrade a contaminant may also be limited due to the presence of a single rate-limiting step in the degradation reaction. The addition of an organism designed to optimise a particular stage of the degradation process, such as the rate-limiting step, may provide an effective strategy for the bioremediation of the pollutant.

2.8 Strategies that involve the degradation of the contaminant are of course restricted to organic pollutants. Strategies to bioremediate sites polluted with metals involve the use of organisms that can convert the metal into another form [17]. This may be less

toxic, more mobile in the environment, with greater bioavailability or immobility. Whilst strategies that involve the conversion of metal pollutants to a more immobile state (stabilisation) do not result in the metal being removed from the site, they do result in the metal becoming effectively 'locked-up' in the environment in a biounavailable state where it is unlikely to have an impact on human health and/or the environment [18]. Contaminants can be adsorbed or sequestered within the soil matrix or within particular organisms. Certain plants for example are able to bioaccumulate metals in above-ground tissues, or sequester them in their roots [19].

2.9 All applications of GMOs in bioremediation strategies are based on the exploitation or utilisation of a particular interaction between the organism and the target pollutant. In order to use that interaction effectively, its genetic basis must be understood. Current applications of GMOs in bioremediation have been developed following the identification and subsequent manipulation of certain genetic sequences. Future applications are likely to arise from the modification of existing processes, and also the use and manipulation of new genetic sequences that are now being identified.

THE USE OF MICROORGANISMS FOR THE BIOREMEDIATION OF POLLUTANTS

2.10 The ability of bacteria and fungi to utilise a wide range of compounds make microorganisms ideal candidates for use in the bioremediation of pollutants [8, 20]. Bacteria and fungi have a broad spectrum of metabolic capabilities [21, 22], which have evolved to enable the microorganisms to utilise natural compounds as sources of nitrogen, carbon and energy. Several hundred of the genetic systems encoding these capabilities are reported to be useful in bioremediation applications [7, 23]. However, because microorganisms have not been exposed to xenobiotic compounds for a sufficient length of time to develop catabolic pathways that are targeted directly at those chemicals, then many of the microorganisms that are able to utilise xenobiotic compounds do so using existing metabolic systems [24, 25]. For example, the enzymes used by *Pseudomonas putida* LB400 and *Alcaligenes eutrophus* H850 to degrade biphenyl and lightly-chlorinated polychlorinated biphenyls (PCBs) are reported to have evolved to degrade terpenes produced naturally by plants in the rhizosphere [26].

2.11 Because the pathways used for the biodegradation of xenobiotic compounds are only modifications of those employed for the utilisation of natural compounds [27], then it is likely that the biodegradation of xenobiotics by microorganisms does not occur at potentially the most optimum rate. This is proposed as one of the reasons for the continuing persistence of some xenobiotics in the environment [4].

2.12 Optimisation of the biodegradative pathway is therefore an important consideration if the bioremediation process is to operate effectively, and be adopted as part of a commercial strategy. The characteristics of the contaminated site such as the types and concentrations of contaminants present (including the existence of any toxic or inhibitory waste types) and the bioavailability of the target compounds are also important considerations and will influence the rate, extent and success of the bioremediation process [22].

2.13 GM technology is reported to offer some solutions to the problems of co-contaminants and bioavailability constraints, and may also be used to develop monitoring tools to detect for the presence of pollutants in the environment [22]. However, it is the use of GM techniques to alter the metabolic pathways used by microorganisms to degrade pollutants that offers the greatest potential application of genetic modification in bioremediation. Few naturally occurring microorganisms possess the pathways required to mineralise the more recalcitrant xenobiotic compounds such as pentachlorophenol (PCP) and PCBs [28]. GM technology has the potential to improve existing catabolic pathways or to extend such pathways to include additional target compounds that may otherwise not be degraded [10, 13], and may also be applied to overcome the toxic or inhibitory effect of a particular pollutant or a metabolite [7].

2.14 Extension of the scope of catabolic pathways through the introduction of additional genetic sequences, or the alteration of existing genes is reported to offer the simplest application of GM techniques to bioremediation strategies [20, 29]. Many of the applications offer a generic approach, particularly in the optimisation of the bioremediation process.

General Strategies for the Optimisation of Bioremediation Applications

2.15 The degradation of organic pollutants by microorganisms typically occurs in a series of stages or steps [30]. Each stage of the catabolic pathway is mediated by enzymes that are produced following the transcription and translation of specific genetic sequences. Depending on the compound involved, the complete catabolic pathway may be encoded by a single microorganism, or may require a consortium of microorganisms, each performing one or more of the stages. Altering various aspects of the gene sequences that make up the catabolic pathway can improve the efficiency and efficacy of the pathway at each of these stages. Such alterations are not specific to individual pathways and may be applied to the biodegradation of all xenobiotic compounds, if sufficient information is available on the genetic basis of the degradative pathway.

Improvements in transcription of the gene sequences

2.16 The genes involved in the degradation of pollutants are usually arranged in operons carried on wide-host range, conjugative or mobilisable plasmids. Arranging the genes in operons ensures that transcription occurs in the correct sequence to produce the required enzymes [27]. Transcription of the catabolic operons is controlled at an individual operon level and also at a 'whole cell' level.

2.17 Individual operons are controlled by positively acting regulatory proteins which are activated by substrates or metabolites (termed effectors) present in the catabolic pathway. The control of transcription in this way ensures that the microorganism will only operate the catabolic pathway in the presence of specific substrates, and will not waste carbon and energy synthesising unnecessary proteins in the absence of those substrates [10].

2.18 The amendment of contaminated sites, with compounds that act as effectors for particular catabolic pathways, may improve the degradation of xenobiotics by activating the respective catabolic pathway. The promoter P_{lac} is activated by the addition of isopropyl-ß-D-thiogalactoside (IPTG), and is reported to function well in *Escherichia coli* under laboratory conditions [10]. The P_{tac} promoter also responds to IPTG and is reported to work better than P_{lac} in a variety of Gram-negative bacteria. However, IPTG is relatively expensive, and this means that the development of genetically modified microorganisms (GMMs) with promoter systems based on this effector are unlikely to be commercially viable for field release applications [10]. The P_m promoter of the *meta*-cleavage pathway of the TOL plasmid functions well in a number of Gram-negative bacteria, and is induced when the XylS regulatory protein is activated by the presence of benzoate and its derivatives [30, 31]. Unlike IPTG, benzoate is relatively cheap and is likely to be produced *in-situ* during the degradation of aromatic pollutants. Because of its existing use as a food preservative and its poor persistence in the environment benzoate has been described as an 'environmentally friendly' inducer [10]. Other systems that are reported to be applicable to field release systems are the salicylate-induced P_{sal}-NahR promoter-regulator, the T7 promoter and the XylR/P_u expression system which controls the upper pathway of the TOL plasmid and is induced by effectors such as toluene, ethylbenzene and xylene (present at many contaminated sites) [30, 32]. The T7 promoter has been incorporated for use in a number of soil bacteria [33, 34]. Future developments in this area are proposed to include expression systems based on responses to metals, such as CadC (cadmium regulatory protein) and ArR (regulatory protein for the arsenic resistance system) [35] and other environmental contaminants [30].

2.19 Operons, at a 'whole-cell' level, are controlled by global regulatory circuits. These are designed to ensure that the operons operate as part of the overall nutrient and energy requirements of the microbial cell. The global regulatory circuit responsible for the coordination of the transcription of catabolic operons is known as catabolite repression and acts to repress the operons used for the catabolism of certain substrates when other preferred substrates are available [10]. Therefore where the target compound is not a preferred substrate, degradation of the target compound may be blocked by catabolite repression.

2.20 Inactivation of the catabolite repression circuit through genetic modification may however lead to a reduction in the competitiveness of the microorganism and for the purposes of bioremediation is therefore undesirable. A preferred approach is to block the catabolite repression of the specific operon used in the degradation of the target compound. This can be achieved through the identification and elimination of the sites in the operon promoter where repression is exerted [10].

2.21 Activation of the catabolic operons occurs when the relevant effector is present at a sufficient concentration (termed the effector concentration threshold). For example, the catabolic promoters of the TOL plasmid are activated at an effector threshold concentration of 5-50 μM [36]. The *Pm* promoter of the TOL plasmid pWWO in *Pseudomonas putida* is activated at a concentration of 1 ppm of benzoate [37]. The *Pm, Pu* and *Psal* promoters, which are activated by alkyl- and halobenzoates, alkyl- and halotoluenes, and salicylates respectively, have a broad host range and can function in a number of genera [37]. However, if the aim of the proposed bioremediation strategy is to degrade the target pollutant to a concentration lower than the effector concentration threshold, then an alternative catabolic pathway may be required. Otherwise, when the pollutant drops to a concentration below the effector concentration threshold, transcription of the catabolic operon will not occur, and no degradation will take place. If an alternative catabolic operon is not available then the microorganism may be genetically modified to place the catabolic genes under the control of a different promoter that is not activated by the target compound [10].

2.22 The use of alternative promoters means that the activation of the catabolic operon can be effectively separated from the needs of the microorganism to use the pollutant as a carbon source. In addition to enabling the pollutant to be degraded to below the level required to support microbial growth, this approach can also be used to degrade pollutants that would provide little or no benefit to the microorganism in terms of carbon or energy, for example trichloroethylene (TCE) [10]. Where the bioremediative function of the microorganism is separated from the requirement to use the pollutant as a carbon source, the microorganism must be able to utilise other

carbon sources to maintain its energy balance. One option that avoids having to add supplementary carbon sources to the contaminated site is the introduction of the desired catabolic trait into microorganisms that naturally colonise the rhizosphere [4, 37]. For example, Mondello (1989) [38] reported the genetic modification of a root-colonising strain of *Pseudomonas* with a *bph* gene cluster used in the biodegradation of lightly chlorinated PCBs.

2.23 The application of genetic modification techniques to insert alternative promoters also increases the range of effectors that may activate the biodegradative pathway, and therefore offers greater flexibility to the bioremediation process [4]. Such effectors may confer greater transcription of the catabolic operon and ultimately improve the efficiency of the catabolic process. However, to ensure that the inserted trait works well in the environment, it is important to use a promoter that is known to function in the target environment, and then fit the modified genes to that promoter. This is likely to be more successful than attempting to modify a promoter that works well under laboratory conditions to operate in the field [37].

2.24 If suitable promoters are not available, then the modified genes can be inserted into the host microorganism using a minitransposon vector. The location of the modified genes adjacent to the terminus of the minitransposon will result in the genes being controlled by a promoter sequence indigenous to the host microorganism. Screening of the GM microorganism is subsequently required to identify the most suitable mutant strain [37].

2.25 The use of an indigenous promoter sequence to control the inserted gene may confer a greater level of expression than the gene's own promoter sequence. For example, insertion of the *opd* gene from *Flavobacterium* sp ATCC 27551 and from *P diminuta* into other Gram-negative bacteria under the control of its own promoter sequence results in the poor expression of the gene. Expression of the *opd* gene (which confers the ability to degrade the pesticides parathion and methyl parathion) is much better if it is placed under the control of the modified organism's own promoter [20].

2.26 Expression of the catabolic genes in contaminated environments that are lacking in sufficient nutrients can be improved if expression of the catabolic genes is linked to starvation-induced promoters such as *groEL* [39, 40] and *tra* [41]. Under conditions of environmental stress, GroEL can constitute 3-4 percent of total cell protein. Starvation linked promoters, which provide a universal signal to activate heterologous gene sequences, are particularly suited to environments where the nutrient conditions are too low to support exponential growth of the microorganism [37]. Promoters responsive to carbon, nitrogen, iron and phosphate starvation have been characterised

in many Gram-negative bacteria [37]. A further advantage of using starvation-induced promoters is that it avoids the problems (technical, biological and financial) associated with the introduction of large quantities of nutrients into contaminated sites. Matin *et al.,* (1995) [42] used starvation promoters from *E. coli* to control synthesis of toluene monooxygenase in pseudomonads, with a resulting 60-90 percent reduction in nutrient demand for transforming a given amount of TCE compared to wild type microorganisms.

Improving translation

2.27 Improving the translation of a genetic sequence is particularly useful in optimising the rate-limiting step of a reaction. The rate-limiting stage is often due to the relative lack of specific protein compared to the others required for the pathway. However, unless the improvements in translation are targeted, any measures are likely to lead to the increased production of all proteins and may therefore not necessarily address the lack of a specific protein (unless that necessary gene is under the control of a separate promoter).

2.28 Alteration of the translation initiation region (TIR) of the relevant gene by site-directed mutagenesis can be performed to ensure that more of the required protein is synthesised in relation to the other proteins [10]. However, as TIRs are reported to behave differently in different hosts, then site directed mutagenesis may not solve the problem when applied from one taxon to another one. Translational enhancers can be used to ensure that translation of the desired sequence occurs in a range of host organisms, and will therefore avoid the potential problems of host specificity encountered with TIRs [37]. Translational enhancers are short sequences (40-50 bp) present in some plant viruses. The insertion of a translational enhancer from tobacco mosaic virus enabled the gene for chloramphenicol acetyl transferase to be expressed in *E. coli, S. typhimurium, Erwinia amylovora, A. tumefaciens, A. rhizogenes, Rhizobium meliloti* and *Xanthomonas campestris* [43].

2.29 Stability of the mRNA will also influence the expression of the desired trait. The mRNA of gene 32 of the bacteriophage T4 is extremely stable (due to structural determinants at its 5' end). The fusion of the native promoter/TIR region of gene 32 to various heterologous genes has been shown to result in the production of more stable transcripts when expressed in heterologous hosts. The fusion of this transcript to the *xylE* gene (encoding catechol 2,3 dioxygenase) has been reported to result in the expression of high levels of the reporter product in Gram-negative bacteria such as *A. tumefaciens* and *X. maltophila* [44].

2.30 The introduction of DNA cassettes into the 5' untranslated region of a gene of interest is reported to improve the stability of the mRNA by introducing hairpin structures at the 5'-end of the mRNA [30]. Because the hairpin-containing mRNA had a half-life three times greater than that of equivalent mRNA with no hairpins, then the introduction of hairpins increases the amount of mRNA and therefore protein that is produced [30].

Improving protein stability and activity

2.31 Improvements in transcription and translation will result in an increased production of the enzymes and proteins required for the biodegradation of the target contaminant. However, poor stability and activity of the proteins and low substrate specificity may still restrict the efficiency of the whole biodegradative process [10].

2.32 The stability of a protein may be improved by altering the sequence of its amino acids [45]. For example, the aromatic ring cleavage enzyme catechol-2,3-dioxygenase is slowly inactivated by its substrates oxygen and catechol derivatives such as 4-ethylcatechol. Ramos *et al.,* (1987) [46], reported that by modifying the catechol-2,3-dioxygenase by two single amino acids caused the enzyme to be less susceptible to inactivation by 4-ethylcatechol and more stable in the presence of this substrate.

2.33 The activity and substrate specificities of enzymes can be altered through the development of hybrid gene clusters. These encode subunits of different enzymes to produce a single enzyme with superior transforming capability. For example, the degradation of TCE by an *E. coli* genetically modified to express a hybrid gene cluster consisting of genes from the toluene metabolic *tod* operon and the biphenyl metabolic *bph* operon, was much faster than non-GM *E. coli* cells expressing either the original toluene dioxygenase genes (*todC1C2BA*) or the original biphenyl dioxygenase genes (*bphA1A2A3A4*) [47]. The hybrid cluster consisted of *todC1* (encoding the large subunit of toluene terminal dioxygenase in *P. putida* F1), *bphA2* (encoding the small subunit of biphenyl terminal dioxygenase in *P. pseudoalcaligenes* KF707), *bphA3* (encoding the ferredoxin in *P. pseudoalcaligenes* KF707) and *bphA4* (encoding the ferredoxin reductase in *P. pseudoalcaligenes* KF707).

2.34 Hybrid enzymes have also been developed to produce a single enzyme system capable of degrading benzene and tetrachlorobenzene. The enzyme TecA chlorobenzene dioxygenase is able to dehalogenate partially 1,2,4,5-tetrachlorobenzene but has no activity against benzene, whereas the TodCBA toluene dioxygenase can dioxygenate benzene but has no activity against tetrachlorobenzene. The genetic modification of *E. coli* to express a hybrid enzyme consisting of the large alpha-subunit of the

TodCBA dioxygenase whose specific todC1 alpha-subunit subsequences had been replaced by equivalent sequences of the tecA1 alpha-subunit, resulted in a GMM with activity towards benzene and tetrachlorobenzene [48].

2.35 Improvements in protein activity can also be developed through 'forced evolution' in which the genes of interest are subjected to random mutagenesis followed by selective screening for desirable properties [7, 49] or by a more rational approach based on an understanding of the three dimensional structure of the protein and its structure-sequence relationship [7, 10]. Site-directed mutagenesis of the *bphA* gene of *Pseudomonas* sp LB400 for example increased the specificity of the expressed biphenyl dioxygenase to include congeners not degraded by the non-GM strain [50].

2.36 However, the information required for the more rational approach exists for relatively few degradative enzymes [7], including cytochrome $P450_{cam}$, haloalkane dehalogenase, dihydroxybiphenyldioxygenase and methane monooxygenase hydroxylase [10].

Extending the scope of existing catabolic pathways

2.37 Extending the scope of existing catabolic pathways to degrade new compounds avoids the requirement to develop wholly new degradative pathways and may therefore offer the most immediate route to the bioremediation of previously non-degradable xenobiotic pollutants. One route for improving the scope of a catabolic pathway is to widen the types of effectors that can regulate that particular pathway. This may allow the catabolic pathway to operate in contaminated sites that do not contain the effectors used in the non-modified pathway. Studies with the XylS regulator of the catabolic operon of the TOL plasmid found that although the pathway was activated by benzoate, some other benzoate analogues such as 4-ethylbenzoate competitively inhibited the effector-mediated activation of XylS. Mutation of the XylS regulator resulted in all benzoate analogues being able to activate the system [31]. The inclusion of new effector compounds may also confer a greater efficiency of transcription [10].

2.38 Extension of the catabolic pathway to include more substrates is described as either lateral or vertical. Lateral extension involves the incorporation of more analogues of existing substrates, and vertical extension describes the addition of totally new substrates into an existing pathway. Due to the modular nature of many catabolic pathways, the substrate range of a pathway can often be extended by adding a biochemically compatible module to the microorganism which enables a new substrate to be channelled into an existing pathway [10]. For example, the addition of

dehalogenase genes to microorganisms able to co-metabolise PCBs can extend existing pathways beyond the chlorobenzoate intermediate and improve the mineralisation of PCBs by single microorganisms [51]. Lateral expansion of a pathway can be achieved through the utilisation of isofunctional routes for the degradation of structurally related compounds, the preferential use of enzymes with relaxed substrate specificities, and site-directed mutagenesis to alter the specificity of proteins for their substrates [10].

2.39 The ease with which protein mutagenesis can be used to alter existing pathways to include previously non-metabolised compounds depends on the number of proteins that need to adapt before catabolism of the new target compound can occur. Where only a single protein needs to be altered, the required changes can be achieved by the direct selection of a mutant able to grow on the target compound [10]. However, where multiple 'non-permissive' proteins require alteration, the necessary mutations are likely to occur at too low a frequency to produce the desired microorganism. In this case, the proteins that are non-permissive for the new target compound must be identified and either sequentially modified to achieve the required specificity, or replaced by 'permissive' proteins from other organisms [10].

2.40 With respect to the catabolism of aromatic compounds, four points have been identified in the relevant catabolic pathways that are likely to require attention or alteration if the scope of the pathway is to be increased (either laterally or vertically) [10]. These are substrate/metabolite-activated transcriptional regulators, the mono- or dioxygenases that mediate the initial attack on the substrate, the ring cleavage enzymes and the enzymes that transform the substituted muconolactones to oxoadipate.

2.41 The inability of *Pseudomonas* sp B13 to degrade 4-chlorobenzoate (4CB) or 3,5-dichlorobenzoate (3,5DCB) was found to be due to the narrow specificity of the first enzyme in the pathway, benzoate 1,2-dioxygenase. This enzyme only allowed the pseudomonad to degrade 3-chlorobenzoate (3CB) [52]. Insertion of the genes encoding the isofunctional enzyme toluate 1,2-dioxygenase enabled the bacterium to degrade 3CB and 4CB. Toluate 1,2-dioxygenase has a much broader substrate specificity than benzoate 1,2-dioxygenase, and includes all alkyl- and chlorobenzoates. To degrade 3,5DCB the pseudomonad had to be further modified by mutation of the XylS regulator, as the existing one was not activated by the dichlorobenzoate [52].

Construction of recombinant microorganisms

2.42 Although potentially less straight-forward than the (minor) modification of existing pathways and/or microorganisms, the construction of recombinant microorganisms has the potential to provide a number of benefits that may not be available from the natural selection of non-GM microorganisms. Genetic modification has the potential to enable both the construction of bacteria with multiple degradative pathways and the creation of microorganisms that are able to degrade previously non-biodegradable compounds.

2.43 The development of recombinant microorganisms with the capability to degrade xenobiotic compounds completely may be preferable to the use of consortia of non-GM microorganisms, where most stages of the catabolic pathway are conducted by different microorganisms. The limitations with a consortium based approach to biodegradation are that pathway intermediates may need to be transported between cells resulting in loss of efficiency of catabolism [53], and that metabolites may be misrouted into dead-end incomplete pathways by some members of the consortium, or transformed into toxic metabolites that may subsequently destabilise the entire process [10]. The use of genetic modification, to ensure that the entire catabolic process is conducted by a single cell may have the potential to avoid these problems.

2.44 The biodegradation of chloro- or methylcatechol (formed during the degradation of chloro- and methylaromatic compounds) is a well-studied example of the potential problems associated with the misrouting of pathway intermediates [53, 54]. Although many soil microorganisms possess the abilities to degrade chlorocatechol by the *ortho* cleavage pathway and methylcatechol by the *meta* cleavage pathway, both pathways are only expressed in the presence of the respective substrate. Very few strains of microorganisms are able to grow effectively on mixtures of methylated and chlorinated aromatic compounds [54].

2.45 Exposure to either group of compounds on their own does not result in the production of dead-end or inhibitory metabolites. However, in contaminated sites the presence of chloro- and methylaromatic compounds means that both pathways are likely to be induced and this can lead to inefficient degradation [54]. Catabolism of methylcatechol by *ortho* cleavage results in the formation of dead-end methyllactone metabolites [54], and the compounds formed during the *meta* cleavage of chlorocatechol inhibit the activity of the aromatic ring cleavage enzyme catechol 2,3-dioxygenase [53, 55].

2.46 To avoid the formation of potentially inhibitory metabolites, Erb *et al.*, (1997) [53] developed a GM pseudomonad (designated *Pseudomonas* sp B13 SN45RE) that was able to degrade both chloro- and methylaromatic compounds by the *ortho* cleavage pathway. Another pseudomonad (*Pseudomonas* sp B13 FR1(pFRC20P)), genetically modified to utilise chloro- and methylaromatic compounds by a constructed *ortho* cleavage pathway was able degrade a mixture of 3-chlorobenzoate and 4-methylbenzoate (25 μM of each) in intact sediment cores with an overlying water column throughout a 4 week period [56], highlighting the potential for GMMs to be used in the environment to overcome problems inherent with non-GM microorganisms.

2.47 Analysis of the degradation of 3CB and 4MB by *Pseudomonas* sp B13 FR1(pFRC20P) in water and in sediment microcosms found that the observed and theoretical half-lives corresponded well, indicating that GM pseudomonad functioned optimally in these environments [57]. The physiological characteristics of the GMM and its performance in aquatic microcosms are assessed to make this microorganism a good candidate for *in situ* bioremediation at sites contaminated with mixtures of chlorinated and methylated aromatics [57].

2.48 Genetic modification can also be used to develop microorganisms that are more tolerant to the environmental stresses likely to be encountered in a contaminated environment. Although microorganisms indigenous to the contaminated site are likely to be adapted to the stresses to some extent, stress factors such as poor oxygen, water and nutrient availability, high concentrations of toxic pollutants and extreme pH may be expected to have some restriction on the level of *in situ* biodegradation that occurs. Genetic modification has the potential to confer improved tolerance or resistance to the likely stresses, and consequently improve the performance of the degradative microorganism [10]. The coupling of the catabolic genes to promoters that are responsive to environmental stresses such as low pH enables the degradative properties of the GM microorganism to be enhanced in environments where those stresses are likely to be realised, such as leachate from acid mine waste. Placing the catabolic genes under the control of such promoters also means that the expression of those genes is restricted to the particular 'stressed' environments (see Chapter 4).

Use of Bacteria for the Bioremediation of Organic Pollutants

2.49 Applications to bioremediate organic pollutants that have been reported to date (GM and non-GM) have focused on the use of bacteria rather than fungi. Although both groups of microorganisms have a number of unique advantages that are applicable to their use in bioremediation strategies, it is likely that the ease of culture of bacteria

and the greater level of understanding of bacterial genetic sequences are the factors that are ultimately responsible for the more widespread application of bacteria in bioremediation strategies. Bacteria are also reported to be more amenable to genetic modification and are capable of metabolising chlorinated organic compounds [20, 58].

2.50 With the exception of the application of cytochrome P450 systems, the use of GM bacteria for the bioremediation of organic pollutants is presented in this report in sections according to the target compound. Applications to use GM bacteria in the environment are likely to be targeted at specific pollutants such as PCBs or nitroaromatic compounds. Cytochrome P450 systems have a broad substrate specificity and potentially have applications in the biodegradation of a wide range of organic pollutants. The information presented reviews the current state of the science regarding the use of GM bacteria to bioremediate organic pollutants, and where possible, provides an indication of future developments in this field.

2.51 The most likely area of future development is the modification of microorganisms with the genes currently being identified as important in the degradation of xenobiotics by indigenous microorganisms. As the level of understanding of the genetic basis of pollutant degradation increases so the potential for GMMs in bioremediation applications will also increase. Other possible developments include the use of genetic modification to provide 'support' to other bioremediation processes (GM or non-GM). Examples include the use of GMMs to prevent shock loading of natural microflora by toxic contaminants in wastewater treatment plants [53] and the genetic modification of drought or desiccation tolerant microorganisms. In addition to their use in dry environments, drought tolerant microorganisms may be more suitable for use in commercial bioremediation applications where the microorganisms are likely to be stored for a period of time until they are required in the field [59].

Cytochrome P450s for the bioremediation of organic pollutants

2.52 Cytochrome P450s are found in microorganisms, plants and animals and perform a range of chemical reactions, many of which are central to the degradation of organic pollutants, such as the cleavage of both ether and carbon-chlorine bonds [10, 49]. Microbial P450s are particularly applicable for the bioremediation of xenobiotics because of their ubiquity and strong reductive and oxidative potential [49]. The $P450_{cam}$ from *P. putida* for example is capable of dehalogenating halogenated methane and ethane substrates such as hexachloroethane and pentachloroethane (to tetrachloroethylene and trichloroethylene respectively) [49].

2.53 The potential of P450s for bioremediation of organic pollutants has developed from an improving understanding of the structure-function relationships in the various cytochrome P450s that have been isolated and characterised to date. The significant difference between the tertiary structure of individual microbial P450s is due to variations in structural interactions that form the substrate-binding pocket of the enzyme [49]. A number of reports have shown that by engineering changes in the design and structure of the substrate-binding pocket, the substrate specificity, range and catalytic efficiency of the enzyme can be altered [49, 60, 61].

2.54 Jones *et al.,* (2000) [62] reported that reducing the volume of the active site of the haem monooxygenase cytochrome $P450_{cam}$ enzyme expressed by *P. putida* through the substitution of amino acids with bulkier side chains improved the enzyme-substrate fit, resulted in extending the substrate range of modified microorganism from chlorophenol to include polychlorinated benzenes. Reducing the size of the active pocket of $P450_{cam}$ has also been found to increase the rate of dehalogenation of 1,1,1,2-tetrachloroethane by up to 150 percent compared to the non-modified wild type (Sligar *et al.,* 1996, cited by) [49]. Altering the substrate-binding pocket to increase the space available is reported to confer a more relaxed substrate specificity [63].

Chlorinated compounds

2.55 Chlorinated compounds have been detected as pollutants in many contaminated sites. The presence of these compounds is a consequence of their use by the chemical industry for a wide variety of applications, and also due to their formation during the degradation of polychlorinated compounds such as PCBs [29]. The principal groups of chlorinated compounds which have been targeted by bioremediation applications are the chlorobenzoates (CBAs) and dichlorobenzoates (DCBs); tetrachloroethene (PCE) and trichloroethylene (TCE); the chlorobenzenes (CB) and chlorinated herbicides such as 2-methyl-4-chlorophenoxyacetate (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D) and atrazine.

2.56 Many of the pathways involved in the biodegradation of chlorinated compounds share similar metabolites, and single enzymes are often able to catalyse the degradation of a number of different compounds. Chlorinated catechols for example are key intermediates in the degradation of chlorinated aromatic compounds, and the enzyme toluene 4-monoxygenase has activity against TCE, toluene, ethylbenzene, acetanilide, 2-phenylethanol and phenol [10, 64, 65]. The similarities of many of the catabolic pathways for chlorinated compounds do provide some advantages in the development of GMMs able to degrade a number of contaminants. However, the presence of a

number of chlorinated compounds in a single contaminated site may inhibit the degradation of particular compounds and result in the production of dead-end or toxic metabolites [53].

2.57 Compounds such as TCE are particularly suitable as targets for bioremediation by GMMs, as non-GM microbial degraders require the addition of specific substrates such as methane, ammonia, cresol or toluene, which are themselves environmental pollutants. The removal of TCE by chemical and/or physical-based processes is expensive and does not result in the complete degradation of the compound, respectively [66].

2.58 A number of studies have been conducted using *E. coli* and *P. putida* genetically modified to express the toluene monoxygenase genes from *Pseudomonas mendocina* KR1, and to degrade TCE in the absence of the types of substrate inducers described above [67]. Winter *et al.,* (1989) [68] constructed a GM *E. coli* to degrade TCE in the presence of glucose. Because the inserted genes were placed under the control of the temperature inducible lambda phage promoter (P_1), the cloned toluene monoxygenase genes were only expressed at a temperature of 42 ºC and above. The use of this GMM was therefore confined to a bioreactor bioreactor-based facility, the inserted genes would not be expressed if the GMM was released into the environment. The use of temperature sensitive promoters therefore represents a form of biological containment for GMMs.

2.59 *E. coli* and *P. putida* have also been genetically modified to degrade TCE through the insertion of a range of phenol catabolic genes (*pheA, pheB, pheC, pheD* and *pheR*) carried on plasmid pS10-45)[2] isolated from *P. putida* BH [66]. Although this microorganism is capable of degrading TCE in the presence of phenol, it is unable to degrade phenol, as were the *E. coli* JM103, *E. coli* HB101 and *P. putida* KT2440 used as host strains for the genetic modification. Modification of the *E. coli* strains with plasmid pS10-45 enabled them to degrade TCE at a concentration of 20 ppm. Comparison of the TCE degradation (1 ppm) by *E. coli* HB101, *P. putida* KT2440 and the non-GM *P. putida* BH showed that the recombinant *E. coli* strain removed the TCE more efficiently and to a greater extent than the pseudomonad strains. This was due to the inhibition of the *pheA* gene in the pseudomonads by the TCE metabolite TCE-epoxide. The *E. coli* was apparently unaffected by this compound. The results highlight the importance of selecting host organisms for genetic modification that are

[2] Genes encoding phenol hydroxylase, catechol-2,3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase, 2-hydroxymuconic semialdehyde hydrolyase and the positive regulation of the phenol catabolic operon respectively.

not sensitive to the formation of metabolites likely to be produced during degradation of the target compound [66].

2.60 The addition of phenol to a contaminated site to induce degradation of TCE may however be undesirable if the phenol itself has a toxic effect to the flora and/or fauna present. GM technology has been applied to develop microorganisms that have a similar capability to degrade TCE, but in the absence of potentially toxic substrate inducers such as phenol. Krumme *et al.,* (1993) [69] compared the degradation of TCE by the non-GM *P cepacia* G4 that was able to degrade TCE in the presence of phenol and tryptophan, with the GM *P cepacia* G4 5223 PR1 that expressed the toluene ortho-monooxygenase constitutively. Both microorganisms degraded TCE in groundwater microcosms from 50 μM to <0.1 μM in 24 h at a cell density of 10^8 cells ml^{-1} and both strains were also able to survive in aquatic sediment microcosms for a period of 10 weeks. In the environment the GM strain is therefore likely to behave in a similar way to the non-GM strain, and should therefore survive and compete with the natural microflora. However, the use of the GM strain may be preferred to the phenol dependent non-GM strain in field based bioremediation applications, should the use of phenol be of environmental or regulatory concern [70].

2.61 Where the GMM is unable or unsuited to survive in the field environment for a sufficient period of time to degrade the target contaminant, then the microorganism may be more suited to a bioreactor based bioremediation application, such as 'pump and treat' where a more contained and regulated environment for the microorganisms can be maintained [71, 72]. The ability of the microorganism to survive in the environment in which it is intended to be used is as important a consideration as the capability of the organism to degrade the target pollutant.

2.62 Many of the applications involving microorganisms in bioremediation (GM and non-GM) use heterotrophic soil microorganisms [73]. Whilst these organisms are likely to be reasonably well suited to survive in most soil and sediment environments, the poor nutrient conditions that are characteristic of many aquatic environments may be more suited to photoautotrophic microorganisms such as cyanobacteria. The use of such organisms would avoid the addition of organic nutrients to the inoculated environment, thereby reducing the costs incurred and maintenance required.

2.63 Two strains of cyanobacteria *Anabaena* sp and *Nostoc ellipsosporum* were genetically modified by insertion of *linA* (from *P. paucimobilus*) and *fcbABC* (from *Arthrobacter globiformis*) respectively. The gene *linA* controls the first step in the biodegradation of lindane (γ-hexachlorocyclohexane), and *fcbABC* confers the ability to biodegrade halobenzoates. The GM *Anabaena* sp showed enhanced degradation of lindane

compared to the wild type strain, and the GM *N. ellipsosporum* was found to able to degrade 4CB, a capability not expressed by the wild type strain [73].

2.64 Strong *et al.,* (2000) [11] avoided the issue of survival of the GMM in the environment by using an inoculum of killed *Escherichia coli* cells to remediate soil contaminated with the *s*-triazine herbicide atrazine under field conditions. The *E. coli* had been genetically modified to overexpress the *atzA* gene, isolated originally from *Pseudomonas* ADP. The *atzA* gene encodes the production of the enzyme atrazine chlorohydrolase that dechlorinates atrazine in a single step to hydroxyatrazine. This product is non-toxic to plants [11].

2.65 GM *E. coli* cells were grown under laboratory conditions and then killed and cross-linked by exposure to 0.3 percent glutaraldehyde. Cross-linking the cells was reported to retain more than 50 percent of the cell's enzyme activity, for up to eight months storage in buffer at room temperature. This was reported to compare favourably with the storage of purified enzymes [11]. Inoculation of the atrazine contaminated soil with 0.5 percent (*w/w*) of killed *E. coli* cells reduced the atrazine concentration by 77 percent (from 6700 ppm to 1450 ppm) in 8 weeks. This level of reduction required the supplementation of the soil with phosphate (300 ppm), although in the absence of the killed cells, the addition of phosphate had no significant effect on atrazine levels.

2.66 The complete degradation of polychlorinated compounds such as pentachloroethane and PCBs requires the sequential occurrence of anaerobic and aerobic reactions [25]. Degradation proceeds by an initial reductive dehalogenation reaction (under anaerobic conditions), which converts the parent compound to the substrates susceptible to attack by bacterial oxygenases produced under aerobic conditions [25]. The requirement for two sets of degradation pathways poses a challenge for the development of a single microorganism with the capability to degrade such compounds completely. (The advantages of using a single microorganism to encode the entire degradative process have been presented earlier in this report). Wackett *et al.,* (1994) [25] used the cytochrome $P450_{cam}$ and toluene dioxygenase (encoded by the *tod*C_1C_2BADE genes) systems to catalyse the consecutive reductive and oxidative dehalogenation reactions (respectively) required to degrade pentachloroethane. In the presence of camphor *P. putida* G786 (pDTG351) converted pentachloroethane to TCE, and aeration of the culture with oxygen resulted in the reduction of TCE levels. Utilisation of TCE by this GMM was improved by placing the *tod* genes under the control of a hybrid *tac* promoters [25].

2.67 The same system has also been applied to the biodegradation of polybrominated and chlorofluorocarbon compounds [74]. Under anaerobic conditions, the GMM (designated *P. putida* G786 (pHG-2)) [74] reduced 1,1,2,2-tetrabromoethane, 1,2-dibromo-1,2-dichloroethane and 1,1,1,2-tetrachloro-2,2-difluoroethane to less halogenated compounds. Under aerobic conditions, the GMM oxidised *cis*- and *trans*-1,2-dibromoethenes, 1,1-dichloro-2,2-difluoroethene and 1,2-dichloro-1-fluoroethene [74].

*Polychlorinated biphenyl*s

2.68 The application of GMMs to the biodegradation of PCBs has only been reported for the aerobic part of the degradation pathway, i.e the degradation of lightly-chlorinated biphenyls. Dehalogenation of the more highly-chlorinated biphenyls, which requires anaerobic conditions [75] is reported to be less amenable to manipulation, by genetic modification, with fewer potential improvements available [76].

2.69 The aerobic biodegradation of PCBs proceeds in a similar manner to the metabolism of biphenyl [77], and consists of an upper pathway involving the oxidation of the PCB congener to the corresponding chlorobenzoate, and a lower pathway through which the chlorobenzoate undergoes complete mineralisation [78]. However, many PCB degrading microorganisms are unable to degrade PCBs beyond the formation of the chlorobenzoate, particularly where the microorganisms are exposed to a number of different PCB congeners[3].

2.70 The initial stages of the aerobic pathway involve the insertion of two atoms of molecular oxygen at the 2 and 3 positions of one of the biphenyl rings, and are catalysed by the enzyme 2,3-dioxygenase. The differing ability of microbial degraders to utilise different PCB congeners may be due to the organisms possessing single non-specific dioxygenases or multiple dioxygenases. Gibson *et al.,* (1993) [79] dismissed the idea of multiple dioxygenases and proposed that differences in substrate specificity of two species of pseudomonad was due to differences in as few as two amino acids in BphA. Further studies have supported this proposal and have demonstrated that the BphA1 subunit of the biphenyl dioxygenase (encoded by $bphA_1$) is responsible for the recognition of the PCB molecule and consequently the range of congeners that can be degraded by the particular microorganism [80]. This observation has enabled the $bphA_1$ gene to be modified by site-directed mutagenesis, to extend the congener specificity of PCB degrading microorganisms. The

[3] PCB congeners differ in the number and position of the chlorine atoms on the biphenyl. Because PCBs were always manufactured as mixtures of different congeners then environments contaminated with PCBs are likely to contain a range of different PCB congeners.

modification altered the Thr-376 to Asn-376 in the biphenyl dioxygenase and extended the congener specificity of the *P pseudoalcaligenes* KF707 [80, 81].

2.71 The genes that encode the capability to degrade PCBs are organised on the *bph* operon, and include *bphA*$_1$, *bphA*$_2$, *bphA*$_3$, *bphA*$_4$, *bphB, bphC* and *bphD* [13]. The genes *bphA*$_1$ to *bphA*$_4$ encode a multicomponent dioxygenase enzyme that catalyses the degradation of the biphenyl to biphenyl-dihydrodiol. This compound is degraded, by the *bphB* gene product, to 2,3-dihydroxybiphenyl. Gene *bphC* encodes another dioxygenase which cleaves the 2,3-dihydroxybiphenyl to form a *meta* cleavage compound which is then converted to benzoic acid and a pentanoic acid derivative by the product of the *bphD* gene [13, 76, 82].

2.72 Overexpression of the *bph* pathway in *Pseudomonas* sp LB400 resulted in the GMM being able to degrade a greater percentage of lightly chlorinated PCBs, compared to the wild type, and also to degrade the normally recalcitrant 2,4,5,2',4',5'-chlorinated derivative [38]. The genetic modification of the toluene degrading microorganism *P. putida* F1, by insertion of the *bphD* gene cluster, allowed the pseudomonad to grow on biphenyl [10], and the fusion of the *bph* and *tod* (toluene-degrading) operons and subsequent insertion into a PCB degrading strain of *Pseudomonas,* enabled the GMM to degrade both toluene and biphenyl.

2.73 Dowling *et al.,* (1993) [83] reported the development of a transposable genetic element TnPCB designed to insert the *bph* operon (from *Pseudomonas* sp LB440) into the chromosome of a range of Gram-negative bacteria, including rhizosphere-competent pseudomonads. Other methods used to insert novel traits into the chromosome of rhizosphere pseudomonads have required homologous recombination between chromosomal sequences, which reduces the range of potential recipient organisms. The introduction of TnPCB into the recipient organism using plasmid pDDPCB is reported to avoid this problem. Once inserted, TnPCB is reported to remain stable in the chromosome, with no detectable lateral transfer of the *bph* genes to other microorganisms [13].

2.74 The insertion of TnPCB into the rhizosphere-competent pseudomonad *P. fluorescens* F113pcb enabled the microorganism to degrade biphenyl as a sole carbon source. The *bph* operon was found to be expressed constitutively in the GM pseudomonad, and importantly had no effect on the rhizosphere fitness (including ability to colonise or be maintained on plant roots) of the GMM compared to the wild type [13]. The genetic modification was also stable within the chromosome for at least 25 days in the absence of positive selection for PCB degrading or biphenyl degrading microorganisms [13].

2.75 The introduction of the *bph*-module into *P. putida* KT2442 and *Pseudomonas* sp B13FR1 expanded the biodegradative ability of these two strains to include biphenyl and 4-chlorobiphenyl. The survival and function of the GMMs in the environment was assessed using lake sediment microcosm. The GMMs were able to survive under simulated natural conditions in the microcosms, and degraded 4-chlorobiphenyl over a five day incubation period [83].

2.76 The accumulation of chlorobenzoates during the degradation of PCBs by some microorganisms is a consequence of the inability of PCB degrading microorganisms to utilise these compounds (particularly chlorobenzoates with a chlorine atom on both rings[4]), and the inhibitory effect of the chlorobenzoate metabolites chlorocatechol and chloromuconate semialdehyde on the enzyme 2,3-dihydroxybiphenyl-1,2-dioxygenase [85]. Inhibition of this enzyme has a negative feedback effect on the degradation of the PCB molecule. Several publications have reported the development of GMMs designed to degrade Cl,Cl'-PCB by pathways that avoid the formation of inhibitory metabolites [84, 85]. The production of inhibitory chlorocatechols and the chloromuconate semialdehydes is due to the activity of broad-substrate specificity benzoate-1,2-dioxygenases. The elimination of the genes encoding these enzymes by mutagenesis, and insertion of the *cba* genes (isolated from *Alcaligenes* BR60) carried on the catabolic transposon Tn5271 caused the GM *E. coli* and GM *Alcaligenes* sp to degrade chlorobenzoate to protocatechuate and chlorodihydroxybenzoate which were not inhibitory to the microorganism [85]. Although the application of these GMMs in the environment was not addressed, the findings demonstrate the importance of considering the formation of toxic intermediates when designing bioremediation strategies, as well as the ability of the microorganism to degrade the parent compound [86].

2.77 An alternative approach to overcoming the accumulation of chlorobenzoates by PCB co-metabolising microorganisms is the introduction of dehalogenase genes into these microorganisms. The rational for this, is that the microorganisms able to degrade PCBs to chlorobenzoate and chloropentadiene are also often able to degrade non-chlorinated benzoate and pentadiene as part of other existing metabolic pathways [51]. The introduction of the genes, necessary to enable these microorganisms to dehalogenate the chlorobenzoates and chloropentadiene, is proposed to allow a more complete degradation of PCBs by a single population of microorganisms, rather than by consortia.

[4] Designated Cl,Cl'-PCB [84]. McCullar MV, Brenner V, Adams RH and Focht DD (1994). Construction of a novel polychlorinated biphenyl-degrading bacterium - Utilization of 3,4'-dichlorobiphenyl by *Pseudomonas acidovorans* M3GY. *Applied and Environmental Microbiology*, 60(10): 3833-3839.

2.78 Hrywna *et al.,* (1999) [51] genetically modified the PCB metabolising *Comamonas testosteroni* VP44, so that it was able to grow on, dechlorinate and completely mineralise *ortho*- and *para*-substituted monochlorobiphenyls. The *C. testosteroni* was modified by insertion of either plasmid pE43 from *Pseudomonas aeruginosa* 142 (encoding the oxygenolytic *ortho*-dechlorination *ohb* gene), or plasmid pPC3 from *Arthrobacter globiformis* KZT1 (encoding the hydrolytic *para*-dechlorination *fcb* gene). The GMM was able grow on and completely dechlorinate high concentrations (up to 10mM) of 4-CBA (chlorobenzoate), 4-CB (pentadiene), 2-CBA and 2-CB depending on the modification. The non-GM parent strain was only able to grow on non-chlorinated benzoate and pentadiene [51].

2.79 Biodegradation of PCBs, particularly in contaminated soils may also be improved through the application of surfactants. The addition of these compounds to contaminated sites has a dual purpose in that they improve the bioavailability of the hydrophobic PCBs, and also provide the PCB degrading microorganisms with a readily available carbon source. Non-ionic surfactants are reported to be more suitable than anionic or cationic surfactants, due to their lower toxicity to microorganisms [82]. Lajoie *et al.,* (1997) [82] genetically modified *P. putida* and *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) with the transposon TnPCB containing the biphenyl/PCB degradative operon (genes *bph*A_1, A_2, A_3, A_4, *B, C, K, H, J, I* and *D*). Both organisms were also capable of utilising surfactants as a source of carbon and energy. The GMMs were reported to be capable of degrading individual congeners in Aroclor 1242 over a 20 day period, and may offer a suitable system for the degradation of PCBs in the environment.

2.80 GMMs designed to utilise a selective substrate such as a surfactant, and express an inserted gene are described as field application vectors (FAVs) [82]. FAVs offer a number of advantages over other bioremediation systems designed to degrade hydrophobic compounds such as PCBs. The addition of surfactants purely to improve the bioavailability of the pollutant may inhibit the activity of the pollutant degrading microorganisms [87]. This may be avoided through the use of microorganisms able to degrade the surfactant and pollutant [88]. Naturally occurring microorganisms are unlikely to be able to utilise synthetic surfactants such as Ipegal CO-720 [88]. Therefore, the ability of the FAV microorganism to use the surfactant as a source of carbon and energy provides the organism with a unique substrate, and increases the chances of survival following addition to the contaminated environment. Also, because the *bph* genes inserted into the FAV are controlled by constitutive promoters, and the microorganism is able to use the surfactant as a growth substrate, then the growth of the microorganism and the degradation of the PCB are effectively decoupled from biphenyl [88]. This avoids the addition of biphenyl to the

contaminated site to induce degradation of the PCBs, and means that in the absence of biphenyl, there in no selective pressure to discard the *bph* genes [88].

2.81 The initial reports of the use of FAVs for the biodegradation of PCBs used *P paucimobilis* IGP4 (subsequently reclassified as *Sphingomonas paucimobilis*) genetically modified to express the PCB degradative genes *bphABC*. Although this organism was able to degrade Aroclor 1242 (a commercial mixture of PCB congeners) in moist agricultural soil, the *bph* genes, which were plasmid encoded, were not stably inserted in the GMM. Attempts to insert transposon-encoded genes in this strain were not successful [88].

2.82 Stable insertion of the recombinant genes into the GMM used in the FAV is important if the system is to be used to bioremediate PCBs in a field situation. Lajoie *et al.,* (1994) [88]compared the two FAV systems for their ability to degrade PCBs, and also the stability of the inserted *bphABCD* genes. The *P. putida* IPL5 was genetically modified using plasmid encoded (pPCB), or transposon encoded (TnPCB) genes. Both FAVs were able to degrade some PCB congeners when inoculated into surfactant amended soil slurries, although the activity in the soil slurries was lower than in liquid culture, suggesting that the FAV system is affected by bioavailability constraints in soil. The growth of GMMs in non-sterile soil slurries (10^5 to 10^9 cells ml^{-1} in two days) indicated that competition with the indigenous microflora was minimal [88].

2.83 In soil, the GMM modified using the plasmid pPCB showed greater activity against the PCB congeners, both in terms of numbers of congeners degraded, and the level of degradation achieved [88]. The plasmid encoded genes were however less stable than the transposon. The greater activity of IPL5(pPCB) towards the PCB congeners was reported to be due to a higher level of expression of the *bph* genes in IPL5(pPCB) than in IPL5::TnPCB, although this could have been due to the presence of two or more copies of pPCB. Higher levels of expression of the degradative genes may also be a consequence of more effective channelling of carbon and energy from the surfactant to the production of the PCB degrading enzymes [88].

2.84 Further studies with PCB degrading FAVs based on the TnPCB transposon found that activity towards PCB congeners was inhibited in the presence of biphenyl, and that the degradation of PCB congeners (Aroclor 1242) was greater using a mixed culture of *P. putida* IPL5::TnPCB and *Ralstonia eutrophus* B30P4::TnPCB than with pure cultures of either strain [82].

2.85 Excess overexpression of the nonadaptive pathways, like that used in the FAV may cause the growth rate of the GMMs to decrease. In a competitive environment this would increase the selective pressure against the maintenance of the *bph* genes. The balance between gene expression and growth is reported to be an important consideration in the development of FAVs for environmental applications [88]. The use of FAVs does however represent a potentially successful application of GMMs in the bioremediation of hydrophobic pollutants such as PCBs or polyaromatic hydrocarbons (PAHs), either in contaminated soils, or soil washing systems [82].

Hydrocarbons

2.86 Environmental contamination by xenobiotic hydrocarbon compounds is largely a consequence of the spillage or improper disposal of oil. Such contamination may consist of a wide range of different compounds, ranging from the low molecular weight unbranched aliphatic hydrocarbons, which are relatively easily biodegraded by naturally occurring microorganisms, to the higher molecular weight branched and aromatic compounds which are more recalcitrant. Bioremediation of hydrocarbons has focused on two groups of the more recalcitrant compounds. These are the PAHs and benzene, toluene, ethylbenzene and xylene (BTEX) compounds [89].

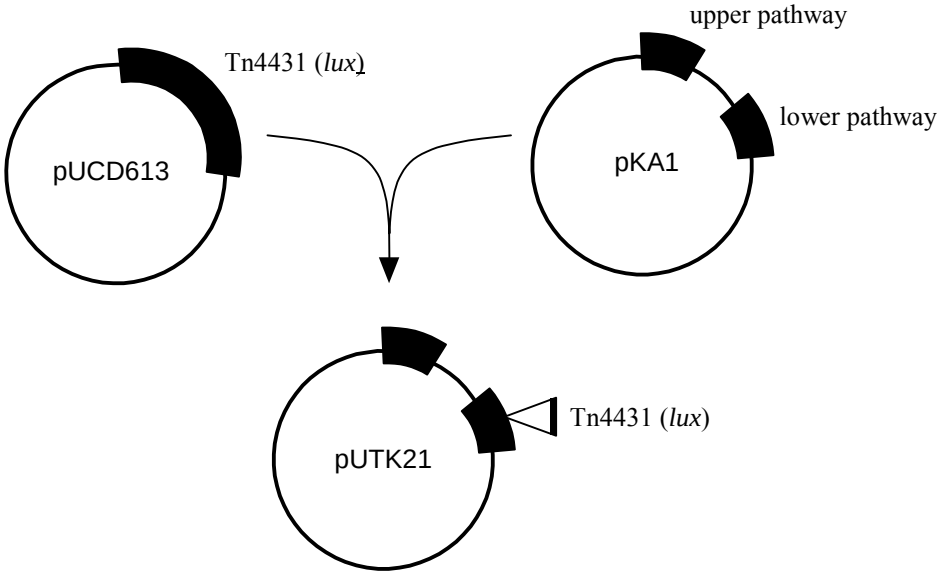
2.87 The PAH naphthalene was the target compound in the only field-based bioremediation application of live GMMs that has been conducted to date [23]. (The only other field release of a GMM conducted to date for a bioremediation application used an inoculum of killed *E. coli* genetically modified to degrade atrazine) [11]. The microorganism used by Sayler *et al.,* (2000) [23] was *Pseudomonas fluorescens* strain HK44 which had been genetically modified to express the naphthalene catabolic plasmid pUTK21 which had itself been mutagenised by a transposon-inserted *lux* gene[5]. [90]. The parent strains of *P. fluorescens* (5R and HK9) from which the GM HK44 had been derived were isolated originally from soil at a manufactured gas plant facility [91].

2.88 The bioluminescent GM *P. fluorescens* HK44 was constructed in two stages. The first involved filter matings between *E. coli* HB101 (containing the *lux* transposon Tn4431 carried on the suicide vector plasmid pUCD623) with *P. fluorescens* 5R

[5] The GMM is reported as the first recombinant microorganism to undergo full USEPA biotechnology risk assessment review and achieve environmental release status for applications in bioremediation [90] Ripp S, Nivens DE, Ahn Y, Werner C, Jarrell J, Easter JP, Cox CD, Burlage RS and Sayler GS (2000). Controlled field release of a bioluminescent genetically engineered microorganism for bioremediation process monitoring and control. *Environmental Science & Technology*, 34(5): 846-853. Further information on the release of this GMM and the regulatory process that was undertaken in order to conduct the release is presented in the report of the workshop at the end of this document (presentation by Prof Sayler).

(containing the upper and lower pathway operons for naphthalene degradation on plasmid pKA1). The bioluminescent construct *P. fluorescens* 5RL (containing plasmid pUTK21) was selected from the matings illustrated in Figure 2.1 on the basis of its ability to produce strong inducible light when exposed to naphthalene vapour or when grown in the presence of salicylate [91].

Figure 2.1 - Construction of bioluminescent reporter plasmid pUTK21) [91]



2.89 The catabolic pathway for naphthalene is encoded by two operons. The upper operon encodes for the conversion of naphthalene to salicylate, and the lower pathway for the oxidation of salicylate to acetaldehyde and pyruvate [91].

2.90 The strain selected from the filter matings was designated *P. fluorescens* 5RL. This GMM was able to degrade naphthalene to salicylate, which then accumulated, indicating that the transposon Tn4431 had inserted itself in the lower operon of the naphthalene pathway (Figure 2.1). The second stage, in the generation of HK44 therefore, involved transferring pUTK21 into another *P. fluorescens* capable of oxidising salicylate [91]. *P. fluorescens* HK9 was able to degrade salicylate but not naphthalene. Transfer of pUTK21 from *P. fluorescens* 5RL to HK9 by conjugation produced *P. fluorescens* HK44 that had a Nah^+Sal^+ phenotype and the same light producing characteristics as strain 5RL [91].

2.91 The construction of the GM HK44 meant that the naphthalene degradation genes and the *lux* gene were under the control of the same naphthalene induced promoter. Exposure of the GMM to naphthalene (or the intermediate metabolites salicylate or 4-methyl salicylate) resulted in the increased expression of the naphthalene catabolic

genes and also caused the microorganism to bioluminesce. The bioluminescence was monitored on-line during the release using fibre optics and photon counting modules. The advantage of this system was that as the level of bioluminescence was assessed to be proportional to the rate of naphthalene degradation, then monitoring levels of bioluminescence allowed the bioremediation rate to be monitored *in situ* without the need for chemical analyses of the contaminated soil. Bioluminescence monitoring is also non-invasive, non-destructive, rapid and population specific [91].

2.92 The GM *P. fluorescens* HK44 were released into subsurface lysimeters 4 m deep and 2.5 m in diameter containing layers of gravel (31 cm) at the base, then coarse sand (61 cm), uncontaminated soil (92 cm), PAH contaminated soil (92 cm) and a cap of uncontaminated soil (61 cm) (Figure 2.2). The PAH contaminated soil contained 1000 mg kg^{-1} naphthalene, 100 mg kg^{-1} anthracene and 100 mg kg^{-1} phenanthrene [90]. The use of lysimeters meant that the release of the GMMs was conducted under replicated and physically contained conditions. The scale of the test, and the location of the lysimeters in the field (Figure 2.3), meant that the GMMs were subjected to environmentally relevant conditions of temperature, water availability and humidity, and that the study was an accurate approximation of the natural subsurface environment [23]. The use of non-sterile soil, both for the uncontaminated and contaminated soil layers meant that the GMMs were exposed to competition from other soil microorganisms. Although the GMM only had activity against naphthalene, the additional presence of phenanthrene and anthracene meant that the GMMs were exposed to a heterogeneous mixture of PAHs which is more representative of a PAH contaminated environment. The PAHs were added to the soil 260 days prior to inoculation with microorganisms to allow for PAH sorption and soil weathering [90].

2.93 The population dynamics of the *P. fluorescens* HK44 were similar in all inoculated soils (in the presence and absence of PAHs). After an initial inoculation density of $1x10^6$ cfu g^{-1} soil, numbers of the GMM decreased rapidly during the first 12 days to a density of approximately $1x10^5$ cfu g^{-1} soil. This reduction was followed by a more gradual decline throughout the duration of the trial, with a final population of approximately $1x10^3$ cfu g^{-1} soil after 660 days. After 444 days, the GMMs still showed activity to PAH contaminants, although only in the presence of inorganic nutrients [90]. The availability of PAHs (used by the GMM as the primary carbon source and electron donor) and oxygen (used by the GMM as the electron acceptor) were found to be the significant factors affecting *in situ* metabolic activity, growth and aerobic biodegradative activity of the GMM [90].

Figure 2.2 - Schematic diagram of the lysimeter containing PAH contaminated soil

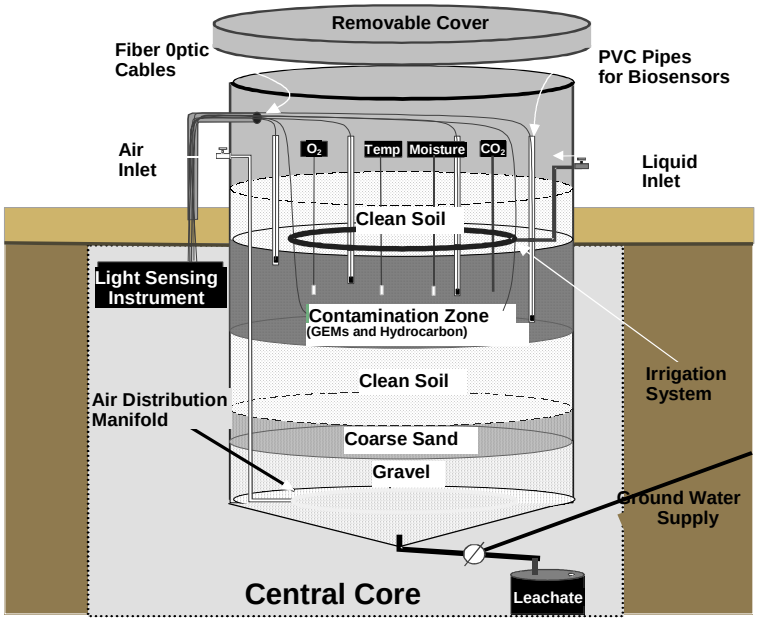


Figure 2.3 - Aerial photograph of lysimeters in the field. Photograph taken prior to filling the lysimeters and demonstrates the scale of the operation

2.94 The results of the lysimeter studies demonstrated that GMMs designed for use in bioremediation applications can be maintained viably in a field environment [90]. However, the application of GMMs for bioremediation in subsurface environments may be restricted by inadequate dissemination of the GMM and mass transfer limitations of electron donors and acceptors [90]. The construction of the lysimeters used by Ripp *et al.,* (2000) [90], meant that these problems were largely avoided, although limitations with transfer of electron donors and acceptors were still encountered.

2.95 The application of GMMs in the bioremediation of hydrocarbons, other than PAHs, has focused on the genetic modification of microorganisms most suited to the target environment, rather than just the design of microorganisms able to degrade the hydrocarbon. The reasons for this may be due to the relative ease by which many hydrocarbons (other than PAHs) may be degraded by microorganisms, and also the relatively good level of understanding of the genetic basis of the biodegradation of many hydrocarbons by microorganisms [29, 92].

2.96 Toluene is reported to be degraded by bacteria by one of five individual pathways [29]. Although toluene is known to be degraded in contaminated sites by non-GM bacteria, the current level of understanding of the toluene degradation pathways may allow the future insertion of the catabolic genes into microorganisms more capable of surviving in contaminated environments, than the non-GM toluene degrading strains.

2.97 Although the biodegradation of BTEX compounds by microorganisms has been well characterised and is mediated by the *tod* and *tol* pathways, many of the studies conducted have focused on the biodegradation of just one of the BTEX compounds, rather than the group of four compounds as a whole [93]. Environmental contamination by BTEX compounds, which are common components of petroleum, is likely to involve mixtures of all four compounds, rather than just benzene or toluene for example [93].

2.98 Lee *et al.,* (1995) [93] reported that biodegradation of benzene, toluene and *p*-xylene (BTX) in the environment does not result in the complete mineralisation of the three hydrocarbon compounds, even by consortia of microorganisms. Biochemical studies showed that the incomplete biodegradation was due to the 3,6-dimethylcatechol (formed during the degradation of *p*-xylene) being a dead-end metabolite in the *tod* pathway, and that the enzyme xylene oxygenase in the *tol* pathway was unable to degrade benzene. The BTX compounds are degraded by toluene dioxygenase (encoded by the *tod* genes) to the corresponding dihydrodiol. If *cis*-*p*-toluate-

dihydrodiol dehydrogenase is present, then this enzyme channels the dihydrodiols into the *tol* pathway and allows the complete mineralisation of the BTX mixture. The genetic modification of *Pseudomonas putida*, by the insertion of the *todC1C2BA* genes (encoding toluene dioxygenase), enabled this microorganism, designated ***P. putida*** TB105, to mineralise BTX without the accumulation of intermediate metabolites [93]. Because of the presence of BTX compounds in contaminated sites as a mixture, and the reported failure of indigenous microorganisms to degrade BTX mixtures completely, then the development of a GMM able to degrade such pollutant mixes may have significant application in the bioremediation of BTX contaminated environments.

2.99 Particular attention has focused on the use of GMMs to bioremediate hydrocarbons in wastewater treatment plants, especially those strains suited to survive and express the desired bioremediative activities in aquatic environments [53, 94]. Consideration of the characteristics of the target environment is an important component in the development of GMMs for bioremediation. A failure to identify this factor may have contributed to the poor performance of many of the initial attempts to develop GMMs for bioremediation applications [95]. Whilst indigenous microorganisms may offer no advantages over non-indigenous microorganisms [96] it is recognised that the non-indigenous organisms are likely to be more effective if they were isolated from a similar environment to the target site.

2.100 The use of GMMs has been reported as one approach to improve the biologically-mediated processes currently applied to the treatment of wastewater [94]. The limitation with some of the GMMs used in wastewater treatment systems is that although the GMMs are designed to degrade the pollutants present such as phenol, and may be inoculated strains directly into the resident microflora, these microorganisms are often rapidly washed-out of the activated sludge or biofilm fairly rapidly, and are therefore not present for a sufficient period of time to degrade the target contaminant. The efficacy of the bioremediation strategy relies on the ability of the GMM to persist as part of the indigenous microflora for a sufficient length of time to remove the target pollutant [94].

2.101 Microorganisms that are able to form flocs or to attach to existing flocs are reported to persist in the wastewater treatment systems for significantly longer periods of time than cells that do not possess either property. However, whilst the genes encoding for the degradation of organic pollutants have been well characterised, the ability to form flocs is more complex and often encoded by multiple genes [94].

2.102 Soda *et al.,* (1999) [94] therefore genetically modified a floc-forming *Sphingomonas paucimobilus* with recombinant plasmid pS10-45 containing phenol catabolic genes from *P. putida* BH. The GM *S. paucimobilus* survived in the wastewater treatment process longer than a non floc-forming *E. coli* (also genetically modified with pS10-45), and conferred better removal of phenol from the wastewater. The plasmid pS10-45 was, however, reported to be unstable in the GM sphingomonad. Transposon-mediated modification of the microorganism was proposed to provide a more stable GMM [94].

2.103 Removal of compounds such as phenol from wastewater treatment systems is important both for ensuring the quality of the plant's effluent and also to protect the *in situ* microflora from the toxic effects posed. The addition of 1 mM methyl- or chlorophenol to wastewater systems is sufficient to eliminate the populations of protozoa and metazoa present, and reduce the numbers of culturable bacteria, by three orders of magnitude [53]. The addition of the GM *Pseudomonas* sp B13 SN45RE to a laboratory scale wastewater treatment plant resulted in the degradation of the phenol compounds present and prevented the toxic effects normally caused by shock loadings (1 mM) of these pollutants [53]. The use of GMMs in this way demonstrates their use as an in-direct application of bioremediation.

2.104 Microorganisms such as GM *P. putida* DOT-T1, that are able to grow in the presence of high concentrations of solvents, may potentially be used in applications to bioremediate xenobiotics in sites where levels of organic solvents such as toluene are sufficient to inhibit the degradative capability of solvent sensitive microorganisms [97, 98]. *P. putida* DOT-T1 can grow in the presence of 90 percent toluene (*v/v*) and other organic solvents whose octanol/water partition coefficient is $>2.3^6$. The capability of *P. putida* DOT-T1 to degrade m- and p-xylene and other related hydrocarbons including toluene was conferred by the insertion of the TOL plasmid pWWO-Km [97]. Marconi *et al.,* (1997) [98] employed a similar strategy to utilise the solvent-resistant property of *P. putida* S12 to degrade naphthalene, toluene and biphenyl, by insertion of the plasmids encoding the catabolism of these contaminants.

2.105 The use of terrestrial mesophilic microorganisms is also restricted in environments containing high concentrations of salt. Although non-halotolerant microorganisms have been used in the bioremediation of crude oil spills in marine environments, the biodegradation of particular hydrocarbons in oil decreases as the salinity of the water increases [99]. This can inhibit the overall bioremediation of the oil spill. For example, the biodegradation of crude oil by a consortium of four pseudomonads was

inhibited in the presence of 1 percent NaCl (*w/v*) [100], and growth of the consortium was inhibited at concentrations of >0.9 M NaCl [101]. Insertion of the *proU* operon, from *E. coli* into each member of the consortium, using a broad-host range plasmid was found to confer a 25-fold improvement in the salt tolerance of the consortium [101], and provides a solution to developing microorganisms to degrade pollutants in hypersaline environments, such as contaminated estuaries and coastal areas.

Nitroaromatic compounds

2.106 One of the most problematic nitroaromatic pollutants is 2,4,6-trinitrotoluene (TNT) that is often present at sites used to manufacture and handle munitions. Nitroaromatic compounds can be biodegraded under both aerobic and anaerobic conditions [89]. No GMMs have been identified in the current literature that have been designed to degrade nitroaromatic compounds, although if the relevant degradative genes have been identified, then GMMs could be developed for use in this field[7].

2.107 Because munitions waste is likely to be present at contaminated sites alongside other xenobiotics, particularly organic solvents, then solvent-degrading GMMs might be employed in the bioremediation of such sites in a bioprotection capacity (similar to that described for wastewater treatment plants [53].

Use of Bacteria for the Bioremediation of Inorganic Pollutants

2.108 Unlike organic pollutants, inorganic pollutants such as mercury cannot be degraded to carbon dioxide and water and consequently removed from the contaminated environment [102]. The objective of strategies to bioremediate inorganic pollutants is therefore to use the bacteria to:

- transform the pollutant into a non-toxic form (biotransformation); or
- precipitate the metal ions at the surface of the cell (bioprecipitation); or
- sequester the metal ions and bioaccumulate the pollutant within the microbial cell (biosorption). The strategy used depends on the characteristics of the pollutant and the level and location of the contamination at the target site.

[6] Such compounds are likely to partition within the lipid bilayer of the microorganism's outer membrane, and are more toxic than those organic compounds with a partition coefficient of <1 or >5.
[7] Further information on the application of GM technology for the bioremediation of nitroaromatic compounds, particularly munitions waste is presented in the report of the workshop at the end of this document (presentation by Dr Bruce).

2.109 The capability of many organisms including microorganisms to biotransform inorganic pollutants from a toxic to a less toxic state, is often a consequence of the strategy developed by the organism to survive in environments contaminated by such pollutants [103, 104]. By transforming the pollutant into a less toxic and/or less soluble state the organism is no longer exposed to the toxic effect the pollutant may have. (Reducing the solubility of the pollutant reduces the bioavailability and therefore the level of exposure of the compound).

2.110 Biosorption and bioprecipitation based strategies are most likely to be applied in *ex situ* processes where the microorganism is contained in some form of bioreactor. Although microorganisms capable of accumulating or precipitating metal compounds could be added to the contaminated site to localise the pollutant *in-situ,* the pollutant will remain in the site, and further measures will still be required to remove the pollutant/microorganism 'complex'.

Metallothioneins

2.111 Metallothioneins (MTs) are a group of a range of proteins that are able to sequester and subsequently accumulate heavy metals [105]. The expression of metallothioneins in bacteria has been used to enhance the metal-retaining capability of microorganisms, and has applications in the removal of heavy metals from wastewater and groundwater if the metallothionein expressing microorganism is immobilised in some form of permeable matrix [105-107].

2.112 For example, the genetic modification of *E. coli* to overexpress the outer membrane protein OmpC, caused the bacterium to display multiple copies of metal-binding poly-histidine peptides on the surface of its outer membrane. The GM *E. coli* was able to adsorb between three and six times more Zn^{2+}, Fe^{3+} and Ni^{2+} metallic ions than non-GM cells expressing the wild type OmpC [108]. The uses of metallothioneins in bioremediation all operate using this same basic strategy. However, a number of recent publications have proposed novel variations on this theme, including:

- the synthesis of new metallothionein proteins that are not produced naturally, and therefore may be able to accumulate different heavy metals;

- the alteration of the system used to express the metallothioneins in the bacterium, and therefore the location in the cell in which they are expressed. This is reported to alter the metal-binding capability of the GMM; and,

- the use of metallothioneins with multiple metal-binding sites.

2.113 The advantages of using metallothioneins with multiple binding sites, are an increased capacity to remove metals from the solution, a greater stability of the expressed protein and the potential to use a single strain of GMMs to target a number of different heavy metals [105]. Mauro and Pazirandeh (2000) [105] genetically modified *E. coli* to express the monomeric Mtt1 metallothionein unit from *Neurospora crassa* in the cell's periplasm, as a fusion with the maltose-binding protein. The *E. coli* was modified to express between 1 and 12 copies of Mtt1 on a single maltose-binding protein, and its ability to sequester cadmium measured. In all cases, the GMM was able to take up approximately 10-fold more Cd^{2+} than the non-GM *E. coli* strain. The relationship between the uptake of Cd^{2+} and the number of Mtt1 subunits was linear up to the trimer. Although cells, with more than three copies of the Mtt1, showed increasing uptake of Cd^{2+} with the number of Mtt1 units, the improvements in terms of removal of Cd^{2+} from the medium were only small.

2.114 The fusion of the Mtt1 metallothionein to the maltose-binding protein meant that the metal-binding protein was expressed in the periplasm (between the inner and outer membranes of the cell). The choice of 'anchor', for the metallothionein determines the location in the cell where the protein is expressed, and is reported to affect the metal-binding capability of the protein [106]. Comparisons between the accumulation of cadmium by GM *E. coli* expressing human or mouse metallothionein fused to LamB, Lpp/OmpA (both membrane associated proteins) and PAL (peptidoglycan-associated lipoprotein) showed that the LamB-MT fusion was the most stable and demonstrated the best metal-binding capability. Fusion of the metallothionein to LamB results in the metal-binding protein being expressed on the outer surface of the outer cell membrane. *E. coli* modified with plasmid pLBMT1 (containing the LamB-MT fusion) adsorbed 30 nmol Cd^{2+} mg^{-1} biomass, whilst the non-GM *E. coli* cells adsorbed only 0.8 nmol Cd^{2+} mg^{-1} biomass [106].

2.115 A possible limitation of the metallothionein-based remediation strategies discussed so far, is that they are not specific to particular metals [106]. Although this non-specific accumulation of metals may be useful for the general removal of metal contaminants from wastewater for example, it is not suitable for the extraction of particular hypertoxic pollutants, such as chromium. Such compounds whilst only comprising a minor percentage of the total contaminant load in an environment may contribute a significant proportion of the total toxicity, and should be targeted specifically in order to reduce the toxic load present at the contaminated site.

2.116 Specificity of human metallothioneins can be improved by just using the α (C-terminal) domain of the metallothionein rather than the whole protein, and also by using shorter synthetic peptides known to have specific metal-binding properties [106]. *E. coli* modified to express a fusion of the LamB protein and the α domain of the human metallothionein showed greater affinity and selectivity for Cd^{2+} than the LamB fused to the entire metallothionein [109].

2.117 Kotrba *et al.,* (1999) [110] genetically modified *E. coli* to express short metal-binding peptides fused to LamB protein. The two sequences (designated HP[8] and CP[9]), showed different affinities to Cd^{2+}, Cu^{2+} and Zn^{2+}, indicating that the use of short metal-binding peptides may be applied to the removal of specific heavy metal pollutants from a contaminated environment. Mejare *et al.,* (1998) [111] reported that the genetic modification of *E. coli* to express a fusion of the peptide His-Ser-Gln-Lys-Val-Phe and the outer membrane protein OmpA, conferred resistance to 1.2 mM cadmium chloride. Such GMMs have applications in the removal of cadmium from contaminated water, and if used as recipient organisms for further genetic modification, may also be as degraders of other pollutants present in cadmium contaminated environments.

2.118 The ability of metallothioneins to accumulate toxic heavy metal pollutants can also be applied in the bioprotection of other organisms from the toxic effects of the metal. Such uses are applicable particularly for the protection of agricultural crops. Valls *et al.,* (2000) [112] genetically modified the metal tolerant *Ralstonia eutropha* CH34 to express the gene *mtb*. This gene encoded a chimeric metallothionein β protein developed from the fusion of a mouse metallothionein 1 protein with an autotransporter β-domain of the IgA protease of *Neisseria gonorrhoeae*. The metallothionein protein confers the ability to sequester metal ions from the environment, and the protease from *N. gonorrhoeae,* ensures expression of the hybrid protein on the outer membrane of the GM *R. eutropha*. The GMM (designated *R. eutropha* MTB) was reported to have an enhanced ability to immobilise Cd^{2+} ions from its surrounding environment, compared to the non-GM strain. The inoculation of the GMM into soil contaminated with Cd^{2+} significantly reduced the toxic effects of cadmium on the growth of tobacco plants (*Nicotiana bentamiana*), indicating that the GMM was able to reduce the bioavailability of the cadmium present in the soil, by sequestration onto the microorganism [112].

[8] Gly-His-His-Pro-His-Gly
[9] Gly-Cys-Gly-Cys-Pro-Cys-Gly-Cys-Gly

Other general strategies for the bioremediation of heavy metals

2.119 Several strategies for the removal of heavy metals from wastewater systems utilise microbial processes indirectly to either biosorb or bioprecipitate heavy metals from the wastewater. These include the use of sulphate-reducing bacteria to precipitate soluble metal species out of solution, as insoluble metals sulphides [113], and the manipulation of polyphosphate metabolism in bacteria, fungi and protozoa [114].

2.120 Sulphate-reducing bacteria are able to remove heavy metals from solution as a by-product of the production of sulphide from inorganic thiosulphate [113]. The gene (*phsABC*) responsible codes for thiosulphate reductase, isolated originally from *Salmonella typhimurium*. The insertion of pSB74 (containing the *phsABC* gene) caused *E. coli* DH5α to overproduce hydrogen sulphide from thiosulphate [115]. Growth of the GMM in the presence of zinc, lead and cadmium chloride (separately and in combination) in liquid culture for 24 h at 37 ºC resulted in the precipitation of the metals as metal sulphides [113]. The GMM was able to remove 99 percent of zinc (500 μM), 99 percent of lead (200 μM) and 98 percent of cadmium (100 μM) from the solution. With the lead and cadmium solutions, insoluble PbS and CdS were visible on the base of the culture flask. At higher concentrations the lead and cadmium were found to have a toxic effect on the GMM with only 9.3 percent of cadmium and 31 percent of lead removed from the 500 μM solution [113].

2.121 To assess the potential of the GMM to remove heavy metals from wastewater, contaminated with a number of heavy metals, the *E. coli* DH5α harbouring pSB74 was grown in the presence of combinations of zinc, lead and cadmium (each 100 μM). Although competition between the metals for sulphide delayed their removal in some combinations, all the heavy metals were removed from the solution within 24 h [113].

2.122 The manipulation of polyphosphate metabolism in microorganisms is another strategy that may prove useful in the development of GMMs with improved metal tolerance or metal accumulation capability. Although the modification of polyphosphate metabolism has yet to prove a realistic option for the bioremediation of heavy metal contaminated water, it is reported that polyphosphate is involved in the storage of and/or tolerance to heavy metals [114]. Some microorganisms are reported to use intracellular polyphosphate to detoxify heavy metals such as lead [116], and others may use cell-surface associated polyphosphate to chelate cations such as uranium [117].

2.123 Keasling *et al.,* (1998) [114], genetically modified *E. coli* CA38 to overexpress the genes for polyphosphatase (*ppk*) and phosphate kinase (*ppx*). Overexpression of just

ppk (*E. coli* CA38 pBC29) resulted in the increased production of intracellular polyphosphate and improved uptake of phosphate, but no greater tolerance to cadmium than the non-GM *E. coli* CA38. However, where the GMM was designed to overexpress both *ppk* and *ppx* (*E. coli* CA38 pBC9), lower intracellular levels of polyphosphate were observed along with secretion of phosphate from the cells. Exposure of *E. coli* CA38 pBC9 to 2 ppm cadmium had no apparent effect on the growth of the GMM. At a concentration of 4 ppm cadmium, the growth of the *E. coli* CA38 pBC9 was reduced, but to a lesser extent than for the non-GM strain and for *E. coli* CA38 pBC29. Exposure of 10 ppm cadmium inhibited the growth of all three strains [114].

2.124 As with the biodegradation of organic pollutants, future applications to bioremediate inorganic pollutants are likely to utilise the current understanding of the genes possessed by indigenous microorganisms that are expressed against environmental contaminants. For example, the soil microorganism *Alcaligenes eutrophus* CH34 is able to survive in areas containing high concentrations of heavy metals because it contains the megaplasmids pMOL28 and pMOL30 which confer resistance to Co^{2+}, Ni^{2+}, CrO_4^{2-}, Hg^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+} [118]. Two of the operons involved in the heavy metal resistance, the *cnr* operon (resistance to cobalt and nickel) on pMOL28 and the *czc* operon on pMOL30 (resistance to cobalt, cadmium and zinc), have been linked to the *lux* reporter system in *A. alcaligenes* to produce a GMM capable of monitoring concentrations of metals in wastewater effluent [118].

Mercury

2.125 To date the majority of the work on the use of GMMs for bioremediation has focused on the treatment of mercury contaminated sites. A limited amount has also been undertaken on the bioremediation of cadmium, arsenic and nickel, although from the information available this has focused on the use of GM plants rather than GMMs. Mercury, cadmium and arsenic have probably been addressed first due to their relatively high toxicity and the widespread contamination of the environment with these metals.

2.126 Mercury is reported to be one of the most toxic heavy metals present as an environmental contaminant. In aqueous environments, mercury present in sediments in its Hg(II) state, is subject to methylation by both microorganisms and abiotic processes to the much more toxic form methylmercury (CH_3Hg^+). This compound can be bioaccumulated through aquatic food chains, and can give rise to toxic effects to human health through the consumption of contaminated fish or shellfish [119]. Other organic species of mercury (including alkyl and aromatic derivatives) are also

capable of bioaccumulating, at potentially toxic levels, in the tissues of higher organisms [102].

2.127 The bacterial mercury resistance system (Hg^r) incorporates a mercuric reductase, a mercury specific transport system and an organomercurial lyase that is able to cleave carbon-mercury bonds. Expression of the Hg^r system confers resistance to both organomercurial compounds and mercurial ions [102]. Many of the strategies reported for the bioremediation of sites contaminated with mercury have used various parts of the Hg^r system, primarily to reduce Hg(II) to the more inert, volatile elemental form (Hg^0).

2.128 The use of bioremediation strategies to remove Hg(II) from polluted sites offers certain advantages over 'conventional processes', such as chemical precipitation, carbon adsorption and ion exchange. These chemical based processes are often restricted by variations in pH and the tendency of Hg(II) to form complexes with various ligands and become associated with suspended solids or sediments.

2.129 The genes conferring resistance to mercury are encoded in a single operon (*merTPABD*) regulated primarily by the product of *merR*. The products of genes *merT* and *merP* encode an integral membrane transport protein and a periplasmic Hg^{2+} binding protein respectively, and are therefore involved in the uptake, transport and accumulation of Hg(II). MerP is reported to sequester extracellular Hg^{2+} and transfer it to MerT which then transports it across the cell membrane [102, 120]. The *merA* gene and *merB* gene encode mercuric reductase and organomercurial lyase respectively, and *merD* has been linked with a transcriptional coregulatory function [102].

2.130 Expression of the *merA* gene enables microorganisms such as *E. coli* to reduce Hg(II) to Hg(0) and thereby survive and grow in environments containing mercury, at concentrations of 50 μM $HgCl_2$ [104]. Survival of the microorganism is important if it is to fulfil its intended bioremediation function. As mercury concentrations in contaminated sites rarely exceed 10 μM, then expression of the *merA* gene offers the potential to remove Hg(II) from even very contaminated environments.

2.131 However, although the microorganisms are able to express *merA* and therefore transform Hg(II) to Hg(0) in their cytoplasm, they may still be restricted by the toxic effects of Hg(II), in the outer membrane and cell wall regions of the cell [104]. If the population of $merA^+$ microorganisms in the contaminated environment is large enough, then sufficient numbers of microorganisms are likely to survive the toxic effects of the mercury and reduce its concentration to below a threshold level. When

this point is reached (defined by the rate of cell growth of *merA*$^{+}$ microorganisms overtaking the rate of cell death of the population) Hg(II) should be successfully removed from the environment [104]. This issue should be considered in deciding the inoculum size required to bioremediate a contaminated site, where the target compound is likely to be toxic to the inoculant.

2.132 *E. coli* cells genetically modified to express *merT* and *merP* and also to over express metallothionein as a glutathione S-transferase fusion protein (GST-MT), were reported to be capable of accumulating Hg^{2+} over a concentration range of 0.2 - 4 mg l^{-1} [120]. The GMMs were designed so that they would specifically target, sequester and accumulate Hg^{2+} in preference to other metal ions and would not be sensitive to changes in ambient conditions. This was achieved by using the MerP/MerT membrane transport system to target and sequester Hg^{2+} specifically, and the overexpressed metallothionein to accumulate the metal ion intracellularly. Bioaccumulation within a cell is reported to be more tolerant of changes in extracellular conditions [121].

2.133 In order to assess the potential for these GM *E. coli* to be used in a bioremediation application, for example in the removal of mercury from contaminated water, the GMMs were immobilised in a hollow fibre bioreactor. Water contaminated with Hg^{2+} was then circulated through the bioreactor at a rate of 150 ml min^{-1} (25 ºC). The system was found to remove Hg^{2+} from the water, reducing the Hg^{2+} concentration from 2 mg l^{-1} to around 5 µg l^{-1} [120]. The system was also resistant to changes in pH (from pH 3-11), ionic strength and the presence of common metal chelators (ethylenediaminetetraacetic acid (EDTA) and citrate) or complexing agents, and may therefore be more suitable to remove Hg^{2+} from the environment than 'conventional methods', particularly where the concentration of Hg^{2+} is low. The combination of the mercury transport system and the metallothionein binding proteins was also found to exhibit good selectivity to mercury in the presence of other metal ions such as magnesium (200 mM) and cadmium (100 µM) [120, 121]. Although the work was conducted at laboratory scale, the basic system and approach is intended as an *ex situ* bioremediation strategy for the removal of Hg^{2+} from contaminated water or soil washings.

2.134 Inorganic pollutants such as mercury are also often present in sites co-contaminated with some form of radioactive pollutant. In these environments, exposure to ionising radiation usually inhibits the activities of microorganisms that, in a non-radioactive environment could be employed to bioremediate the toxic inorganic pollutants present. Riley *et al.,* (1992) [122] reported the existence of ~1000 sites in the USA which were contaminated with inorganic and organic pollutants such as mercury and

toluene, and had radiation levels exceeding 10 mCi l^{-1}. The bioremediation of such sites requires microorganisms that are able to survive and function under radiation stress [104].

2.135 The most radiation resistant organism discovered to date is the bacterium *Deinococcus radiodurans* [123]. This microorganism is a non-pathogenic, solvent tolerant soil bacterium capable of growing continuously in the presence of 60 Gy h^{-1} without an effect on its growth rate or ability to express foreign genes [15, 104].

2.136 Brim *et al.*, (2000) [104] utilised the high tolerance of *D. radiodurans* to radiation to develop a GM *D. radiodurans* designed to transform Hg(II) and degrade toluene in contaminated sites with high levels of ionising radiation. A wild type strain of *D. radiodurans* was genetically modified with the *merA* locus from *E. coli* BL308 and *tod* genes (encoding the ability to degrade toluene or chlorobenzene) from *P. putida*.

2.137 Resistance of the GM *D. radiodurans* to Hg(II) correlated positively with the copy number of *merA* genes in the microorganism, with the most mercury resistant strain (MD737) having 150 copies of plasmid containing the *merA* gene (plasmid pMD731). Although the genome of strain MD737 was ~3 Mbp larger than that of the wild type *D. radiodurans,* the additional genetic material had no detectable effect on the survival or growth of the GMM (compared to the wild type). The presence of multiple copies of the *merA* gene also had no effect on the ability of the GM strain to degrade toluene [104]. The report concluded that *D. radiodurans* offers a unique system to bioremediate inorganic and organic pollutants concomitantly in the presence of high concentrations of ionising radiation.

Nickel

2.138 In bacteria resistance to nickel is conferred by the gene *ncc-nre*. Dong *et al.*, (1998) [124] reported that the genetic modification of a range of eubacteria with plasmid pMOL222 (containing *ncc-nre* isolated from *Alcaligenes* sp 31A) conferred an increased resistance to nickel. Where the plasmid was transferred to activated sludge bacteria in a pilot-scale activated sludge plant, the microflora were able to survive the shock loading of waste contaminated with nickel at a concentration of 0.25 mM.

Use of Fungi for the Bioremediation of Pollutants

2.139 Although bacteria may be easier and faster to culture and more amenable to genetic modification, fungi do have several inherent advantages over bacteria that may be applicable in the bioremediation of pollutants. In addition to being more tolerant of

environments with low pH, fungi are able to degrade and utilise a wide range of complex natural substrates such as cellulose, hemicellulose, lignin and pectin [20]. Because the ability to degrade these compounds is conferred by extracellular enzymes with a relatively low substrate specificity, then fungi are also able to degrade a range of complex xenobiotic organic pollutants including chlorinated phenols, PCBs, dichlorodiphenyltrichloroethane (DDT), dioxins, PAHs, alkyl halides and nitrotoluenes [125-127].

2.140 Fungi also exhibit a number of capabilities that make them suitable for the bioremediation of inorganic pollutants. These include a tolerance to relatively high concentrations of metals [128], the ability to bioaccumulate metals by active and passive processes [129, 130] and the production of extracellular compounds, such as oxalates and citrates that improve the solubility and therefore the mobility of metals in the environment [131, 132].

2.141 However, although fungi have been reported to be capable and in some cases uniquely suitable for the bioremediation of pollutants, particularly heavy metals and high molecular weight aromatic organic compounds [133-135], no reports of the use or development of GM fungi have been identified. To date, the only GM fungi that have been developed that are relevant to bioremediation applications, are those that have been modified to express particular reporter genes. These include the bacterial gene *uidA* (encoding ß-glucuronidase (GUS)) and the gene for green fluorescent protein (GFP) [136, 137]. Such modifications would enable the GM fungus to be monitored if released into the environment.

2.142 Other studies of relevance to the application of fungi in bioremediation include work designed to improve understanding of the degradation of lignocellulose [138], a complex organic compound whose degradation may be appled to xenobiotic pollutants with similar structural similarities [139]. Broda *et al.,* (1996) [138] have reported studies in which individual proteins involved in the degradation of lignocellulose have been expressed in recombinant systems to determine their mechanistic use both singly and in combination.

2.143 A number of reasons are proposed for the limited genetic modification of fungi for environmental applications:

- because of their mycelial structure and consequently non-homogenous growth in liquid culture, fungi are inherently more difficult to work with than bacteria. The mycelial growth also makes it difficult to ensure an even inoculation across a contaminated environment;

- many fungi are plant pathogens, and therefore their release into the environment may not be desirable, due to the potentially adverse effects a release might have on resident flora;

- fungi require a primary growth substrate in order to co-oxidise some aromatic compounds, and because they are unable to metabolise the products of co-oxidation, then complete mineralisation of the contaminant does not occur [58];

- the genetic basis for the biodegradation of compounds (including pollutants) by fungi is relatively complex. Rather than the specific individual genes present in bacteria, the degradation of compounds by fungi is usually encoded by multiple gene systems that are induced by a number of different effectors. This makes the transfer of degradative pathways from one fungus to another by genetic modification more difficult than a similar transfer in bacteria; and

- the enzymes produced by fungi are largely extracellular and have a low substrate specificity. This means that the fungi used in bioremediation strategies are likely to have a degradative activity to a range of compounds in the environment, in addition to the target pollutant. This lack of focus may have an adverse effect on other biological processes such as nutrient recycling, and is therefore less desirable than a strategy which just degrades the target pollutant and its metabolites.

2.144 Although no reports of the use of GM fungi for bioremediation have been identified, the use of non-GM fungi in the biodegradation of complex organic pollutants suggests that GM fungi may have a role in bioremediation in the future. Work with non-GM fungi has focused on the white-rot fungi such as *Phanerochaete chrysosporium, Irpex lacteus* and *Coriolus versicolor*. Due to their ability to degrade chlorinated phenols, PCBs, chlorinated pesticides, dioxins, PAHs, alkyl halides and nitrotoluenes, this group of fungi may therefore be the most likely candidates for genetic modification in the future.

2.145 In addition to the white-rot fungi, the mycorrhizal fungi have also been reported as potentially playing an important role in bioremediation applications [140, 141]. Although no work on GM mycorrhizal fungi has been reported, the importance of these fungi in the rhizosphere, as an interface between plants and soil means that they may offer particular benefits to phytoremediation applications.

2.146 Arbuscular mycorrhizal fungi for example are important symbionts of plant root systems and are able to play both a passive and active role in phytoextraction, phytodegradation or phytostabilisation. The mechanism of remediation is reported to involve the fungi's extracardial mycelium (ERM) which radiates out from the plant's root system into the soil, and may involve either the biosorption of the pollutants onto the ERM or the use of the ERM as a site to harbour specific degradative bacteria [141].

2.147 The most suitable strategies identified for mycorrhizal fungi in bioremediation are the development of stress-tolerant indigenous strains rather than a generic strain that may not be adapted to the target environment [141]. The relatively untapped application of non-GM mycorrhizal fungi in bioremediation may however mean that work may focus on the application of non-GM strains before GM mycorrhizal fungi are developed.

THE USE OF MICROORGANISMS FOR THE MONITORING OF POLLUTANTS

2.148 Microorganisms can be used to monitor the degradation, presence and toxicity of contaminants in the environment [12]. Because of their ease of culture, rapid response to toxins and ability to survive in environments in which the pollutants are likely to be found, microorganisms are the ideal organisms for pollutant monitoring. Also, because microorganisms are involved in the degradation of a wide range of environmental pollutants, they can be used to monitor specific contaminants, as well as indicating overall metabolic status of the cell, and also the actual level of toxicity in a contaminated environment [142, 143].

2.149 Genetic modification techniques have enabled reporter genes, such as *luc* (from the firefly *Photinus* sp and *Phyrophorus* sp.) and *lux* to be inserted into a range of microorganisms, and have consequently increased the type of applications for biomonitoring and biosensing significantly. The *lux* genes were isolated originally from the marine bacterium *Vibrio fischeri* (formerly *Photobacterium phosphoreum*) [143], and cause the recombinant microorganism to bioluminesce when they are expressed.

2.150 Although *V. fischeri* has been used extensively to assess the toxicity of pure compounds in liquid media, this microorganism is sensitive to the pH and osmotic changes and requires a high saline concentration in the analyte under test [142, 143]. Methods such as Microtox and Lumintox, which are based on the response of

naturally luminescent marine microorganisms such as *V. fischeri* are therefore unsuitable for the monitoring of pollutants in non-marine environments [142].

2.151 Other bioluminescent reporter genes that have been inserted into microorganisms include *luc* and *Rluc* from the Click Beetle (*Renila reniformis*) [144, 145]. The *gfp* gene (from *Aequorea victoria* and *Renilla reniformis*) encodes for the formation of GFP (which fluoresces under illumination with blue light) has also been used as a reporter gene to study the survival and efficacy of GMMs used as inocula for bioremediation [144].

2.152 The use of bioluminescent reporter genes, such as *lux*, can be designed for different applications by the choice of promoter to which the gene is linked. Fusion of the *lux* reporter genes, to appropriate heavy metal promoters means that the GMM will emit light when the heavy metal promoters are induced by the presence of particular heavy metals. The application of such biosenor technology has been reported for arsenic, cadmium, chromium, cobalt, copper, mercury, nickel and zinc (cited by) [142], and organic pollutants including napthalenenaphthalene [23, 91] and PCBs [146]. However, because expression of the genes, encoding the degradation of organic compounds, is often induced by more than one compound, then biosensing for organic compounds is usually less compound specific than for heavy metals [142]. For example, the GM *P. fluorescens* HK44 used by Sayler and Ripp (2000) [23] to monitor for the degradation of naphthalene emitted light in the presence of naphthalene, 4-methyl salicylate and salicylate, although the *lux* genes were under the control of the promoter for just the naphthalene catabolic genes.

2.153 If the *lux* gene is linked to a constitutive promoter, then the system can be employed to report on the overall metabolic status of the cell, and consequently the level of environmental stress experienced by that cell or population. The presence of toxic compounds in the environment will increase the level of environmental stress and reduce the amount of light emitted by the *lux*-modified microorganism [147].

2.154 The significant advantage of biosensors compared to conventional chemical assessment techniques, is that because the control of the sensor is biological it is only able to respond to the fraction of pollutant that is bioavailable [142]. Determination of the concentrations of pollutants that are in a bioavailable state is a more environmentally relevant measurement of the actual toxicity at a contaminated site, than measurements based on the total amount of pollutant present [23, 147]. Chemical analyses can only determine the total amount of a particular pollutant(s) present, and are also unable to provide an indication of the level of toxicity where the pollutants interact to produce a cumulative toxic effect [147]. Cumulative effects are

likely to be greater than those determined by summing the toxicities of the individual pollutants present.

2.155 Although, bioluminescence based monitoring for the presence of toxic pollutants is significantly cheaper than existing chemical based analyses [23], and has a number of advantages over chemical techniques; the most accurate picture of the toxicity characteristics of a particular contaminated site is likely to be provided by a combination of biological, chemical and physical based analyses.

2.156 The use of a *lux*-modified microorganism in the preliminary assessment of a contaminated site can identify the location, concentration and type of toxic pollutant(s) present at the site, enabling subsequent bioremediation or chemical treatment processes to be targeted at the required areas. This of course improves the efficiency and reduces the costs of the remediation process.

2.157 Sousa *et al.,* (1998) [147] used *Pseudomonas fluorescens* 10586s pUCD607 (genetically modified with the *lux CDABE* genes on a multicopy plasmid), in combination with chemical treatment methods, to determine the environmental constraints affecting the remediation of a BTEX contaminated site[10]. Sediment supernatant and groundwater samples were taken from the BTEX contaminated site and inoculated with a cell suspension of the GMM. The level of bioluminescence recorded from these untreated samples reflected the overall toxicity of the pollutants in the sample to the GMM. As pseudomonads are ubiquitous to terrestrial environments, then the results obtained for the GMM were assessed to be applicable to the indigenous microflora present in the contaminated site.

2.158 Chemical analysis of the BTEX contaminated site identified the presence of heavy metals, PAHs and chlorinated alkanes at the site in addition to BTEX. To determine the contribution of each of these groups of compounds to the overall toxicity, samples were treated chemically and then tested again with the GMM. For example, to determine the influence of the volatile organic compounds (VOCs) on overall toxicity, the bioluminescence of the untreated samples was compared with samples that had been air-sparged to remove the VOCs present. Muffle furnacing can be used to remove the non-volatile organic compounds such as the PAHs [147]. If the toxicity due to non-volatile organics and heavy metals is high, then the inoculation of the site with BTEX degrading microorganisms will not be successful in reducing the ecotoxicity of the site significantly. If the BTEX degraders are sensitive to heavy

[10] Further information on the application of GM biosensor technology for the detection and assessment of contaminants in a particular environment is presented in the report of the workshop at the end of this document (presentation by Prof Killham).

metals for example, then bioremediation of the BTEX pollutants present may also be unsuccessful [147].

2.159 Although *lux*-modified GMMs can provide a very sensitive method for the detection of specific compounds in the environment, any application of this technology needs to ensure that the insertion of the reporter gene has no effect on both the interaction between the GMM and the target pollutant, and its overall ecological fitness [142]. The GMM must also be representative of the types of microorganisms present in the indigenous microbial community [147]. If the GMM behaves differently in the environment, compared to the wild type strain (with the exception of being able to bioluminesce), then the activity of the GMM will not be representative of the indigenous microflora and may provide inaccurate information on the levels and/or toxicity of the pollutants in the target environment. Sousa *et al.*, (1998) [147] noted that at some contaminated sites, some taxa within the indigenous microflora may have adapted to the unique conditions present at the site. Therefore, in order to ensure that the *lux*-modified GMM is representative of the indigenous microbial community, it should ideally be isolated from that specific site, and then be genetically modified.

2.160 Layton *et al.,* (1999) [143] reported a potential limitation of some *lux*-modified microorganisms used to monitor the presence of hydrophobic contaminants such as PAHs and PCBs in terrestrial environments. Because of their low water solubility and strong lipophilicity, compounds such as PAHs and PCBs tend to absorb to the particulate matter in soils and sediments. To improve the aqueous solubility and consequently the biodegradation of these compounds, surfactants are often added to the contaminated environment. However, if the surfactant is toxic to the *lux*-modified GMM, then the amount of bioluminescence recorded will be due to the toxic effects of both the target pollutant (e.g naphthalene) and the surfactant. Therefore, surfactant resistant microorganisms such as *Stenotrophomonas* sp 3664 and *Alcaligenes eutrophus* 2050 should be used for toxicological evaluation of pollutants in the presence of surfactants [143].

2.161 The applications involving the use of *lux*-modified microorganisms, described above, have not involved the release of the GMM into contaminated sites. In most of the applications described, samples of contaminated material are added too a solution containing the *lux*-modified microorganisms, and the bioluminescence recorded. Because the GMMs are not released into the environment, the potential risks of the use of this type of GM technology to the environment and human health may be described as essentially zero.

2.162 Because the *lux*-modified microorganisms are usually strains that are capable of surviving in the environment, they could potentially be released directly into the contaminated site. The detection of bioluminescence in contaminated sites *in situ* does require additional electronic equipment and is probably therefore more applicable to long-term monitoring strategies, for example, the two year study reported by Ripp *et al.,* (2000) [90]. In this study, the *lux*-modified *P. fluorescens* HK44 enabled the biodegradation and bioavailability of the naphthalene, and the optimal degradation conditions to be determined *in situ*. The *lux*-modified GMMs were added to the contaminated soil as a direct inoculum and also in fibre optic biosensors where they were immobilised in alginate[11] [90].

2.163 The immobilised cells were used to monitor the presence of naphthalene in the soil vapour phase with the bioluminescence relayed by a fibre optic cable to a photomultiplier tube (PMT). The immobilised GMMs survived for approximately one week, although they could be easily replaced when necessary. Light emitted by the inoculated GMMs was detected by either PMTs or fibre optic cables, buried throughout the contaminated soil. The fibre optic cables were however found to be ineffective at detecting bioluminescence from the GMMs [90]. However, the biomonitoring system employed to study *P. fluorescens* HK55 was reported to provide a suitable online system able to quantify growth and activity of the GMMs in the soil. Improvements in the encapsulation materials used to immobilise the microorganisms in the biosensors may increase the survival of the microorganisms in the biosensors and thereby improve the described system [90].

THE USE OF PLANTS FOR THE BIOREMEDIATION OF POLLUTANTS

2.164 The use of plants for the bioremediation of pollutants is described collectively as phytoremediation, and has applications both for the removal of contaminants from the environment and the conversion of pollutants into a less toxic state [148-150]. Recent advances in molecular biology and biotechnology, coupled with a need for sustainable technologies, have allowed the further development of phytoremediation as a potentially cost-effective and environmentally friendly technology [150-154]. Developments in these fields have also provided a faster and more targeted alternative to traditional plant breeding, and have extended the types of traits and properties that can be introduced into individual plant varieties [155, 156].

2.165 The use of plants for the bioremediation of pollutants has been presented in this report as two sections covering the phytoremediation of metals and the treatment of organic

[11] Further information on the online monitoring system developed to measure bioluminescence *in situ* is

contaminants. However, from the information identified in the scientific literature, the majority of the work into the use of plants for bioremediation has focused on the phytoremediation of metal pollutants [151]. This is in contrast to the application of microorganisms in bioremediation and reflects the unique physiological and biochemical characteristics of plants.

2.166 Because of the different biological processes involved in the phytoremediation of metals and organic compounds, it is likely that applications to use plants for bioremediation purposes will be targeted towards either metal or organic pollutants, but not both types of contaminant. The biological mechanisms involved in phytoremediation are addressed in this report as a background to the application of GM technology in this field. Although applications are likely to be targeted towards the phytoremediation of either organic or metal pollutants, there are a number of approaches and advantages that are common to all phytoremediation-based processes.

Approaches and Advantages to Phytoremediation

2.167 As described already in this report, the application of bioremediation-based strategies is determined by the properties of the organisms employed. Plant-based strategies are therefore most suitable for the bioremediation of pollutants where:

- the pollutants are relatively close to the surface (within reach of the plant's root system), probably within the top 1 m of the soil profile. This is the case for many, and probably the majority, of anthropogenically derived pollutants, which tend to be deposited from above (e.g atmospheric emissions, application of contaminated sludge, etc.). Moreover, metal pollutants normally bind tightly to clay particles in the soil and so frequently remain in the upper part of the soil, in principle accessible to plant root systems;

- the pollutants are relatively non-leachable and likely to pose a low risk to the environment or human health. The comparatively slow growth of plants compared to microorganisms means that phytoremediation strategies are only suitable for the treatment of contaminated sites where the pollutant is likely to remain on-site until the plant has grown [156, 157]. Phytoremediation strategies may, however, be applicable to the treatment of more mobile pollutants if the plants are grown and in place prior to the site being contaminated, for example as a biobarrier 'downstream' of a shallow contaminated aquifer; and

presented in the report of the workshop at the end of this document (presentation by Prof Sayler).

- the pollutants are present in a contaminated site at low levels or across a wide surface area [18, 158]. The relatively low cost of phytoremediation strategies means that compared to chemical- and/or physical-based techniques, they can be applied for the treatment of large sites, and on sites where the pollutant concentration is very low.

2.168 The significant advantage of phytoremediation-based strategies is one of cost. Although reports on the potential savings of phytoremediation applications compared to traditional methods for the remediation of contaminated land such as 'dig and dump' (and replacement of soils lost), incineration and soil-washing vary, phytoremediation is proposed as between 10 and 10,000 times cheaper than existing 'conventional techniques' [156, 159, 160]. For example, growing a crop plant capable of phytoremediation on an acre of land can be achieved at a cost of between two and four orders of magnitude less than the current cost incurred by the physical excavation and re-burial of the contaminated material [160], and even where the phytoremediation strategy requires several sequential crops to be grown, overall costs are still reported to be up to an order a magnitude cheaper than 'dig and dump' methods [159].

2.169 Where sites are contaminated with toxic metals, phytoremediation strategies offer the potential to remove the metals from the site physically. This approach is likely to be significantly cheaper than the mechanical bulk excavation of the contaminated soil and re-burial at a designated landfill site, as it involves the removal of significantly less material from the site [148, 161]. Phytoremediation-based strategies may offer the only effective and/or financially viable solutions for the remediation of very extensive metal-contaminated sites (cf. Chernobyl exclusion zone).

2.170 The phytoremediation of some organic pollutants such as TNT may also be more cost effective than the 'conventional' excavation and thermal processing technique most commonly used to date [162]. In some cases plants may also prove cheaper for the remediation of organic pollutants than microorganisms [150], particularly where the microbial-based approach requires the addition of supplementary carbon sources to stimulate growth and biodegradative activity (although the use of plant-microbe combined strategies offer a number of alternatives).

2.171 Growing plants is also significantly cheaper than the culture of an equivalent weight of microbial biomass. The large amounts of biomass that can be produced by plants is one of the principal advantages of phytoremediation strategies over microbial-based approaches [163]. Plants do not require the sterile and specific growth conditions, or the specific organic nutrients needed to culture large volumes of microorganisms, and

are also generally easier to propagate and to harvest [148, 158]. Bioremediation of explosives using microorganisms for example, requires the excavation of the contaminated soil and treatment in bioreactors. Where these methods are as expensive as more 'conventional' soil incineration processes, plants may offer a cheaper approach, particularly since contaminants from munitions production are often spread over the large areas near to the soil surface [164].

2.172 Although the use of plants for the remediation of contaminated environments may offer a number of financial benefits, all phytoremediation strategies are limited by the properties of the plants involved. As with microorganisms, different plants are susceptible to different types and concentrations of contaminants, although plants are reported to be able to survive higher concentrations of certain hazardous compounds than most microorganisms used for bioremediation [157]. Plant-based strategies are also depth-limited, as pollutants can only be phytoremediated if they are within reach of the plant's root system. Different plants are also restricted to particular growth climates, with length of the growing season and soil characteristics also having a bearing on the effectiveness of phytoremediation strategies [165, 166]. Despite these limitations, in areas where contamination is spread over a large surface area, phytoremediation may offer a novel, effective solution [18].

Types of Phytoremediation

2.173 Phytoremediation is a process that uses plants to remove, transfer, stabilise or degrade contaminants present in the growing medium. Different strategies are available for the phytoremediation of metals and organic compounds in the environment. The choice of strategy depends on the identity and characteristics of the target compound, the type of environment to be remediated and the overall objective of the phytoremediation programme. The general strategies of types of phytoremediation available are:

- for metal pollutants - phytoextraction (also often referred to as phytoaccumulation); phytostabilisation; rhizofiltration and phytovolatilisation;; [151, 152, 155, 156, 167]; and

- for organic contaminants - phytodegradation and phytostabilisation [151, 156, 157].

Phytoextraction

2.174 Phytoextraction (or phytoaccumulation) refers to the use of plants capable of removing metals from the soil and accumulating them in the above-ground parts of the plant, that can then be harvested [151, 152, 154, 168].

Rhizofiltration

2.175 Rhizofiltration is a similar process to phytoextraction except that plant roots are grown hydroponically to adsorb and, to some extent, absorb metals directly from polluted aqueous environments [151, 154, 155]. Although both phytoextraction and rhizofiltration can also be used for the phytoremediation of organic pollutants [168], to date this is only a minor application of these processes, and this report will therefore focus on their use for the treatment of metal pollutants by GM plants.

Phytostabilisation

2.176 Phytostabilisation is a process which uses plants to either reduce the bioavailability of the metal pollutants in the environment, or to transform them into a less toxic form. Unlike phytoextraction, phytostabilisation does not result in the removal of the pollutant(s) from the soil [151, 152, 155]. However, this process can be very important in preventing dispersal of the pollutant(s) from the contaminated site by wind erosion or leaching [156].

2.177 Phytostabilisation can also be applied to the remediation of organic pollutants. This area of phytoremediation research has however been less well studied than phytoextraction, rhizofiltration or phytovolatilization for the treatment of metal pollutants [152].

Phytovolatilisation

2.178 Phytovolatilisation uses the ability of some plants to convert specific pollutants into volatile and usually less toxic forms, and is particularly relevant for the removal of mercury and selenium from the soil [151, 155, 156].

Phytodegradation

2.179 Phytodegradation is the process where plants are used to degrade organic pollutants, either through the uptake of the pollutants and their subsequent break-down within the plant, or by the secretion of degradative enzymes from the plant into the environment

[151]. The exact role of the plant in the phytodegradation of an organic pollutant is still somewhat controversial [169]. To date, studies have been unable to demonstrate whether the plant is able to degrade the pollutant in the absence of rhizosphere microorganisms, or if the plant is required to stimulate or assist in the degradation of the pollutants by the microorganisms. Where microorganisms are not involved directly in the biodegradation process, some synergistic process may still be involved [169]. GM plants are reported to have applications in both types of phytodegradation strategy.

Phytoremediation of Metals

2.180 The phytoremediation of metals is restricted to the sorption of the metal onto a solid matrix or the conversion of the metal into a less toxic or non-toxic state [170]. The advantage of phytoremediation-based processes is that, unlike other *in situ* remediation strategies (with the exception of vitrification- or concretisation-type approaches), the phytoremediation process does not always require the conversion of the metal pollutant into a more mobile form. Increased mobility of the pollutant is likely to represent a greater hazard to the environment [171].

2.181 As discussed in the microbial section of this report, the application of microorganism-based bioremediation strategies to the *in situ* treatment of metal-contaminated sites, is limited to the immobilisation of the metal through precipitation, or its reduction into a less toxic or non-toxic state [159]. Plants are able to bioremediate metals in this way but, most importantly, can also accumulate the metal into above-ground parts of the plant allowing the metal to be removed physically from the site following harvesting.

2.182 The application of phytoremediation-based strategies for the treatment of environments contaminated with metals is based on the ability of naturally occurring (non-GM) plants to extract and concentrate elements and compounds from their environment. In particular, plants require a number of metals for use as electrolytes, solutes, cofactors and essential components of proteins, and possess specialised strategies to obtain these compounds from the environment. These include calcium, copper, iron, potassium, magnesium, manganese, molybdenum, nickel and zinc. To protect themselves from any potentially harmful effects of accumulating these compounds, plants have developed mechanisms that inactivate or chelate the metal ion upon its entry into the plant cytosol [172]. This system prevents the metal from inactivating active or structural proteins, whilst at the same time allowing elements essential for the plant's metabolic function to be taken up and transformed into forms that are tolerable to the plant [103]. However, this system is proposed only to protect the plants up to a certain level, as soils that contain high concentrations of metals such

as potassium, magnesium, manganese and molybdenum are usually toxic to plants (Thurman and Hardwick cited by) [173].

2.183 The genetic basis of the ability of plants to tolerate metals is not yet well understood compared to other species such as microorganisms [161, 163, 174]. However, the introduction into plants of genes that confer resistance (or greater tolerance) to pollutants in other species, have been reported [152]. The biological mechanisms and genes involved and their potential application in phytoremediation of metals by GM plants, are discussed below[12].

Phytoextraction

2.184 The ideal plant for use in a phytoextraction application would have a large biomass, be able to grow rapidly and accumulate the target metal pollutant to a higher concentration than that found in the soil. Unfortunately, naturally occurring plants with all three of these desired characteristics do not exist [168]. Some plants which occur naturally on metalliferous soils are able to accumulate very high concentrations of metals such as nickel, cobalt, copper, zinc and lead [148], with metal concentrations in above-ground plant structures reaching between 0.1 and 3 percent of their shoot dry biomass [168]. Several *Thlaspi* species have been found to accumulate nickel and zinc to between 1 and 5 percent of their dry biomass [155].

2.185 However, although these plants are capable of hyperaccumulating metals, their low biomass and relatively slow growth rate means that they are not suitable for phytoextraction applications [17, 159]. Naturally occurring hyperaccumulators are in general further limited by their ability to only accumulate a specific metal (with the exception of *Thlaspi* sp.) and not the range of metals likely to be present on a single contaminated site. The metals that are accumulated primarily by these plants are nickel, zinc, manganese and cobalt, which are not among the more hazardous environmental pollutants [154], although there are occasional reports of phytoaccumulation of lead, copper and arsenic [175, 176]. Natural hyperaccumulating plants are also often rare, often of limited population sizes and growing in remote regions that may be threatened by mining, development and other activities. Little is known, therefore, about the ability to cultivate these plants for phytoextraction purposes. However, due to their patchy natural growth habits, it is unlikely that they would be suitable for monoculture [18, 154].

[12] Further information on the natural mechanisms employed by plants to accumulate metals, and the application of GM technology to improve or modify these processes is presented in the report of the workshop at the end of this document (presentations by Prof Smith and Prof Meagher).

2.186 The transfer of the genes responsible for metal hyperaccumulation from natural metal hyperaccumulating plants to faster-growing, high-biomass plants, without a consequent reduction in plant yield or metal accumulating ability is therefore one approach to the use of GM plants in phytoextraction strategies.

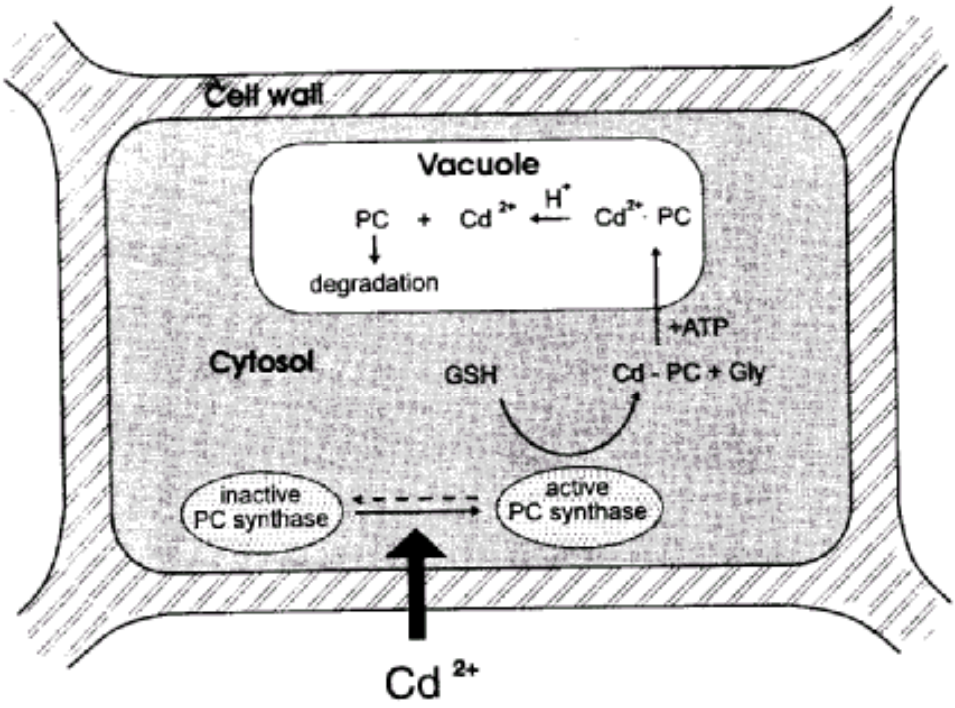
2.187 The most likely mechanism by which hyperaccumulating plants are able to tolerate the relatively high concentrations of metals that they accumulate is reported to be the production of a compound(s) that binds to (coordinates) the metal in the plant's cytoplasm [172]. Once bound, further interference between the metal and the plant's normal metabolic processes is prevented, and the metal complex may stay within the cytoplasm or move to another part of the cell, such as the plant vacuole [173]. Current applications of this approach are however restricted to those compounds whose genetic and biochemical pathways have been elucidated. Effective genetic engineering to develop a range of plants for use in phytoextraction applications will depend on an understanding of the mechanisms of metal uptake, transport and tolerance, and the rate limiting steps involved in these processes [17-19, 164, 168, 177, 178].

2.188 Although not related directly to the bioremediation of contaminated sites, plants capable of extracting metals from their environment also have applications in the recovery of 'precious metals' from the environment. The use of plants in this way is particularly relevant for the removal of metals from spoil waste, where the target compounds are often present at relatively low concentrations across a wide surface area [179].

- Metal-binding compounds in plants - Phytochelatins

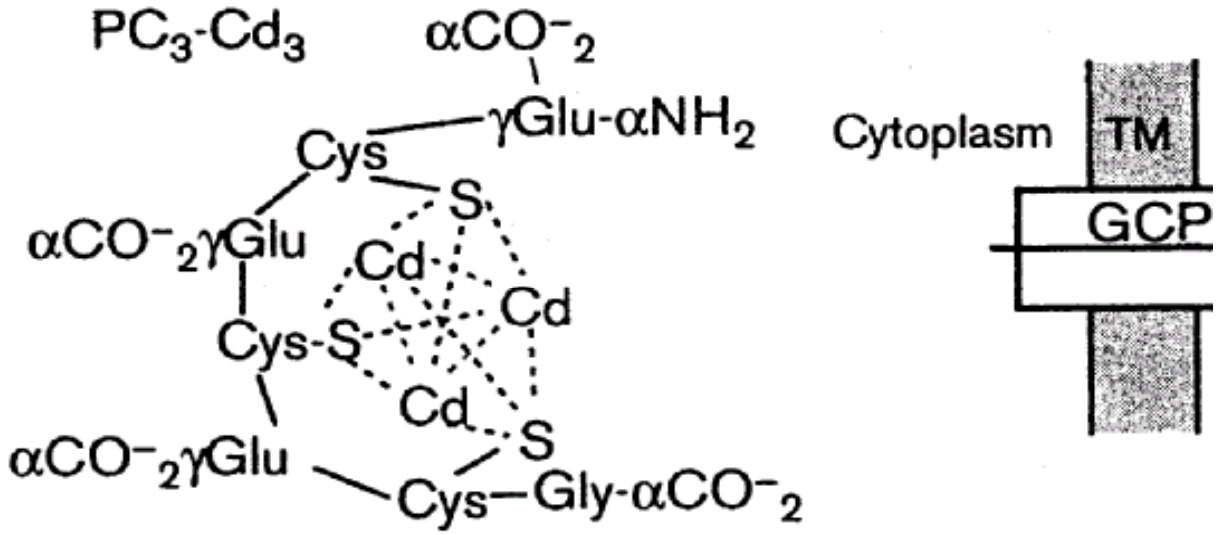
2.189 Most of the work reported in the scientific literature that has addressed the development of GM plants for phytoextraction applications has focused on the use of phytochelatins (PCs) by plants to remove metals from their environment. Phytochelatins are a family of heavy metal-inducible peptides that have been identified as important in the detoxification of heavy metals in plants and some microorganisms [180]. They are produced by a wide range of plants in response to exposure to metal pollutants, and are proposed to form ligand complexes with the metal thereby aiding its transport into the cell vacuole, where the metal is sequestered and the phytochelatin degraded (Figures 2.4 and 2.5) [19, 103, 181].

Figure 2.4 - Cadmium ions entering the cell activate PC synthase that catalyses the transformation of GSH to PC [103]



Cadmium ions entering the cell activate PC synthase that catalyses the transformation of GSH to PC. The Cd^{2+}-PC complex is actively taken up into the vacuole. Within the vacuole the Cd^{2+}-PC complex eventually dissociates. The metal is stored there while the PC peptide is degraded.

Figure 2.5 - Possible structure of a phytochelatin (n=3, X=Gly) binding three Cd(II) ions [19]



Phytochelatins, in this case a trimeric PC3, form tetrahedral complexes with thiol-reactive metals like cadmium (Cd^{2+}) enhancing tolerance. These structures should aid in the transport, and sequestration of, metals into vacuoles via the glutathione *S*-conjugate pump (GCP).

2.190 Cadmium ions have been reported to be the strongest inducers of phytochelatin formation *in vivo*, with differentiated plants and suspension cultures of mosses, ferns and angiosperms all found to detoxify Cd^{2+} ions through the production of phytochelatins of varying chain length [103].

2.191 In mesophyll protoplasts derived from tobacco plants that had been exposed to cadmium, almost all of the accumulated cadmium and phytochelatins were identified as being confined to the vacuole, thereby confirming the sequestration of the toxic metal within the plant. The identification of an ATP-dependent, proton- gradient-independent active mechanism, capable of transporting both phytochelatins and PC-Cd complexes into tonoplast vesicles derived from oat roots has also been reported [178].

- Structure and biosynthesis of phytochelatins

2.192 Phytochelatins have the structure $(\gamma\text{-Glu Cys})_nX$ where n is between 2 and 11 (but is generally in the range 2-5), and X is commonly Gly (but can be β-Ala, Ser or Glu in some plant species) [19, 178, 182]. Phytochelatins are just one family of the γ-Glu Cys peptides (also known as class III metallothioneins) [182]. The direct precursor of phytochelatin is the reduced form of glutathione (γ-Glu-Cys-Gly, GSH). Glutathione (GSH) is synthesised from its constituent amino acids in two sequential, ATP-dependent enzymatic reactions catalysed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS), respectively. PC synthase subsequently catalyses the elongation of the $(\gamma\text{-Glu-Cys})_n$ by transferring a γ-Glu-Cys group to glutathione or to phytochelatins (Figure 2.6). The proposal that phytochelatins arise from glutathione is based on:

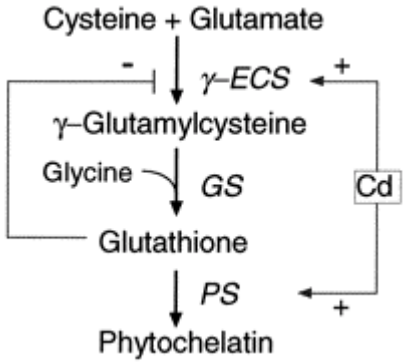
- the structural resemblance of phytochelatins and glutathione;
- the appearance of phytochelatins at the same time as the disappearance of glutathione; and
- the inhibition of PC synthesis by buthione sulfoximine, an inhibitor of γ-ECS which can be reversed by the addition of glutathione to the growth medium [178, 182].

2.193 The final stage of the synthesis of a phytochelatin is strictly dependent on the presence of metal ions, since the PC synthase ion is only activated by metal ions. The enzyme is also self-regulating since the reaction product, phytochelatin, chelates the

metal responsible for activating the enzyme [103, 182]. Evidence for this mechanism comes from the fact that the synthesis of PC is halted abruptly through the introduction of another metal-chelating agent, such as EDTA [103].

2.194 Because PC synthase is only activated in the presence of metals, it is unlikely that this enzyme is the rate-limiting step in phytochelatin synthesis [17]. It is thought that the rate-limiting step in this process is a combination of the reactions catalysed by γ-ECS and GS, depending on whether the plant is under metal stress or not [17].

Figure 2.6 - Regulation of GSH/PC biosynthesis in plants [17]



Regulation of GSH/PC biosynthesis in plants: cadmium enhances the transcription of ECS and activates the PC synthase enzyme, leading to the production of PCs and the depletion of GSH. γ-ECS is also subject to feedback inhibition by GSH.

- Evidence for importance and function of phytochelatins

2.195 Correlatory evidence for the importance of phytochelatins came from work by Speiser *et al.,* (1992) [183], which showed that selenium-tolerant *Brassica juncea* produced two types of PC-Cd complex on exposure to cadmium. These included a more stable high molecular weight PC-Cd-sulphide form, which could contribute to higher metal tolerance by more effective metal sequestration. Increased cadmium tolerance in tomato cell lines was found to be accompanied by increased production of Cd-binding phytochelatins, most of which were higher molecular weight compounds. At least 90 percent of the cadmium in most tolerant cells was found to be associated with Cd-PC complexes [184].

2.196 The importance of phytochelatins for tolerance to cadmium has been demonstrated by the isolation of a Cd-sensitive mutant (designated *cad1*) of *Arabidopsis thaliana* that appeared to be unable to accumulate or sequester cadmium [185]. Further experiments revealed an allelic series of *cad1* mutants that were all deficient both in their ability to accumulate phytochelatins when exposed to cadmium and in PC synthase activity compared to the wild type [186]. The level of phytochelatins

observed correlated with the level of sensitivity of the mutant. The mutant strains had wild type levels of glutathione, suggesting that *cad1* mutants were defective in the gene for PC synthase. Further experiments found that all four, independent *cad1* mutants had base-pair substitutions on the same *cad1* gene [180]. This result was confirmed by extracts of *E. coli* cells expressing the *cad1* gene product being able to catalyse glutathione-dependent, metal-activated synthesis of phytochelatins [180].

2.197 A second cadmium-sensitive *Arabidopsis* mutant (designated *cad2*) was affected at a different locus to the *cad1* mutants [187]. The *cad2* mutant was also deficient in its ability to sequester cadmium compared to the wild type. The accumulation of phytochelatins was also found to be only about 10 percent of that in the wild type and glutathione levels were also lower. The deficiency in phytochelatin synthesis was proposed to be due to a deficiency in glutathione [187], and was supported by the observation that phytochelatins are synthesised from glutathione. Further experiments on the *cad2* mutant found that this mutant was actually deficient in the first enzyme in the pathway of glutathione biosynthesis, γ-ECS. Enzyme assays showed that the *cad2* mutant had only 40 percent of γ-ECS activity compared to the wild type, and that the activity of the second enzyme in the pathway (GSH synthetase) was unchanged compared to the wild type. In particular, the *cad2-1* mutant was partially deficient in glutathione and γ-ECS activity (the first of the two glutathione biosynthetic enzymes). The *cad2-1* mutation was found to be a six base pair deletion within an exon of the γ-ECS gene, which affected residues in the vicinity of the presumed active site of the enzyme [188].

2.198 These findings were reported to demonstrate the importance and function of phytochelatins in protecting plants from toxic metals, particularly cadmium. The sensitivity of the *cad1* mutants to specific heavy metals gives some indication of the importance of phytochelatins for the detoxification of metals *in vivo* (for *Arabidopsis* at least) [180].

- Application of phytochelatins in the development of GM plants for bioremediation

2.199 The manipulation of glutathione and PC concentrations therefore appears to hold significant potential for increasing the accumulation of toxic metals by plants [19]. None of the γ-Glu-Cys peptides are synthesised on ribosomes, and all are formed through enzymatic reactions [182]. Because of their identification as possible rate-limiting steps in the synthesis of phytochelatins, the over-expression of GS or γ-ECS may have the potential to improve metal accumulation in plants [189]. The

overexpression of the bacterial γ-ECS gene in hybrid poplar for example resulted in increased levels of foliar glutathione [190]. Unlike other genetic modifications where the modified gene is designed to have a direct phytoremediation application, for example the insertion of the *mer* genes, the manipulation of the expression of GS or γ-ECS is intended to have a more indirect effect. The levels of phytochelatins in plants are not increased directly as a result of overexpression of GS or γ-ECS, but are a secondary result of the modification.

2.200 Alteration of the phytochelatin biosynthetic pathway of Indian mustard (*Brassica juncea*) by genetic modification was reported to demonstrate the possibility of producing GM plants with superior phytoextraction capability [17, 191]. This plant was modified to overexpress the *gshII* gene (encoding for GS and isolated from *E. coli*) in its cytosol. *B. juncea* was selected for genetic modification because the wild type has a rapid biomass production and a high trace element accumulation capacity [17]. *B. juncea* has also been shown to produce a high molecular weight PC-Cd-sulphide complex. Such complexes have been found to have greater stability compared to low molecular weight complexes and could therefore contribute to higher metal tolerance due to more effective sequestration [183].

2.201 Overexpression of the *gshII* gene in the GM *B. juncea* resulted in enhanced production of glutathione and phytochelatins and improved accumulation of, and tolerance to cadmium [17]. Increased levels of both compounds by the plant correlated positively with the *gshII* expression levels [17]. The corresponding increase in phytochelatin levels and improved accumulation of cadmium in the GM plant was expected as greater levels of phytochelatins mean an improved ability to bind and sequester the metal pollutant in the vacuole. Further complexation of the cadmium with sulphide is reported to occur in the vacuole [17, 103].

2.202 In unstressed GM plants expressing *gshII*, GS was found not to be rate limiting for the synthesis of glutathione, as the glutathione levels were not significantly different in the unstressed GM plants compared to the wild type. There was also no detectable phytochelatin in either the unstressed GM plants or the wild type variety [17]. Under situations of cadmium stress however, the GS enzyme appeared to become rate limiting for the biosynthesis of glutathione and consequently phytochelatins. In the roots of wild type plants exposed to cadmium, glutathione levels were three-fold lower than in similar plants in the absence of cadmium. This was reported to be due to depletion of glutathione following the synthesis of phytochelatins by the stressed plant. In the GM plants the modification meant that the rate limitation was removed

and the glutathione levels were found to be the same irrespective of the presence of cadmium [17].

2.203 Similar experiments have also been reported using *B. juncea* genetically modified to overexpress *gshI* (encoding for γ-ECS) in the plant's chloroplasts [191]. Overexpression of the *gshI* gene (isolated from *E. coli*) in the GM plant resulted in increased γ-ECS activity compared to the wild type. In plants treated with cadmium, overexpression of the γ-ECS gene increased the formation of γ-EC (its direct product), and also glutathione and phytochelatins further down the biosynthetic pathway [191].

2.204 In unstressed transgenic plants (those not treated with cadmium) there was increased production of γ-ECS and glutathione, compared to the wild type, although production of phytochelatins remained unchanged [191]. The findings from this work were reported to suggest that γ-ECS was limiting for glutathione and therefore production of phytochelatins. The enzymes γ-ECS and GS were only reported to co-limit glutathione production under conditions of cadmium stress [191].

2.205 The work reported with *B. juncea* demonstrates the potential of genetic modification for the development of cadmium accumulating plants to treat contaminated land [17, 191]. The application of this type of genetic modification to other metal pollutants depends on the ability of plant ligands to be induced and sequester other metals. Although various other metals, for example copper, nickel and zinc, have been found to induce the formation of ligands in plants, their function in sequestering the metal *in vivo* has only been demonstrated extensively for cadmium, with comparatively little work reported for other metals [182, 192]. Therefore, the determination of the functional importance of plant ligands in cellular metal sequestration and the elucidation of the genes involved in metal tolerance should improve the applications of phytoremediation in the treatment of contaminated environments [182].

2.206 Studies on the *cad1* mutants of *Arabidopsis* have shown that PC synthase is activated in the wild type *in vivo* and *in vitro* by a number of metal ions to which the *cad1* mutants are not hypersensitive. For example, in the wild type, PC synthesis *in vivo* is activated effectively by copper. However, the *cad1* mutant is only slightly more sensitive to copper than the wild type [180].

2.207 Therefore, although synthesis of phytochelatins may be activated by a number of different metal ions, this may not necessarily mean that phytochelatins play a major role in their detoxification. From the information available, it appears that

phytochelatins are a major component of certain heavy metal detoxification, but that the increased tolerance of plants to metals other than cadmium may involve other as yet undetermined aspects of phytochelatin function, or may involve the operation of other more effective biological pathways for the detoxification of these metals [180, 186]. For metals other than cadmium there are few studies demonstrating the formation of phytochelatin-metal complexes either *in vitro* or *in vivo* [178].

2.208 Studies of both naturally occurring metal-tolerant plants and laboratory-selected metal tolerant plant cell lines do not show a clear correlation between increased resistance and increased production of phytochelatins [186]. Although phytochelatins have been shown to be able to bind more than one metal, most of the naturally occurring plant species that are hyperaccumulators are only able to tolerate high levels of just one metal [173], suggesting that hyperaccumulation is a consequence of more than just the production of phytochelatins. Chaney *et al.,* (1997) [155] proposed that the properties identified in plants as cadmium tolerance mechanisms are in fact incidental biochemical phenomena. An improved understanding of the genetic basis of natural hyperaccumulating mechanisms is required to enable their manipulation in a wider variety of plant species [19].

- Metal binding compounds in plants - Metallothioneins

2.209 Metallothioneins (MTs) have been proposed as the mammalian equivalent of phytochelatins [193]. However, further studies of metal-binding complexes in plants have revealed that some plants have metallothionein-like genes and proteins in addition to phytochelatins, suggesting that these two groups of compounds may have different roles [178, 186]. Kawashima *et al.,* (1991) [194] identified a metallothionein-like protein from soybean using a synthetic oligonucleotide probe that corresponded to part of the nucleotide sequence of the mammalian metallothionein.

2.210 A number of reports have proposed that metallothioneins and phytochelatins may have relatively independent or overlapping functions in metal detoxification and/or metabolism, although it has not been determined to what extent their individual roles are complementary or redundant in plants [19, 178, 180, 186]. In animal cells and in some fungi, metallothioneins appear to play a major role in heavy metal detoxification [186]. The expression of the mouse metallothionein 1 gene (*mt1*) in tobacco plants, found that the cadmium concentration in the transformed plants was 24 percent less in the shoots, and approximately 5 percent more in the roots compared to non-GM tobacco seedlings [195]. Although the results demonstrated that insertion and expression of the mouse metallothionein did affect the sequestration of cadmium in

the roots and shoots of the plant, the relatively low increase in cadmium accumulation meant that this modification is not applicable to phytoextraction applications. However, the modification may be useful in the development of a crop plant that has lower concentrations of the accumulated metal pollutant in the consumable parts of the plant.

2.211 In a similar study a gene construct encoding the α-domain of the human metallothionein gene I_A (MT-I_A) was introduced into tobacco cells on a disarmed Ti plasmid of *Agrobacterium tumefaciens*. The transgenic plants were tolerant to levels of cadmium that were toxic to the non-GM control plants, and suggested that the transgene was involved in metal detoxification and/or sequestration in the tobacco plant [196]

- Other strategies for the development of GM plants for phytoextraction

2.212 Harmens *et al.,* (1993) [197] found that increased tolerance to zinc in the plant *Silene vulgaris*, which is naturally either sensitive or tolerant to zinc, was not related to an increased accumulation of phytochelatins. The relatively low affinity of phytochelatins for zinc compared to cadmium meant that the findings were not unexpected. The results were reported to support the hypothesis that zinc detoxification in the roots of plants involves other ligands such as organic acids, for example citrate or malate, that may facilitate the transport of zinc through the xylem and its ultimate storage in the vacuoles of cells in the shoot.

2.213 In the nickel hyperaccumulator *Alyssum lesbiacum*, histidine has also been identified complexed with a proportion of the nickel present in roots, shoots and xylem [168, 178]. Moreover, the free amino acid histidine is produced in the roots of *A. lebiacum* as a direct and proportional response to nickel exposure [198]. However, it is not yet known at what level (e.g transcriptional, post-translational), the regulation of histidine biosynthesis occurs, at least in *Thlaspi goesingense* [168].

2.214 Since the genetic basis for the above observations is unknown, it is difficult to use this knowledge to devise genetic engineering strategies to improve the suitability of. plants for the phytoextraction of nickel and/or zinc from contaminated sites. No work has been reported in the scientific literature on the over-expression of genes encoding the enzymes involved in histidine biosynthesis, although this field would be expected to hold considerable promise for future work on phytoextraction of zinc and nickel by certain plant species.

2.215 The transfer of the gene(s) encoding the 'hyperaccumulating' phenotype into higher biomass plants has been restricted up to now by a lack of fundamental knowledge regarding the molecular, physiological and biochemical basis of hyperaccumulation [169]. However, recent research into the genes encoding for proteins involved in the transport of metals has improved the knowledge base in this area and is reported to show some promise for its application to phytoextraction [177].

2.216 Radiotracer studies with *Thlaspi caerulescens* and a closely related non-hyperaccumulating species, *T. arvense*, have suggested that the uptake of zinc is controlled by the number (area density) of active transporters located in the membranes of the root cells. Once in the plant's root, zinc is proposed to be transported into the xylem and taken up by the leaf cells, thus preventing the build-up of toxic levels of the metal in the cytoplasm [199-201]. These observations are proposed to indicate that zinc transporter systems exist within plants and operate to transport the metal between cells and into subcellular compartments within the plant.

2.217 The first plant transporter genes for zinc were successfully isolated and characterised from *Arabidopsis thaliana* [202]. Four genes encoding a zinc transporter were identified (designated *zip1, zip2, zip3* and *zip4*). The genes *zip1, zip2* and *zip3* were found to encode for transporters that showed unique sensitivities to metal ions other than zinc (Mn, Fe, Co, Cd and Cu were tested). This was proposed to be due to differences in their substrate specificity [202]. Multiple zinc transporters are likely since after the metal ion has entered the plant it must cross cell and organelle membranes as it is distributed in the plant. Each of the transporters identified may perform different roles in different parts of the plant. For example *zip1* and *zip3* were found to be most strongly expressed in the roots of zinc-deficient plants. Little or no mRNA of these genes was detected in the roots of zinc-sufficient plants or in the shoots of zinc-deficient or zinc-sufficient plants [202]. The mRNA from *zip4* however, also responds to zinc deficiency like *zip1* and *zip3,* but is induced in the shoots as well as the roots. These results were consistent with the theory that *zip1* and *zip3* are involved in the uptake of zinc from the rhizosphere and *zip4* is involved in the transport of zinc in the plastids [202]. Histidine residues were identified in nearly all the variable regions of these ZIPs, as well as within the transmembrane regions of the protein, suggesting that these residues play a role in metal recognition and/or in the transport of zinc through the membrane [202].

2.218 A gene encoding a zinc transporter has also been identified in *Arabidopsis thaliana* (designated *zat*). Compared to the wild type transgenic *Arabidopsis* plants over-expressing *zat* were found to have enhanced resistance to zinc and the ability to

accumulate higher concentrations of zinc in the roots when exposed to high concentrations of zinc [203].

2.219 A set of mammalian genes closely related to *zat* that are also involved in the uptake of zinc have been found to encode proteins that are involved in the facilitation of the vesicular sequestration of zinc. Expression of these proteins was found to result in an increased resistance to otherwise toxic levels of extracellular zinc. The *zat* gene may also encode for a protein that has a similar function, possibly transporting zinc into the central vacuole of the plant cell, thereby contributing to the resistance of zinc in plants [203].

2.220 A set of genes coding for a reported calcium transporter system have also been identified in *Arabidopsis* [204]. These genes (designated *cax1* and *cax2*) were identified by their ability to suppress mutants of a yeast defective in vacuolar calcium ion transport [205]. The expression of *cax1* in tobacco plants resulted in the disturbance of normal vigour, including necrotic lesions, chlorosis and a reduction in root mass, compared to the non-GM plants. All of the symptoms identified are characteristic of calcium ion deficiency. The GM plants were also more sensitive than the non-GM plants on exposure to other ions and to cold-shock. When the GM tobacco plants were grown in a media containing supplements of calcium ions, the number of transformed plants showing abnormalities decreased dramatically [204]. The altered phenotype and increased stress sensitivities emphasised the importance of *cax1* for normal growth and several biological responses [204].

2.221 Similar altered phenotypes were observed in tobacco plants genetically modified to express *cax2*, although the majority of the GM plants were as vigorous as the non-GM controls. T_2 plants from the healthy *cax2* modified plants had a slight reduction in root mass but the majority of plants appeared normal [206]. Studies showed that the transporter encoded by *cax2* was localised in the plant's vacuolar membrane and was responsible for transporting divalent cations, including Ca^{2+}, Cd^{2+} and Mn^{2+} into the vacuole [206]. The expression of *cax2* was also found to result in increased accumulation of calcium, cadmium and manganese ions in the roots and shoots of the GM plants compared to the control. The transgenic plants were also slightly more tolerant to manganese ion stress than the non-GM controls [206].

2.222 Studies with the *cax2* modified tobacco plants [206] suggested that the expression of *cax2* in transgenic crops could alleviate Mn^{2+} toxicity problems and aid the phytoremediation of Cd^{2+} in contaminated soils through the accumulation of the metal ions within the plant vacuoles. Since various plant transporters appear to have a broad selectivity in ion transport, *cax2* may also be capable of conferring increased

resistance/accumulation of other metal ions [206]. The *cax2* modified plants however showed no enhanced Cd^{2+} tolerance, and only limited increased tolerance to Mn^{2+} [206].

2.223 However, in addition to the genetic modification of the plant to sequester the required metal pollutants, modifications may also be required in the control of metal uptake in the roots, long distance transport of the metal within the plant and additional tolerance factors to accommodate high concentrations of these metal ions.

2.224 An important trait of natural hyperaccumulating species is enhanced translocation of the absorbed metal to the shoot [168, 175]. It is proposed that continued research in the elucidation of the molecular basis for heavy metal transport in natural hyperaccumulators, the tools to allow genetic modification of high biomass metal accumulating plants for phytoextraction will become apparent. For example, a recent study on the natural hyperaccumulator *Thlaspi caerulescens* identified another zinc transporter, encoded by the gene *znt1*. This transporter is expressed at very high levels in the roots and shoots of the plant and was shown to mediate high-affinity Zn^{2+} uptake as well as low-affinity Cd^{2+} uptake [201].

Phytostabilisation

2.225 The main distinction between phytostabilisation and other types of phytoremediation is that phytostabilisation does not actually reduce the amount of pollutant present at a site. Phytostabilisation uses methods such as the secretion of compounds into the soil to alter the soil chemistry, formation of humic matter and accumulation of other organic phases to reduce the bioavailability of the pollutant [18].

2.226 The ideal plants for phytostabilisation applications are therefore those capable of tolerating high levels of heavy metals and having the capability to immobilise those metals in the soil [159]. In biologically active soils, organic and inorganic contaminants can form chemical and biological associations of varying intensity. These associations can decrease the bioavailability of a contaminant, therefore effectively reducing its risk of causing a toxic effect [18].

2.227 Plants are particularly well suited to the sequestration and/or immobilisation of pollutants as they produce dense root systems that infiltrate large volumes of soil and help to stabilise disturbed ecosystems [174]. Root densities equivalent to 4.8×10^8 km of roots per hectare have been reported [149]. Because of their large spreading root system, trees are particularly suited for phytostabilisation purposes [149, 158]. Because trees are perennial and relatively slow growing, plant establishment will

occur in parallel with soil stabilisation for a period of years with little maintenance required [158].

2.228 No work has been identified in the scientific literature into the use of GM plants in phytostabilisation. However, studies have been conducted that have demonstrated the potential for phytostabilisation as a treatment for contaminated sites. The infection of trees with the bacterium *Agrobacterium rhizogenes* results in the formation of larger than normal root masses. Preliminary results showed that these non-GM trees had higher growth rates with greater potential to establish on and stabilise the soil of a contaminated site more quickly [158].

2.229 Deep-rooted plants have been shown to reduce the highly toxic form of chromium. (Cr^{6+}) to the more insoluble and significantly less toxic Cr^{3+} form [155]. The roots of *Agrostis capillaris* have been found to cause the formation of pyromorphite, an insoluble and bio-unavailable form of lead, in soil containing concentrations of lead and phosphate. However, the mechanism involved remains unknown [155].

2.230 Investigation into the various mechanisms that plants use to render metal species unavailable may provide useful insights into using molecular modification techniques for those metals that cannot be remediated in other ways. Alternatively, stabilisation of the soil surface could be achieved using metal-tolerant GM, or non-GM, species in order to at least achieve stabilisation of a contaminated area to prevent erosion and/or leaching of the pollutant and spreading of the pollutant into the surrounding area.

Rhizofiltration

2.231 Rhizofiltration has applications in the removal of pollutants from aqueous environments through the adsorption of the metal compounds onto the plant's roots. Hydroponic plants therefore offer the greatest potential for rhizofiltration applications. The ideal plant for rhizofiltration should be able to produce large amounts of fine root biomass rapidly, and be able to remove toxic metals from solution over an extended period of time [152, 159, 168]. Rhizofiltration applications include the treatment of surface waters and groundwater aquifers, industrial and residential effluents, storm waters, acid-mine drainage, agricultural run-off, diluted sludges and radionuclide-contaminated solutions [207].

2.232 Research on the use of plants for rhizofiltration has been limited predominantly to non-GM plants. The only application of GM plants in this field is the use of GM plants modified to express a bacterial enzyme capable of detoxifying and removing

mercury from experimental solutions. This is addressed further in the following section of this report.

2.233 Young seed-lines of certain species grown in aerated water have been shown to be effective at removing metals from water. Indian mustard seeds grown in this way rapidly generate a large seedling biomass, which is capable of accumulating various metals including Cd, Pb, Sr, Ni and Cr [159]. In order to improve the performance of such systems, the affinity of the plant roots for metals will need to be increased. This is reported to be possible using molecular techniques to produce GM plants with the capacity to express high-affinity metal binding peptides on their roots [148]. Continued studies of the mechanisms of heavy-metal uptake by root tissue should also provide important insights into increasing the efficiency and applications of rhizofiltration [207].

Phytovolatilisation

2.234 Phytovolatilisation strategies are applicable for the treatment of volatile metal pollutants, such as mercury and selenium. Plants used in phytovolatilisation applications are able to sequester the metal, convert it into a less toxic form and then volatilise the metal into the atmosphere, thereby removing the pollutant from the contaminated site[13].

- Phytovolatilisation of mercury

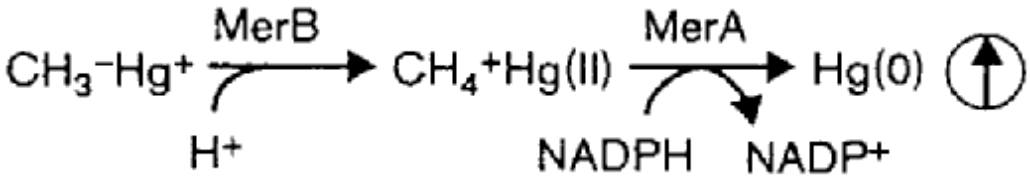
2.235 In the environment mercury exists mainly as a divalent cation (Hg^{2+}), but may bioaccumulate as methylmercury. Conversion of ionic mercury to its elemental state reduces the risk posed to the environment due to the lower toxicity, aqueous solubility and reactivity of elemental mercury compared to the other forms [171, 208]. Elemental mercury and Hg^{2+} are released into the environment as a result of gold mining, various industrial processes, burning of fossil fuels and the disposal of medical waste. On entering the lower trophic levels of the food chain, Hg^{2+} can be converted to the even more toxic compound methylmercury by microorganisms. Methylmercury is also bioaccumulated through the food chain [149, 156, 174]. Bioaccumulation can lead to mercury poisoning in organisms in upper trophic levels [19]. Although releases of mercury to the environment have decreased since the 1960s, there are still large areas contaminated with mercury which are likely to remain hazardous to the environment for many years, unless remediated [208].

[13] Further information on the application of GM technology to phytovolatilise pollutants from contaminated sites, particularly mercury is presented in the report of the workshop at the end of this document (presentation by Prof Meagher).

2.236 Mercury contaminated sites can be remediated 'conventionally' through 'dig and dump' processes, although this simply transfers the contamination to a land-fill site and also is severely disruptive to the contaminated area. With respect to the potential for the phytoremediation of mercury, plants cannot detoxify mercury naturally, and their tolerance to mercury is generally low. Therefore the potential to use naturally occurring plant species for the phytoremediation of mercury contaminated sites is limited [156]. Microorganisms have been isolated from mercury contaminated environments, and have been found to have some natural resistance to mercury. Resistance of microorganisms to mercury is encoded by the *mer* operon[14]. The two *mer* genes that have been used in phytovolatilisation systems are the *merA* and *merB* genes [208].

2.237 The *merA* gene codes for an NADPH-dependent mercuric ion reductase that converts ionic mercury (Hg^{2+}) to elemental mercury (Hg(0)), which is then volatilised into the atmosphere. The *merB* gene encodes an organomercurial lyase that degrades methylmercury to methane and Hg^{2+} (Figure 2.7).

Figure 2.7 - The bacterial enzymes MerA and MerB catalyse the detoxification of methyl and ionic mercury respectively to produce volatile Hg(0) [19]



2.238 Although microorganisms have been proposed for use in the bioremediation of mercury contaminated sites, the limited sphere of influence of individual cells means that the treatment of large-scale sites with microorganisms requires a large inoculum density and would therefore be unlikely to be financially viable [149, 208]. Since plants are autotrophic and have large root systems, they should be able to increase the rate at which mercury is eliminated from the soil by orders of magnitude over and above the rate of mercury remediation by bacteria expressing the *mer* operon [19]. In theory, plants genetically modified to express the *mer* genes should be able to extract organomercuric compounds from the environment and convert them using the same system conducted by *mer*$^+$ microorganisms. The modified plants should then be able to transpire Hg (0) from their leaves [174].

[14] For further information on the bioremediation of mercury by microorganisms, see section on the use of bacteria for the bioremediation of inorganic pollutants - mercury.

2.239 Initial work on the genetic modification of plants to express the *mer* genes was conducted with *Arabidopsis thaliana* [208]. Initial attempts to express the *merA* gene in the *Arabidopsis* plant were unsuccessful. The coding sequence of the bacterial *merA* is guanine and cytosine rich, contains 218CpG dinucleotides and is skewed toward GpC-rich codons, which are uncommon in plants. In order to express the *merA* gene, a modified version of the gene was constructed so that nine percent of the original coding region was replaced with nucleotide combinations and codons that were more similar to those in highly-expressed plant genes. In addition, 15 bp of the 5′ region immediately upstream from the initiator codon of the *merA* gene was replaced with a plant translation signal code [171]. Plants expressing the modified *merA* gene were reported to grow in an environment containing concentrations of mercury normally toxic to most plant species, including the wild type *Arabidopsis*. The GM plants also reduced Hg^{2+} to Hg (0) several times more efficiently than the non-GM control plants [148, 171, 208].

2.240 Similar studies were conducted with the *merB* gene using *A. thaliana.* As with *merA,* the bacterial *merB* gene had to be modified in order for it to be expressed efficiently in the plant. Flanking regions were added containing plant and bacterial translation signals. The transgenic plants were able to germinate and grow well in environments containing concentrations of methylmercury that caused adverse effects and death to non-GM plants [209].

2.241 Although the GM *Arabidopsis* was found to be capable of removing mercury from a contaminated environment, the small biomass of this plant and its unsuitability for large-scale cultivation means that it would not be effective for the phytovolatilisation of mercury in a field situation [148]. Rugh *et al.,* (1998) [149] selected yellow poplar (*Liriodendron tulipifera*) for genetic modification using *merA* gene constructs. The transgenic poplars expressing *merA* were found to be resistant to levels of Hg^{2+} that were toxic to the wild type. High levels of Hg(0) were found to be released from GM poplar plantlets rooted in a medium containing Hg^{2+}. However, these transgenic plants were not tested in model phytoremediation experiments using mercury contaminated soils [149].

2.242 Plants expressing the *merA* construct are therefore reported to have some potential for the remediation of Hg^{2+} from soil. Reduction in concentrations of Hg^{2+} also has the great benefit of slowing the formation and accumulation of methylmercury, although the use of $merB^{+}$ plants would be able to reduce the levels of methylmercury present. Plants expressing both *merA* and *merB* should therefore be capable of removing both methylmercury and Hg^{2+} from mercury contaminated environments [208].

2.243 *Arabidopsis* genetically modified to express the modified *merA* and *merB* genes had a higher level of resistance to methylmercury concentrations than either *merB* plants or the wild type. The GM plants were also able to convert methylmercury to Hg(0), which was subsequently volatilised from the plant [209]. Further experiments found that the rate of evolution of Hg(0) from individual GM plants correlated positively with MerB concentrations. Regression analysis confirmed this relationship [174]. These experiments were carried on culture media containing methylmercury; a real test of this technology would be to grow these plants on methylmercury contaminated soils from the field.

2.244 Because methylmercury poses a greater environmental hazard in aquatic and marine sediments (due to its higher toxicity to aquatic organisms), it has been proposed that the modification of aquatic and saltmarsh plants would provide the most effective solution to bioremediate contaminated aquatic habitats. Target species for the insertion of the *mer* genes are wetland species and water-tolerant trees, such as cordgrass (*Spartina*), cat-tail (*Typha*), bulrush (*Scirpus*), poplar (*Populus*) and willow (*Salix*). These species could be planted in aquatic and/or wetland environments where methylmercury pollution is most prevalent [156, 210].

- Phytovolatilisation of selenium

2.245 Another element that can be volatilised by plants is selenium. This metal is a common contaminant in oil-refinery wastewater and can cause death and deformities in wildlife [211]. Microorganisms are known to be involved in the volatilisation of selenium from soils, and the ability of a naturally occurring plant to volatilise this compound has only recently been identified in *Brassica juncea*. This species has high rates of selenium accumulation and volatilisation, which combined with the fast growth rate and high productivity make *B. juncea* a very suitable species for the bioremediation of selenium by phytovolatilisation [211].

2.246 Volatilisation of selenium in the form of methyl selenate has been proposed as a mechanism of selenium removal by plants. Volatile forms of selenium have been reported to be 500 to 600 times less toxic than the inorganic forms [211]. Some plants can also remove selenium from the soil by accumulating non-volatile selenium compounds in their foliage. Naturally occurring plants such as *Astralagus* sp hyperaccumulate high concentrations of selenium, although the mechanism for how these plants are able to cope with toxic levels of selenium is unknown [19, 152].

2.247 Selenium has very similar chemical properties to sulphur. Both of these compounds are taken up and assimilated by plants through a common pathway activated by ATP

sulphurylase [19]. The reduction of selenate, mediated by ATP sulphurylase, has been proposed as the rate-limiting step for the assimilation of selenate [211]. This proposal was tested by modifying *B. juncea* to overexpress ATP-sulphurylase, and resulted in the increased reduction of supplied selenate, compared to the wild type. The findings supported the theory that the enzyme ATP-sulphurylase mediates selenate reduction *in vivo* and that the enzyme is rate-limiting for the uptake and assimilation of selenate to organic selenium [211].

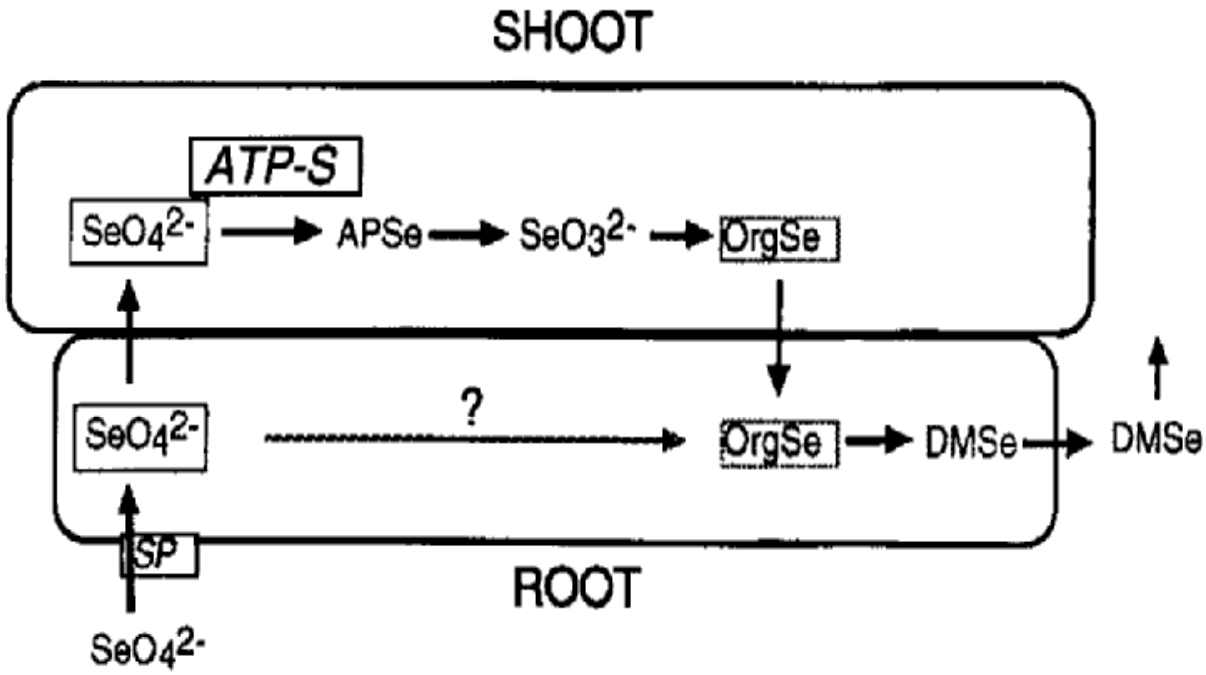
2.248 Earlier studies found that, in a second detoxification mechanism, selenate can be converted to dimethylselenide, which is 100 times less toxic than selenate, and can be volatilised from the leaves and the roots (predominantly the roots) (Figure 2.8) [19, 211]. It was proposed that in the transgenic plants expressing the gene for ATP sulphurylase, the rate-limiting step for selenium-volatilisation shifts from the reduction of selenate to the volatilisation of organic selenium [211]. The development of a GM plant capable of volatilising selenium from the soil into the atmosphere was, however, not achieved (Terry, cited by Black, 1995) [166].

Phytoremediation of Organic Pollutants

2.249 The application of plants for the bioremediation of organic pollutants can be divided into phytodegradation and phytostabilisation strategies. The concept of using plants to bioremediate soils contaminated with organic compounds stems from the observations that organic chemicals disappear faster from vegetated soils compared to non-vegetated ones [151]. As with microbial-based strategies, the phytodegradation of organic pollutants is intended to result in the breakdown of the pollutant to its relatively non-toxic constituents [19]. Organic compounds that are potential targets for phytoremediation include persistent organic pollutants that are also known to be toxic, teratogenic and carcinogenic [19, 150]. Suggested appropriate organic targets for phytoremediation include:

- petroleum products and by-products. These compounds probably represent the largest volume of organic pollutants requiring remediation;
- industry-specific chlorinated organics (PCBs, dichlorobenzenes and TCE);
- industry-specific nitroaromatic compounds (TNT and dinitrotoluene (DNT)); and
- pesticide residues that are historic “off-label” or accidental spills [212]

Figure 2.8 - A proposed model for the selenium flow in Indian mustard plants [211]



The compounds shown in boxes are the Se forms that accumulate in selenate-supplied plants. Selenate is translocated rapidly from root to shoot, and is accumulated in shoots and roots of wild type plants because ATP-sulphurylase activity is limiting. When ATP sulphurylase is overexpressed (in APS plants), an organic form of Se (possibly SeMet) is accumulated in shoots and roots. Because detopped roots of APS plants do not accumulate organic Se, selenate assimilation appears to be a predominantly shoot-specific process and there must be a flow of organic Se from shoot to root. (SP, sulphate permease; ATP-S, ATP sulphurylase; OrgSe, organic selenium).

2.250 With the exception of some PAHs that are acquired by plants from the atmosphere, organic compounds are taken up by naturally occurring plants in the liquid phase [151, 213]. Although plants can transform and mineralise a wide variety of complex organic compounds, only a few of these chemicals appear to be completely mineralised by naturally occurring plants to water and carbon dioxide [19]. This puts plants at a disadvantage compared with some bacteria in providing an effective mechanism for the degradation of organic pollutants.

2.251 The modification of plants with microbial genes that confer the ability to remediate pollutants may, however, provide a more effective method for the phytoremediation of organic pollutants in the environment [163]. An important consideration in the bioremediation of all organic pollutants by both plants and microorganisms is the toxicity of the compounds produced as intermediate end products of metabolism [151].

Phytodegradation

2.252 The ideal plant for phytodegradation would have a high growth rate and be able to mineralise the target organic compound without accumulating or releasing toxic metabolites. Only a limited number of studies have been reported that have used GM plants for the phytodegradation of organic compounds.

2.253 French *et al.,* (1999) [163] genetically modified tobacco plants to express pentaerythritol tetranitrate (PETN) reductase, a microbial enzyme responsible for the denitration of TNT and GTN. The bacterial gene encoding for the reductase was modified to include a plant consensus start sequence [163, 164, 214].

2.254 Although certain naturally occurring plants are able to degrade TNT, their application for the treatment of contaminated sites is limited as the principal products of the degradation are aminodinitrotoulenes, which are potentially more toxic than the parent compound [163]. Transgenic plants producing PETN reductase were able to germinate and grow in media containing higher levels of glycerol trinitrate (GTN) or TNT compared to the wild type seeds [163]. The transgenic tobacco seedlings were able to denitrify GTN. The ultimate products of TNT reduction by PETN reductase have not been identified, but the results indicate that the products are less inhibitory to plant growth than aminodinitrotoulenes [163].

2.255 The degradation of TNT is undergoing further investigation since this compound is a more significant environmental pollutant that GTN, due predominantly to its wider use and greater recalcitrance and toxicity. Much of the land polluted by TNT and other types of munitions contamination is spread over large areas in near-surface soils; conditions ideally suited for phytodegradation-based treatments [164]. However, numerous sites exist where TNT and GTN are just two components of a diverse mixture of explosive contaminants. For phytodegradation to provide an effective remediation solution at these sites, plants must be able to take up and degrade effectively a number of compounds [164]. Further study is also required to see if these transgenic plants are able to degrade explosive residues in soils in field conditions, rather than in plant growth media [163].

2.256 Work on the phytodegradation of TNT and GTN demonstrates the potential for the introduction of transgenes into a plant genome, to enhance the natural capacity of plants to break down organic compounds. Similar applications of other bacterial

degradative pathways in plants, give an indication of the potential for phytodegradation applications[15] [163].

2.257 Studies have also been reported for the use of GM plants for the treatment of TCE contamination. Non-GM yellow poplar plants were found to be able to take up and degrade TCE to several known metabolic products [215]. The introduction of a transgene such as the mammalian cytochrome P450 2E1, into the plant may however improve the rate and or extent of the biodegradation of this compound [150]. The mammalian cytochrome P450 2E1 is a reported to be capable of oxidising a wide range of compounds including TCE, ethylene dibromide (EDB), carbon tetrachloride, benzene, styrene, chloroform, 1,2-dichloropropane and vinyl chloride [216], (Guengerich *et al*., 1991 cited by) [150].

2.258 After exposure to TCE for five days the GM plants were found to contain significantly higher concentrations of the TCE metabolite trichloroethanol in their tissues than the non-GM wild type. The greatest difference in concentrations of trichloroethanol between the GM and non-GM plants was in the roots, with the smallest difference in the leaves. The metabolite was further degraded in the plant, with the fastest removal in the roots. The reason for these differences between the plant tissues was not determined, but was proposed to be due to transport of the trichloroethanol from the roots to the leaves, loss of trichloroethanol from the roots into solution, or because of faster metabolism of the trichloroethanol in the roots and stem of the plant compared to the leaves [150].

2.259 Tobacco plants were genetically modified with a gene construct containing a plant promoter and terminator and the P450 2E1 cDNA. The transgenic tobacco plants expressing human P450 2E1 metabolised TCE and EDB at an enhanced rate compared to the wild type. However, further work is reported to be required for these transgenic plants to identify the downstream metabolic products of the reaction and ensure that no toxic intermediates are released into the environment [150].

Phytostabilisation

2.260 No applications for the phytostabilisation of organic pollutants as a strategy for the treatment of contaminated land have been reported to date. However, as with metal contaminants, organic pollutants form chemical and biological associations of varying intensity within the soil and plants. These associations can decrease the

[15] Further information on the application of GM technology to phytoremediate nitroaromatic compounds from munitions contaminated sites is presented in the report of the workshop at the end of this document (presentation by Dr Bruce).

bioavailability of the contaminant and therefore effectively reduce its risk of causing a toxic effect. For example, organic compounds can be incorporated into lignin, thereby becoming irreversibly trapped in plant cell wall constituents [18]. One realistic benefit of the use of plants on contaminated sites is to stabilise the soil to prevent erosion and/or leaching of the pollutant and further spreading of the pollutant into the surrounding area [157].

Technical Problems Encountered with Transgene Expression in Plants

2.261 The purpose of this section is to address briefly the technical problems of transgene expression in plants that are relevant to phytoremediation applications. This section is not intended as an exhaustive consideration of the issues, which would be best addressed in a separate report, and is only intended to highlight the potential problems that could be encountered.

2.262 The genetic basis of contaminant degradation and/or accumulation is not as well studied in plants as in bacteria. Therefore much of the work conducted to date in this field has focused on the use of bacterial genes to enhance the bioremediation abilities of plants. The modification of plants with non-plant genes is not technically straightforward. It has been shown that in plants, (trans)gene expression is affected to a large extent by the codon composition [217]. For example, certain bacterial genes were not expressed in transgenic plants, in spite of their presence in very efficient plant expression systems (*Agrobacterium tumefaciens* mediated transformation) [171]. This was thought to be because the coding sequence of the bacterial gene was GC-rich, and used codon sequences that are rarely found in highly expressed plant genes [149]. Although these problems can be resolved by making changes to the sequence of the bacterial gene, and creating a new gene construct which is more compatible for plant expression without losing the resistance encoded for in the original bacterial gene, such changes may be required for all bacterial genes intended for use in plant systems.

2.263 In another example in which the bacterial gene has required modification, the gene encoding PETN reductase was modified to introduce a plant consensus start sequence so that the gene was expressed efficiently within the plant [163]. Similarly, the mammalian P450 2E1 cDNA was placed between a plant promoter and plant terminator sequence in order to effect efficient expression within the recipient plant [150].

2.264 Work with *merA* and *merB* demonstrated that a plant's ability to bioremediate pollutants can be expanded beyond the plant's natural capacity, by the incorporation

of multi-gene pathways from other organisms [174]. Both the *mer* genes were modified in order to facilitate their efficient expression in the transgenic plants. A number of reports have stated that in order for phytoremediation to be a truly successful technology then it must be able to remediate sites that have a number of pollutants present, including those with a mixture of organic and or inorganic pollutants. Such technologies will require the identification, characterisation and cloning of the coding regions of genes for a number of enzymes from plants and other organisms involved in pollutant transformation. These will then have to be introduced into the recipient organism at the same time. The introduction of multiple genes is likely to be important for the development of phytoremediating plants capable of remediating sites that are contaminated with more than one type of pollutant.

2.265 The *merA* and *merB* genes were introduced to a single plant by crossing independently transgenic *merA* and *merB* plants. However, Chen *et al.*, (1998) [218] demonstrated that it was possible to introduce at least 13 different genes into the rice genome using the co-bombardment method, where the genes carried on separate plasmids are mixed prior to transfer by particle bombardment.

2.266 Several other factors related to the integration and structure of transgenic DNA may influence the expression of transgenes within plants, as well as environmental and developmental factors. These include the transgene copy number, the number of rearranged and truncated transgene copies, their position in the genome and level of methylation [149, 219-221]. Desirable new phenotypes created in plants can become unstable following propagation, leading to the loss of the newly acquired trait (a phenomenon is known as gene silencing). The way in which plants recognise and specifically inactivate foreign DNA is unknown, and several different mechanisms are probably involved [220, 222]. This will also affect the potential for the development of effective phytoremediative plants.

2.267 Another example of loss of transgene, not related to gene silencing, was seen in the *Arabidopsis thaliana* plants that were engineered with *merA* and *merB* gene constructs independently. These were crossed to produce independent *merA* and *merB* alleles within the same genetic background. The F1 plants were heterozygous for one or more *merA* and *merB* insertions, having received one haploid chromosome set from each parent. The F1 generation plants were then selfed to produce an F2 population containing both homozygous and heterozygous plants. Some of the plants of the F2 generation were found to lack *merA* and/or *merB,* probably due to the loss of the alleles through segregation (Mendel's first law) [174].

COMBINED STRATEGIES FOR THE BIOREMEDIATION OF POLLUTANTS

2.268 Combined strategies for the bioremediation of pollutants are defined as those processes that require the use of more than one organism to bioremediate the contaminant successfully. Not all of the organisms involved are required to be genetically modified. Combined strategies, particularly those involving plants and microorganisms offer some particular advantages not available to strategies involving single GMOs.

Multi-plant Strategies

2.269 Plants are able to infiltrate the soil to provide soil surface stabilisation. Where GM trees are used in the phytoremediation of a site (e.g transgenic yellow poplars expressing *merA* gene constructs), the treatment process may be more efficient if the trees are grown in combination with smaller herbaceous species [149]. These smaller species will fill the gaps between the larger trees. This will promote further stabilisation of the soil surface and, if the herbaceous layer was also genetically engineered to express the *merA* construct, increase remediation of the site's pollution.

Plant-microbial Strategies

2.270 Plant-microbial strategies have been identified involving either GM plants or GMMs. In the absence of plants, soil is a relatively oligotrophic environment, with the lack of bioavailable nutrients having a significant negative effect on the activity of microorganisms present (with respect to the level of microbial activity in the rhizosphere). The lack of substrates is also likely to have an adverse effect on bioremediation applications, particularly where the degradation of the target compound provides the microorganism with little or no source of energy, for example PCBs. An insufficient supply of nutrients may result in either the population of inoculated GMMs dropping to below an effective level to degrade the pollutant, or the GMMs losing the degradative trait (particularly where the recombinant genes are present on a mobilisable plasmid) [13].

2.271 Plants could participate in the bioremediation of a contaminated site indirectly through their support of symbiotic, root-associated microorganisms that carry out the actual bioremediation of the contaminant [158]. In cases where plants promote the microbial breakdown of the pollutant(s), the process is described as phytostimulation or enhanced rhizosphere degradation [156-158]. The plant's role in this type of process could be through the release of exudates and/or enzymes into the rhizosphere

that stimulate microbial activity and biochemical transformations. Plants are reported to secrete 10 to 20 percent of their photosynthate in root exudates [151, 154, 157].

2.272 In the rhizosphere, the exudation of potential microbial growth substrates from plant roots into the surrounding environment means that microbial activity is significantly higher than in plant-free soil. Recalcitrant compounds such as TCE and the herbicide mecoprop are reported to be less persistent in the rhizosphere [223, 224]. The successful modification of two rhizosphere-competent GM strains of *P. fluorescens* to degrade biphenyl (following insertion of *bph* genes on transposon TnPCB) demonstrates that these microorganisms can be suitable recipients for genetic modification to degrade environmental pollutants [13]. Plants also release oxygen from their roots into the rhizosphere, thereby ensuring good aeration of the soil. Penetration of plant roots through the soil not only improves the aeration, but also water availability [149, 157]. Because the macro-movement of many microorganisms and nutrients through soil is mediated by the flow of water, then root growth is also likely to promote the spread of microbial degraders through a contaminated site. It should also be noted, however, that exudation of organic compounds from plant roots may (at least transiently) retard the degradation of a xenobiotic if the plant products are preferred substrates for microbial growth.

2.273 The success of the GMMs reported by Brazil *et al.,* (1995) [13] was due to the ability of the microorganisms to use the root exudates as a general source of carbon and energy. However, the discovery that some plant exudates may be able to induce the degradation of pollutants such as PCBs means that a more targeted use of rhizosphere-competent microorganisms may be employed. Terpenes are compounds exuded by some plants into the rhizosphere, and are reported to induce co-metabolism of PCBs by *P. putida* LB400, *A. eutrophus* H850 and *Rhodococcus globerulus* P6 to the same degree as when the microorganisms were grown in the presence of biphenyl [26]. Although these three strains were not genetically modified, recombinant versions of these microorganisms have been designed for the enhanced degradation of PCBs. The combination of terpene producing plant species and PCB degrading GMMs at PCB-contaminated sites may avoid the addition of biphenyl supplements to the site to promote the degradation of the PCBs.

2.274 The use of GM plants in plant-microbial strategies is likely to be based on the development of plants capable of producing particular compounds required by microorganisms to degrade or chelate specific pollutants. Although no such GM plants have been produced to date, future applications may include the plants able to secrete phytochelatins to concentrate metal pollutants in the rhizosphere for subsequent bioremediation by GM or non-GM microorganisms. GM plants may also

be designed to reduce the pH of the environment, which release metals from fixed cation-exchange sites and will improve the bioavailability of metal pollutants for microorganisms. It is unknown whether the action of these phytochelatins or pH-reducing compounds would be restricted to the rhizosphere, or may potentially have a more widespread action.

2.275 Improvements in phytoremediation by GM plants e.g through rhizofiltration or phytoextraction, may also be achieved through a better understanding of the role of rhizosphere microorganisms in metal uptake and precipitation by plant roots [207]. Roots may be able to employ rhizospheric organisms (mycorrhizal fungi or root-colonising bacteria) to increase the bioavailability of metals. Mycorrhizal fungal associations with plant roots have been found to affect plant tolerance to heavy metals, metal uptake and translocation and plant growth parameters (e.g root: shoot ratio) in a number of plant species [152, 158]. For example, the volatilisation of selenium by plants is enhanced by the inoculation of the plants with rhizospheric bacteria, as the presence of the bacteria affected the root surface area of the plant and the rate of selenium uptake [225]. It has also been shown that natural microbial populations can be manipulated to improve the metal accumulation abilities of plant roots [168]. The ability of plant-leaf microflora and endophytic organisms are also being investigated to determine their effect on phytoremediation. However, the significance of microorganisms for the phytoremediation of pollutants remains mostly unknown [18, 212].

2.276 Strategies involving both GM plants and GMMs may include the use of GMMs to improve the mobility of xenobiotic compounds, particularly heavy metal contaminants by changing their state. This could increase the uptake and bioremediation of the contaminant by the GM plant. GM plants could be engineered to exude specific molecules required to induce GMM bacteria to degrade pollutants [160]. Rhizosphere-competent microorganisms can of course be added to the existing rhizosphere at a contaminated site by inoculation directly into the soil. However, if the site does not contain sufficient plant cover, then both the plant and GMM must be added. The inoculation of the microorganism onto the outside of the seeds used to sow the site reduces the amount of labour required and ensures a close interaction between the GMM and the plant. Wheat seeds coated with a GM pseudomonad were used as a plant-microbial strategy to remove TCE from contaminated soil [226]. The GM *P. fluorescens* expressed the *tomA*(+) genes (encoding toluene monooxygenase) isolated from *Burkolderia cepacia* PR1(23)(TOM23C). The efficacy of the system was tested by adding TCE to soil microcosms containing wheat plants and the rhizosphere associated GMM. The level of TCE in the soil was reduced by 63 percent in 4 d (20.6 nmol TCE d^{-1} $plant^{-1}$) in the soil containing the GMM, compared to only a

9 percent reduction in soil microcosms containing wheat plants and the non-GM wild type *P. fluorescens* 2-79 [226].

Multi-microbial Strategies

2.277 The application of consortia of microorganisms is a common strategy for the biodegradation of pollutants by non-GM microorganisms. However, as described in the section on 'General strategies for the optimisation of bioremediation', the use of consortia may not be the most efficient method of biodegrading pollutants in the environment, if genetic modification techniques can be applied to enable single taxa of microorganisms to degrade a pollutant. No reports have been identified during the compilation of this review that have used GMMs in consortia with other microorganisms (GM or non-GM) to bioremediate pollutants. Indeed, many of the GMMs reported have been designed to replace consortia [51, 53, 93].

3. ASSESSMENT OF THE RISKS OF THE USE OF GMOs FOR THE BIOREMEDIATION TO THE ENVIRONMENT AND HUMAN HEALTH

3.1 Assessment of the risks posed by the use of GMOs in bioremediation strategies to the environment and human health is a key component of the regulatory process that must be completed before any release of a GMO can take place (in either the field or contained facilities).

3.2 For the purposes of this report, the risk assessment has been divided into two parts to address the risks posed by GMMs and GM plants separately. This has been done due to the inherent differences between the two groups of organisms, the bioremediation applications in which they are employed and consequently the risks posed to the environment and/or human health.

3.3 For each group of organisms, the risk assessment has been conducted in two stages; the identification of the characteristics or properties of the GMO which may cause an adverse effect(s) to the environment and/or human health (the hazard identification stage), and the assessment of the likelihood of each of the adverse effects identified being realised (the risk assessment stage). A hazard or adverse effect of a GMO is defined as an intrinsic property or characteristic of the GMO that could result in harm to the environment and/or human health.

3.4 Although the hazards posed by a particular GMO are likely to vary depending on the characteristics of the GMO and the environment in which it is intended to be used, there are a number of key hazards common to GMOs involved in bioremediation applications:

- transfer of the inserted genetic material from the GMO to other organisms in the environment;
- accumulation of above ambient concentrations of toxic compounds such as heavy metals by the GMO;

- production of toxic metabolites or by-products by the GMO during the degradation of the target pollutant; and

- disruption of indigenous organisms by the GMO, and the potential knock-on effects on food-chains and biological processes such as decomposition and nitrification. It should be noted that the diversity of organisms present in a polluted environment is likely to be a consequence of the contamination present, and is unlikely to be representative of an equivalent non-contaminated environment. This may need to be addressed in assessing the level of disruption caused by the introduction of the GMO.

3.5 The risk posed by the GMO to the environment and/or human health is determined by the likelihood of the particular hazard being realised, and the magnitude of the effects if they occur. Therefore the level of risk will vary between GMOs depending on whether they possess a specific hazardous property and are able to express it in the environment.

3.6 It should be noted that not all of the hazards described above apply to all GMOs used in bioremediation applications. For example, GMMs modified by insertion of the *lux* gene will not accumulate toxic compounds or produce toxic metabolites, whereas all of the hazards described may be realised with GMMs designed to degrade organic pollutants and bioaccumulate heavy metals. Where the GMO is used in such a way so that it is not released to the environment, then it is likely that none of the hazards identified will be realised. Examples of such applications include *lux*-based biosensors where samples of the contaminated site (soil or water) are added to a suspension of the biosensor *ex situ* and the level of bioluminescence quantified.

3.7 The level of risk posed by a particular GMO (defined by the likelihood of the hazard being realised) is expected to vary between different GMOs, and also potentially for the same GMOs used in different environments or in different applications.

3.8 Although a number of hazards may potentially be associated with the use of a GMO for the bioremediation of a pollutant(s), it should be noted that in many cases, the use of a GMO may provide a number of advantages (or fewer potential hazards) compared to the other options available, namely:

- the use of physical and/or chemical remediation strategies;

- the use of non-GM based bioremediation strategies; or

- leaving the contaminant in place.

3.9 As discussed previously in this report, physical and/or chemical remediation strategies may pose their own inherent hazards to the environment, through for example, the increased mobilisation of the contaminant(s) or the use of compounds that are themselves environmental pollutants. A potential limitation with non-GM based strategies is that the organism involved may not be capable of remediating the more recalcitrant or toxic pollutants. In situations where the pollutant is relatively immobile in the contaminated site, or is non-toxic, then it may be less hazardous to leave the contaminant untreated. However, changes in land-use or climate, for example, may alter the mobility and level of likely exposure of the compound.

3.10 In addition to the potential direct applications for plants in bioremediation discussed in the previous chapter, plants also offer a secondary advantage in reducing erosion at the contaminated site which may otherwise result in the increased exposure of the surrounding environment to the contaminant(s). The growth of plants with no direct ability to degrade or sequester pollutants at a contaminated site may be regarded as some form of phytoremediation strategy [212].

3.11 The other issues that will be considered in selecting the appropriate remediation strategy include cost and public acceptability. Whilst these subjects are outside the scope of this technical report, it has been reported that bioremediation-based process may offer significant financial savings compared to physical/chemical-based techniques for particular sites [151, 152, 154, 156, 159, 160]. The choice of whether or not to leave the pollutant on-site is likely to depend on the intended future use of the area, but is unlikely to be publicly acceptable if the site is intended for domestic or recreational usage, irrespective of the potential for pollutant exposure[16].

THE USE OF MICROORGANISMS

3.12 The use of GMMs in the bioremediation of pollutants is only likely to pose a risk to the environment and/or human health if the microorganism can survive in the environment into which it is released. The only exceptions to this statement are:

- where genetic material is released into the environment prior to or during cell death and remains in the environment in a state where it can be transformed into other microorganisms; and

[16] The issue of public perception of contaminated sites, and the measures available to remediate them was dicussed during the workshop. The outputs from this discussion are presented in the report of the workshop at the end of this document (General discussion section).

- where the GMM is designed to accumulate toxic inorganic pollutants such as heavy metals. The use of such organisms usually results in the localisation of comparatively high concentrations of the toxic compound in an environment where the contamination was originally more dispersed. Because such compounds are not biodegraded, then these localised accumulations may remain even after the death of the microorganism.

3.13 Survival of GM microorganisms in the contaminated environment is important if they are to perform their bioremediative function to the desired level [57, 227]. Early reports on the use of GMMs in the environment suggested that the microorganisms were unlikely to survive, grow and compete with the indigenous microflora, due to the greater energy demands imposed by the foreign genes and the consequent reduction in the fitness of the GMM [8, 228-230]. However, the findings from various releases of GMMs in the environment have not supported this proposal, and have found that GMMs are able to survive for long periods of time (up to 6 years) [231] in the environment in the presence of natural microbial populations [90, 232-234].

3.14 Ripp *et al.,* (2000) [90] found that the GM *P. fluorescens* HK44 introduced into PAH contaminated soil persisted for up to 660 days in the soil under field conditions. Assessment of the risks posed by the GMM should assume that the microorganisms are capable of surviving in the environment, although determining the level and duration of the survival of GMMs in the environment is not easy to predict [23].

3.15 Roberts (1989) [95] proposed that many of the earlier studies that were conducted to assess the survival of GMMs in the environment were poorly designed and many ecological aspects of the release were not taken into account in the design of the GMM. Consequently, many of the GMMs tested were inherently less fit than the indigenous microflora and therefore unlikely to persist in the environment. Much of the recent work in the development of GMMs for use in the field have focused on the modification of microorganisms capable of surviving in the target environment, as well as being able to degrade the target contaminant [94, 101].

3.16 Crozat *et al.,* (1987) [235] reported that the inoculation of a bacterial population (GM or non-GM) into soil is often followed by a decline in viable numbers until the population reaches a 'survival' population. This reduction occurs irrespective of the initial inoculum size and therefore needs to be taken into account when calculating the size of the population of GMMs required to bioremediate the pollutant. The rate of reduction is reported to vary between different taxa of microorganisms, depending on their ability to survive in the environment [232]. Enteric microorganisms such as GM

E. coli are likely to exhibit a rapid reduction in cell numbers down to an undetectable level within three days of inoculation into the contaminated environment. Microorganisms such as GM *Bacillus subtilis* exhibit an exponential decrease over a 7-10 day period down to a population density of 1-100 cells g^{-1} of soil, and GMMs such as *Rhizobium* sp exhibit a rapid exponential decrease within 1-14 days of inoculation [232].

3.17 The initial decrease in microbial numbers leads to a reduction in the population of the inoculated microorganisms, by between one and four orders of magnitude, and in some cases may be followed by a complete and progressive decrease of culturable cells. Studies involving GM pseudomonads were reported to be more variable, with the inoculated population decreasing by 0.2 to 1 orders of magnitude in 10 days [232]. However, no single model has been applied successfully to determine the survival of inoculated microorganisms (including GM strains) in a particular environment. This is due to the successful colonisation of an environment by a GMM being dependent on a wide variety of biotic (competition and predation) and abiotic (temperature, pH, moisture and adsorption) factors [23]. Those which can or may be controlled such as strain selection, the genetic modification, soil aeration and moisture content should be addressed as part of the initial design of the bioremediation strategy [90].

3.18 Blumenroth and Wagner-Döbler (1997) [96] found that the use of microorganisms indigenous to the target environment for bioremediation and as hosts for genetic modification did not provide any inherent advantage over microorganisms isolated from other environments, and that the ability of the microorganism to compete successfully for available nutrients was the principal factor in determining survivability. Blumenroth and Wagner-Döbler (1997) [96] concluded that the use of well characterised strains (taxonomically, physiologically and pathologically) as hosts for genetic modification would present a lower level of risk to human health and the environment, compared to the isolation and genetic modification of microorganisms indigenous to the contaminated site. Any microorganisms selected for genetic modification would however have to be able to compete successfully for available nutrients. However, where the genetic modification enables the GMM to utilise a nutrient source that is not available to the indigenous microflora, for example the target pollutant or in the case of FAVs, the surfactant [88], then the ability to compete for available nutrients may not be as significant a factor in the survival of the GMM in the environment.

Transfer of Genetic Material

3.19 Transfer of recombinant genetic material from the GMM to other organisms in the environment, is not desirable. This is due to the knock-on effects the transfer might have on other organisms and biological processes. The level of hazard posed by the transfer of genetic material is dependent on the properties conferred by the modification on the GMM, and on any recipient organisms in the environment. The properties that may have the greatest potential to cause adverse effects to other organisms or biological processes are those that increase the virulence, pathogenicity and survival of the GMM so that it is able to out compete other organisms for nutrients and/or habitat. The transfer of reporter genes such as *lux* are unlikely to be hazardous to the other organisms or biological processes, as they do not encode for any selective advantage in the environment.

3.20 Therefore, the important factors in the assessment of the risk posed by the transfer of genetic material are the potential for the genetic material to be transferred, and the likely effect that the transfer might have on other organisms and biological processes.

Potential for genetic material to be transferred

3.21 Three basic mechanisms exist whereby genetic material can be transferred from the GMM to other organisms [232, 236]. Whilst the potential for transformation, transduction and conjugation between GM and non-GM microorganisms, and between GM microorganisms and non-GM plants has been addressed extensively in other publications [232, 233], some information that is relevant to the use of GMMs in bioremediation is included in this review. With the exception of the Ti plasmid of *Agrobacterium tumefaciens* that can enable the transfer of genetic material from microorganisms to plants, the transfer of genetic material from GMMs is restricted to other microorganisms in the environment.

3.22 Both transformation and transduction of genetic material between microorganisms relies on good homology between the 'foreign' DNA from the GMM and the DNA of the potential recipient [232]. The transfer of DNA by transduction is mediated by bacteriophages with a narrow host range, and is therefore restricted to microorganisms of the same species or between closely related species [232]. This has potential implications to the use of GMMs in bioremediation, where in order to improve the survival of the GMM in the target environment, a microorganism that is indigenous to that environment is used as the recipient for the genetic modification. In such cases the potential for transduction should be addressed, although the risk is likely to be low

due to the narrow host specificity range of bacteriophages capable of transduction [237].

3.23 The potential for transformation to result in the transfer of 'foreign' DNA from a GMM into the indigenous microbial community is restricted both by the requirement for good DNA homology and the restriction-modification systems operated by individual cells. These are reported to reduce transformation efficiency in laboratory systems by a factor of 10^4 [232].

3.24 Conjugation represents the most ecologically significant mechanism to transfer plasmid encoded genetic material between microorganisms, and in the case of the Ti plasmid from *Agrobacterium tumefaciens*, between microorganisms and plants [232]. The transmission of recombinant genes from the GMM is therefore increased if the gene(s) is encoded in the GMM on a transmissible plasmid vector [10, 37]. However, the transfer frequency of large degradative plasmids such as pJP4 (confers resistance to mercury and the degradation of 2,4-D) between microorganisms is lower in sterile soil than in liquid and solid growth media, and is further reduced by the biotic stresses encountered in non-sterile soil [238].

3.25 The transfer of conjugative or mobilisable plasmids between microorganisms is most likely to occur in environments where the plasmid encodes some ability to improve survival in that environment. Horizontal transfer of genes of selective value in a polluted environment has been found to occur up to six years after the introduction of GMMs to a phenol contaminated site [239]. Because many of the genes responsible for the degradation of organic pollutants and the resistance to inorganic pollutants are plasmid encoded [22], then the selective pressure for transfer of such plasmids is high in contaminated environments [232, 240]. The frequency of plasmid transfer is however lower if narrow-host range plasmids are used instead of broad-host range plasmids. Because narrow-host range plasmids can replicate in a restricted number of microbial taxa then such plasmids are more biologically contained than those with a broad-host range [30]. Narrow-host range plasmids also confer improved structural and segregational stability in the host strain [241].

3.26 Conjugative plasmid transfer is reduced in environments where the microorganisms are in a nutrient limited state. In this state, which is characterised by virtual absence of protein synthesis, the replication of plasmids and expression of plasmid genes are reported not to occur [232]. Therefore the addition of various substrates to contaminated sites to support the growth of the GMM or to induce expression of the modified genes, may also reduce any nutrient limitations in the environment and consequently increase the potential for plasmid transfer [232].

3.27 The transfer of chromosomal genes between microorganisms is reported to be very rare and is only likely to occur between organisms of the same specie or between closely related species with a high degree of DNA homology [232].

Effect of transfer of genetic material to other organisms or biological processes

3.28 The transfer of genetic material from the GMM is therefore only likely to occur through conjugation, and only then if the recombinant genes in the GMM are located on a mobilisable plasmid. Assuming that transfer of genetic material does occur between the GMM and the indigenous microflora then the potential for any adverse effects occurring (and therefore the level of risk) depends on the properties or characteristics conferred by the transferred genes.

3.29 The most significant effects are likely to be incurred through the transfer of genes that alter the virulence, pathogenicity or survivability of the recipient organism. Because virulence or pathogenic properties are unlikely to provide any useful purpose in bioremediation applications then they are not expected to be present in GMMs used for bioremediation and are therefore unlikely to be transferred. Although the ability to grow at 37 ºC is not assessed to be a pathogenic trait, it would enable the microorganism to grow in the human body. It may therefore be preferable to avoid the use of microorganisms that possess such traits, for bioremediation. .

3.30 However, as described previously in this report, the GMMs used for bioremediation are usually designed to be able to survive in the environment. The most significant 'survival' traits are those that would give the recipient microorganism such a significant advantage against other microorganisms in the environment that it would be more competitive than other species for nutrients and/or habitats, and consequently alter the microbial diversity of the resident microflora. This may subsequently affect certain biological processes. The addition of any new population of microorganisms to an environment containing an existing microflora may be expected to alter the composition of that environment's microflora to some degree.

3.31 In assessing the effect the inoculation of GMMs may have on the existing microflora, the state and biodiversity of microorganisms in contaminated sites should first be addressed. In environments, not exposed to pollutants, the composition of the microbial community is relatively stable and the microorganisms present are specialists in terms of their nutritional and physiological requirements [242]. Because of the specialised nature of the microorganisms present, then the addition of a new population of microorganisms is likely to have an effect on the composition of the microflora.

3.32 In polluted environments microbial communities are stressed due to the presence of the pollutant, and changes in physiological factors such as pH and the types of energy sources available [243, 244]. These changes select for opportunistic microorganisms with more general nutritional and physiological requirements [242, 244]. Therefore, the microbial community in contaminated sites is less specialised and more adaptable to change. The addition of a new population of microorganisms is therefore likely to have less of an effect on microbial biodiversity than in a non-contaminated environment.

3.33 The level of risk associated with the transfer of genetic material from GMMs to members of the indigenous microbial community is therefore assessed to be low.

Accumulation of Toxic Compounds

3.34 The use of GMMs designed to accumulate toxic compounds can result in the localisation of contaminants at significantly higher concentrations than those likely to have been present throughout the untreated contaminated site. Therefore, whilst the original level of contamination may have been too low to cause significant effects to human health and the environment (particularly adverse acute effects), the localisation of the contaminant(s) through the use of pollutant-accumulating GMMs may result in the concentration of toxic pollutants at levels sufficient to have a significant adverse effect to biota.

3.35 It should be noted that the accumulation of toxic compounds by GMMs may be the only mechanism to remove the pollutant from the contaminated environment, and is usually only a transient stage in the overall bioremediation strategy. After accumulation of the contaminant, the GMM-pollutant 'complex' has to be removed from the contaminated site.

3.36 The level of risk associated with the accumulation of toxic compounds depends on the ability of the GMM to accumulate toxic compounds, the state in which those compounds are accumulated and the environment in which the GMM is applied.

3.37 As discussed in Chapter 1 in this report, there has been limited application of GMMs for the accumulation of toxic compounds such as heavy metals. GM-based bioremediation strategies designed to accumulate toxic pollutants have utilised GM plants rather than microorganisms. The most likely reason for this is that metal-accumulating plants are more suitable for use in *in-situ* bioremediation strategies. Although metal-accumulating GMMs can be (and are) applied directly to the contaminated environment, their small size means that further procedures are required

to remove the microorganism-pollutant 'complex' from the site. GM plants, particularly those designed to accumulate the toxic compound in above-ground plant material allow significantly easier removal of the toxic compound from the environment after accumulation.

3.38 The use of GMMs for accumulation of toxic compounds has therefore been confined largely to bioreactors or other types of physically contained systems. These are used to remove heavy metals from wastewater or groundwater (pump and treat technology) [120], although the majority of the GMMs reported to date are still only at the laboratory design stage [105-107]. Because GMMs used in these systems are physically contained from the wider environment then there is likely to be only negligible exposure of the biota to localised increases in concentrations of toxic pollutants.

3.39 Metal-accumulating GMMs are also used in bioprotection applications, as well as in direct bioremediation processes. In the bioprotection strategy the GMM is released into the contaminated environment specifically to accumulate or degrade a particular contaminant, so that it does not affect the activity of other organisms present. Such microorganisms may have applications in protecting agricultural crops from the inhibitory effects of any particular contaminant present in the environment [112], or the protection of the microflora in wastewater treatment plants [124]. Because the GMMs are released directly into the environment in bioprotection applications, then there is a risk of localised higher concentrations of toxic compounds affecting other organisms present. However, it should be noted that any pollutants that are accumulated would be present in the environment anyway, and it is only the concentration of pollutant that varies.

3.40 The risk posed to other organisms depends on the bioavailable concentration of the toxic compound that is accumulated by the GMM, and how this compares with the threshold tolerance concentration of the surrounding biota to that compound.

Production of Toxic Metabolites or By-products

3.41 The use of bioremediation strategies to remove pollutants from the contaminated environment may result in the formation of toxic metabolites or by-products during the degradation or biotransformation of the pollutant. The level of risk incurred is dependent on the toxicity and concentration of any metabolites formed, and also the length of time that any toxic metabolites are likely to persist in the environment.

3.42 From the information obtained from the review of the scientific literature, no GMMs have been identified whose use in the bioremediation of pollutants is likely to result in the production of toxic metabolites or by-products.

3.43 The only exception to this is the use of GMMs as FAVs for the biodegradation of hydrophobic pollutants such as PCBs. Although toxic metabolites or by-products are not produced from the degradation of the target pollutant, the incomplete degradation of the surfactants used to support the growth of the FAV and improve the bioavailability of the pollutant may result in the formation of compounds that may have a toxic effect to other organisms in the environment [82]. For example, the degradation of alkylphenol polyethoxylates results in the formation of persistent short-chain mono-, di- and triethoxylates. These degradation products are more toxic than the parent compound, particularly to aquatic fauna and are potentially oestrogenic [245].

3.44 The level of risk, posed by degradation of the surfactant used with the FAV, depends on the type of compound used as the surfactant. The possible adverse effects caused by the accumulation of surfactant degradation products may be avoided by maximising the degradation of surfactants in the environment in which they are used. Alkyl ethoxylate surfactants are more readily biodegradable in the environment than alkylphenol ethoxylates and their degradation products are less likely to accumulate [82]. The *P. putida* IPL5::TnPCB used in the FAV designed to degrade PCBs in the environment [88] was able to degrade both alkylphenol ethoxylate and alkyl ethoxylate surfactants as growth substrates. However, the GMM was only able to degrade the ethoxylate moiety of these compounds, leaving potentially toxic metabolites [88]. The *Ralstonia eutrophus* B30P4::TnPCB FAV was able to degrade both of the surfactants without the production of potentially toxic compounds. Although this GMM grew more slowly than *P. putida* IPL5::TnPCB [82] it may be more suitable to the bioremediation of PCBs in the environment, particularly those where aquatic organisms may be at risk of exposure to any surfactant degradation products.

3.45 If suitable (i.e highly biodegradable) surfactants are used with the FAVs, then the risk posed to the biota is assessed to be negligible. No other GMMs have been identified whose use in bioremediation applications is likely to result in the formation of toxic metabolites or by-products.

Disruption of Other Organisms and Biological Processes

3.46 A number of studies have addressed the effect of the inoculation of a GMM in a terrestrial environment on the numbers and activities of the indigenous microflora [246-248]. In all three studies, soil microcosms (with or without 500 μg g^{-1} 2,4-D) were inoculated with *P. putida* PPO301(pRO103) which had been genetically modified by the insertion of plasmid pRO103 which was expressed constitutively. The plasmid encoded resistance to both the antibiotic tetracycline (25 μg ml^{-1}) and mercury (25 μg ml^{-1}) and the ability to mineralise phenoxyacetate and partially degrade 2,4-D to chloromaleylate. Control microcosms (with or without 500 μg g^{-1} 2,4-D) were inoculated with the non-GM parental organism *P. putida* PPO301.

3.47 Both Short *et al.,* (1991) [246]and Doyle *et al.,* (1991) [247] found that in a nutrient poor, semi-arid soil the degradation of 2,4-D by the GMM caused a significant reduction in numbers of fungal propagules. The formation of the metabolite 2,4-dichlorophenol during the degradation of 2,4-D was found to be inhibitory to the fungal community. However, when the same GMM was inoculated into more nutrient rich, less arid soil no degradation of 2,4-D by the GMM was observed, and there was no effect on the fungal community [248].

3.48 Ingham *et al.,* (1995) [248] also reported that there was no significant difference in numbers of culturable bacteria, bacterial biomass and numbers of nitrifying and denitrifying bacteria present in the microcosms inoculated with the GMM, compared to the non-GM strain. Although the GM *P. putida* PPO301(pRO103) retained the inserted plasmid for the duration of the 90 day trial, no degradation of 2,4-D was detected. The continuing presence of 2,4-D in the soil had a significant effect on the size of the microbial population (bacteria, fungi, amoeba and flagellates) compared to the controls.

3.49 The inoculation of GMMs into contaminated sites may also have a positive effect on the size and/or biodiversity of the indigenous microbial community. In contaminated environments where the natural microflora is being inhibited by the presence of a particular pollutant, or is restricted by a lack of nutrients, then the addition of a GMM designed to degrade the inhibitory compound may allow the natural population to grow. The degradation of previously non-utilisable pollutants into compounds that can be used by the indigenous microflora may also alleviate the nutrient limited status of the cells.

3.50 The inoculation of *P. cepacia*, genetically modified to degrade the compound 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), into soil resulted in the increase in the

taxonomic and genetic diversity of the indigenous microbial community due to increased nutrient availability [249]. However, if the degradation of a pollutant results in the formation of toxic or inhibitory compounds, then an adverse effect on the indigenous microbial community is likely to be realised [232]. This scenario can of course be achieved following the inoculation of a GMM or non-GMM into an environment as part of a bioremediation strategy.

THE USE OF PLANTS

3.51 The use of plants for the bioremediation of pollutants offers a number of advantages and disadvantages to the environment and human health compared to the other options available. Although the same basic hazards need to be addressed in assessing the use of plants and microorganisms for the bioremediation of pollutants, the different physiological and morphological characteristics of plants compared to microorganisms, and the alternative approaches applied to their use in bioremediation means that a different emphasis on the assessment of the likely hazards may be required.

Transfer of Genetic Material

3.52 As with GMMs, the transfer of recombinant genetic material from the GM plant to other organisms in the environment is not desirable due to the knock-on effects the transfer might have on other plants and biological processes [208, 250]. With plants, the properties most likely to have an adverse affect on the surrounding flora are those that confer a selective advantage to survive, such as drought resistance, tolerance to frost and altered pH, improved weedy characteristics (e.g rapid growth) and the survival in the presence of particular toxic compounds.

3.53 Transfer of genetic material between plants is restricted largely to cross-pollination. The potential for transfer to occur is therefore greater in those plants that are able to undergo sexual hybridisation with resident flora and produce large quantities of relatively mobile pollen, for example brassicas. The assessment of the risks associated by the transfer of genetic material through cross-pollination (by wind or insects) has been addressed by other reports, and is not restricted to those plants used in phytoremediation. This subject will therefore not be addressed further in this report. Particular reference is made towards the report on the 'Guidance on best practice in the design of genetically modified crops' produced by the Best Practice sub-group of the UK's Advisory Committee on Releases to the Environment (ACRE) [251].

3.54 All of the genetic modifications identified to date that may be applied in phytoremediation have been directed towards the sequestration, degradation or resistance to particular compounds in the environment. The modified properties are only of benefit to a plant within the contaminated site and are not expected to confer any more general traits that may give the modified plant a selective advantage outside the contaminated site [156], for example frost tolerance or drought resistance. Therefore, expression of the modified trait is likely to be effectively restricted to the contaminated environment. Outside the contaminated site in the absence of the target pollutant, the modified properties transferred from the GM plant are likely to confer a negative selection pressure and may therefore not be maintained in the population [208].

3.55 Rugh *et al.,* (1996) [171] reported that several of the GM plant lines expressing the modified *merA* gene (conferring mercury resistance) actually grew better on a mercury-containing medium than on the control medium without mercury. The findings suggested that the mercury-resistant transgenic plants would not compete well in areas that were un-contaminated with mercury, and that this would effectively promote the self-containment of the transgenic plants within the mercury polluted sites. Indeed, this is equivalent to the scientific view that natural metal tolerance in plants is associated with a metabolic 'cost' that makes these plants less competitive (fit) than non-tolerant genotypes when growing on uncontaminated soils [175].

Accumulation of Toxic Compounds

3.56 Any risks associated with the accumulation of toxic compounds by GM plants in phytoremediation are restricted to those plants that are able to hyperaccumulate pollutants. The plants capable of removing compounds such as mercury and selenium, from contaminated sites by phytovolatilisation are not addressed in this section, as the compounds are only accumulated transiently within the plant before being released through the leaves.

3.57 The ability of some plants (GM and non-GM) to sequester toxic compounds from their environment and accumulate them within their tissues, is a key application of the use of plants for bioremediation, especially in the removal of heavy metals from contaminated sites. However, because the target pollutants are accumulated at concentrations that may be significantly higher than in the surrounding environment, the plants may pose a hazard to the environment and/or human health if they are eaten, not harvested or not disposed of correctly after harvest. For example, the compound(s) accumulated by the plant may be lost during plant senescence and returned to the environment. Therefore the use of plants in phytoextraction and

rhizofiltration applications needs to ensure that the sections of the plants containing the accumulated compounds (usually the above ground structures) [175] are removed from the site before any plant senescence occurs. At the same time, there is good evidence that metal hyperaccumulation acts as an effective feeding deterrent to help protect the plant from herbivores [252], so this would help to minimise the risk of metal transfer from such plants into food chain.

Production of Toxic Metabolites

3.58 As mentioned in the previous chapter, phytoremediation strategies may result in the production of toxic metabolites, or lead to the greater exposure of the target parent contaminant. These hazards are however not restricted to phytoremediation using GM plants, although the improved properties of the GM plant compared to the wild type strain may result in a potentially larger hazard to the environment and/or human health.

3.59 *In situ* approaches to the phytoremediation of metal pollutants may involve the conversion of the metal to a different more mobile and more bioavailable state. Although this conversion is required in order to remove the metal from the environment, the more mobile metal forms are likely to have a potentially greater toxicity to other organisms [168, 171].

3.60 Potential applications for the phytoremediation of pollutants have been proposed in which the GM plant is designed to secrete metal-selective ligands into the rhizosphere to solubilise the target compound [19, 155]. Some non-GM plants are known to release substances that are able to reduce certain species of metal, to facilitate their uptake by the plant, and also to produce compounds capable of chelating metals and promoting their mobility in the environment. The addition of synthetic chelating agents to soil under laboratory conditions has been reported to result in the enhanced accumulation of metals such as lead in the shoots of Indian mustard [159, 253, 254].

3.61 The conversion of metal pollutants into a more mobile and potentially more toxic state is however only likely to result in a transient exposure of the more toxic metal to the environment. Once the plant has sequestered the metal (either within the plant or immobilised in the rhizosphere), further environmental exposure is unlikely to occur.

3.62 The production of toxic compounds is more likely to occur during the phytodegradation of organic pollutants, especially where the pollutant is degraded outside the plant. For example, the expression of a gene encoding the mammalian cytochrome P450 2E1 in tobacco plants resulted in the increased metabolism of TCE,

compared to the wild type variety [150]. Although this modification was not reported to result in the production of any toxic metabolites during the breakdown of TCE, a thorough study of these transgenic plants would need to be undertaken to verify that no toxic intermediates were released into the environment, as a result of the phytoremediation process conducted under field conditions [150].

3.63 The assessment of many of the potential risks that may be associated with the use of plants for phytoremediation has been restricted to date by the lack of research being conducted under field conditions involving GM plants designed for phytoremediation applications. Although no releases of GM plants for phytoremediation have occurred to date, one of the more advanced areas of research is the use of GM plants for the phytovolatilisation of pollutants such as mercury and selenium. Plants used for phytovolatilisation are designed to sequester a volatile metal such as mercury and emit it from their leaves into the atmosphere in a less toxic form. Although the mercury released from the plant is significantly less toxic than the form present at the contaminated site, such plants may be described as releasing toxic compounds into the environment.

3.64 However, in order to assess the potential implications of the phytovolatilisation of mercury from contaminated sites, the relative scale of such releases compared to atmospheric emissions of mercury from other sources must be addressed.

3.65 The residence time of Hg (0) in the atmosphere is approximately two years before it is re-deposited onto the earth's surface, usually through precipitation. Therefore, mercury released through phytovolatilisation is likely to be diluted to trace concentrations in the atmosphere before being re-deposited [174]. Any quantity of mercury released from a contaminated site is likely to be small in comparison with the atmospheric mercury load (~4 x 10^6 kg) [174] and would be negligible compared to other sources of mercury emission, e.g burning of fossil fuels and medical waste [156]. Even if the levels of volatile mercury produced during phytovolatilisation were 400-fold higher than normal background levels, they would still be 25 times below most regulatory limits. Bizily *et al.,* (1999) [209] proposed that the rate of mercury volatilisation from the GM plants could be designed to ensure that the quantities of mercury released from the plant were within government regulations.

3.66 Although the amount of mercury released from GM plants is likely to be relatively low compared to other emissions of mercury to the atmosphere, it has been proposed that the direction of prevailing winds, the location of nearby population centres, and the magnitude of total site Hg(0) emission would need to be considered prior to determining the level of risk posed to the environment and/or human health [208].

3.67 Similarly, for GM plants capable of phytovolatilising selenium, it is thought that the amount of selenium added to the atmosphere through phytovolatilisation would be negligible when compared to the atmospheric inputs of this compound from volcanoes, soil and non-GM plants [166].

Disruption of Other Organisms and Biological Processes

3.68 Disruption of other organisms and biological processes by GM plants used for phytoremediation is most likely to be a consequence of the plants having a selective ecological advantage and consequently out-competing other flora in a particular environment, or by having a toxic effect on other organisms, particularly those likely to ingest parts of the plant.

3.69 As discussed in the section assessing the risks posed by the transfer of genetic material from GM plants, the transgenic species used in phytoremediation are unlikely to have any ecological selective advantage outside the contaminated site, and are therefore not expected to disrupt other flora or biological processes outside the contaminated environment. It should be noted that within the polluted site, the types of flora present are likely to be determined by their tolerance to the contaminants present, and may therefore be less affected by any selective advantage expressed by the GM plants.

3.70 The accumulation of toxic compounds within the tissues of GM plants designed for phytoextraction or rhizofiltration, has the potential to cause harm to organisms that consume parts of the plant. The uptake of heavy metals such as lead by crop plants, for example, is reported to be a major source for the accumulation of such toxic ions within the human body [177]. However, this can be avoided by preventing consumption of the GM plants used [212].

3.71 Consumption of GM plants designed to phytoremediate metal contaminants is likely to be low because of the presence of the metal within the plant. One of the main functions of metal hyperaccumulation in non-GM plants is to prevent disease and herbivory [154]. Therefore, the genetic modification of a plant to hyperaccumulate metals is also likely to result in reduced herbivory. It has also been reported that some species of insects that would normally be expected to eat certain species of plant, avoid the plants when they contain metal contaminants in their tissues (Ensley (*pers comm*) in) [166]. However, there are concerns that insects, particularly pest species, could become adapted to feeding on hyperaccumulating plants containing

high levels of metal which may lead to foodchain contamination [166]. To date no research has been reported investigating these possibilities in GM plants.

3.72 Where genetic modification is used to improve an existing phytoremediation capability of a particular plant, then the disruption of other organisms or biological processes may be less likely. For example, the GM poplars designed to degrade TCE are proposed to utilise this compound using the same pathway as employed by the non-GM poplars for the degradation of TCE [150]. Consumption of leaves from non-GM poplars exposed to TCE had no harmful effects on herbivorous insects, suggesting that the degradation of TCE by the non-GM trees resulted in the production of non-harmful metabolites. If the GM trees use the same pathway for the biodegradation of TCE, then no toxic metabolites would be expected to be produced by the GM trees [150]. However, the greater rate of phytodegradation of TCE by the GM poplars means that higher concentrations of metabolites are likely to be present. Such metabolites may be toxic at higher concentrations, and further research is required to address this issue.

3.73 As well as affecting external systems, such as interactions between the plant and its environment, the introduction of novel genes into a plant can also disrupt systems within the plant itself. The disruption may be significant enough to prevent the GM plant from germinating, or may only cause the plant to be more susceptible to environmental effects. In GM tobacco plants designed to overexpress γ-ECS, it was found that foliar levels of γ-ECS and GSH were increased, but that the increased GSH appeared to result in greatly enhanced oxidative stress. The γ-ECS transformed plants were reported to suffer from oxidative stress due to a failure in the redox-sensing process in the chloroplast [255]. However similar findings were not reported when the gene for γ-ECS was over-expressed in Indian mustard [17].

3.74 In addition to their direct application for removing metal pollutants from contaminated sites, GM hyperaccumulating plants have been proposed as having secondary benefits to other organisms. For example, plants could be modified to extract desired, beneficial micronutrients such as selenium from the environment, at specified levels. Such plants could then be fed to livestock as pellets to provide the recommended daily requirement of the micronutrient [256]. Canola plants, grown for the phytoremediation of selenium, were found to be safe to feed to marginally selenium-deficient lambs and cows, in order to meet their normal selenium intake requirements [257].

4. POTENTIAL MANAGEMENT STRATEGIES FOR THE USE OF GMOs IN BIOREMEDIATION

4.1 The purpose of this section of the report is to identify and review the possible management strategies that could be employed to reduce any of the risks identified in the previous chapter.

MICROORGANISMS

4.2 The purposes of the management strategies designed for the use of GMMs in bioremediation are to ensure that any of the risks, identified in Chapter 3, are minimised and that the GMM functions as intended. Of the risks identified, the most significant are those resulting from the transfer of genetic material from the GMM to other microorganisms, and the disruption of other organisms and biological processes. The use of GMMs in bioremediation is not assessed to result in a significant level of risk, in terms of the accumulation of toxic compounds and/or the production of toxic metabolites.

4.3 The level of risk associated with the transfer of genetic material and the disruption of organisms and other biological processes can be minimised through addressing the biological and/or physical containment of the GMM, and ensuring that only recombinant DNA that is required for the bioremediation application is present in the GMM. Management strategies can be developed to prevent or minimise transfer of genetic material from the GMM, and also to contain the populations of GMMs to a particular location, determined by specific environmental or physiological parameters.

4.4 The presence of extraneous recombinant genetic material in the GMM is not desirable if the GMM is intended for use in the environment. In addition to potentially reducing the activity and competitiveness of the GMM in the environment, there is a greater chance of recombinant DNA being transferred to other microorganisms. Selectable marker genes are often inserted into the GMM, with the other transgenes, to assist in the detection and selection of the microorganisms that have been genetically modified. If possible it may be preferable to use a selectable marker gene

that is appropriate to introduced modification, such as resistance to organic or inorganic pollutants.

4.5 Virtually all available plasmids carry gene encoding resistance to antibiotics as selectable markers [37]. However, such markers are undesirable for environmental applications [4], and can be replaced with other selectable markers (Table 4.1), or eliminated from the microorganism after gene transfer [258].

Table 4.1 - Alternative selectable genetic markers [37]

Selectable marker gene	Origin	Selectable phenotype
bar	*Streptomyces hygroscopicus*	Resistance to the herbicide bialophos
aroA CT7	*Salmonella typhimurium*	Resistance to the herbicide glyphosate
merTPAB	*Serratia marcescens*	Resistance to mercuric salts/organomercurials
arsAB	*Escherichia coli* R773	Resistance to arsenite
luc	firefly	Bioluminescence
lacZY	*Escherichia coli*	Growth on lactose
teh	RP4 plasmid	Resistance to potassium tellurite

Transfer of Genetic Material

4.6 The risks incurred by the transfer of genetic material from the GMM to other organisms are assessed as most likely to occur if the recombinant genes are located on a mobilisable plasmid. The transfer of plasmids between microorganisms, by conjugation, can be reduced by using non-mobilisable or mobilisation defective plasmids, or narrow-host range plasmids. However, transfer of the genetic material can still occur and the use of such plasmids does not ensure containment of the recombinant genetic material within the GMM.

4.7 To avoid the problems of transfer of plasmids between the GMM and resident microflora, minitransposon vectors can be used to modify the host microorganism genetically. Transposons can also be designed to contain a number of selectable markers that can be useful to detect and select for the modified cells. The minitransposons that are based on Tn5 and Tn10 lack the transposase gene (encodes the enzyme that catalyses the movement of transposon) and only contain a selection marker between the minimal inverted repeats needed for transposition. These minitransposons are available on a plasmid suicide delivery system, and provide very stable constructs with minimal horizontal transfer of cloned genes [10]. Tn5 based transposons have been developed containing selectable markers encoding resistance

to herbicides such as bialaphos and glyphosate, and heavy metals such as mercury, arsenic and tellurite [37].

4.8 Minitransposon vectors have been applied successfully in the genetic modification of a range of bacteria, including *E. coli, Klebsiella, Salmonella, Proteus, Vibrio, Bordetella, Actinobacillus, Rhiziobium, Rhodobacter, Agrobacterium, Alcaligenes* and several pseudomonads [37]. In addition to providing a very stable phenotype, minitransposons can be engineered into microorganisms with a minimal number of manipulations. This is important with respect to the development of GMMs able to survive in the field, as keeping the amount of manipulation low minimises the loss of competitiveness usually observed in laboratory-designed microorganisms [259].

4.9 Although the transfer of genetic material is not desirable after the GMM has been released into the environment, the spread of conjugative or mobilisable plasmids from the inoculated GMMs to other microorganisms has been proposed as a possible tool to ensure more effective and/or more immediate degradation of the target contaminant [64], and could conceivably be used as part of the development of the microorganism that would be used in the bioremediation strategy. The transfer of the degradative plasmid to a representative sample of the target environment's indigenous microflora, under contained conditions, may result in the formation of a microorganism that is more effective in degrading the pollutant due to its greater ability to survive and compete in the contaminated environment. The inoculation of GM *P. putida* UWC1 (containing plasmid pD10 which confers the ability to utilise 3-chlorobenzoate as a sole carbon source) into freshwater sediment microcosms did not improve the intensity of biodegradation of the 3-chlorobenzoate present. However, following conjugative transfer of pD10 from the GMM to members of the indigenous microflora, the degradation of 3-chlorobenzoate improved [260].

4.10 It was proposed that instead of using GMMs to degrade the target pollutant directly, they should be used as donor organisms to introduce the necessary genetic capability into a member of the indigenous microbial community that could then be inoculated into the environment [260]. Transfer of the plasmid encoded genes from the GMM in a contained environment such as a microcosm means that the recipient microorganisms can be identified and characterised before their possible release into the environment [64]. Due to the wide biodiversity of microorganisms in the environment it is likely that any recipient microorganism is likely to be unknown and therefore poorly characterised.

Biological Containment Systems

4.11 Biological containment systems provide a mechanism to restrict the population of GMMs (and the recombinant DNA) to a particular environment, as defined by specific physical or chemical parameters [261]. Some biological containment systems also enable the population of GMMs to be killed, following the addition of a chemical trigger to the environment. Should the GMMs have a disruptive effect on other organisms or biological processes, then the availability of biological containment systems means that such disruption can be limited to the contaminated environment, and potentially to a limited period [261]. As discussed in Chapter 3, microbial communities in contaminated environments are likely to be more tolerable and therefore less affected by such disruption.

4.12 Biological containment systems are applicable particularly as an alternative to the physical containment systems available for GMMs used in laboratory type applications [23]. The purpose of biological containment systems for GMMs is to minimise or prevent the transfer of the genetic modification from the GMM to other members of the indigenous microbial community. This can be achieved either by reducing the likelihood of gene transfer of the inserted genes, by ensuring that they are inserted into the chromosome of the recipient microorganism, or by designing the GMM so that it is only able to survive in environments that have particular chemical or physical characteristics [23, 262, 263]. For example, the recombinant genes in the GM *E. coli* described by Winter *et al.,* (1989) [68] were under the control of a temperature inducible promoter and would therefore only be expressed at temperatures >42 ºC.

4.13 Although the use of mini-transposon vectors for example to modify the recipient microorganism is likely to reduce the potential for transfer of the recombinant genes, the reliance on such systems for biological containment does mean that the GMM may remain in the environment for a significant length of time. Although this may not pose a risk to the environment or human health, the persistence of the GMMs after completion of their intended function may not be desirable, both in terms of public perception, and possibly because of the potential for unanticipated consequences arising [264].

4.14 Two basic types of systems have been proposed that enable the GMM to become inactivated at a specified time, and therefore effectively removed from the environment when required. These are the attenuation of the microorganism so that survival in the environment is dependent on the presence of a specific compound

(usually a growth substrate), and the use of controllable suicide systems that are able to kill the cell when expressed under specific conditions [263].

Attenuation based biological containment systems

4.15 Attenuation based containment systems are designed so that the GMM is only able to survive in particular environments. These systems are used widely in physically contained processes such as industrial fermenters. The GMMs are designed so that they are unable to synthesise key compounds, which must be added to their culture medium in order for them to survive. *E. coli* X1776, for example, is unable to synthesise the cell wall component D-amino pimelic acid. Because this compound does not occur naturally, then *E. coli* X1776 is unlikely to survive, should it be released into the environment (Curtiss *et al.*, 1977, cited by) [263].

4.16 With respect to their use in bioremediation, such attenuation systems are really only applicable to GMMs used in physically contained facilities such as bioreactor systems used to treat contaminated groundwater. Amending contaminated sites with key nutrients or growth compounds is likely to be prohibitive both in terms of cost and time, although the use of plants to provide the required compounds in the rhizosphere is a possible option that could be applied to large scale contaminated sites. However, because the GMM may still persist in the environment in a dormant state in the absence of the required substrate, then attenuation systems are unlikely to result in the removal of the GMMs from the environment.

4.17 The use of *recA* mutants has been proposed as an attenuation based biological containment system that does not depend on the addition of specific compounds and should result in the inactivation of the microorganism after a period of time. The *recA* system in bacteria is involved in the repair of DNA following exposure of the bacterium to ultraviolet light. If the *recA* system is mutated then the bacterium is unable to repair the radiation damage and will eventually be eradicated. However, although *recA* is easily identified, isolated and manipulated in most bacteria, most *recA* mutants are often too disabled to survive in the environment for the length of time required in bioremediation applications [263].

Controllable suicide systems

4.18 Controllable suicide systems (also referred to as active biological containment systems) consist of a killing element, designed to induce cell death, and a control element designed to modulate the expression of the killing element within the modified microorganism [264]. Expression of the killing gene(s) causes the

inactivation of the GMM and the destruction of the recombinant genetic material. The advantages of controllable suicide systems, over the other types of biological containment systems, are that they are designed to have no effect on the behaviour or ability of the GMM to survive in the environment, but when activated cause the GMM to be removed from the environment as a biological entity. This prevents the GMM persisting in the environment in a dormant state.

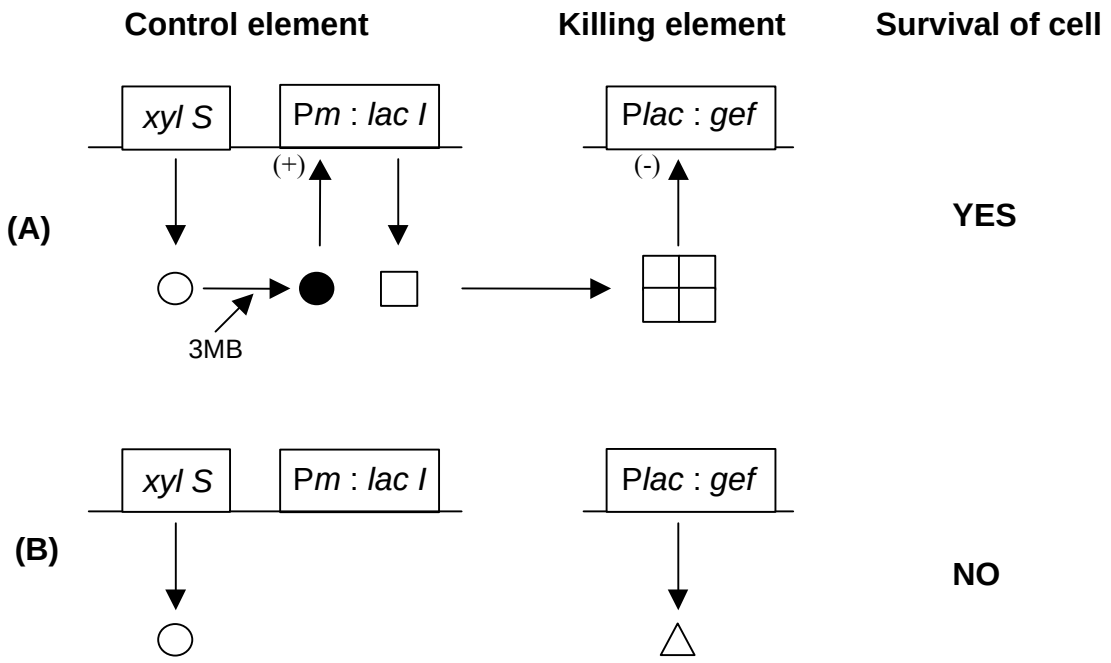
4.19 Although a number of gene products have been identified that are toxic to certain strains of bacteria and therefore potential candidates as killing elements in controllable suicide systems, many of these products are growth inhibitors and are only effective at high concentrations and towards a limited number of microbial taxa. For the purposes of containing GMMs, the ideal suicide systems are based on gene products that affect cellular functions common to most bacteria and whose toxicity is high at low concentrations [263].

4.20 The *gef* gene is one of several genes that have been used as the basis of controllable suicide systems for GMMs. Expression of the *gef* gene (isolated originally from *E. coli*) results in the formation of the membrane protein Gef. This porin like protein becomes inserted in the cell membrane [264], where it generates pores, causing the membrane potential across the cell membrane to collapse and consequently killing the microorganism [265]. The use of different promoter and regulator sequences for the control element of the system, allows the *gef* gene to act as the killing element in GMMs used to biodegrade a wide range of organic compounds. Because the *gef* gene product targets the cell membrane (a common component of all bacterial cells), then it should be able to kill most, if not all species of bacteria [263]. Some species of bacteria have been found to be less sensitive to Gef than others, and may therefore require a higher level of expression of *gef* to kill the cell.

4.21 The design of the control element determines under what conditions the killing element is activated. To date, all of the controllable suicide systems incorporated into GMMs designed for bioremediation applications have been developed so that the GMM is inactivated in the absence of a particular pollutant [261, 263-265]. These GMMs are therefore restricted to environments containing that pollutant, and following the degradation of the pollutant, the GMM is destroyed. To date *gef*-based systems have been designed that are activated in the absence of benzoate, alkyl-, dimethyl-, chloro-, dichloro-, methyl-, ethyl- and methoxybenzoates; salicylate; and methyl- and chlorosalicylate [266]. Such systems are therefore applicable to the biological containment of GMMs designed to be able to bioremediate these compounds.

4.22 An example of a *gef*-based controllable suicide system is presented in Figure 4.1. The use of the XylS regulator, as part of the control element, means that the system can be controlled by the presence of effectors of XylS. These include the alkyl-substituted, chloro-substituted and other halo-substituted benzoates [264]. In the system illustrated in Figure 4.1, the regulatory gene *xylS* is expressed constitutively in both (A) and (B). In scenario (A), the presence of an effector of the *xylS* gene causes the product of *xylS* to be activated. This promotes transcription from P_m and leads to the synthesis of the LacI repressor. The action of the LacI repressor is to prevent expression from P_{lac}, which is required to achieve expression of *gef*. However, in the absence of an effector of *xylS,* the expression from P_{lac} is not repressed and Gef is produced, killing the cell [261].

Figure 4.1 - Elements and functioning of a biological containment system [261]



In both (A) and (B) the regulatory *xylS* gene is expressed constitutively. However, in the presence of 3-methylbenzoate (3MB) (A) the product of *xylS* is activated and causes transcription of the *lacI* repressor (□) that forms tetramers that prevent expression from P*lac* and formation of Gef. In the absence of 3-methylbenzoate (B) expression from P*lac* occurs and Gef is formed thus killing the cell.

4.23 Ronchel *et al.,* (1995) [261] inserted the *gef* system illustrated in Figure 4.1 into *Pseudomonas putida* EEZ29. The recombinant pseudomonad (designated EEZ30), was modified so that the killing element was located on the chromosome and the control element on a mobilisable plasmid (pCC102). The inserted genes were found to be stable genetically, and pCC102 was not mobilised from the GMM during the transfer of the TOL plasmid from the GMM to other pseudomonads in soil microcosms. Both contained (*gef*$^+$) and uncontained (*gef*$^-$) versions of *P. putida*

EEZ29 survived well in soils amended with 0.1 percent (*w/w*) *m*-methylbenzoate. However, in the absence of the *m*-methylbenzoate numbers of the contained GMM (EEZ30) declined markedly, compared to the *gef* strains [261].

4.24 The genes *hok* and *relF* have also been proposed as the 'kill' genes for controlled suicide systems [266, 267], as has the streptavidin gene (*stv*) isolated from *Streptomyces avidinii* [268]. In the presence of a particular growth substrate or target pollutant the *stv* gene is repressed using a similar system to the one described for *gef*. However, in the absence of the particular substrate, the *stv* gene is expressed and streptavidin is produced. This compound has a particularly high binding affinity for the ubiquitous essential prosthetic group, D-biotin (vitamin H). Inactivation of D-biotin by streptavidin kills the microorganism [268]. Because the streptavidin system targets cell metabolic processes, then it should complement cell suicide systems which target cell membranes and walls, or nucleic acids [268].

4.25 Destruction of the cell will expose the DNA to extracellular nucleases that are likely to inactivate the recombinant genetic material. The use of nucleases as the killing agent in controlled suicide systems will result in the destruction of the DNA and the inactivation of the GMM [263].

4.26 The nucleases produced by *Staphylococcus aureus* and *Serratia marescens* are reported to be suitable, as they have sufficient activity to override the ability of the GMM to repair the damage to its DNA. However, the use of nucleases to control GMMs in the field may be limited by the intracellular stability of the enzymes (half-life of two minutes for *S. marescens* and two hours for *S. aureus*) and the conflicting activities of the GMM's DNA repair mechanisms [263].

4.27 A limitation of all suicide systems is that they are significantly inefficient. Even under optimal laboratory conditions, up to 10^{-4} microorganisms in a population are not killed [8]. The primary reasons for the low effectiveness of suicide systems are mutational inactivation of the suicide gene and the selection of mutants with a defective suicide system [267]. Although suicide systems are designed not to affect the normal growth of the microorganism, even a small basal level of expression of these genes is enough to confer a selective advantage to cells that have a mutated and therefore non-expressed suicide system [263, 269]. If suicide systems are intended to control large populations of GMMs then the basal level of expression of the killing gene must be as low as possible [263].

4.28 Insertion of the suicide genes into the GMM in multiple copies was found to reduce the mutation rate, especially when the insertions were made so that no single mutation

event, particularly a deletion, would result in the inactivation of both copies of the suicide system [269]. A single copy of a suicide system is reported to have a mutation rate of approximately 10^{-6} per generation, whereas a double system, both using the same control element will have a mutation rate of 10^{-8}. With the double system, if it is designed with independent control elements then the rate may be reduced to as low as 10^{-12} per generation [269]. The mutation rate is also reduced if the killing element and control elements are located on the chromosome of the GMM [261, 264]. The reason for this was reported to be unknown, although it was possibly due to the overall basal rate of transcription of the P_{lac}::*gef* being lower on the chromosome than on a high copy number plasmid [261].

4.29 Atlas (1992) [262] reported that variations in the stability of the vector, used to insert and maintain the suicide constructs in the GMM, may also effect the effectiveness of the biological containment system. Improvements in vector stability, through the use of stably-inherited plasmids or chromosomal insertion, in combination with tightly-controlled promoters and duplicate/multiple insertions is proposed as the solution for using suicide elements for the biological containment of GMMs in the environment.

Application of Microorganisms to the Contaminated Site

4.30 The method used to apply the GMMs to the contaminated site may have implications to the immediate containment of the GMMs to the target area. Microorganisms are most easily applied to contaminated soils in solution as a spray or mist. Although this method allows an even distribution of the microorganism across the target area, it does have the potential to form bacterial aerosols, and may result in the dispersion of the microorganisms beyond the designated site. Ford *et al.,* (1999) [270] reported that the meteorological conditions encountered during the inoculation of the field lysimeters with the GM *P. fluorescens* HK44 did affect the survival and dispersion of the GMMs.

4.31 A relative humidity of 50 percent has been reported to produce a far higher death rate of microorganisms in aerosols than either higher or lower humidities, and the death rate was especially high during the first 5-20 min of exposure (Dunklin and Puck, 1948; cited by) [270]. High ultraviolet light also reduces bacterial counts in aerosols (Barnthouse and Palumbo, 1986; cited by) [270]. Therefore to reduce 'spray drift' of the GMMs from the application area, it was proposed that the optimum humidity for spray inoculating contaminated soils was 45-60 percent, and that the GMMs should be dispersed in a saline solution [270].

PLANTS

4.32 To date, a large number of research trials and commercial growing of GM plants have taken place worldwide in the field. The majority of these GM plants have been crop plants grown for agricultural purposes. No releases of GM plants for bioremediation have so far taken place in the field. However, due to the possibility that many of the transgenic plants designed for agricultural applications may be used for phytoremediation, particularly where rapid growth or ease of harvest is required, then the management strategies developed and/or proposed for GM plants in agriculture are applicable to GM plants in phytoremediation.

Transfer of genetic material

4.33 The management strategies that have been developed or proposed to limit the transfer of the recombinant genes between the GM plants and other flora in the environment have largely been addressed for GM plants designed for agricultural purposes [251], and are therefore not covered in more detail in this review. However, as discussed in the risk assessment, the transfer of genetic material from GM plants designed for phytoremediation may not confer any selective advantage to other plants, and will therefore be effectively self-contained within the area of the contaminated site. If this biological containment is effective, and the GM plant only contains transgenes to confer the desired trait (and no other 'superfluous' recombinant material such as herbicide tolerant selective marker genes), then no other management strategy is likely to be required.

Accumulation of toxic compounds

4.34 The requirement for management strategies for GM plants designed to hyperaccumulate pollutants depends on the concentration and potential toxicity of the pollutant that is accumulated, and the location in the plant where the accumulation occurs. The hazards associated with GM plants designed for rhizofiltration or phytoextraction are only likely to be realised if the GM plant is consumed. Therefore, any release of such plants should consider the potential for herbivory of the plants and take steps to prevent this from occurring, such as physical containment or using non-palatable or inedible plants.

4.35 If herbivory was a possibility, then one management strategy to reduce exposure to the grazer is to produce GM plants with organ-specific overexpression of the protein(s) involved in phytoaccumulation. This could lead a reduction of the potentially toxic metals in the consumable parts of the plant, and the partitioning of

the hazardous metals into non-consumable sections, such as below ground structures [103, 196]. Yeargen *et al.,* (1992) [195] demonstrated that tobacco seedlings expressing the mouse metallothionein I gene, exhibited a difference in the tissue partitioning of cadmium within the plant, compared to the wild type. This was reported to demonstrate the potential for reducing the cadmium content of above-root tissues of certain plants. This would however only be of use in reducing the potential exposure of organisms feeding on the above-ground parts of the plant.

4.36 Alternative suggestions to prevent organisms from feeding on GM hyperaccumulating plants have included the use of 'clean crops' adjacent to GM plants, along with fences and/or other repellents. The non-accumulating 'clean crops' are designed to attract organisms away from consuming the transgenic plants, with the use of repellents and fences to further reduce the attractiveness of the hyperaccumulating plants [208].

4.37 Because the pollutant is only accumulated within the plant and not degraded, then plants used for phytoextraction or rhizofiltration will need to be harvested to remove the accumulated pollutants from the contaminated site. After harvesting, it may be possible and commercially viable to employ a biomass processing system to recover the metal contaminant from the plant material. Possible marketable metals include nickel, zinc and cobalt [173]. If this were not possible or cost-effective, then it may be beneficial to reduce the harvested plant material by weight and/or volume by thermal, microbial, physical or chemical means. This step would decrease the handling, processing and potential subsequent landfill costs [160].

4.38 Salt *et al.,* (1995) [159] investigated methods for the further concentration of metals in plant tissues, to reduce the weight/volume of material that would ultimately need to be disposed of. Possible methods included sun, heat or hot-air drying; environmentally-safe ashing or incineration; composting; pressing or compacting and leaching.

4.39 The type of plant species used and the time of harvest also need to be considered for plants used for phytoextraction. For example the use of annual plants or deciduous tree species for phytoextraction would result in the return of accumulated compounds to the soil if the plant was not harvested prior to the end of the growing season and the start of senescence (e.g through leaf fall) [157]. For phytoextraction processes a fast growing plant that is easily harvested would be preferable, making sure that the plant is harvested prior to senescence and/or leaf-fall. This would ensure the maximum possible recovery of the target contaminant from the soil, and could be designed to involve more than one harvest per growing season [160].

Production of toxic metabolites

4.40 The production of toxic metabolites by GM plants developed for phytoremediation is not desirable. Where possible the breakdown of the contaminant by the transgenic plant should be followed completely to ensure that no toxic metabolites are produced during the reaction pathway [165]. The production of a toxic metabolite that is subsequently converted into a non-toxic compound is likely to be less of an issue than the formation of a dead-end toxic compound, although this will depend on the toxicity and persistence of the transient toxic metabolite within the plant.

4.41 However, it should be noted that with GM plants designed to phytovolatilise toxic pollutants such as mercury, the objective of the phytoremediation process is for the plants to produce a toxic compound (although one that is significantly less toxic than the target contaminant), and to result in the overall abatement of the environmental hazard represented by the metal. With these plants, the potential management options may include reducing the residence time of the mercury in the plant, and the design of plants from which the quantities of mercury released can be predicted in advance.

Disruption of Other Organisms and Biological Processes

4.42 From the information presented in the risk assessment, the most likely mechanisms by which GM plants developed for phytoremediation may disrupt other organisms or biological processes is following the transfer of genetic material, or by toxicity caused by ingestion of hyperaccumulating plants. The transgenic traits applicable to phytoremediation strategies are unlikely to provide the GM plant with any characteristics that would confer a selective ecological advantage over other plants outside the contaminated area. Whilst the GM plant may become the dominant floral specie within the contaminated site, this dominance would only be expected to persist for the duration that the target contaminant remained on the site.

5. REPORT OF THE WORKSHOP

INTRODUCTION

5.1 This section of the report consists of the technical report of a one-day workshop held at Magdalen College, Oxford on 31st March 2001.

5.2 The purpose of the workshop was to create a technical forum to discuss the applications, advantages, limitations, risks and potential management strategies associated with the use of GMOs in field-based and contained bioremediation programmes. By bringing together researchers in the range of disciplines that encompass plant and microbial-based bioremediation strategies, the workshop was designed to build on the information compiled in the previous sections of this report, and in particular to discuss the most likely applications for this technology, the major issues involved and to make recommendations for future research.

5.3 The meeting was comprised of six paper presentations followed by a general discussion on the main issues raised. The morning session, which covered the use of genetically modified (GM) microorganisms in bioremediation was chaired by Professor Chris Knowles (Oxford Centre for Environmental Biotechnology, University of Oxford). The afternoon session, which addressed the use of GM plants in phytoremediation applications, was chaired by Professor Chris Leaver (Department of Plant Sciences, University of Oxford). Professor Leaver also acted as overall chair for the workshop and led the general discussion following the afternoon session.

5.4 The six papers presented at the meeting were:

- Prospects and challenges for bioremediation with genetically modified microorganisms (GMMs)

  – Professor Kenneth N Timmis, National Research Centre for Biotechnology, Braunschweig, Germany.

- Reporter gene based biosensors – risk-based management support for remediation of contaminated land

  – Professor Ken Killham, Department of Plant & Soil Science, University of Aberdeen, UK.

- Field release of *Pseudomonas fluorescens* HK44:. Long term persistence and field performance of a bioremediation bioluminescent bioreporter

  – Prof Gary S Sayler, Center for Environmental Biotechnology, University of Tennessee, Knoxville, Tennessee, USA

- Metal accumulation by plants

  – Professor Andrew Smith, Department of Plant Sciences, University of Oxford, UK

- Phytoremediation of toxic chemicals in our environment

  – Professor Richard B. Meagher, Department of Genetics, University of Georgia, Athens, Georgia, USA.

- Defusing the environment: engineering transgenic plants to degrade explosives

  – Dr Neil Bruce, Institute of Biotechnology, University of Cambridge, UK

5.5 Copies of the abstracts for each of the paper presentations are provided in Appendix A at the back of this report. A list of the delegates at the workshop is in Appendix B.

PROSPECTS AND CHALLENGES FOR BIOREMEDIATION WITH GMMs

Presentation by Professor Kenneth N Timmis

5.6 The presentation focused on the wider issues associated with the use of microorganisms in bioremediation. Because the challenges posed by the use of genetically modified microorganisms (GMMs) in bioremediation were proposed as being no different from the other biocatalysts carrying out useful activities in polluted environments, then understanding the role and application of microorganisms in

contaminated environments would provide the information required to address the use of GMMs in bioremediation applications.

5.7 It was recognised that there is an enormous knowledge deficit in our understanding of what is going on in polluted environments, and that this constitutes a major barrier in applying the information obtained from studies optimising a single bioremediation process to developing a more generic understanding of how to optimise bioremediation processes.

5.8 A significant area of knowledge deficit is in understanding how underlying processes such as the microbe-microbe and microbe-contaminant leads to bioremediation in the environment. Information is required on the interactions that are important in certain processes and those which are not. This is an important area that needs to be resolved if what is happening in bioremediation processes can be understood and subsequently optimised or improved.

5.9 The lack of knowledge in these areas is partly a consequence of the understanding of microbiology and microbial processes being based on the study of pure homogenous cultures, and the view that catabolism occurs as a series of linear reactions taking place in a single cell. In the environment, microorganisms are likely to inhabit surfaces and exist in heterogeneous communities such as biofilms.

5.10 In addition to improving our understanding of basic microbial processes, another major issue that needs to be addressed is bioavailability. It is recognised that all life takes place in aqueous systems, and that this limits the biodegradation of many pollutants due to their extremely hydrophobic nature. How microorganisms extract nutritional benefits from these hydrophobic compounds in the environment is, however, poorly understood.

5.11 Results were presented from work performed on a polychlorinated biphenyl (PCB) contaminated site in Germany that had soil concentrations of PCBs from 0-50 g kg^{-1}. The lack of vegetation on the site and the low carbon content of the soil suggested that whatever microorganisms were present and active in the site were dependent on the PCBs present. The community was described as 'PCB-driven'. Analysis of the metabolically active component of the microbial community through the generation of rRNA libraries identified a surprisingly diverse range of microorganisms, including *Burkholderia* sp and *Sphingomonas* sp (both of which are known degraders of aromatic compounds). However, microorganisms such as *Acetobacter* sp that have no previous record as aromatic degrading organisms were also detected at the site.

5.12 During the study of the interaction between the microorganisms and PCBs in soil samples taken from the site, the microorganisms were observed to form biofilms directly on the PCB droplets (when exposed to them in an aqueous environment), and also to collate clay particles and PCBs as a composite biofilm in which the clay particle was proposed to act as a nutrient shuttle between the microorganism and the PCB.

5.13 Although information on the role of biosurfactants and bioemulsifiers was not presented, these are likely to be involved in interactions between microorganisms and PCBs, for example in altering the toxicity and solvent properties of the PCB, and in changing the structure of the interaction faces between the microorganism and this hydrophobic substrate.

5.14 The basis of the diversity of microorganisms at PCB contaminated sites was proposed to be due to a number of different possibilities. The primary carbon source at the site was PCBs. However, PCB contamination consists of a large number of different congeners, none of which can be degraded by a single microorganism. Therefore the diversity of microorganisms present may have been due to:

- specialist substrate utilisation with individual microbial taxa using a different group of congeners as a carbon source;
- competition between different microbial taxa for the same substrates;
- sharing of substrates or metabolites between microorganisms; or
- a diversity of physiological or metabolic optima between different microbial taxa towards PCB congeners.

5.15 The basis of the observed diversity of microorganisms was addressed by chemostat culture that consisted of a stable community of four bacteria isolated from a PCB contaminated site. One of the isolates degraded eighty percent of the 4-chlorosalicylate substrate, and generated large quantities of 4-chlorocatechol and proteoanemonine. When this isolate was grown on its own, production of the 4-chlorocatechol and proteoanemonine ultimately killed the cell. However, in the community with the other three microorganisms, this isolate survived as two of the other organisms sequestered and metabolised either 4-chlorocatechol or the proteoanemonine. This system was proposed as an example of substrate sharing

between microorganisms and demonstrated that parent substrates, in this case 4-chlorosalicylate are not metabolised linearly but rather as a network.

5.16 Therefore in order to further understand and utilise the abilities of microorganisms in the bioremediation, it was concluded that a number of key areas need to be investigated:

- what is the 'face' of the substrate in the environment from the perspective of the microorganism?. This is likely to be in a less well defined physiochemical form to that in the laboratory;

- what is the 'face' of the microorganism in the environment?. Interactions between microorganisms and hydrophobic pollutants may involve the production of biosurfactants. These have been identified as being produced in a free form or bound to the surface of the outer membrane. Microorganisms producing bound biosurfactant are likely to have a different 'face' and consequently a different interaction with a pollutant than those that do not produce a biosurfactant or secrete one in a free (non-membrane bound) form;

- what is the 'face' of the microbial community?. The pollutant is likely to have an effect on the structure and physiology of the community in the environment; and

- what is the 'face' of the catabolic route in the environment?. Although there is a relatively good level of understanding of the enzymatic reactions involved in the degradation of compounds, there is relatively little knowledge of the routes taken by individual metabolites in microbial communities during the mineralisation of the parent substrate.

Questions

5.17 The questions following this presentation addressed the potential benefits of bioaugmentation and the survival of DNA in soil.

5.18 With respect to bioaugmentation, this was suggested to only improve the biodegradation of a particular compound if the biology associated with the degradation is in some way limiting. Although bioaugmentation was recognised as a highly promising technique it is currently limited because of the lack of knowledge regarding the survival of microorganisms in the environment. Sometimes a

microorganism introduced into a particular environment will survive very well, whilst in other cases it will not, even if it originated from the same site. Improved understanding of the survival of microorganisms in the environment is required to increase the success of bioaugmentation as a bioremediation strategy.

5.19 Regarding the fate of DNA in soil, much of the work reported to date has studied the persistence of DNA in unnatural substrates. Where more natural systems have been used, the half-life of DNA is much lower.

REPORTER GENE BASED BIOSENSORS – RISK-BASED MANAGEMENT SUPPORT FOR REMEDIATION OF CONTAMINATED LAND

Presentation by Professor Ken Killham

5.20 The presentation focused on the use of reporter gene based biosensors as a tool for providing key information on contaminant bioavailability. Such information is required to develop an informed risk based management strategy for a contaminated site, from assessment through to cleanup.

5.21 The main driver for the biosensor technology was proposed to be new legislation, namely Part IIA of the Environmental Protection Act 1990. Such legislation requires the development of new (preferably rapid and reliable) methods that enable contaminant availability within a site to be determined, and allow an assessment to be made as to whether site contaminants are risk chemicals and how they map into the source-pathway-receptor model.

5.22 To date, assessments have relied largely on chemical models that have been devised from data generated from appropriate analyses. Although such techniques are powerful and well-proven technologies, they can be time consuming and do not measure hazard directly. Chemical analyses are particularly time consuming if information on the processes and the likely pollution occurred on a site are not available at the time of the assessment. The lack of such information means the chemical analysis of a site cannot be targeted towards particular areas or particular compounds.

5.23 In addition, because chemical analyses are unable to address toxicity issues directly, it is more difficult to use them to derive an informed remediation strategy for the contaminated site.

5.24 The reporter gene based biosensors addressed in this presentation involved the use of *lux*-modified bacteria to monitor the presence of toxic contaminants in soil and groundwater. Such biosensors are designed to work as complements to chemical analyses, primarily to determine both the spatial and temporal characteristics of the pollutant in the site, and assist in the development of a sensible site-specific remediation strategy. Because the remediation of any contaminated site in a commercial context is likely to be conducted by 'non-scientists', any technique must be suitable for operation or application by 'non-scientists'.

5.25 Although *lux* is only one of the biosensor systems available that have good *in-situ* application (others include *luc* (luciferase) and the unstable green fluorescent protein (GFP)), the key to all such systems is not just the type of biosensor but the method of its application. Importantly, the means by which the sensor is placed in contact with the contaminants present in a site, in particular when there are many compounds with different physiochemical and toxicological characteristics present, has a real bearing on site characterisation. For contaminants that are associated closely with solid substrates it is necessary to ensure that the biosensor is added to the sample so that it comes into contact with the contaminant in an environmentally relevant way.

5.26 The *lux* system used in the bacterial biosensor consisted of the *CDABE* genes and therefore includes the *lux* structural genes and those required for the synthesis and recycling of the enzyme. The gene cassette was placed downstream of a strong general constitutive promoter, which ensures that the metabolic activity of the biosensor microorganism can be tracked reliably and quickly. The non-specific status of the promoter means that the system can be used against metals and organic pollutants.

5.27 The two main approaches to generating biosensors containing *lux*, are to mark the microorganisms chromosomally with the appropriate cassette or to use plasmids to introduce the genetic material. Chromosomal marking was presented as the preferred choice of the regulatory authorities, in order to reduce the risk of spreading genes. The bacteria used as biosensors are all designated as Class I GMOs under the current regulations, and therefore require a license for use at a particular location. This was proposed as potentially restricting their use in online operations, at individual contaminated sites, as a license would be required for each site. It was questioned whether this requirement should be reviewed for what is now a proven technology.

5.28 The advantage of the biosensor microorganisms is that once they have been produced they can be freeze-dried and stored on the shelf until required. The system used with these biosensors is described as 'lights-off' as there is a reliable dose-response

relationship between the biosensor and the contaminant, with a diminution of light output from the reporter gene biosensor with increased concentration of the toxic contaminant(s). The biosensors can be used in batch assay to provide information on acute toxicity by exposing the biosensor to the sample for a brief period. Alternatively they can be used online to monitor site contamination temporally to assess the success of a specific remedial approach.

5.29 The reporter gene based biosensors addressed in this presentation have been commercialised using a range of different microorganisms, selected on the basis of their intended application. For example, for environmental or ecological assessments *lux* modified *Pseudomonas fluorescens* or *Rhizobium leguminosarum* biovar *trifolii* are likely to be the most relevant, whereas the sewage sludge microorganism *Escherichia coli* HB101 may be more applicable for assessments in wastewater treatment plants.

5.30 As discussed at the start of this presentation, the key application of biosensor technology is that it is able to provide information on contaminant bioavailability. In the environment, this is determined by a wide range of biological, physical and chemical factors. Because of the variety of parameters involved, it is very difficult to model bioavailability across a contaminated site using chemical analyses. Microbial biosensors, however, respond to all of the parameters operating at a particular site and integrate these into a single signal (light output).

5.31 To date, biosensor technology is well developed for addressing environmental risk assessment issues and has achieved good correlation with other analytical processes. However, the technology is less well developed to address human risk assessment, and there is still some way to go before it is able to address all human toxins, although it was suggested that GM biosensors may eventually be developed for all types of human risk assessments.

5.32 Although this presentation focused on the use of microbial biosensors, the choice of biosensor organism is important in order to achieve relevant information to address such issues as ecosystem toxicity. However, the simplest and therefore the easiest biosensor to apply, including molecular and cellular systems, may not always be relevant to address toxicity at an ecosystem or macropopulation level.

5.33 The specific properties of particular organisms, either in terms of their ecological niche or ecophysiological predisposition, can be exploited to address specific issues of contaminant toxicity. For example the high sensitivity of *Rhizobium* sp to organic

compounds means that this microorganism is an ideal choice as a biosensor to determine the toxicity of organic contaminants in environmental samples.

5.34 The case study used to demonstrate the application of reporter gene based biosensors in a contaminated environment was an 8-9 hectare paint manufacture site. The site was still partly in operation at the time of assessment and was located on top of 30 m deep sediments, some of which were contaminated with BTEX and chlorinated pollutants. The depth of the sediments and on-going usage of the site meant that some form of *in-situ* remediation strategy was required. The biosensors were used initially as a primary screen to produce a toxicity map of the site. On completion this map was found to include an additional toxic hotspot not identified by previous chemical analyses. This was due presumably to this area not being sampled previously because of a lack of documentary evidence that contamination had occurred at that location.

5.35 The biosensors were then used to identify the possible constraints to intrinsic bioremediation. This was performed by exposing environmental samples to a range of remedial procedures designed specifically to remove any potential bioremediation bottlenecks. For example, air sparging samples removed the volatile organic compound fraction, and muffle furnacing removed the non-volatile organic compounds present. By comparing the biosensor response in the sample before and after each process the significant constraints to bioremediation were identified.

5.36 In this case air sparging was found to have the greatest effect and this result enabled the subsequent management of the remediation process to be more focused. This example was used to demonstrate biosensors as an effective management tool for contaminated sites.

5.37 The latest development with biosensor technology has been to design online monitoring of contaminated sites. This allows sites to be monitored permanently to study the process of a bioremediation campaign, by continual sampling through groundwater wells of lysimeters. The hardware involved is now down to a more manageable benchtop size and offers great potential for the remote monitoring of contaminated sites.

5.38 In conclusion, the new contaminated land legislation that is now in force requires innovative techniques that are capable of assessing contaminants in relevant bioavailable forms. Biosensors are able to detect available contaminants and can therefore assess the potential risk directly as prescribed in Part IIA of the Environmental Protection Act.

Questions

5.39 The questions following the presentation focused on what was actually involved with online sensing and the promoter used to control expression of the *lux* cassette.

5.40 The online sensing system that has been used to date was described as involving some automated mechanism that takes samples from across the site, for example a pump linked to the boreholes on the site to extract samples of groundwater. After the sample has been retrieved it is mixed with a resuscitated biosensor sample and the result recorded. Therefore, although the process is described as online there is a delay of a few minutes between sample extraction and sample analysis.

5.41 Regarding the control of the *lux* cassette within the GMM, the microorganisms used are generated by minitransposon based modification of the organism's chromosome. This results in the random insertion of the *lux* genes and the generation of a large number of isolates that are then screened to identify the most suitable biosensor. Because the biosensor is only exposed to the contaminant for a short period (no more than 5 minutes) then suitable modified microorganisms are selected from the initial screen on the basis of the enzyme activity of the *lux* expressed system rather than gene expression. Therefore, the inserted *lux* cassette is not under the control of a prespecified promoter, although the screening system ensures that whichever promoter is involved, it is a strong general promoter, resulting in constitutive expression.

FIELD RELEASE OF *P. fluorescens* HK44:. LONG TERM PERSISTENCE AND FIELD PERFORMANCE OF A BIOREMEDIATION BIOLUMINESCENT BIOREPORTER

Presentation by Professor Gary S Sayler

5.42 The purpose of the presentation was to demonstrate how a research/laboratory based study, using genetic engineering technology, would scale up to a large-scale field application.

5.43 The overall aim of the study was to assess how effective the application of GMMs would be for monitoring bioremediation activity in the field. The specific issues that were addressed using GMM containing bioluminescence genes included (1) the physiologically state of introduced cells, (2) the ability of the introduced population to promote biodegradation of the target compound, (3) the effectiveness of bioluminescence for monitoring biodegradation within the environment and (4) the

identification of the environmental conditions that optimised biodegradation rates. Improving the rate and effectiveness of biodegradation was proposed as the key goal of bioremediation strategies.

5.44 The organism used in the study was *Pseudomonas fluorescens* HK44. The donor and recipient strains used in the generation of this microorganism were isolated originally from a heavily contaminated town gas site. The organism that was the fundamental donor strain for HK44 contained a NAH7-like plasmid which was almost identical to the archetypal NAH7 plasmid known to confer hydrocarbon degradation capability.

5.45 The NAH plasmid is a fairly large plasmid and transmissible at low rates. (Transmission was however was not addressed in this study). Within the plasmid the genes are arranged into an upper and lower pathway, with the *nah* regulatory gene in the middle.

5.46 In order to use the GMM as a process-monitoring tool, the *lux* gene cassette (comprising of *lux CDABE* genes) was inserted into the lower pathway in the NAH plasmid using transposon Tn4431. This of course knocked out the lower pathway, and required several more mating experiments (with other pseudomonads also isolated from the contaminated gasworks site) to generate strain HK44, that was able to degrade naphthalene and salicylate and had a good bioluminescent response to the target compound.

5.47 The GM *Pseudomonas fluorescens* HK44 was reported to be able to degrade a variety of mono-, di- and tricyclic aromatic compounds including naphthalene, although not all of the compounds were degraded to completion. (The incomplete biodegradation of compounds by microorganisms was highlighted as an issue that should be addressed as part of the risk assessment for microorganisms, both the GM and non-GM for use in field trials). When exposed to naphthalene, strain HK44 exhibited a linear production of light with increasing naphthalene concentration within the range of 50 µg/l to 10 mg/l. The GMM also responded to pulsed exposure to naphthalene or salicylate when immobilised, and also when in dirty samples in the presence of other compounds.

5.48 Having generated the desired microorganism, the next stage of the research was to obtain funding from the Department of Energy to use facilities at Oakridge National Laboratory for the field trial. These facilities consisted of five lysimeters, each of which extended five metres into the ground. The lysimeters were intended for use in this study to simulate the subsurface environment and to determine degradation of PAHs in the vadose (unsaturated) zone of the soil.

5.49 Following agreement with the Department of Energy, a proposal was submitted to the USEPA[17] for the release of the GMM. This proposal was submitted in the form of a full-scale review (termed pre-manufacturing notification (PMN) application), which is the same as that required by the USEPA for new industry-based activities. The reason for this was that in addition to the research objectives of introducing and maintaining a GMM in the environment, the programme was also intended to overcome a perceived regulatory barrier within the USEPA towards GMMs release into the environment. A final objective was to develop a field site for use by other investigators[18].

5.50 The PMN application was submitted to the USEPA in July 1995 and approval was granted in March 1996, with work finally starting in October that year. Because the GMM contained an antibiotic resistance gene and is part of a group of microorganisms that contains opportunistic plant pathogens (as determined by RNA classification), the USEPA granted a consent order for the work. This was not a full and unlimited approval of the use of the microorganism, but instead required the work to be conducted exactly as specified in the PMN.

5.51 Each of the five lysimeters were packed with a base layer of gravel/aquifer matrix, a layer of clean soil, then soil (1 m) contaminated with hydrocarbons (except in lysimeters 3 and 4) and a finally layer of clean soil (1 m). The GMMs were added to lysimeters 1 to 4, with lysimeter 5 acting as the negative control (containing hydrocarbons but no GMMs).

5.52 Each of the lysimeters were also modified by the addition of a number of sensors and manipulation systems that allowed the microorganisms and chemical conditions within the lysimeter to be studied remotely for the duration of the programme, and also to alter the conditions if required. During packing of the lysimeters, an aeration system was inserted into the gravel matrix to allow bioventing; oxygen and soil moisture sensors[19] were added to the soil layers, and fibre optic cables were inserted into the contaminated layer to allow the direct observation of the microorganisms on the soil surfaces. The addition of a sub-surface irrigation system within each lysimeter meant that additional nutrients and more contaminants could be added to the soil if required.

5.53 The soil used in the experiment was artificially contaminated specifically for the trial. (Soil was mixed with the hydrocarbons and aged for several months prior to use).

[17] United States Environmental Protection Agency.
[18] The site is still available for use for other investigations.
[19] The availability of oxygen was found to be more important than soil moisture in this study.

The GMMs used were grown up in a 500 l fermenter and then added to the soil by spray application. The original application submitted to the USEPA had proposed to mix the microorganisms and soil outside the lysimeters and then pour it into the lysimeter. However, due to the height of the lip of the lysimeter above ground level this was not possible and so the contaminated soil and GMMs were added to the relevant lysimeters in layers.

5.54 At the time of the loading the wind speed at the trial site was 25 mph, and although the GMMs were added by spray, none of the modified microorganisms could be detected outside the lysimeters by either Anderson sampling of the air or from nasal swabs from the people present.

5.55 With respect to the population dynamics of the GMMs added to the soil, the initial inoculum density was approximately $1x10^7$ GMMs g^{-1} soil. The population declined fairly rapidly and this was found to be due to a loss of the hydrocarbons from the soil. The addition of further naphthalene, anthracene and phenanthrene to the soil increased the population of GMMs back up to near inoculation levels. After fourteen months the GMMs could still be detected in the soil at a population density of $1x10^2$ GMMs g^{-1} soil, and could still be recovered and cultivated by standard selective isolation techniques.

5.56 The heterogeneity and relatively low population density of the GMMs in the soil meant that the original fibre optic system added to the lysimeters during loading did not work. The insertion of additional fibre optics[20] to the soil after 300 days enabled light production to be detected directly from the soil. The level of light production was responsive to the addition of further spikes of naphthalene and was visible to the naked eye when viewed via boreholes cut into the soil.

5.57 The GMMs added to the soil were found to utilise the hydrocarbons present, and further degradation occurred following the addition of more naphthalene. Interestingly, the GMMs remained within the layer of contaminated soil for the two year duration of the project and were not detected in either the upper or lower layers of clean (non-contaminated) soil. There was however no hydraulic gradient through the lysimeter.

5.58 In conclusion, studies of microorganisms inoculated into a field environment typically results in the decline of population densities. In those cases where the aim is to sustain desired degradation rates then steps may have to be taken to maintain

[20] Vapour phase sensor and downhole photomultiplier.

introduced populations. A population density of 1 x 10^8 cells g^{-1} soil has been proposed as a minimum requirement to achieve biodegradation of target contaminants. Levels of $1x10^5$ g^{-1} or $1x10^6$ g^{-1} are unlikely to result in a level of biodegradation significantly greater than that achieved naturally.

5.59 In terms of obtaining approval from the USEPA to conduct such work, it is important to provide as much information on the GMM as possible. For the strain used in this study the USEPA required the full 16S RNA sequence homology and absolute ancestry of all of the transposon fragments. As much microcosm data should also be provided with the application, including information on the lowest detection/monitoring level for the microorganism in its intended environment.

5.60 Because of concerns by regulators with the possible use/release of pathogenic microorganisms, it was recommended that any potentially pathogenic phenotype should be avoided. For example, the ability to grow at 37 ºC and able to produce exudates could be considered as phenotypic traits indicative of human and plant pathogenicity, respectively. It was also recommended that GMMs designed for field use should not contain mobile elements or antibiotic resistance selective marker genes.

Questions

5.61 The questions following the presentation addressed the issue of gene transfer from the GMMs during the period of study, and the advantages of bioaugmentation over *in-situ* biostimulation. Gene transfer, was not studied during the trial as the focus of the investigation was the bioremediation of the PAHs and monitoring of the activity of the GMMs. However, additional funding is now being sought to analyse some of the samples taken during the study to address whether gene transfer had occurred.

5.62 Questions were also raised on whether the addition of transposon Tn4431 into strain HK44 conferred transposon immunity, and the current position of the USEPA towards GM based field studies. The insertion of Tn4431 was thought not to confer transposon immunity, and it was suggested that following the work reported in this presentation it would now be easier to get approval from the USEPA for this type of work, and that the USEPA were very keen for further studies to take place, even at the level of a full field release.

METAL ACCUMULATION BY PLANTS

Presentation by Professor Andrew Smith

5.63 The presentation was intended as an introduction to the application of plants in bioremediation. Compared to microbially based strategies, the use of plants is still developing and was described as a relatively young field of expertise. However, many plants have been identified that demonstrate a high degree of tolerance to potentially toxic metals, and some of these plants are able to accumulate metals to high concentrations. Such plants, could in principle, be used to remove metals from a contaminated soil.

5.64 Although metal-tolerant and metal-accumulating plants have been identified, further information is now required on the natural basis of plant adaptations to metals in their particular environments. It was proposed that only through a detailed and mechanistic understanding of how plants deal with and survive exposure to toxic compounds, can a rational understanding of the potential for genetic engineering technologies be developed. A report by the US Department of Energy in 1994 identified a number of areas of further research requirements, some of which are still relevant today:

- greater understanding of the uptake, transport and accumulation of metal ions by plants;
- greater use of genetic screens to identify the variability within hyperaccumulation traits. The purpose of this is to identify whether natural breeding could improve hyperaccumulation capabilities of naturally occurring plant species;
- better understanding of the interactions between plant root systems and the immediate rhizosphere; and
- greater number of trials of potential phytoremediating plants under relevant field conditions.

5.65 Naturally occurring metal-tolerant and metal-accumulating plants are thought to have developed in response to the presence of particular metals in the environment. These have been generated from the erosion and weathering of natural deposits as well as through pollution from anthropogenic activities. Examples of such naturally occurring plants include the lead violet (*Viola lutea*) which is able to tolerate quite

high concentrations of lead, zinc and cadmium, and can be found in areas with long histories of mining for metal ores. The plant *Alyssum bertolonii* (a member of the Brassica family) was identified growing on serpentine ultramafic soils in Tuscany, and has been found to accumulate nickel at concentrations of three to four percent of plant dry biomass.

5.66 The highest concentrations of metal hyperaccumulation by plants have been reported for zinc, nickel, manganese and more recently for cadmium, with concentrations of these elements capable of exceeding one percent of plant dry biomass. Hyperaccumulation of copper, cobalt and lead has also been reported but at lower concentrations.

5.67 Although a number of plants have been identified that are capable of growing in the presence of relatively high concentrations of metals, only a minority of these plants are able to hyperaccumulate the metal(s). The majority of the plants identified are able to grow in the presence of the metal because of their ability to exclude the metal from their cells. Such plants are described as metal excluders and include the *Viola lutea* described earlier. These plants are able to exclude the metal(s) up to a relatively high concentration, above which acute toxicity occurs and the plant is unable to survive. Work with arsenate tolerance in grasses found that genetic variations in the plant's phosphate transport system[21] conferred differential resistance to arsenate.

5.68 In contrast to metal excluders, metal hyperaccumulating plants are very effective at sequestering and accumulating metals, even when the concentrations of that metal(s) in the environment are relatively low (although saturation of the metal is reached eventually). The general consensus within the scientific community is that metal exclusion and metal hyperaccumulation are distinct strategies for growth in environments with high metal concentrations, rather than opposite ends of a continuum.

5.69 With respect to the application of metal hyperaccumulation to the treatment of metal contaminated sites, there are a number of traits expressed by metal hyperaccumulators that may be of interest. These include:

- effective mobilisation and transport of the metal from the soil into the root system. The mechanism(s) involved are however poorly understood due

[21] Arsenate is thought to enter plant cells through the phosphate transport system because of similarities between the ions.

partly to a lack of understanding of the soil system and factors determining the bioavailability of different metals;

- very effective root-to-shoot translocation system, since most of the metal accumulated by these plants is concentrated in above-ground structures (stem and leaves). This has potentially advantageous implications for the removal of the metal from contaminated sites, through harvesting/cropping of the hyperaccumulating plants (and subsequent retrieval of the metal from the plant matter[22]). However, the mechanistic basis of this translocation is poorly understood; in particular, it is not yet known why certain metal ions can be transported from the roots to the shoot so effectively in different species; and

- the mechanism by which the plants chelate the toxic metal within the plant cells. There is still inadequate information on the cellular basis of metal tolerance in plants, although it is accepted that this must rely on complexation or chelation of the metal ion within the cell. The most toxic form of most metals is likely to be the free or hydrated metal cation, and the key to the detoxification of this compound within the plant is the chelation or binding of the ion to a particular ligand. Further identification of the ligands involved and an improved understanding of their production will provide better knowledge of metal tolerance at a cellular level. The metals hyperaccumulated by the plant are thought to be stored predominantly in the central cell vacuole (which can occupy ninety percent of cell volume) and also in the apoplastic phase of the cell wall outside the cell membrane.

5.70 Many of the metals hyperaccumulated by plants are essential micronutrients, required by the plant for effective growth, albeit at much lower concentrations. Hyperaccumulation may therefore be the overexpression of a trait possessed by all plants for the acquisition of essential nutrients.

5.71 In addition to the hyperaccumulation applications discussed so far, there are a number of other phytoremediation strategies that are envisaged. These include rhizofiltration which has applications particularly in the treatment of aqueous environments, and may be more appropriate with metal-tolerant plants rather than metal-accumulating plants, due to their greater biodiversity and growth rate. Metal-tolerant plants would

[22] Where the harvested material is incinerated to reduce the quantity of plant matter and extract the metal, the ash produced may contain up to 30 percent by mass of the metal. Where the plant material is burnt in a waste to energy system, the entire process becomes a highly sustainable and environmentally friendly strategy for the treatment of contaminated land.

potentially be employed to adsorb the metal(s) onto their roots, either through the sediment or onto the cells directly.

5.72 Other strategies include phytovolatilisation and plant-assisted degradation of organic pollutants. Metal tolerant plants also have potential application in land reclamation and landscaping programmes on contaminated sites. The mining sector for example is now required to direct significant proportions of their budget to site rehabilitation and revegetation.

5.73 To date a number of field trials have been conducted using plants to remediate contaminated sites. None of these trials to date, however, have involved GM plants.

5.74 The ideal metal-hyperaccumulating plants for field application are those with a high growth rate/biomass production, good accumulation of the target compound within the biomass and a high planting density. Whilst naturally occurring metal hyperaccumulators have the advantage of being able to accumulate high concentrations of particular metals in their biomass, the majority of them have low growth rates. Poor growth rates are a consequence of these plants being adapted to growing on relatively poor and infertile soils, typically with low levels of organic matter and particular nutrients as well as the presence of metal contaminant(s).

5.75 The exceptions to the slow-growing metal hyperaccumulators are some members of the Brassica family, which whilst not true hyperaccumulators, exhibit good growth rates and reasonable metal accumulation. These plants were proposed as representing the best targets for genetic modification for metal phytoremediation.

5.76 The only example of a metal-hyperaccumulating plant in the UK is the slow-growing and low biomass forming alpine pennycress (*Thlaspi caerulescens*), that is able to hyperaccumulate between five or six different metals. This species is unusual amongst metal hyperaccumulators, which usually are only able to target one or two specific metals. For example, nickel hyperaccumulators usually demonstrate some capacity to tolerate and accumulate cobalt, but are very sensitive to copper. Multiple metal accumulation, such as that exemplified by *Thlaspi caerulescens,* is likely to be an important trait in plants developed for phytoremediation applications.

5.77 Most metal hyperaccumulators are tropical species, and in the case of nickel hyperaccumulators the greatest diversity are present on the island of New Caledonia. Most of these New Caledonian plants are trees, one of which, *Sebertia acuminata* (the blue sap tree) has in its sap the highest nickel content of any biological fluid, with nickel concentrations of up to 26 percent by dry weight of nickel. The nickel in this

tree is complexed with carboxylic acids, mainly citrate, which gives the sap its distinctive blue/green colour.

5.78 Work with other nickel hyperaccumulators has identified the importance of the free amino acid histidine in chelating the nickel within the plant immediately after uptake from the soil[23]. With the hyperaccumulating members of the genus *Alyssum* a linear relationship has been identified between the level of nickel exposure and production of histidine by the plant. This system is currently being applied on a field scale using *Alyssum* sp for the phytoextraction and subsequent recovery of cobalt from contaminated soil. The increased production of histidine by this plant has been found to be specific for nickel and cobalt, and was not observed in the case of hyperaccumulation of other metals by different species (e.g zinc hyperaccumulation by *Thlaspi caerulescens*).

5.79 A further advantage of using hyperaccumulating plants to remove metals from soil, compared to non-accumulating plants is that the hyperaccumulating plants are able to mobilise and sequester the metal at a localised level, so avoiding subsequent environmental contamination issues. This was demonstrated during a field trial on a former lead battery production site in the USA, where the metal chelator EDTA[24] was added to the site at the start of the trial to improve the bioavailability of lead in the soil. The increased availability of the lead meant that the plants could sequester and take up this metal. Although this resulted in the death of the plants, the plants were harvested within several days of EDTA application, so that the accumulated lead was removed from the site before it could leach back out of the decaying plants. However, the increased availability and therefore the mobility of the lead meant that there was increased risk of leaching of the metal to runoff or groundwater. Use of hyperaccumulator plants able to accumulate lead without EDTA applications would be a more desirable strategy for soil clean-up by phytoremediation.

5.80 The final issues that have to be addressed regarding the use of metal hyperaccumulating plants in bioremediation are the potential risks of grazing animals consuming plants containing high concentrations of metals, and the potential for the metal subsequently to enter the foodchain. Although this could occur should such plants be grown in the field, previous studies have shown that high metal concentrations within the plant actually deter grazing herbivores. Indeed, it seems likely that metal hyperaccumulation has evolved naturally as a mechanism by which the plants can protect themselves from grazing animals.

[23] Immediate chelation is important to prevent any toxic effects of the metal to the plant being realised.
[24] Ethylenediaminetetraacetic acid.

Questions

5.81 The issue of foodchain contamination was addressed during the questions that followed the presentation. The question was raised as to whether it was less hazardous to the environment and human health to have a bare untreated but contaminated site, or to introduce metal hyperaccumulating plants to that site with the risk of some metal entering the food chain through herbivores grazing on the plants. This issue was addressed further during the general discussion at the end of the workshop.

5.82 Other questions addressed the number of genes likely to be involved in hyperaccumulation; the phytoremediation of aluminium, and the potential for mutant screens to help identify genes involved in metal hyperaccumulation using well-characterised genetic models such as *Arabidopsis thaliana*. Although work is known to be underway to address the latter point using mutant screens, no details have yet been published, probably because mutations are more likely to cause an impairment of metal tolerance rather than produce a hyperaccumulator phenotype.

5.83 Regarding the number of genes involved, this was currently unknown and would pose a challenge to genetic modification technology. It was likely that the system used by plants to chelate metals and to translocate them through the plant was under the control of many different genes.

5.84 With respect to aluminium, this metal has a distinct chemistry and is characteristic of acid soil. Work is currently being conducted in Mexico using GM plants designed to overexpress the citrate synthase gene; these plants secrete elevated amounts of citrate from their roots, which serves to chelate and detoxify the soil aluminium.

PHYTOREMEDIATION OF TOXIC CHEMICALS IN OUR ENVIRONMENT

Presentation by Professor Richard B Meagher

5.85 The basis of the presentation was the application of plants for the bioremediation of pollutants in the environment. The presentation focused on the use of GM plants for the treatment of heavy metal contaminated environments.

5.86 Compared to inorganic pollutants, strategies to degrade organic contaminants are able to mineralise or reduce the organic pollutant into smaller constituents. Naturally occurring plants have already been used at a number of sites to degrade (or stimulate the degradation of) organics, and even where the plant does not degrade the

compound directly, the plant may have an indirect role in stimulating the rhizosphere microorganisms to break down the contaminants.

5.87 Results from trials at two contaminated sites using naturally occurring plants were presented. At a coal gas manufacturing site, trials with 40 plant species found that two to three plant species showed good activity against the principal contaminant benzopyrene (present at concentrations of 50 ppm). The trials were conducted by growing the plants in pots containing soil from the site. Degradation of the benzopyrene to below detectable limits was found to coincide with the plant's roots filling the pot. Trials using soil from a floor tile manufacturing site that contained 0.4 percent phthalates and 25 percent casein identified four plant species that were able to grow successfully and reduce the phthalate content in the soil significantly.

5.88 The major limitation with both of these trials was that the identification and screening for suitable plants was an extremely time consuming and labour intensive activity, taking four months to select the final plants. Although the work with naturally occurring plants has shown promising results, the use of plants genetically modified with genes from other organisms may offer a potential advance in the use of phytoremediation at contaminated sites.

5.89 Metal contaminants cannot be mineralised and are effectively immutable by any available remediation strategy. According to the USEPA, mercury is now the number one metal pollutant, with large numbers of contaminated sites across the USA. In the environment, mercury is present in the environment in one of three states:

- elemental mercury (Hg(0)) - this is the least toxic of the three forms of mercury. It is volatile and accumulates in the atmosphere where it has a residence time of several years and is not subject to wet deposition;

- ionic mercury (Hg(II)) - this has moderate toxicity and has a transit time in the atmosphere of several weeks. Hg(II) is subject to wet deposition; and

- methylmercury - this is the most toxic of the three forms, and is approximately 1000 times more toxic than Hg(0). Methylmercury is produced from ionic mercury in the environment by microbial activity, particularly in wetland sites. The compound is also bioaccumulated through the foodchain, with a 50-fold increase in toxicity at each trophic level. As with ionic mercury, methylmercury is also subject to wet deposition from the atmosphere where it has a transit time of only a few weeks.

5.90 The potential application of bioremediation for the treatment of mercury contaminated sites has developed from the identification the *mer* operon in bacteria. Two of the genes *merB* and *merA* are known to be able to confer on bacteria the ability to convert methylmercury to ionic mercury (*merB*) and Hg(II) to elemental mercury (*merA*).

5.91 The ability of bacteria to detoxify methylmercury and ionic mercury was proposed to have developed as a consequence of the prokaryotic nature of bacteria and the fact that their electron transport chain, which is particularly sensitive to mercury, is located in the outer membrane of these organisms. In order to avoid the toxic effects of the mercury, bacteria are proposed to use a *mer* operon encoded pump to sequester and pump Hg(II) into the bacterial cell where it is converted into the less toxic Hg(0), which then diffuses passively out of the cell.

5.92 By extending this biotechnical hypothesis to plants, it was proposed that because the electron transport chains in plants are located within organelles, protected by a layer of cytoplasm, then plants are likely to be less immediately sensitive to the toxic effects of mercury. Plants may therefore be more suitable to bioremediate mercury, using the plant's systems to sequester and extract the mercury from the environment and recombinant bacterial *merA* and *merB* genes to detoxify the mercury within the plant to Hg(0) which would then be volatilised from the plant into the atmosphere.

5.93 Initial work with GM plants has focused on the development of plants modified with the bacterial *merA* gene. Due to the high guanine/cytosine (GC) content of this gene and the strange codon usage, the bacterial gene had to be reconstructed before it could be expressed in the plant. However, GM ($merA^+$) tobacco were found to be able to grow in soil containing Hg(II) at 500 ppm. Growth of the GM tobacco plants was slow initially, although this was attributed to the small size of the plants at this stage. Once the plants initiate operation of photosystem II, their growth improved significantly and levels of Hg(II) were reduced. Similar findings were obtained using $merA^+$ yellow poplar.

5.94 Although the GM plants were shown to be successful against high concentrations of ionic mercury (the non-GM control plants did not survive), the majority of mercury contamination in the environment is low level. For example, light bulb manufacturing plants have mercury in their waste streams at levels of 1 ppm.

5.95 Tobacco plants modified to express *merA* and developed to grow hydroponically were found to be able to remove 70 percent of the Hg(II) from a 1 ppm solution within one week. Both the $merA^+$ plants and the non-GM control plants reduced the mercury content in the solution to below detectable levels within several hours. This was a

consequence of the strong sorption of mercury to the root systems. Although they were able to sequester the mercury, the control plants did not survive the experiment.

5.96 Although these experiments demonstrated the potential for GM plants to convert ionic mercury to the less toxic elemental mercury, the environmental contamination of methylmercury is a more significant pollution issue. It was proposed that by converting methylmercury to ionic mercury, this would prevent the more toxic methylmercury from entering the foodchain.

5.97 In laboratory mesocosm studies, tobacco genetically modified to express *merB* were able to survive in the presence methylmercury at concentrations approximately 20 times greater than those detected in the environment, and much higher than the 0.1 μM concentration that was sufficient to kill the non-GM control plants. In the absence of methylmercury, the GM plants grew almost as fast as the non-GM plants.

5.98 Where the tobacco plants were modified to express both *merA* and *merB* they were able to grow at even higher concentrations of methylmercury than plants expressing just *merB*. The $merA^{+}merB^{+}$ plants were also observed to be healthier than the $merB^{+}$ plants in the presence of the same concentrations of methylmercury. Alteration of the level of expression of the *merA* gene in the $merA^{+}merB^{+}$ plants was found to have no effect on the detoxification of methylmercury by the modified plant. However, increasing expression of *merB* did increase the detoxification, although not in a linear fashion. A ten-fold increase in *merB* activity only produced a two-fold increase in the *merA*/*merB* coupled reaction.

5.99 In the original experiments, the *merB* gene was expressed in the plant cytoplasm. When the plants were modified to express the gene either in the endoplasmic reticulum (ER) or in the cell wall, no changes were observed in the coupled reaction. However, the level of expression of *merB* in either the ER or cell wall was 20 to 100 times lower than in the cytoplasm, indicating a much greater specific activity of the merB enzyme following expression of the gene in the ER or cell wall. Studies to investigate the broader implications of these findings are now underway.

5.100 With respect to applying all experimental studies using plants to treat contaminated sites, it is essential that the range of plant species studied to date is extended to include plants other than tobacco and *Arabidopsis*. Because of the high bioaccumulation of methylmercury within wetland environments, the goal of any plant selection needs to be to identify plants that can thrive in a wetland habitat. Therefore work is now underway to trial GM rice, cottonwood, willow, sweetgum and yellow poplar.

5.101 It was concluded that by using these types of plants it should be possible to harness GM technology to target ionic and methylmercury in the environment and to block the formation of methylmercury and its subsequent bioaccumulation within the foodchain. Although this conclusion had yet to be supported by field data, the results from the laboratory and mesocosm work certainly suggest that it should be possible. Because of the severity of the pollution of many sites with extremely recalcitrant compounds such as mercury, the use of non-GM plants is unlikely to be sufficient to deal with the contamination. Therefore, for such compounds, GM plants pose the most realistic *in situ* remediation system.

5.102 In addition to the work on the phytovolatilisation of mercury, a number of other applications of GM plants for phytoremediation were also presented, including the:

- use of plants for phytomining - the *merA* gene for example has good activity towards gold;

- development of above ground accumulators for mercury - such plants would hyperaccumulate mercury rather than volatilise it off during transpiration. The *merA*$^{+}$ plants have little mercury in their above ground biomass as they volatilise it. However, by grafting a wild type plant onto a GM *merA*$^{+}$ root system, reasonable hyperaccumulation was achieved in the above ground parts of the plant. It was proposed that the level of accumulation could be improved further by generating a bigger mercury sink in the above ground part of the plant through for example, the addition of a phytochelatin system.

  The use of grafted plants was reported to be unsuitable for field use. Using non-grafted plants would require root specific promoters for example; and

- development of GM plants to target arsenic and cadmium - initial work in this area has focused on the generation of GM plants expressing the gamma-glutamyl cysteine synthetase system (thought to be the rate limiting step of the three part phytochelatin system). The GM plants were slightly retarded compared to the wild type in the absence of arsenite, but were able to survive exposure to arsenite, unlike the wild type.

5.103 It was concluded that different strategies may be expected for different metal pollutants, but that based on the work conducted to date within the field, then even six gene systems such as the one involved in arsenic detoxification could now be addressed.

Questions

5.104 The questions raised after the presentation addressed the importance of indigenous rhizosphere microorganisms in the sequestration and detoxification of the mercury and arsenic, and also the identification of plant homologues to the *merA* and *merB* genes. These have been identified in plants using genomic screenings, but are not sequence homologues.

5.105 The studies presented were all based on non-sterile soils. Rhizosphere microorganisms were present in each experiment and thus may have been expected to have played a role in the phytoremediation process at some level. Non-sterile soil was used in the experiments in order to replicate field conditions.

DEFUSING THE ENVIRONMENT: ENGINEERING TRANSGENIC PLANTS TO DEGRADE EXPLOSIVES

Presentation by Dr Neil Bruce

5.106 The presentation addressed the work that has been conducted to date on the use of enzymes, microorganisms and plants to degrade explosives. Explosives were described as true xenobiotic compounds and in some cases have been introduced to the biosphere for only tens of years. This is an insufficient period of time for microorganisms to evolve metabolic pathways to utilise and consequently degrade these compounds.

5.107 To date microorganisms have been isolated that are able to grow on all of the major classes of explosives, usually using the explosive as a nitrogen source. However, due to their high recalcitrance and toxicity, explosives are proposed as one group of pollutants that may require genetic engineering techniques to remediate them.

5.108 The most significant sources of explosives contamination are the ordnance manufacturing and disposal sites. An ordnance manufacturing plant for example generates 500,000 gallons of explosive contaminated waste water a day which is discharged to on-site reed beds. The relatively short shelf-life of ordnance means that levels of disposal are potentially high. Some contaminated sites are so polluted with explosives that they are bare of vegetation.

5.109 The initial interest in identifying microorganisms capable of growing on explosives was the development of biosensors for these compounds. By isolating microbial degraders, the relevant enzymes can be identified and used as the recognition

components in the sensors. These enzymes can however also be used as the basis for bioremediation strategies.

5.110 Microorganisms such as *Rhodococcus* sp have been isolated that grow on RDX (Royal Demolition Explosive) as a sole nitrogen source and an *Enterobacter cloacae* has been isolated that can use PETN (pentaerythritol tetranitrate) and TNT (trinitrotoluene) as a sole source of nitrogen. The structures of the enzymes involved in these pathways have been determined and this information can potentially be used to engineer the enzyme and improve their activity against the explosive.

5.111 Enzymes similar to those isolated from *Rhodococcus* sp and *Enterobacter cloacae* are present in a wide range of microorganisms. Due to the short time explosive compounds have been present in the biosphere, these enzymes could not have evolved in response to explosives and are thought to have been developed as part of the organism's antioxidant defence mechanism. All of the enzymes show activity against nitrate ester explosives such as GTN (glycerol trinitrate), but only those very closely associated to PETN reductase will degrade TNT.

5.112 The potential for direct evolution using gene shuffling to improve activity against explosives was also investigated. This technique involves cutting, mixing and reannealing genes from different organisms to generate chimaeric mutants. Gene shuffling is a very powerful technique, but is limited by the screening system available to select the mutants generated. Gene shuffling was described as a means of effectively speeding up the evolutionary process. Once potentially useful genes have been identified these can be reinserted into organisms for use in the environment.

5.113 As has been discussed in the previous two presentations, plants and phytoremediation offer a number of advantages (scientific and 'presentational') over the use of bacteria to degrade pollutants. Plants generate a large quantity of biomass, have large root systems, are cheap to grow and have a relatively good public perception. It was proposed that the public were more likely to accept the release of plants for bioremediation than microorganisms. It was also suggested that in the short term at least there was a higher chance of releasing GM plants for bioremediation than GM microorganisms. Rhizosphere interactions mean that plants grown on a contaminated site have the added advantage of being able to stimulate the natural microflora to assist in the bioremediation process.

5.114 However, although plants such as pondweed (*Lemna* sp.) have been used to degrade organic pollutants[25], the phytoremediation of organics is limited by the relatively low metabolic diversity of plants towards these compounds, certainly when compared to microorganisms. Therefore, the genetic modification of plants with bacterial genes may offer the best solution to the phytoremediation of organic pollutants.

5.115 The final part of the presentation focused on the development and potential of plants modified with the PETN reductase system or the nitro-reductase system. To date only GM tobacco[26] has been produced, although trials with more robust plants such as poplar are planned.

5.116 Studies have shown that modification of tobacco with PETN reductase resulted in high levels of expression of the enzyme. Germination of the GM tobacco was unaffected by exposure to 1 mM nitroglycerine. The GM plant grew as well in the presence of nitroglycerine as the wild type plant did in the absence of this compound. The non-GM wild type control plant did not germinate in the presence of 1 mM nitroglycerine.

5.117 Tobacco modified with the nitro-reductase system grew well in the presence of TNT at concentrations ranging from 0.1 mM to saturation. At sites contaminated with high levels of explosives, compounds such as TNT can be present at saturation levels, highlighting the application of this technology to bioremediating explosives waste in the environment.

5.118 The results of studies with plants modified with the nitro-reductase system were reported to be surprising, as they were expected to be less resistant to TNT than those modified with the PETN reductase. This is because the degradation pathway of TNT by nitro-reductase is reported to generate toxic metabolites. However, on exposure to 0.25 mM TNT both the wild type and PETN reductase modified plants died, whilst the transgenic nitro-reductase plants remained healthy.

5.119 The results with tobacco plants were used as an indication of the potential for GM-based phytoremediation strategies to target sites contaminated with explosives. To date only incineration and landfill have been available for reducing the contamination levels in these sites. (Techniques such as biopiling are probably too costly). In the USA, plants used to phytoremediate explosives waste are disposed of by composting.

[25] Trials in the USA have used pondweed to remove TNT from contaminated aqueous environments.
[26] Tobacco is used as a model system as it is easy to transform and generates large quantities of biomass.

Unlike metal phytoremediation, plants are able to degrade toxic organics, although some toxic metabolites may remain in the plant.

5.120 Further work is now underway to develop, through gene stacking, plants capable of degrading several classes of explosives. Sites contaminated with explosives typically contain more than one class of these toxic compounds.

DISCUSSION ON THE USE OF GMOs FOR THE BIOREMEDIATION OF POLLUTANTS

Chaired by Professor Chris Leaver

5.121 The final session of the workshop was intended as a general discussion forum on the use of GMOs in bioremediation. The issues discussed in this session can be summarised as:

- the development of bioremediation applications incorporating both plant and microbial based strategies;
- the potential for plants employed in phytoremediation strategies to have secondary effects on the environment and/or human health;
- the perception of the risk of bioremediation strategies by the general public;
- the advantages of GMOs over non-GMOs for bioremediation strategies; and
- the information that is required for the further development of bioremediation applications.

Incorporation of plant and microbial based strategies

5.122 In the environment, terrestrial plants do not exist in the absence of a rhizosphere microbial community. However, although the importance of the rhizosphere is accepted, there is only very limited understanding within the scientific community of the interactions between plants and microorganisms, even at the most basic level. Because of the range of different processes and reactions involved, the interactions between plants and microorganisms in the rhizosphere are likely to be complex. It was suggested that experiments were needed that would identify these interactions and assess how important they were in phytoremediation.

5.123 The introduction of plants to the environment will increase the metabolic activity of the rhizosphere microbial community by the exudation of carbon into the soil. By introducing the appropriate plant into an environment, the rhizosphere may effectively be 'fine tuned' so that the rhizosphere community operates to its greatest potential in terms of remedial activity. A potential application of genetic modification technology is to develop plants that are able to introduce specific carbon substrates, for example, benzoate analogues into the environment. This will have the effect of increasing the relative abundance and activities of those microbial populations that are effective at degrading the target pollutant(s). Because naturally occurring microorganisms such as those in the rhizosphere are more suited to survival in their particular environment, then the combination of GM 'supporter' plants and non-GM microorganisms may be a successful approach for phytoremediation.

5.124 For example, field trials are now underway in Mexico involving plants genetically modified to secrete citrate from their roots as a mechanism to block uptake of aluminium by the plants, and ensure their survival in acidic soils (which tend to be high in aluminium). A naturally occurring aluminium tolerant plant has also been identified with a mutated hydrogen ion pump that causes an increase in the pH of the rhizosphere by 0.7 units. This is likely to have a significant impact on the biodiversity of the rhizosphere community and demonstrates the potential effect different plants could have on the soil microorganisms.

Secondary effects of organisms used in bioremediation

5.125 The organisms employed in bioremediation strategies (plants and microorganisms) are constructed to do a specific job or have a primary effect, for example degrade or accumulate the pollutant, or support the activities of other organisms present. Many of the organisms designed for use in the field are, however, developed and assessed as a monoculture in a controlled environment. In the field these GM organisms are likely to exist as part of a heterogeneous community, so their activities should never be thought of as one of a mono-culture, but as part of a complex and dynamic community.

5.126 Therefore, there is the potential for the introduced organisms to have secondary effects that were not identified during their development in a monoculture. For example, a microorganism designed to degrade a particular pollutant may also have activity in the environment against a second compound that is required as a growth substrate by another organism that has a pivotal role in its habitat.

5.127 This was recognised as a possibility by the panel of speakers, particularly because many pollutants have not been present in the biosphere for sufficient time for organisms to develop effective metabolic pathways to degrade them. Many catabolic pathways therefore have kinetic bottlenecks and involve the misrouting of metabolites to unproductive end points. The development of genetically modified organisms has been proposed as possibly amplifying the potential for such misrouting to occur. However, it was recognised that this should be addressed when designing the GMO. It was important to determine what was going to happen at the target environment (or type of target environment) before any large scale release, and not to rely solely on the results from laboratory monoculture experiments.

5.128 With plants, the production of pollen and fruit, herbivory of the plants by other organisms and the accumulation of toxic compounds, may all have secondary effects on the surrounding environment and/or human health. The risks posed by the transfer and dissemination of pollen, seed and fruit may be avoided by using, for example, male sterile plants or plants that reproduce by vegetative means.

Perception of risk by the general public

5.129 With respect to the perception of risk by the general public of using GMOs for the bioremediation of pollutants, there was considerable discussion on the commitment of various groups (general public, industry, regulators, NGOs[27]) to remediate contaminated sites, and at a more basic level, in answering the question "can we afford to do nothing?" (with respect to chemical pollution). Although the use of GMOs may have some adverse effects on the environment, the risk of these possible effects must be balanced against the risk of leaving the contaminants untreated or remediating the site using other technologies. It is also important to address whether significant resources should be employed to remove every last trace of a contaminant from a site, or to use less effort to remove the bulk of contaminants present, and then to use the resources saved to remediate tens of other contaminated sites.

5.130 In the UK, 'dig and dump' treatment of contaminated material is currently the most cost effective strategy and is encouraged by landfill tax exemptions. Therefore, the remediation of contaminated sites may be expected to be treated in this way until the economics change or there is pressure from NGOs or the general public, for example, to use alternative and more sustainable technologies. Pressure to use different technologies is likely to arise from a change in the perceived costs/benefits of the

[27] Non-government organisations.

respective methodologies and their risks to the environment and human health, as well as concerns about sustainability.

5.131 In the USA, the driving force behind the choice of remediation strategy is economic rather than regulatory. In particular, remediation technologies are selected on the basis that they offer the opportunity to clean-up a site for the lowest expense. Bioremediation has the potential to provide the cheapest option for the remediation of contaminated sites, and on a site specific basis may offer potential benefits and/or less risk to the environment than other technologies. However, since bioremediation is a biologically based process and is rate limited, often being slower than alternative approaches such as dig and dump. If bioremediation is to be a feasible option, it will be essential to improve long term management and planning of land use. Better long term planning should provide more time for the biological process to become a feasible option.

5.132 At a fundamental level, irrespective of the specific purpose of the plants (GM or non-GM) used in phytoremediation applications, the action of covering what is often a poorly vegetated or barren contaminated site with healthy vegetation should be expected to receive a positive public perception. If the site is intended for housing, then it was proposed as possibly beneficial to 'green' the site for a period of time before developing the housing. In the long term it may then be easier to sell the houses if there is a less immediate link between the site as a contaminated area and the site as a residential area. This green amenity type of phytoremediation application is most suited to naturally occurring plants, or possibly GM plants designed to optimise rhizosphere activity.

5.133 Regarding the field release of the GM *Pseudomonas fluorescens* HK44 at Oakridge National Laboratory described at this workshop, the release encountered virtually no adverse public reaction. The general public within the vicinity of the site were kept informed of what the trial would entail and there was extensive communication with the press. However, it was noted that because Oakridge National Laboratory also make material for nuclear weapons, the local population around the site may be relatively ambivalent to the field trial of some GMMs.

5.134 A similar situation however was reported at a contaminated site in Long Island Sound, New York. Here there was a highly vociferous NGO trying to get the site cleaned up. Although the regulators favoured excavation and re-burial of the contaminated material, residents in the vicinity of the site wanted the option of using of GM plants to bioremediate the site rather than a re-burial strategy.

5.135 Approval for field trials of tobacco genetically modified with *merA* was reported to have been received from the USEPA with relatively little resistance (six week approval time), although the USEPA required that the site be well fenced and away from the general public. (The GM tobacco trial was approximately 100 miles away from the nearest commercial tobacco plantations). As with the Long Island Sound site, the owners of the contaminated material had approached the phytoremediators as they (the site owners) felt it was morally unacceptable to dig and dump the contaminated sediment (although they had approval from the regulators to do so).

5.136 It was concluded that improving the public perception of all applications of GM technology should increase the acceptance and demand for bioremediation based strategies for the clean-up of contaminated land. This would be particularly the case when the public was made aware that for many contaminated sites GM technology may be the only feasible approach other that dig dump, to clean-up a contaminated sites. GMOs have potential application for use in end of pipe treatment of contaminated waste and even for the production of added value products such as specific polymers from low level waste materials. Other GMO applications that may be envisaged may include the use of plants and/or microorganisms for carbon sequestration to combat global warming, and for biomining to recover or extract metals such as cobalt from mining waste or other contaminated material.

Specific advantages of GMOs for bioremediation

5.137 The specific advantages and applications of GMOs in bioremediation have been addressed in the report that accompanies this workshop report. However, it was proposed at the workshop that recalcitrant and newly synthesised compounds represented the most likely targets for direct bioremediation by GMOs.

5.138 In addition to the use of GMOs to directly sequester or degrade the target pollutant, it may be more effective to manipulate the organisms already present at the contaminated site to target the pollutant(s). These organisms are more predisposed to bioremediating the contaminants for a wide variety of reasons compared to any organism that is introduced into the environment (GM or non-GM). It is recognised that the necessary catabolic genes to degrade the pollutant(s) are likely to be present in the contaminated site. Therefore it should be possible, by manipulating the environment, to reshuffle those genes within the biota and generate whole new catabolic pathways so that the natural flora and fauna are able to bioremediate the pollutant(s). Similarly, it should be possible to preferentially stimulate beneficial activities and populations. However, it was recognised that where a large amount of gene space is required to be manipulated then the natural biota may be expected to

take a long time to evolve to do the job. In these situations, bioaugmentation with GM or non-GM organisms may be required.

5.139 Bioremediation of pollutants by the natural biota is often restricted by certain key metabolic bottlenecks. It was proposed that GMOs could be applied to identify those bottlenecks and alleviate them in the contaminated environment.

5.140 With respect to the use of biosensors, these were described as being very good at assessing the bioavailability of pollutants across several scales of organisms. However, as sensors to determine human toxicity, it was recognised that microbial biosensors should only be applied as a rapid pre-screening system. Whilst they were useful to indicate a possible problem for human health they should not be applied further.

What information is required?

5.141 Although a large amount of knowledge is available to develop and employ organisms to bioremediate contaminated sites, it was highlighted several times during the workshop that there were still a number of basic research questions that needed to be addressed, and that the commitment to remediating contaminated land needed to be resolved. Many of these questions are due to the fundamentally poor understanding of the diversity and activity of microbial communities in the environment, both GM and non-GM.

5.142 At the fundamental level, information was required on the function of microbial processes in the environment, and in particular in the rhizosphere. It was recognised that is was important to identify which activities were important to bioremediation and how to optimise them. These information requirements were applicable to both GM and non-GM organisms.

5.143 In addition to requiring further information to answer these fundamental base-line questions, the most pressing requirement was to determine the commitment to clean-up contaminated sites. Bioremediation applications were recognised as having the ability to treat contaminated environments now, and that further research would optimise existing processes and identify new ones. However, it was concluded that:

- the questions that asked whether the use of a particular organism in bioremediation may or may not have an effect on the environment could only be answered by conducting environmentally relevant experiments and field trials; and

- the potential for bioremediation to cause adverse effects to the environment and/or human health should be addressed in the context of the alternatives, namely leaving the contamination untreated or using other remediation technologies. Such assessment may require a form of cost/benefit analysis comparing the different methods available.

6. CONCLUSIONS

6.1 The purpose of this report has been to identify current and future applications of GMOs in bioremediation and to address the potential risks and available management strategies related to their use in the field. From the information reviewed in this report, it may be concluded that GMOs offer a wide number of potential applications for the bioremediation of contaminated environments. GMOs offer the ability to bioremediate a large range of pollutants, including organic and inorganic compounds, in both terrestrial and aquatic environments.

6.2 Existing applications of GMOs in bioremediation may be divided into:

- GMMs designed to degrade organic pollutants, either *in situ* in terrestrial environments, or in *ex situ* applications such as bioreactors or as part of 'pump and treat' systems;

- GMMs employed as biosensors to determine the presence and potential toxicity of pollutants present in a contaminated site (aquatic or terrestrial). Depending on the system used, the biosensor can be designed as a general screening system to determine the combined toxicity of all pollutants present, or as a more targeted system to detect the presence of specific compounds; and

- GM plants designed to hyperaccumulate or volatilise metal pollutants. Such systems also have applications in aquatic and terrestrial environments.

6.3 Although the development of other GMOs for bioremediation applications has been reported, for example the generation of GM plants to degrade organic pollutants such as nitroaromatic compounds, the three areas outlined above offer the most immediate application of GM technology to the treatment of contaminated land.

6.4 Indeed, although no *in situ* applications of GMOs for bioremediation have been conducted to date in the UK, field trials of GM plants and GMMs have been and are underway in other countries (see workshop report). The use of GMMs as biosensors in *ex situ* applications has been applied in the UK as, for example, a preliminary

screening system to identify the presence of pollutants within a large contaminated site. It should be noted that the use of GMMs in this way does not involve any release of GMMs into the environment and is essentially risk free.

6.5 The application of GMOs for the bioremediation of pollutants offers a number of advantages (and disadvantages) over bioremediation strategies using non-GM organisms and compared to physical or chemical remediation strategies. An advantage of bioremediation strategies (using GM or non-GM organisms) compared to physical or chemical processes is one of cost. This is due, in part, to the low maintenance costs of using the technology (once the organism has been identified and tested), and also to the ability of bioremediation processes to target particular pollutants selectively, without the need for the wholesale removal of all the material from the site (irrespective of the level of contamination present).

6.6 The disadvantages of bioremediation applications are a consequence of physiological characteristics of the organism(s) used. Phytoremediation of contaminated soils, for example, has huge potential for the low-cost treatment of large sites where the contaminants are present at relatively low levels, but throughout the first few metres of the soil across the site. In such cases the pollutants will be well within the vicinity of root systems of the plants and available for phytoremediation.

6.7 However, careful selection of the organism(s) used for the bioremediation strategy should avoid any significant disadvantages posed by particular organisms, and should improve the success of the bioremediation process.

6.8 Although existing applications of GMOs in bioremediation are limited largely to the three areas highlighted at the start of this section, there are a significant number of publications highlighting the future applications of GMOs in this field. These may be divided into three broad areas:

- the identification of the genetic basis of the processes involved in the bioremediation of pollutants. This includes the identification of the particular genes responsible, for example, the degradation of a particular compound, as well as the identification of the necessary promoter and terminator sequences;

- the application of GM technology to improve non-GM bioremediation processes. Many of the applications to use GMOs have developed by altering or improving existing biochemical processes. Developments include the identification and modification of rate limiting steps of a catabolic pathway, or

the alteration of the pathway to avoid the production of toxic or dead end metabolites; and

- the improvement and development of existing GMOs used in bioremediation. The initial applications of GMOs for bioremediation focused on the development of organisms capable of bioremediating the target pollutant(s). The effectiveness of many of these applications has, however been restricted by the poor performance of the GMO in the environment. The environment in which the GMO is intended to function is an important consideration as is the nature of the target pollutant. This is now being addressed for example, through the genetic modification of aquatic microorganisms, rather than terrestrial ones, for the treatment of pollutants in aquatic environments; and the development of GMOs capable of degrading the target pollutant as well as tolerating toxic concentrations of other pollutants, such as radionuclides.

6.9 The development of each of these three areas is restricted by a lack of information, both at the genetic and metabolic level, and also at the population and environmental level. As discussed during the workshop, information on how microorganisms, plants and substrates (including pollutants) interact in the environment will assist in the development of GMOs, and indeed non-GM organisms for bioremediation immeasurably.

6.10 The risks posed by the use of GMOs in bioremediation depend on the characteristics of the particular organism and those of the site in which it is used. For GMMs, the most significant risks were determined to result from the transfer of the recombinant genes to other organisms, and the disruption of other organisms and biological processes. In both cases the level of risk that might actually occur can be minimised by using GMMs with stable genetic insertions, and with no superfluous recombinant material, that may confer a selective advantage over organisms already present in the environment.

6.11 For plants, the transfer of the inserted genes can be minimised, for example, by managing the plants so that flowering and transmission of pollen does not occur. Other possible risks that may be associated with GM plants, such as the accumulation of toxic compounds may be reduced by ensuring that the herbivory of the plants does not take place, or by developing plants where the compounds are accumulated in non-grazed areas of the plant.

6.12 In assessing the risks posed by the use of GMOs in bioremediation, the nature of the site and the alternatives to bioremediation should be considered. As a result of their

contamination, sites targeted for bioremediation are relatively inhospitable habitats, characterised by a limited number of micro and macroorganisms. In many cases, the level of contamination is so high as to render the site barren of all vegetation. Therefore, the implications of using GMOs on such sites, with respect to the effect they may have on natural flora, fauna and biological processes, may not be great, particularly where the site has a very low level of biological activity.

6.13 As discussed in the risk assessment in Chapter 3 of this report, the risks posed by the use of GM plants in bioremediation are similar to those encountered with GM plants used in agriculture. However, the differences between arable environments and contaminated sites will have a significant effect on the level of risk that is likely to be realised.

6.14 In addition to offering the potential to remove or sequester specific pollutants from a contaminated site, GMOs also have indirect applications for bioremediation. These may include the use of pollutant tolerant plants in phytostabilisation strategies, and the application of GMOs (microorganisms or plants) to support and supplement the bioremediation activities of non-modified organisms. It is these combined strategies for bioremediation, involving a number of organisms (plants and microorganisms, GM and non-GM) that offers very significant potential for the bioremediation of a wide range of sites contaminated with a cocktail of pollutants.

7. RECOMMENDATIONS FOR FUTURE WORK

7.1 The purpose of this section of the report is to identify and propose areas of future work that are required in order to develop the application of GMOs for the bioremediation of organic and inorganic pollutants and assess the potential risk of their use in the environment. During the production of this report, two broad areas of work have been identified. These relate to the development of the GMOs themselves, and also the understanding of the processes that underlie the survival, behaviour and activities of organisms in the environment. Although this latter area is not confined to GMOs, information on how microorganisms and plants behave in the environment, and interact with themselves, each other and the pollutants, will only improve the efficiency of GMOs in bioremediation, as well as reducing the level of any potential risk to the environment and human health.

7.2 The application of GMOs for bioremediation has, to date, focused on the use of single species of microorganisms or plants to target and bioremediate the contaminant. However, the use of combined strategies, involving a number of GM plants and/or microorganisms may offer a more powerful approach for treating contaminated sites. This is particularly the case for those sites where the contaminants present offer little nutritional benefit on their own, either due to their high toxicity or low concentrations. Because the effectiveness of such combined strategies will depend on maximising the interaction between the different organisms involved, then further research into these areas would be extremely useful. This was one of the recommendations from the workshop. One important example of such research is:

- the investigation of how indigenous microbial communities influence the acquisition and accumulation of heavy metal pollutants by GM plants.

7.3 With respect to the development of the GMOs themselves, there are areas for further work at almost every stage of the developmental process. These include:

- the identification of the metabolic pathway(s) involved in the degradation or accumulation of pollutants;

- the identification of the genes involved and the promoter sequences and effectors that control them;

- the identification of organisms suitable for modification with the relevant genes; and

- the best approach to using those organisms to bioremediate the contaminated site, to ensure that any risks to the environment and human health from the GMO or the pollutant are minimised.

7.4 Further work is also recommended to test the activity of the GMOs in their target environment, and to assess the effect their use may or may not have on indigenous organisms present. Examples include:

- proof of concept studies. Are the GMOs effective at site clean-up, and what can they achieve or perform in the field that indigenous (non-GM) organisms cannot; and

- cost/benefit analyses of using GMOs for bioremediation, rather than indigenous plants and/or microorganisms.

7.5 The more advanced applications of GMOs developed to date are those where the GMO has been selected for its ability to survive and grow in the contaminated environment, as well as expressing the recombinant genes. These include floc-forming aquatic microorganisms designed to biodegrade PAHs in wastewater treatment systems, and wetland plants and trees modified to remove methylmercury or Hg(II) from contaminated marshlands.

7.6 For any bioremediation strategy to work effectively, the organism must survive and compete in the contaminated environment and be able to degrade or accumulate the target pollutant. Many contaminated sites are characterised by a cocktail of organic and inorganic pollutants. Any organisms released into the site must therefore be tolerant or resistant to a wide range of compounds, in addition to being able to degrade the intended pollutant. Further work will almost certainly be needed to identify suitable organisms and bioremediation strategies for the more contaminated sites.

7.7 However, based on the information already available, and the organisms and strategies that have been developed to date, it is recognised that the use of GMOs for

the bioremediation of contaminated sites offers and extremely powerful suite of strategies for the low cost and potentially low-risk removal of toxic compounds from polluted soils and aquatic systems. Further work into any aspect of the use of GMOs in bioremediation can only improve the development of this field.

8. REFERENCES

1. Leisinger T (1983). Microorganisms and xenobiotic compounds. *Experentia*, 39: 1183-1191.
2. Cervantes C and Gutierrezcorona F (1994). Copper resistance mechanisms in bacteria and fungi. *FEMS Microbiology Reviews*, 14(2): 121-137.
3. Vanbeelen P, Fleurenkemila AK, Huys MPA, Vanmontfort ACP and Vanvlaardingen PLA (1991). The toxic effects of pollutants on the mineralization of acetate in subsoil microcosms. *Environmental Toxicology and Chemistry*, 10(6): 775-789.
4. Timmis KN and Pieper DH (1999). Bacteria designed for bioremediation. *Trends in Biotechnology*, 17: 201-204.
5. MAFF, *A feasibility study on the potential for exploiting genetic variability in crop and non-crop species to remove contaminants for the food chain*. 1998.
6. Korda A, Santas P, Tenente A and Santas R (1997). Petroleum hydrocarbon bioremediation: sampling and analytical techniques, in situ treatments and commercial microorganisms currently used. *Applied Microbiology and Biotechnology*, 48(6): 677-686.
7. Mason JR, Briganti F and Wild JR, *Protein engineering for improved biodegradation of recalcitrant pollutants*, in *Perspectives in Bioremediation*, Wild JR *et al.*, Editor. 1997, Kluwer Academic Publishers: Netherlands. p. 107-118.
8. Garbisu C and Alkorta I (1999). Utilization of genetically engineered microorganisms (GEMs) for bioremediation. *Journal of Chemical Technology and Biotechnology*, 74(7): 599-606.
9. Head IM (1998). Bioremediation: towards a credible technology. *Microbiology*, 144: 599-608.
10. Timmis KN, Steffan RJ and Unterman R (1994). Designing microorganisms for the treatment of toxic wastes. *Annual Reviews Microbiology*, 48: 525-557.
11. Strong LC, McTavish H, Sadowsky MJ and Wackett LP (2000). Field-scale remediation of atrazine-contaminated soil using recombinant *Escherichia coli* expressing atrazine chlorohydrolase. *Environmental Microbiology*, 2(1): 91-98.
12. Sayler GS (1991). Contribution of molecular-biology to bioremediation. *Journal of Hazardous Materials*, 28(1-2): 13-27.
13. Brazil GM, Kenefick L, Callanan M, Haro A, de Lorenzo V, Dowling DN and Ogara F (1995). Construction of a rhizosphere pseudomonad with potential to degrade polychlorinated-biphenyls and detection of *bph* gene- Expression in the rhizosphere. *Applied and Environmental Microbiology*, 61(5): 1946-1952.

14. Gallardo ME, Ferrandez A, de Lorenzo V, Garcia JL and Diaz E (1997). Designing recombinant *Pseudomonas* strains to enhance biodesulfurization. *Journal of Bacteriology*, 179(22): 7156-7160.

15. Lange CC, Wackett LP, Minton KW and Daly MJ (1998). Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments. *Biotechnology*, 16: 929-933.

16. DiToro DM, Zarba CS, Hansen DJ, Berry WJ, Swartz RC, Cowan CE, Pavlou SP, Allen HE, Thomas NA and Paquin PR (1991). Technical basis for establishing sediment quality criteria for nonionic organic-chemicals using equilibrium partitioning. *Environmental Toxicology and Chemistry*, 10(12): 1541-1583.

17. Zhu YL, Pilon-Smits EAH, Jouanin L and Terry T (1999). Overexpression of glutathione synthetase in *Brassica juncea* enhances cadmium accumulation and tolerance. *Plant Physiology*, 119: 73-79.

18. Cunningham SD, Berti WR and Huang JWW (1995). Phytoremediation of contaminated soils. *Trends in Biotechnology*, 13: 393-397.

19. Meagher RB (2000). Phytoremediation of toxic elemental and organic pollutants. *Current Opinions in Plant Biology*, 3(2): 153-162.

20. Kumar S, Mukerji KG and Lal R (1996). Molecular aspects of pesticide degradation by microorganisms. *Critical Reviews in Microbiology*, 22(1): 1-26.

21. Drobník J (1999). Geneticall modified organisms (GMO) in bioremediation and legislation. *International Biodeterioration and Biodegradation*, 44: 3-6.

22. Romantschuk M, Sarand I, Petanen T, Peltola R, Jonsson-Vihanne M, Koivula T, Yrjala K and Haahtela K (2000). Means to improve the effect of in situ bioremediation of contaminated soil: an overview of novel approaches. *Environmental Pollution*, 107(2): 179-185.

23. Sayler GS and Ripp S (2000). Field applications of genetically engineered microorganisms for bioremediation processes. *Current Opinion in Biotechnology*, 11(3): 286-289.

24. Ramos JL, Diaz E, Dowling D, de Lorenzo V, Molin S, Ogara F, Ramos C and Timmis KN (1994). The behavior of bacteria designed for biodegradation. *Bio-Technology*, 12(13): 1349-1356.

25. Wackett LP, Sadowsky MJ, Newman LM, Hur HG and Li SY (1994). Metabolism of polyhalogenated compounds by a genetically-engineered bacterium. *Nature*, 368(6472): 627-629.

26. Donnelly PK, Hegde RS and Fletcher JS (1994). Growth of PCB-degrading bacteria on compounds from photosynthetic plants. *Chemosphere*, 28(5): 981-988.

27. van der Meer JR, Devos WM, Harayama S and AJB Z (1992). Molecular mechanisms of genetic adaptation to xenobiotic compounds. *Microbiological Reviews*, 56(4): 677-694.

28. Wilson M and Lindow SE (1993). Rrelease of recombinant microorganisms. *Annual Reviews Microbiology*, 47: 913-944.

29. Johri AK, Dua M, Singh A, Sethunathan N and Legge RL (1999). Characterization and regulation of catabolic genes. *Critical Reviews in Microbiology*, 25(4): 245-273.

30. Keasling JD and Bang SW (1998b). Recombinant DNA techniques for bioremediation and environmentally-friendly synthesis. *Current Opinion in Biotechnology*, 9(2): 135-140.

31. Ramos JL, Stolz A, Reineke W and TIMMIS KN (1986). Altered effector specifities in regulators of gene expression: TOL plasmid *xylsS* mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 83: 8467-8471.

32. de Lorenzo V, Fernandez S, Herrero M, Jakubzik U and Timmis KN (1993). Engineering of alkyl-responsive and haloaromatic-responsive gene-expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*. *Gene*, 130(1): 41-46.

33. Tabor S and Richardson CC (1985). A bacteriophage-T7 RNA-polymerase promoter system for controlled exclusive expression of specific genes. *Proceedings of the National Academy of Sciences of the United States of America*, 82(4): 1074-1078.

34. Davison J, Chevalier N and Brunel F (1989). Bacteriophage-T7 RNA polymerase-controlled specific gene-expression in *Pseudomonas*. *Gene*, 83(2): 371-375.

35. Endo G and Silver S (1995). Cadc, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid Pi258. *Journal of Bacteriology*, 177(15): 4437-4441.

36. Mermod N, Ramos JL, Lehrbach PR and Timmis KN (1986). Vector for regulated expression of cloned genes in a wide-range of Gram-negative bacteria. *Journal of Bacteriology*, 167(2): 447-454.

37. de Lorenzo V (1994). Designing microbial systems for gene-expression in the field. *Trends in Biotechnology*, 12(9): 365-371.

38. Mondello FJ (1989). Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. *Journal of Bacteriology*, 171(3): 1725-1732.

39. Little CD, Fraley CD, McCann MP and Matin A, *Use of bacterial stress promoters to induce biodegradation under conditions of environmental stress.*, in *On-Site Bioreclamation*, Hinchee RE and Olfenbuttel RF, Editors. 1991, Butterworth-Heinemann: Stoneham. p. 493-498.

40. Matin A (1992). Genetics of bacterial stress response and its applications. *Annals of the New York Academy of Sciences*, 665: 1-15.

41. Davison J (1999). Genetic exchange between bacteria in the environment. *PLASMID*, 42(2): 73-91.

42. Matin A, Little CD, Fraley CD and Keyhan M (1995). Use of starvation promoters to limit growth and select for trichloroethylene and phenol transformation activity in recombinant *Escherichia coli*. *Applied and Environmental Microbiology*, 61(9): 3323-3328.

43. Gallie DR and Kado CI (1989). A translational enhancer derived from tobacco mosaic-virus is functionally equivalent to a Shine-Dalgarno sequence. *Proceedings of the National Academy of Sciences, USA.*, 86(1): 129-132.

44. Frey J, Mudd EA and Krisch HM (1988). A bacteriophage-T4 expression cassette that functions efficiently in a wide-range of gram-negative bacteria. *Gene*, 62(2): 237-247.

45. Murdock D, Ensley BD, Serdar C and Thalen M (1993). Construction of metabolic operons catalyzing the *de-novo* biosynthesis of indigo in *Escherichia coli*. *Bio-Technology*, 11(3): 381-386.

46. Ramos JL, Wasserfallen A, Rose K and Timmis KN (1987). Redesigning metabolic routes - Manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. *Science*, 235(4788): 593-596.

47. Furukawa K, Hirose J, Hayashida S and Nakamura K (1994). Efficient degradation of trichloroethylene by a hybrid aromatic ring dioxygenase. *Journal of Bacteriology*, 176(7): 2121-2123.

48. Beil S, Mason JR, Timmis KN and Pieper DH (1998). Identification of chlorobenzene dioxygenase sequence elements involved in dechlorination of 1,2,4,5-tetrachlorobenzene. *Journal of Bacteriology*, 180(21): 5520-5528.

49. Kellner DG, Maves SA and Sligar SG (1997). Engineering cytochrome P450s for bioremediation. *Current Opinion in Biotechnology*, 8(3): 274-278.

50. Erickson BD and Mondello FJ (1993). Enhanced biodegradation of polychlorinated-biphenyls after site-directed mutagenesis of a biphenyl dioxygenase gene. *Applied and Environmental Microbiology*, 59(11): 3858-3862.

51. Hrywna Y, Tsoi TV, Malteva OV, Quensen JF and Tiedje JM (1999). Construction and characterisation of two recombinant bacteria that grow on *ortho*- and *para*-substituted chlorobiphenyls. *Applied and Environmental Microbiology*, 65(5): 2163-2169.

52. Reineke W and Knackmuss H-J (1980). Hybrid pathway for chlorobenzoate metabolism in *Pseudomonas* sp. B13 derivatives. *Journal of Bacteriology*, 142: 467-473.

53. Erb RW, Eichner CA, Wagner-Döbler I and Timmis KN (1997). Bioprotection of microbial communities from toxic phenol mixtures by a genetically designed pseudomonad. *Nature Biotechnology*, 15(4): 378-382.

54. Mars AE, Kasberg T, Kaschabek SR, vanAgteren MH, Janssen DB and Reineke W (1997). Microbial degradation of chloroaromatics: Use of the meta-cleavage pathway for mineralization of chlorobenzene. *Journal of Bacteriology*, 179: 4530-4537.

55. Bartels I, Knackmuss H-J and Reineke W (1984). Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. *Applied and Environmental Microbiology*, 47: 500-505.

56. Pipke R, Wagner-Döbler I, Timmis KN and Dwyer DF (1992). Survival and function of of a genetically engineered pseudomonad in aquatic sediment microcosms. *Applied and Environmental Microbiology*, 58: 1259-1265.

57. Heuer H, Dwyer DF, Timmis KN and Wagnerdobler I (1995). Efficacy in aquatic microcosms of a genetically-engineered pseudomonad applicable for bioremediation. *Microbial Ecology*, 29(2): 203-220.

58. Bouwer EJ and Zehnder AJB (1993). Bioremediation of organic-compounds - Putting microbial metabolism to work. *Trends in Biotechnology*, 11(8): 360-367.

59. Weekers F, Jacques P, Springael D, Mergeay M, Diels L and Thonart P (1999). Improving the catabolic functions of desiccation-tolerant soil bacteria. *Applied Biochemistry and Biotechnology*, 77-9: 251-266.

60. Filipovic D, Paulsen MD, Loida PJ, Sligar SG and Ornstein RL (1992). Ethylbenzene hydroxylation by cytochrome P450-cam. *Biochemical and Biophysical Research Communications*, 189: 488-495.

61. Loida PJ and Sligar SG (1993). Engineering cytochrome P450cam to increase the stereospecificity and coupling of aliphatic hydroxylation. *Protein Engineering*, 6: 207-212.

62. Jones JP, O'Hare EJ and Wong LL (2000). The oxidation of polychlorinated benzenes by genetically engineered cytochrome P450(cam): potential applications in bioremediation. *Chemical Communications*, 3: 247-248.

63. Jones NE, England PA, Rouch DA and Wong LL (1996). Engineering the selectivity of aliphatic C-H bond oxidation catalysed by cytochrome P450cam. *Chemical Communications*, (21): 2413-2414.

64. McClure NC, Fry JC and Weightman AJ (1991). Genetic engineering for waste-water treatment. *Journal of the Institution of Water and Environmental Management*, 5(6): 608-616.

65. Yen KM, Karl MR, Blatt LM, Simon MJ, Winter RB, Fausset PR, Lu HS, Harcourt AA and Chen KK (1991). Cloning and characterization of a *Pseudomonas mendocina* KR1 gene-cluster encoding toluene-4-monooxygenase. *Journal of Bacteriology*, 173(17): 5315-5327.

66. Fujita M, Ike M, Hioki JI, Kataoka K and Takeo M (1995). Trichloroethylene degradation by genetically-engineered bacteria carrying coned phenol catabolic genes. *Journal of Fermentation and Bioengineering*, 79(2): 100-106.

67. Ward TE, Bulmer D and Walton MR (1998). Development of genetically engineered bacteria for trichloroethylene degradation. *Journal of Environmental Science and Health Part A-Toxic/Hazardous Substances & Environmental Engineering*, 33(2): 179-193.

68. Winter RB, Yen KM and Ensley BD (1989). Efficient degradation of trichloroethylene by a recombinant *Escherichia coli*. *Bio-Technology*, 7(3): 282-285.

69. Krumme ML, Timmis KN and Dwyer DF (1993). Degradation of trichloroethylene by *Pseudomonas cepacia* G4 and the constitutive mutant strain G4-5223 Pr1 in aquifer microcosms. *Applied and Environmental Microbiology*, 59(8): 2746-2749.

70. MunakataMarr J, McCarty PL, Shields MS, Reagin M and Francesconi SC (1996). Enhancement of trichloroethylene degradation in aquifer microcosms bioaugmented with wild type and genetically altered *Burkholderia* (*Pseudomonas*) *cepacia* G4 and PR1. *Environmental Science & Technology*, 30(6): 2045-2052.

71. Zylestra GJ, Wackett LP and Gibson DT (1989). Trichloroethylene degradation by *Escherichia coli* containing the cloned *Pseudomonas putida* F1 toluene dioxygenase genes. *Applied and Environmental Microbiology*, 55: 3162-3166.

72. Harker AR. *The potential use of genetically engineered microorganisms in the remediation of environmental pollution.* in *Proceeding of Symposium on Ground Water*. 1991. Nashville: American Society of Civil Engineers.

73. Kuritz T and Wolk CP (1995). Use of filamentous cyanobacteria for biodegradation of organic pollutants. *Applied and Environmental Microbiology*, 61(1): 234-238.

74. Hur HG, Sadowsky MJ and Wackett LP (1994). Metabolism of chlorofluorocarbons and polybrominated compounds by *Pseudomonas putida* G786(Phg-2) via an engineered metabolic pathway. *Applied and Environmental Microbiology*, 60(11): 4148-4154.

75. Kaštánek F, Demnerová K, Pazlarová J, Burkhard J and Maléterová Y (1999). Biodegradation of polychlorinated biphenyls and volatile chlorinated hydrocarbons in contaminated soils and ground water in field condition. *International Biodeterioration and Biodegradation*, 44: 39-47.

76. Focht DD (1995). Strategies for the improvement of aerobic metabolim of polychlorinated biphenyls. *Current Opinions Biotechnology*, 6: 341.

77. Boyle AW, Silvin CJ, Hassett JP, Nakas JP and Tanenbaum SW (1992). Bacterial PCB biodegradation. *Biodegradation*, 3: 285-298.

78. Robinson GK and Lenn MJ (1994). The bioremediation of polychlorinated biphenyls (PCBs): Problems and perspectives. *Biotechnology and Genetic Engineering Reviews*, 12: 139-188.

79. Gibson DT, Cruden DL, Haddock JD, Zylestra GJ and Brand JM (1993). Oxidation of polychlorinated-biphenyls by *Pseudomonas* sp strain LB400 and P*seudomonas pseudoalcaligenes* KF707. *Journal of Bacteriology*, 175(14): 4561-4564.

80. Kimura N, Nishi A, Goto M and Furukawa K (1997). Functional analyses of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally. *Journal of Bacteriology*, 179(12): 3936-3943.

81. Mondello FJ, Turcich MP, Lobos JH and Erickson BD (1997). Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. *Applied and Environmental Microbiology*, 63(8): 3096-3103.

82. Lajoie CA, Layton AC, Easter JP, Menn FM and Sayler CS (1997). Degradation of nonionic surfactants and polychlorinated biphenyls by recombinant field application vectors. *Journal of Industrial Microbiology & Biotechnology*, 19(4): 252-262.

83. Dowling DN, Pipke R and Dwyern DF (1993). A DNA module encoding *bph* genes for the degradation of polychlorinated-biphenyls (PCBs). *FEMS Microbiology Letters*, 113(2): 149-154.

84. McCullar MV, Brenner V, Adams RH and Focht DD (1994). Construction of a novel polychlorinated biphenyl-degrading bacterium - Utilization of 3,4'-dichlorobiphenyl by *Pseudomonas acidovorans* M3GY. *Applied and Environmental Microbiology*, 60(10): 3833-3839.

85. Nakatsu CH and Wyndham RC (1993). Cloning and expression of the transposable chlorobenzoate-3,4-dioxygenase genes of *Alcaligenes* sp. strain BR60. *Applied and Environmental Microbiology*, 59(11): 3625-3633.

86. Stratford J, Wright MA, Reineke W, Mokross H, Havel J, Knowles CJ and Robinson GK (1996). Influence of chlorobenzoates on the utilisation of chlorobiphenyls and chlorobenzoate mixtures by chlorobiphenyl/chlorobenzoate-mineralising hybrid bacterial strains. *Archives of Microbiology*, 165(3): 213-218.

87. Laha S and Luthy RG (1991). Inhibition of phenanthrene mineralisation by nonionic surfactants in soil-water systems. *Environment Science and Technology*, 25: 1920-1930.

88. Lajoie CA, Layton AC and Sayler GS (1994). Cometabolic oxidation of polychlorinated-biphenyls in soil with a surfactant-based field application vector. *Applied and Environmental Microbiology*, 60(8): 2826-2833.

89. Holliger C, Gaspard S, Glod G, Heijman C, Schumacher W, Schwarzenbach RP and Vazquez F (1997). Contaminated environments in the subsurface and bioremediation: organic contaminants. *FEMS Microbiology Reviews*, 20(3-4): 517-523.

90. Ripp S, Nivens DE, Ahn Y, Werner C, Jarrell J, Easter JP, Cox CD, Burlage RS and Sayler GS (2000). Controlled field release of a bioluminescent genetically engineered microorganism for bioremediation process monitoring and control. *Environmental Science & Technology*, 34(5): 846-853.

91. King JMH, DiGrazia PM, Applegate B, Burlage R, Sanseverino J, Dunbar P, Larimer F and Sayler GS (1990). Bioluminescent reporter plasmid for napthalene exposure and biodegradation. *Science*, 249: 778-781.

92. Staples CA, Peterson DR, Parkerton TF and Adams WJ (1997). The environmental fate of phthalate esters: a literature review. *Chemosphere*, 35: 667-749.

93. Lee J-Y, Jung K-H, Choi SH and Kim H-S (1995). Combination of the *tod* and the *tol* pathways in redesigning a metabolic route of *Pseudomonas putida* for the mineralisation of a benzene, toluene, and *p*-xylene mixture. *Applied and Environmental Microbiology*, 61(6): 2211-2217.

94. Soda S, Uesugi K, Ike M and Fujita M (1999). Application of a floc-forming genetically engineered microorganism to a sequencing batch reactor for phenolic wastewater treatment. *Journal of Bioscience and Bioengineering*, 88(1): 85-91.

95. Roberts L (1989). Ecologists wary about environmental releases. *Research News*, 3: 1141.

96. Blumenroth P and Wagner-Dobler I (1998). Survival of inoculants in polluted sediments: Effect of strain origin and carbon source competition. *Microbial Ecology*, 35(3): 279-288.

97. Ramos JL, Duque E, Huertas MJ and Haidour A (1995). Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high-concentrations of aromatic-hydrocarbons. *Journal of Bacteriology*, 177(14): 3911-3916.

98. Marconi AM, Kieboom J and deBont JAM (1997). Improving the catabolic functions in the toluene-resistant strain *Pseudomonas putida* S12. *Biotechnology Letters*, 19(7): 603-606.

99. Ward DM and Brock TD (1978). Hydrocarbon biodegradation in hypersaline environments. *Applied and Environmental Microbiology*, 35: 353-359.

100. Ajisebutu SO (1988). Effects of sodium-chloride on biodegradation of crude oils by 2 species of aeromonas. *Applied Microbiology and Biotechnology*, 28(2): 203-208.

101. Kapley A, Purohit HJ, Chhatre S, Shanker R, Chakrabarti T and Khanna P (1999). Osmotolerance and hydrocarbon degradation by a genetically engineered microbial consortium. *Bioresource Technology*, 67(3): 241-245.

102. Horn JM, Brunke M, Deckwer WD and Timmis KN (1994). *Pseudomonas putida* strains which constitutively overexpress mercury resistance for biodetoxification of organomercurial pollutants. *Applied and Environmental Microbiology*, 60(1): 357-362.

103. Zenk MH (1996). Heavy metal detoxification in higher plants - a review. *Gene*, 179: 21-30.

104. Brim H, McFarlan SC, Fredrickson JK, Minton KW, Zhai M, Wackett LP and Daly MJ (2000). Engineering *Deinococcus radiodurans* for metal remediation in radioactive mixed waste environments. *Nature Biotechnology*, 18(1): 85-90.

105. Mauro JM and Pazirandeh M (2000). Construction and expression of functional multi-domain polypeptides in *Escherichia coli*: expression of the *Neurospora crassa* metallothionein gene. *Letters in Applied Microbiology*, 30(2): 161-166.

106. Valls M, Gonzalez-Duarte R, Atrian S and de Lorenzo V (1998). Bioaccumulation of heavy metals with protein fusions of metallothionein to bacterial OMPs. *Biochimie*, 80(10): 855-861.

107. Hong SH, Gohya M, Ono H, Murakami H, Yamashita M, Hirayama N and Murooka Y (2000). Molecular design of novel metal-binding oligomeric human metallothioneins. *Applied Microbiology and Biotechnology*, 54(1): 84-89.

108. Cruz N, Le Borgne S, Hernandez-Chavez G, Gosset G, Valle F and Bolivar F (2000). Engineering the *Escherichia coli* outer membrane protein OmpC for metal bioadsorption. *Biotechnology Letters*, 22(7): 623-629.

109. Kotrba P, Pospisil P, de Lorenzo V and Ruml T (1999). Enhanced metallosorption of *Escherichia coli* cells due to surface display of beta- and alpha-domains of mammalian metallothionein as a fusion to LamB protein. *Journal of Receptor and Signal Transduction Research*, 19(1-4): 703-715.

110. Kotrba P, Doleckova L, de Lorenzo V and Ruml T (1999). Enhanced bioaccumulation of heavy metal ions by bacterial cells due to surface display of short metal binding peptides. *Applied and Environmental Microbiology*, 65(3): 1092-1098.

111. Mejare M, Ljung S and Bulow L (1998). Selection of cadmium specific hexapeptides and their expression as OmpA fusion proteins in *Escherichia coli*. *Protein Engineering*, 11(6): 489-494.

112. Valls M, Atrian S, de Lorenzo V and Fernandez LA (2000). Engineering a mouse metallothionein on the cell surface of *Ralstonia eutropha* CH34 for immobilization of heavy metals in soil. *Nature Biotechnology*, 18(6): 661-665.

113. Bang SW, Clark DS and Keasling JD (2000). Cadmium, lead, and zinc removal by expression of the thiosulfate reductase gene from *Salmonella typhimurium* in *Escherichia coli*. *Biotechnology Letters*, 22(16): 1331-1335.

114. Keasling JD, Van Dien SJ and Pramanik J (1998). Engineering polyphosphate metabolism in *Escherichia coli*: Implications for bioremediation of inorganic contaminants. *Biotechnology and Bioengineering*, 58(2-3): 231-239.

115. Bang SW, Clark DS and Keasling JD (2000). Engineering hydrogen sulfide production and cadmium removal by expression of the thiosulfate reductase gene (*phsABC*) from *Salmonella enterica* serovar *typhimurium* in *Escherichia coli*. *Applied and Environmental Microbiology*, 66(9): 3939-3944.

116. Sicko-goad L and Lazinsky D (1986). Quantitative ultrastructural-changes associated with lead-coupled luxury phosphate-uptake and polyphosphate utilization. *Archives of Environmental Contamination and Toxicology*, 15(6): 617-627.

117. Rothstein A and Meier R (1951). The relationship of the cell surface to metabolism. VI. The chemical nature of uranium-complexing groups of the cell surface. *Journal of Cellular and Comparative Physiology*, 38: 245-270.

118. Collard JM, Corbisier P, Diels L, Dong Q, Jeanthon C, Mergeay M, Taghavi S, Vanderlelie D, Wilmotte A and Wuertz S (1994). Plasmids for heavy-metal resistance in *Alcaligenes eutrophus* CH34 - Mechanisms and applications. *FEMS Microbiology Reviews*, 14(4): 405-414.

119. Chen SL and Wilson DB (1997). Genetic engineering of bacteria and their potential for Hg^{2+} bioremediation. *Biodegradation*, 8(2): 97-103.

120. Chen SL, Kim EK, Shuler ML and Wilson DB (1998). Hg^{2+} removal by genetically engineered *Escherichia coli* in a hollow fiber bioreactor. *Biotechnology Progress*, 14(5): 667-671.

121. Chen SL and Wilson DB (1997). Construction and characterization of *Escherichia coli* genetically engineered for bioremediation of Hg^{2+} contaminated environments. *Applied and Environmental Microbiology*, 63(6): 2442-2445.

122. Riley RG, Zachara JM and Wobber FJ, *Chemical contaminants on DOE lands and selection of contaminant mixtures for subsurface science research*. 1992, US Department of Energy, Office of Energy Research, Subsurface Science Programe: Washington DC.

123. Murray RGE, *The family Deinococcaceae*, in *The Prokaryotes*, Balows A, Truper HG, Dworkin M, Harder W and Schleifer K-H, Editors. 1992, Springer Verlag: New York. p. 3732-3744.

124. Dong QH, Springeal D, Schoeters J, Nuyts G, Mergeay M and Diels L (1998). Horizontal transfer of bacterial heavy metal resistance genes and its applications in activated sludge systems. *Water Science and Technology*, 37(4-5): 465-468.

125. Evans CS, Veness RG and Ullah M (1998). Breakdown of plant polymers by fungi and their potential for use in bioremediation. *Journal of Chemical Technology and Biotechnology*, 71(4): 357-359.

126. Grotenhuis T, Field J, Wasseveld R and Rulkens W (1998). Biodegradation of polyaromatic hydrocarbons (PAH) in polluted soil by the white-rot fungus *Bjerkandera. Journal of Chemical Technology and Biotechnology*, 71(4): 359-360.

127. Maloney SE (1998). Degradation of insecticides and herbicides by fungi. *Journal of Chemical Technology and Biotechnology*, 71(4): 360-362.

128. Ow DW (1996). Heavy metal tolerance genes: Prospective tools for bioremediation. *Resources Conservation and Recycling*, 18(1-4): 135-149.

129. Tobin JM, White C and Gadd GM (1994). Metal accumulation by fungi - Applications in environmental biotechnology. *Journal of Industrial Microbiology*, 13(2): 126-130.

130. Blackwell KJ and Tobin JM (1999). Cadmium accumulation and its effects on intracellular ion pools in a brewing strain of *Saccharomyces cerevisiae. Journal of Industrial Microbiology & Biotechnology*, 23(3): 204-208.

131. Sayer JA and Gadd GM (1997). Solubilization and transformation of insoluble inorganic metal compounds to insoluble metal oxalates by *Aspergillus niger. Mycological Research*, 101: 653-661.

132. White C, Sayer JA and Gadd GM (1997). Microbial solubilization and immobilization of toxic metals: key biogeochemical processes for treatment of contamination. *FEMS Microbiology Reviews*, 20(3-4): 503-516.

133. Aiken BS and Logan BE (1996). Degradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium* grown in ammonium lignosulphonate media. *Biodegradation*, 7(3): 175-182.

134. Eggen T and Majcherczyk A (1998). Removal of polycyclic aromatic hydrocarbons (PAH) in contaminated soil by white rot fungus *Pleurotus ostreatus. International Biodeterioration and Biodegradation*, 41(2): 111-117.

135. Gadd GM, Gharieb MM, Ramsay LM, Sayer JA, Whatley AR and White C (1998). Fungal processes for bioremediation of toxic metal and radionuclide pollution. *Journal of Chemical Technology and Biotechnology*, 71(4): 364-366.

136. Thomma BPHJ, Tadesse YSH, Jacobs M and Broekaert WF (1999). Disturbed correlation between fungal biomass and ß-glucuronidase activity in infections of *Arabidopsis thaliana* with transgenic *Alternaria brassicicola. Plant Science*, 148: 31-36.

137. Yeoung-Seuk B and Knudsen GR (2000). Cotransformation of *Trichoderma harzianum* with ß-glucuronidase and green fluorescent protein genes provides a useful tool for monitoring fungal growth and activity in natural soils. *Applied and Environmental Microbiology*, 66(2): 810-815.

138. Broda P, Birch PRJ, Brooks PR and Sims PFG (1996). Lignocellulose degradation by *Phanerochaete chrysosporium*: Gene families and gene expression for a complex process. *Molecular Microbiology*, 19(5): 923-932.

139. Pointing SB (2001). Feasibility of bioremediation by white-rot fungi. *Applied Microbiology and Biotechnology*, 57(1-2): 20-33.

140. Perotto S and Martino E (2001). Molecular and cellular mechanisms of heavy metal tolerance in mycorrhizal fungi: what perspectives for bioremediation? *Minerva Biotecnologica*, 13(1): 55-63.

141. Vosatka M (2001). A future role for the use of arbuscular mycorrhizal fungi in soil remediation: a chance for small-medium enterprises? *Minerva Biotecnologica*, 13(1): 69-72.

142. Paton GI, Rattray EAS, Campbell CD, Cresser MS, Glover LA, Meeussen JCL and Killham K, *Use of genetically modified microbial biosensors for soil ecotoxicity testing*, in *Biological indicators of soil health*, Pankhurst CE DB, Gupta VVSR, Editor. 1997, CAB International. p. 397-418.

143. Layton AC, Gregory B, Schultz TW and Sayler GS (1999). Validation of genetically engineered bioluminescent surfactant resistant bacteria as toxicity assessment tools. *Ecotoxicology and Environmental Safety*, 43(2): 222-228.

144. Jansson JK, Bjorklof K, Elvang AM and Jorgensen KS (2000). Biomarkers for monitoring efficacy of bioremediation by microbial inoculants. *Environmental Pollution*, 107(2): 217-223.

145. DETR - unpublished report, *Methods for Detecting Specific Bacteria in the Environment - report of workshop held at University of Liverpool, UK*. 1997.

146. Springael D, Diels L, Hooyberghs L, Kreps S and Mergeay M (1993). Construction and characterization of heavy metal-resistant haloaromatic-degrading *Alcaligenes eutrophus* strains. *Applied and Environmental Microbiology*, 59(1): 334-339.

147. Sousa A, Duffy C, Weitz H, Glover LA and Bar E (1998). Use of a *lux*-modified bacterial biosensor to identify constraints to bioremediation of BTEX-contaminated sites. *Environmental Toxicology and Chemistry*, 17(6): 1039-1045.

148. Raskin I (1996). Plant genetic engineering may help with environmental cleanup. *Proceedings of the National Academy of Sciences of the United States of America*, 93: 3164-3166.

149. Rugh CL, Senecoff JF, Meagher RB and Merkle SA (1998). Development of transgenic yellow poplar for mercury phytoremediation. *Nature Biotechnology*, 16: 925-928.

150. Doty SL, Shang TQ, Wilson AM, Tangen J, Westergreen AD, Newman LA, Strand SE and Gordoon MP (2000). Enhanced metabolism of halogenated hydrocarbons in transgenic plants containing mammalian cytochrome P450 2E1. *Proceedings of the National Academy of Sciences of the USA*, 97(12): 6287-6291.

151. Salt DE, Smith RD and Raskin I (1998). Phytoremediation. *Annual Review of Plant Physiology and Plant Molecular Biology*, 49: 643-668.

152. Raskin I, Smith RD and Salt DE (1997). Phytoremediation of metals: using plants to remove pollutants from the environment. *Current Opinions in Biotechnology*, 8: 221-226.

153. Purvis W (2000). Plant power against pollution. *Nature*, 407(6802): 298-299.

154. Gleba D, Borisjuk NV, Borisjuk LG, Kneer R, Poulev A, Skarzhinskaya M, Dushenkov S, Logendra S, Gleba YY and Raskin I (1999). Use of plant roots for phytoremediation and molecular farming. *Proceedings of the National Academy of Sciences of the United States of America*, 96: 5973-5977.

155. Chaney RL, Malik M, Li YM, Brown SL, Brewer EP, Angle JS and Baker AJ (1997). Phytoremediation of soil metals. *Current Opinions in Biotechnology*, 8(3): 279-284.

156. Pilon-Smits E and Pilon M (2000). Breeding mercury breathing plants for environmental cleanup. *Trends in Plant Science*, 5(6): 235-236.

157. Schnoor JL, Licht LA, McCutcheon SC, Wolfe NL and Carreira LH (1995). Phytoremediation of organic and nutrient contaminants. *Environmental Science and Technology*, 29: 318-323.

158. Stomp AM, Han KH, Wilbert S, Gordon MP and Cunningham SD (1994). Genetic strategies for enhancing phytoremediation. *Annals of the New York Academy of Sciences*, 721: 481-491.

159. Salt DE, Blaylock M, Kumar NPBA, Viatcheslav D and Ensley BD (1995). Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. *Biotechnology*, 13: 468-474.

160. Cunningham SD and Ow DW (1996). Promises and Prospects of Phytoremediation. *Plant Physiology*, 110: 715-719.

161. Persidis A (1999). Agricultural biotechnology. *Nature Biotechnology*, 17(6): 612-614.

162. Hughes JB, Shanks J, Vanderford M, Lauritzen J and Bhadra R (1997). Transformation of TNT by aquatic plants and plant tissue cultures. *Environmental Science and Technology.*, 31(1): 266-271.

163. French CE, Rosser SJ, Davies GJ, Nickin S and Bruce NC (1999). Biodegradation of explosives by transgenic plants expressing pentaerythritol tetranitrate reductase. *Nature Biotechnology*, 17(5): 491-494.

164. Hooker BS and Skeen RS (1999). Transgenic phytoremediation blasts onto the scene. *Nature Biotechnology*, 17: 428.

165. Black H (1999). Phytoremediation, a growing field with some concerns. *The Scientist*, 13(5): 1.

166. Black H (1995). Absorbing possibilities: phytoremediation. *Environmental Health Perspectives*, 103: 1106-1108.

167. Salt DE, *Phytoextraction: present applications and future promise.*, in *Remediation of hazardous waste contaminated soils*, Wise DL, Trantolo DJ, Inyang HI and Cichon EG, Editors. 2000, Marcel Dekker inc. p. 729-744.

168. Salt DE and Baker AJM, *Phytoremediation of metals.*, in *Biotechnology*, Return HJ and Reed G, Editors. 2000, Wiley VCH. p. 386-397.

169. Smith JAC, *personal communication*. 2001: Department of Plant Sciences, University of Oxford.

170. Walker CH, Hopkin SP, Sibly RM and Peakall DB, *Principles of ecotoxicology*. 1997: Taylor and Francis.

171. Rugh CL, Dayton Wilde H, Stack NM, Thompson DM, Summers AO and Meagher RB (1996a). Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial *merA* gene. *Proceedings of the National Academy of Sciences of the United States of America*, 93: 3182-3187.

172. Clemens S (2001). Molecular mechanisms of plant metal tolerance and homeostasis. *Planta*, 212: 475-486.

173. Baker AJM, Brooks RR and Reeves R (1988). Growing for gold…and copper….and zinc. *New Scientist*, 117: 44-48.

174. Bizily SP, Rugh CL and Meagher RB (2000). Phytodetoxification of hazardous organomercurials by genetically engineered plants. *Nature Biotechnology*, 18(2): 213-217.

175. Baker AJM, McGrath SP, Reeves RD and Smith JAC, *Metal hyperaccumulator plants: a review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils*, in *In Phytoremediation of Contaminated Soil and Water*, N. Terry and G. Bañuelos, Editors. 1999, Lewis Publishers: Boca Raton, Florida. p. 85-107.

176. Ma LQ, Komar KM, Tu C, Zhang WH, Cai Y and Kennelley ED (2001). A fern that hyperaccumulates arsenic: a hardy, versatile, fast-growing plants helps to remove arsenic from contaminated soils. *Nature*, 409: 579.

177. Arazi T, Sunkar R, Kaplan B and Fromm H (1999). A tobacco plasma membrane calmodulin-binding transporter confers Ni^{2+} tolerance and Pb^{2+} hypersensitivity in transgenic plants. *The Plant Journal*, 20(2): 171-182.

178. Cobbett CS (2000). Phytochelatins and their roles in heavy metal detoxification. *Plant Physiology*, 123: 825-832.

179. Baker AJM, Li Y-M, Angle JS and Chaney RL, *Method for phytomining of nickel, cobalt and other metals from soil*. 1998.

180. Ha S-B, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB and Cobbett CS (1999). Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. *Plant Cell*, 11: 1153-1164.

181. Kneer R and Zenk MH (1992). Phytochelatins protect plant enzymes from heavy metal poisoning. *Phytochemistry*, 31: 2663-2667.

182. Rauser WE (1995). Phytochelatins and related peptides. *Plant Physiology*, 109: 1141-1149.

183. Speiser DM, Abrahamson SL, Banuelos G and Ow DW (1992). *Brassica juncea* produces a phytochelatin-cadmium-sulphide complex. *Plant Physiology*, 99: 817-821.

184. Gupta SC and Goldsbrough PB (1991). Phytochelatin accumulation and cadmium tolerance in selected tomato cell lines. *Plant Physiology*, 97: 306-312.

185. Howden R and Cobbett CS (1992). Cadmium-sensitive mutants of *Arabidopsis thaliana*. *Plant Physiology*, 100: 100-107.

186. Howden R, Goldsbrough PB, Andersen CR and Cobbett CS (1995a). Cadmium sensitive *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. *Plant Physiology*, 107: 1059-1066.

187. Howden R, Andersen CR, Goldsbrough PB and Cobbett CS (1995b). A cadmium sensitive, glutathione-deficient mutant of *Arabidopsis thaliana. Plant Physiology*, 107: 1067-1073.

188. Cobbett CS, May MJ, Howden R and Rolls B (1998). The glutathione-deficient, cadmium-sensitive mutant cad2-1 of *Arabidopsis thaliana* is deficient in gamma-glutamylcysteine synthetase. *Plant Journal*, 16(1): 73-78.

189. Noctor G, Arisi ACM, Jouanin L, Kunert KJ, Rennenberg H and Foyer CH (1998). Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. *Journal of Experimental Botany*, 49(321): 623-647.

190. Arisi ACM, Noctor G, Foyer CH and Jouanin L (1997). Modification of thiol contents in poplars (*P. tremula* x *P.alba*) overexpressing enzymes involved in glutathione synthesis. *Planta*, 203: 362-372.

191. Zhu YL, Pilon-Smits EAH, Tarun AS, Weber SU, Jouanin L and Terry N (1999). Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing gamma-glutamylcysteine synthetase. *Plant Physiology*, 121(4): 1169-1177.

192. Rauser WE (1999). Structure and function of metal chelators produced by plants: the case for organic acids amino acids phytin and metallothioneins. *Cell Biochemistry and Biophysiology*, 31(1): 19-48.

193. Robinson NJ, Tommey AM, Kuske C and Jackson PJ (1993). Plant metallothioneins. *Biochemical Journal*, 295: 1-10.

194. Kawashima I, Inokuch Y, Chino M, Kimura M and Shimizu N (1991). Isolation of a gene for a metallothionein-like protein from soybean. *Plant Cell Physiology*, 32: 913-916.

195. Yeargen R, Maiti IB, Nielsen MT, Hunt AG and Wagner GJ (1992). Tissue partitioning of cadmium in transgenic tobacco seedlings and field grown plants expressing the mouse metallothionein I gene. *Transgenic Research*, 1: 261-267.

196. Pan A, Tie F, Duau Z, Yang M, Wang Z, Li L, Chen Z and Ru B (1994). Alpha domain of human metallothionein I-A can bind to metals in transgenic tobacco plants. *Molecular Genetics, Gene Transfer, and Therapy*, 242: 666-674.

197. Harmens H, Den Harog PR, Ten Bookum WM and Verleij JAC (1993). Increased zinc tolerance in *Silene vulgaris* (Moench) Garke is not due to increased production of phytochelatins. *Plant Physiology*, 103: 1305-1309.

198. Krämer U, Cotter-Howells JD, Charnock JM, Baker AJM and Smith JAC (1996). Free histidine as a metal chelator in plants that accumulate nickel. *Nature*, 379: 635-638.

199. Lasat MM, Baker AJ and Kochian LV (1998). Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in Thlaspi caerulescens. *Plant Physiology*, 118(3): 875-883.

200. Lasat MM, Pence NS, Garvin DF, Ebbs SD and Kochian LV (2000). Molecular physiology of zinc transport in the Zn hyperaccumulator *Thlaspi caerulescens*. *Journal of Experimental Botany*, 51(342): 71-79.

201. Pence NS, Larsen PB, Ebbs SD, Letham DLD, Lasat MM, Garvin DF, Eide D and Kochian LV (2000). The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens. Proceedings of the National Academy of Sciences of the United States of America*, 97(9): 4956-4960.

202. Grotz N, Fox T, Connolly E, Park W, Guerinot ML and Eide D (1998). Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. *Proceedings of the National Academy of Sciences of the United States of America*, 95: 7220-7224.

203. van der Zaal BJ, Neuteboom LW, Pinas JE, Chardonnens AN, Schat H, Verkleij JAC and Hooykas PJJ (1999). Overexpression of a novel *Arabidopsis* gene related to putative zinc transporter genes from animal can lead to enhanced zinc resistance and accumulation. *Plant Physiology*, 119: 1047-1056.

204. Hirschi KD (1999). Expression of *Arabidopsis* CAX1 in tobacco: Altered calcium homeostasis and increased stress sensitivity. *The Plant Cell*, 11: 2113-2122.

205. Hirschi KD, Zhen RG, Cunningham KW, Rea PA and Fink GR (1996). CAX1, an H^+/Ca^{2+} antiporter from *Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America*, 93(16): 8782-8786.

206. Hirschi KD, Korenkov VD, Wilganowski NL and Wagner GJ (2000). Expression of *Arabidopsis* CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. *Plant Physiology*, 124: 125-133.

207. Dushenkov SPBA, Kumar N, Motto H and Raskin I (1995). Rhizofiltration: the use of plants to remove heavy metals from aqueous streams. *Environmental Science and Technology*, 29: 1239-1245.

208. Heaton ACP, Rugh CL, Wang NJ and Meagher RB (1998). Phytoremediation of mercury and methylmercury polluted soils using genetically engineered plants. *Journal of Soil Contamination*, 7: 497-509.

209. Bizily SP, Rugh CL, Summers AO and Meagher RB (1999). Phytoremediation of methylmercury pollution: *merB* expression in *Arabidopsis thaliana* confers resistance to organomercurials. *Proceedings of the National Academy of Sciences, USA.*, 96(12): 6808-6013.

210. DeWitt N (2000). Phytoremediation of organic mercury. *Nature Biotechnology*, 18(2): 136.

211. Pilon-Smits EA, Hwang S, Mel Lytle C, Zhu Y, Tia JC, Bravo RC, Chen Y, Leustek T and Terry N (1999). Overexpression of ATP sulfurylase in Indian Mustard leads to increased selenate uptake reduction and tolerance. *Plant Physiology*, 119(1): 123-132.

212. Cunningham SD, Anderson TA, Schwab AP and Hsu FC (1996). Phytoremediation of soils contaminated with organic pollutants. *Advances in Agronomy*, 56: 55-114.

213. Simonich SL and Hites RA (1994). Importance of vegetation in removing polycyclic aromatic hydrocarbons from the atmosphere. *Nature*, 370: 49-51.

214. DeWitt N (1999). Phytoremediation blasts off. *Nature Biotechnology*, 17(6): 612-614.

215. Newman LA, Strand SE, Choe N, Duffy J and Ekuan G (1997). Uptake and biotransformation of trichloroethylene by hybrid poplars. *Environmental Science and Technology*, 31: 1062-1067.

216. Guengerich FP, Kim DH and Iwasaki M (1991). *Chemical Research in Toxicology*, 4: 168-179.

217. Guerineau F, Lucy A and Mullineux P (1992). Effect of two consensus sequences preceding the translation initiator codon on gene expression in plant protoplasts. *Plant Molecular Biology*, 20: 1203-1207.

218. Chen L (1998). *Nature Biotechnology*, 16: 1060-1064.

219. Pawlowski WP and Somers DA (1996). Transgene inheritance in plants genetically engineered by microprojectile bombardment. *Molecular Biotechnology*, 6: 17-30.

220. Finnegan J and McElroy D (1994). Transgene inactivation: Plants fight back! *Biotechnology*, 12: 883-888.

221. Ingelbrecht I, Van Houdt H, Van Montagu M and Depicker A (1994). Post-transcriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. *Proceedings of the National Academy of Sciences, USA.*, 91: 10502-10506.

222. Matzke MA and Matzke AJM (1995). How and why do plants inactivate homologous (trans)genes. *Plant Physiology*, 107: 679-685.

223. Lappin HM, Greaves MP and Slater JH (1985). Degradation of the herbicide mecoprop [2-(2-methyl-4-chlorophenoxy) propionic acid] by a synergistic microbial community. *Applied and Environmental Microbiology*, 49(2): 429-433.

224. Walton BT and Anderson TA (1990). Microbial-degradation of trichloroethylene in the rhizosphere - Potential application to biological remediation of waste sites. *Applied and Environmental Microbiology*, 56(4): 1012-1016.

225. de Souza MP, Chu D, Zhao M, Zayed AM, Ruzin SE, Schichnes D and Terry T (1999). Rhizosphere bacteria enhance selenium accumulation and volatilization by Indian mustard. *Plant Physiology*, 119: 565-573.

226. Yee DC, Maynard JA and Wood TK (1998). Rhizoremediation of trichloroethylene by a recombinant, root- colonizing *Pseudomonas fluorescens* strain expressing toluene ortho-monoooxygenase constitutively. *Applied and Environmental Microbiology*, 64(1): 112-118.

227. Krumme ML, Smith RL, Egestorff J, Thiem SM, Tiedje JM, Timmis KN and Dwyer DF (1994). Behavior of pollutant-degrading microorganisms in aquifers - Predictions for genetically-engineered organisms. *Environmental Science & Technology*, 28(6): 1134-1138.

228. Brill WJ (1985). Safety concerns and genetic-engineering in agriculture. *Science*, 227(4685): 381-384.

229. Lenski RE (1993). Evaluating the fate of genetically modified microorganisms in the environment: are they inherently less fit? *Experentia*, 49: 201-209.

230. Giddings G (1998). The release of genetically engineered microorganisms and viruses into the environment. *New Phytologist*, 140: 173-184.

231. Hirsch PR and Spokes JD (1994). Survival and dispersion of genetically-modified *Rhizobia* in the field and genetic interactions with native strains. *FEMS Microbiology Ecology*, 15(1-2): 147-159.

232. Velkov VV (1994). Introduction of genetically-modified microorganisms into the environment - Prospects and risk. *Genetika+*, 30(5): 581-592.

233. Bailey JM and Whipps JM, *Risk assessment and the release of genetically modified microorganisms into the environment.*. 1995, Department of the Environment, Transport and the Regions (DETR) Research Report no.7. p. 1-66.

234. Thompson IP, Lilley AK, Ellis RJ, Bramwell PA and Bailey MJ (1995). Survival, colonisation and dispersal of genetically modified *Pseudomonas fluorescens* SBW25 in the phytosphere of field grown sugar beet. *Nature Biotechnology*, 13: 1493-1497.

235. Crozat Y, Cleyet-Marcel JC and Corman A (1987). *Biology and Fertility of Soils*, 4: 85-90.

236. Neidhart FC, Ingraham JL and Schaechter M, *Physiology of the bacterial cell: a molecular approach.*1990, Sunderland: Sinauer Associates, Inc.

237. Germuda J and Khachatourians GG (1988). Transduction of *Escherichia coli* in soil. *Canadian Journal of Microbiology*, 34: 190-193.

238. Neilson JW, Josephson KL, Pepper IL, Arnold RB, Digiovanni GD and Sinclair NA (1994). Frequency of horizontal gene-transfer of a large catabolic plasmid (Pjp4) in soil. *Applied and Environmental Microbiology*, 60(11): 4053-4058.

239. Peters M, Heinaru E, Talpsep E, Wand H, Stottmeister U, Heinaru A and Nurk A (1997). Acquisition of a deliberately introduced phenol degradation operon, *pheBA*, by different indigenous *Pseudomonas* species. *Applied and Environmental Microbiology*, 63: 4899-4906.

240. Fry JD and Day Mj, *Bacterial genetics in natural environments*. 1990, London: Chapman and Hall.

241. Bovin R, Bellemare G and Dion P (1994). Novel narrow-host-range vectors for direct cloning of foreign DNA in *Pseudomonas*. *Current Microbiology*, 28: 41-47.

242. Mills AL and Mallory LM (1987). The community structure of sessile heterotrophic bacteria stressed by acid-mine drainage. *Microbial Ecology*, 14: 219-232.

243. Leung K, Strain SR, Debruijn FJ and Bottomley PJ (1994). Genotypic and phenotypic comparisons of chromosomal types within an indigenous soil population of *Rhizobium leguminosarum* bv trifolii. *Applied and Environmental Microbiology*, 60: 416-426.

244. Andrews JH and Harris RF (1986). R-selection and k-selection and microbial ecology. *Advances in Microbial Ecology*, 9: 99-147.

245. Mann RM and Boddy MR (2000). Biodegradation of a nonylphenol ethoxylate by the autochthonous microflora in lake water with observations on the influence of light. *Chemosphere*, 41(9): 1361-1369.

246. Short KA, Doyle JD, King RJ, Seidler RJ, Stotzky G and Olsen RH (1991). Effects of 2,4-dichlorophenol, a metabolite of a genetically engineered bacterium, and 2,4-dichlorophenoxyacetate on some microorganism-mediated ecological processes in soil. *Applied and Environmental Microbiology*, 57(2): 412-418.

247. Doyle JD, Short KA, Stotzky G, King RJ, Seidler RJ and RH O (1991). Ecologically significant effects of *Pseudomonas putida* Ppo301(Pro103), genetically engineered to degrade 2,4-dichlorophenoxyacetate, on microbial-populations and processes in soil. *Canadian Journal of Microbiology*, 37(9): 682-691.

248. Ingham ER, Doyle JD and Hendricks CW (1995). Assessing interactions between the soil foodweb and a strain of *Pseudomonas putida* genetically-engineered to degrade 2,4-D. *Applied Soil Ecology*, 2(4): 263-274.

249. Bej AK, Perlin M and Atlas RM (1991). Effect of introducing genetically engineered microorganisms on soil microbial community diversity. *FEMS Microbiology Letters*, 86: 169-176.

250. DoE, *Genetically modified crops and their wild relatives - a UK perspective*. 1994, Department of the Environment.

251. DETR, *Guidance on Best Practice in the Design of Genetically Modified Crops*. 2000, ACRE Best Practice Subgroup; Department of the Environment, Transport and the Regions: www.environment.detr.gov.uk/acre/bestprac/consult/guidance/bp/index.

252. Pollard AJ (2000). Metal hyperaccumulation: a model system for coevolutionary studies. *New Phytologist*, 146: 179-181.

253. Ma JF and Nomoto K (1996). Effective regulation of iron acquisition in graminaceous plants. *Physiologia Plantarum*, 97: 609-617.

254. Blaylock MJ, Salt DE, Dushenkov S, Zakharova O and Gussman C (1997). Enhanced accumulation of Pb in Indian mustard by soil-applied chelating agents. *Environmental Science and Technology*, 31: 860-865.

255. Criessen G, Firmin J, Fryer M, Kular B, Leyland N, Reynolds H, Pastori G, Wellburn F, Baker N, Wellburn A and Mullineux P (1999). Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. *Plant Cell*, 11: 1277-1291.

256. Orser CS, Salt DE, Pickering IJ, Prince RC, Epstein A and Ensley BD (1999). Brassica plants to provide enhanced human mineral nutrition: selenium phytoenrichment and metabolic transformation. *Journal of Medicinal Food*, 1(4): 253-261.

257. Banuelos GS and Mayland HF (2000). Absorption and distribution of selenium in animals consuming canola grown for selenium phytoremediation. *Ecotoxicology and Environmental Safety*, 46(3): 322-328.

258. Kristensen CS, Eberl L, Sanchezromero JM, Givskov M, Molin S and de Lorenzo V (1995). Site-specific deletions of chromosomally located DNA segments with the multimer resolution system of broad-host-range plasmid Rp4. *Journal of Bacteriology*, 177(1): 52-58.

259. de Lorenzo V and Timmis KN (1994). Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5-derived and Tn10-derived minitransposons. *Bacterial Pathogenesis, Pt A*, 235: 386-405.

260. McClure NC, Weightman AJ and Fry JC (1989). Survival of *Pseudomonas putida* UWC1 containing cloned catabolic genes in a model activated sludge unit. *Applied and Environmental Microbiology*, 55: 2627-2634.

261. Ronchel MC, Ramos C, Jensen LB, Molin S and Ramos JL (1995). Construction and behaviour of biologically contained bacteria for environmental applications in bioremediation. *Applied and Environmental Microbiology*, 61(8): 2990-2994.

262. Atlas RM (1992). Molecular methods for environmental monitoring and containment of genetically engineered microorganisms. *Biodegradation*, 3: 137-146.

263. Molin S, Boe L, Jensen LB, Kristensen CS, Giviskov M, Ramos JL and Bej AK (1993). Suicidal genetic elements and their use in biological containment of bacteria. *Annual Reviews Microbiology*, 47: 139-166.

264. Molina L, Ramos C, Ronchel MC, Molin S and Ramos JL (1998). Construction of an efficiently biologically contained *Pseudomonas putida* strain and its survival in outdoor assays. *Applied and Environmental Microbiology*, 64(6): 2072-2078.

265. Jensen LB, Ramos JL, Kaneva Z and Molin S (1993). A substrate dependent biological containment system for *Pseudomonas putida* based on the *Escherichia coli gef* gene. *Applied and Environmental Microbiology*, 59(11): 3713-3717.

266. Contreras A, Molin S and Ramos JL (1991). Conditional-suicide containment system for bacteria which mineralise aromatics. *Applied and Environmental Microbiology*, 57: 1504-1508.

267. Knudsen SM, Saadbye P, Hansen LH, Collier A, Jacobsen BL, Schlundt J and Karlstrom OH (1995). Development and testing of improved suicide functions for biological containment of bacteria. *Applied and Environmental Microbiology*, 61: 985-991.

268. Szafranski P, Mello CM, Sano T, Smith CL, Kaplan DL and Cantor CR (1997). A new approach for containment of microorganisms: Dual control of streptavidin expression by antisense RNA and the T7 transcription system. *Proceedings of the National Academy of Sciences of the United States of America*, 94(4): 1059-1063.

269. Knudsen SM and Karlstrom OH (1991). Development of efficient suicide mechanisms for biological containment of bacteria. *Applied and Environmental Microbiology*, 57: 85-92.

270. Ford CZ, Sayler GS and Burlage RS (1999). Containment of a genetically engineered microorganism during a field bioremediation application. *Applied Microbiology and Biotechnology*, 51(3): 397-400.

9. APPENDICES

APPENDIX A - ABSTRACTS OF WORKSHOP PRESENTATIONS

APPENDIX B - LIST OF WORKSHOP DELEGATES

APPENDIX C - WORKSHOP PROGRAMME

APPENDIX A - ABSTRACTS OF WORKSHOP PRESENTATIONS

Reporter gene based biosensors – risk-based management support for remediation of contaminated land

Professor Ken Killham, Department of Plant & Soil Science, University of Aberdeen, Aberdeen.

The use of microbial biosensors for risk-based site assessment, monitoring and remediation is now providing a completely new and powerful form of support for the management of contaminated land. Construction of the biosensors is based on the insertion of luminescence-based (*lux*) or unstable *gfp* reporter genes into a range of environmentally relevant bacteria, and allows for both specific contaminant and overall toxicity-based contaminant detection. The latter approach is particularly relevant to the new environmental legislation in the UK – Part IIA of the Environmental Protection Act defines site contamination in terms of significant harm. The toxicity biosensors offer a reliable and rapid means of diagnosing the harm caused by site contamination.

Case studies are presented where reporter-gene based biosensors were first used to map the sites for toxic contaminants. This was carried out for both groundwater and for soils/sediments and proved to be able to identify all risk-based contaminants (organic and inorganics) at the sites. The sensors were then used to identify the potential for bioremediation and, using a series of sample manipulations coupled to biosensor-based assays, identify any constraints to bioremediation across the site. Finally the sensors were used to provide risk-based monitoring of the sites to provide the basis for advice to the regulatory authorities on sign-off.

Recent incorporation of reporter genes into a range of single and multi-cell eukaryotes has provided an opportunity to extend toxicity-based biosensing of site contaminant towards human risk assessment. Such biosensors are insensitive to contaminants such as zinc (which strongly inhibit biosensors based on environmental bacteria), but responsive to contaminants such as certain PAH's (which have no effect on, or stimulate bacterial sensors).

Reporter gene based biosensors are highly compatible with on-line instrumentation, where biosensor response can be used for automated, and often remote, groundwater or surface water monitoring. Mathematical analysis of the kinetic response of the sensors on-line can even be used to diagnose the site contaminants.

References

Hollis, R.P., Killham, K & Glover, L.A. 1999. Design and application of a biosensor for monitoring toxicity of compounds to eukaryotes. Applied and Environmental Microbiology, 66, 1676-1679.

Shaw, L.J., Sousa, S., Beaton,Y., Glover, L.A., Killham, K., Meharg, A.A. 2000. Lux based biosensing of 2,4-dichlorophenol biodegradation and bioavailability in soil. In: Proceedings of the International In situ and on-site Bioremediation Symposium, San Diego, California, 5, 247-252.

Beaton, Y., Shaw, L.J., Glover, L.A., Meharg, A.A & Killham, K 1999. Biosensing 2,4-dichlorophenol toxicity during biodegradation by *Burkolderia* sp RASC C2 in soil. Environmental Science and Technology, 33, 4086-4091.

Preston, S., Hall, J.M., Rattray, E.A.S., Chaudri, A. M., McGrath, S.P., Killham, K. and Paton, G I 1999. Assessing metal bioavailability in soils using luminescence-based microbial biosensors. In: *In situ* and On-Site Bioremediation, Battelle Press, Columbus, Ohio.

Phytoremediation of toxic chemicals in our environment

Professor Richard B. Meagher, Department of Genetics, University of Georgia, Athens, GA 30602, USA.

Phytoremediation is the use of plants to extract, sequester, and/or detoxify pollutants in soil, water, and air. At present, phytoremediation is widely viewed as the most promising, ecologically responsible alternative or amendment to the environmentally destructive excavation and reburial methods currently used to remediate toxic sediments. Sediment excavation and reburial not only destroys the environment of the original contaminated site, but create new sites that must be monitored indefinitely. Another serious drawback to physical remediation methods is that they are proving too costly to be applied on the imposing scale that is required. One measure of these drawbacks is that most chemical waste and spill sites larger than a few acres have never been cleaned up.

Phytoremediation is generating great excitement, because its technologies offer a efficacious means of restoring the hundreds of thousands of square miles of land and water now polluted by human activities. Most phytoremediation strategies reestablish a normally functioning ecosystem concomitant with cleansing chemical waste sites. Phytoremediation technologies should also make it possible for manufacturing and agricultural wastes to be processed immediately as they are leaving their point sources, preventing their entering the environment. These same approaches may also help forestry better utilise traditionally marginal lands that are pour in nutrients and contain toxic elements from natural or anthropomorphic sources.

Plants have numerous natural genetic, biochemical, and physiological properties that make them ideal agents to remediate soil and water (Meagher and Rugh, 1996). Among the many plant attributes useful to phytoremediation, there are three specific characteristics that can be directly manipulated for remediation purposes. First, many plant species can grow 100 million miles of roots per acre per year that normally extract nutrients from soil and water. Clearly, this provides an enormous surface area through which contaminants can be contacted and extracted from sediment. Second, plants are photosynthetic and thus control more than 80% of the energy in most ecosystems; this energy can be devoted to phytoremediation. Third, plants are autotrophs with numerous biochemical pathways for degrading or storing complex organics and elements that can be enhanced for phytoremediaton. To understand more specificallyhow plants might be used to degrade or sequester environmental contaminants, it is helpful to distinguish between the strategies for phytoremediating organic and elemental pollutants (Meagher, 2000).

Organic pollutants are composed of carbon usually complexed with nitrogen, sulfur, and/or chlorine. Organic pollutants on every country's priority list include polychlorinated biphenyls (PCBs) like dioxin, polycyclic aromatic hydrocarbons (PAHs) like benzoapyrene, nitroaromatics like trinitrotoluene (TNT), linear halogenated hydrocarbons like trichloroethylene (TCE), and halogenated aromatic pesticides like DDT. All of these compounds are toxic and some are also teratogenic and carcinogenic. Plants have the potential to degrade such organic pollutants to much less toxic forms or even to completely mineralise them into harmless constituents like carbon dioxide, ammonia, or chloride ion. The right plant species can accelerate degradation 100- or even 100,000-fold over natural attenuation. These plants use endogenous enzymes that are either part of their normal metabolism or are part of the biochemical defenses they use against predators and parasites. However, in many cases the fast growing, deeply rooted plants that would be ideal for phytoremediation, like hybrid poplars, willow species, or grasses, do not have enough of the right enzymes to degrade target compounds. Genetically modifying these plants with genes from bacterial, animal, or other plant sources can greatly enhance their phytoremediation properties. For example, trinitrotoluene (TNT) is widely distributed in the environment from use in munitions manufacture. A particular bacterial nitroreductase gene expressed in plants can greatly accelerate the rate of TNT degradation and enhance plant growth by reducing its toxic effects (French *et al.,* 1999).

Elemental pollutants include toxic heavy metals and radionuclides such as arsenic, cadmium, cesium, chromium, lead, mercury, strontium, technetium, tritium, and uranium. Unfortunately, elemental pollutants are essentially immutable by any biological or physical process short of nuclear fission and fusion, and thus their remediation presents special scientific and technical problems. Phytoremediation strategies for elemental pollutants focus first on reducing toxicity; second on sequestering the element in roots to prevent leaching from the site; and third on hyperaccumulation in above-ground organs for later harvest. Only rare and generally exotic native plants have been found that can efficiently extract heavy metals into above-ground tissues. Most of these are so fastidious in their growth habits that they are seldom useful in phytoremediation strategies. Put another way, most ubiquitous, rapidly growing native plants do not naturally express the right genes in the right tissues to be effective agents for elemental remediation. However, several significant experiments demonstrate that the phytoremediation of elemental pollutants can be greatly enhanced using genetically modified plants (Meagher, 2000). My own laboratory's work on the remediation of mercury will serve as a useful example.

Mercury primarily enters the environment from industrial and defense related accidents. Although a few hectare-sized sites have been excavated, tens of thousands of mercury sites worldwide have never been cleaned up, and several of these cover hundreds of square kilometers (Meagher *et al.,* 2000). Ionic mercury Hg(II) is relatively toxic, but with rare

exceptions neither it nor metallic Hg(0) have been involved in serious incidents of human mercury poisoning without first being transformed into methylmercury (Keating *et al.,* 1997). Various mercury species are efficiently converted to methylmercury by anaerobic bacteria, especially in anaerobic sediments (Choi *et al.,* 1994). One of the most dangerous aspects of methylmercury is that it is biomagnified up the food chain by several orders of magnitude and has a greater toxicity than most other mercury compounds (Meagher *et al.,* 2000). As a result, the fish-eating predatory animals and humans at the top of long aquatic food chains suffer the most sever methylmercury poisoning (Boischio and Henshel, 1996; Keating *et al.,* 1997). The world first became aware of the extreme dangers of methylmercury after a large, tragic incident of human mercury poisoning in Japan in the 1950s at Minamata Bay (Harada, 1995). It is likely that a new methylmercury poisoning incident of even greater proportions is victimizing native populations in the tributaries of the Amazon River in South America due to the ongoing use of mercury in the gold mining industry (Barbosa *et al.,* 1995; Cleary, 1996). Because native bacteria at aquatic sites synthesise methylmercury from other forms of mercury, eliminating all forms of mercury contamination from lakes, rivers, and wetlands should largely prevent methylmercury formation.

Our laboratory has used two genes from the well-characterised bacterial *mer* operon, *merA* and *merB,* in order to engineer a mercury transformation and remediation system in plants (Meagher, 2000; Rugh *et al.,* 2000). The bacterial *merB* gene encodes an organomercurial lyase that degrades methylmercury to methane and Hg(II). In plants, expression of *merB* alone confers high levels of methylmercury resistance, allowing the transgenics to grow normally on methylmercury concentrations 10 times higher than those that kill wild type plants (Bizily *et al.,* 1999). At levels of methylmercury as high as or much higher than found in the environmental these transgenic plants accumulate and are able to outgrow Hg(II), the product of the MerB catalyzed reaction. The bacterial *merA* gene encodes a mercuric ion reductase that converts ionic mercury (Hg(II)) to elemental, metallic mercury (Hg(0). Metallic mercury is nearly two orders of magnitude less toxic than ionic mercury and is readily eliminated due to its volatility. Diverse plant species expressing *merA* constitutively are resistant to at least 10-fold higher levels of Hg(II) than those that kill non-transgenic controls (Rugh *et al.,* 1996; Rugh *et al.,* 1998). Plant expression of *merA* and *merB* together results in the two-step conversion to volatile Hg(0) and produces resistance to 50-fold higher levels of MeHg than is required to kill control plants and 5-fold higher than the levels that kill *merB* plants (Bizily *et al.,* 2000). The *merA/merB* plants eliminate mercury 1000 times faster from methylmercury-containing growth medium than control plants. Neither product of these enzymes, Hg(II) or Hg(0), are biomagnified. Because methylmercury is such an extreme human health hazard it is important that a number of phytoremediation strategies that block its flow into the environment be adopted.

Phytoremediation holds great promise as an environmentally friendly approach to cleaning sediments, soils, and water supplies of toxic chemicals. Physical remediation methods are usually very environmentally destructive and create thousands of newly contaminated sites. Natural attenuation of typical contaminated sites is often estimated to take 100-10,000 years, while well-designed phytoremediation strategies function in the range of 3-20 years. Genetically modified plants will greatly accelerate the rates of organic remediation and are essential for the phytoremediation of most elemental pollutants. Applying modern genetic manipulation techniques to phytoremediation will reverse the ongoing deterioration of our environment.

Bibliography

Barbosa, A.C., Boischio, A.A., East, G.A., Ferrari, I., Goncalves, A., Silva, P.R.M., and da Cruz, T.M.E. (1995). Mercury contamination in the Brazilian Amazon. Environmental and occupational aspects. Water, Air, and Soil Pollution 80, 109-121.

Bizily, S., Rugh, C.L., and Meagher, R.B. (2000). Phytodetoxification of hazardous organomercurials by genetically engineered plants. Nature Biotech. 18, 213-217.

Bizily, S., Rugh, C.L., Summers, A.O., and Meagher, R.B. (1999). Phytoremediation of methylmercury pollution:. *merB* expression in *Arabidopsis thaliana* confers resistance to organomercurials. Proc. Natl. Acad. Sci. USA 96, 6808-6813.

Boischio, A.A., and Henshel, D.S. (1996). Mercury contamination in the Brazilian Amazon. Environmental and occupational aspects. Water, air and soil pollution 80, 109-107.

Choi, S.C., Chase, J.T., and Bartha, R. (1994). Metabolic pathways leading to mercury methylation in *Desulfovibrio desulfuricans* LS. Appl. & Env. Microbiol. 60, 4072-4077.

Cleary, D. (1996). Mercury contamination and health risk in the Brazilian Amazon. An ethical dilemma (editorial). Rev Inst Med Trop Sao Paulo 38, 247-8.

French, C.E., Rosser, S.J., Davies, G.J., Nicklin, S., and Bruce, N.C. (1999). Biodegradation of explosives by transgenic plants expressing pentaerythritol tetranitrate reductase. Nat Biotechnol 17, 491-4.

Harada, M. (1995). Minamata disease:. Methylmercury poisoning in Japan caused by environmental pollution. Critical Reviews in Toxicology 25, 1-24.

Keating, M.H., Mahaffey, K.R., Schoeny, R., Rice, G.E., Bullock, O.R., Ambrose, R.B., Swartout, J., and Nichols, J.W. (1997). Mercury study report to congress, I, 2:1-2:9.

Meagher, R.B. (2000). Phytoremediation of toxic elemental and organic pollutants. Curr. Opin. Plant Biol. 3, 153-162.

Meagher, R.B., and Rugh, C.L. (1996). Phytoremediation of heavy metal pollution: Ionic and methyl mercury. In:. OECD Biotechnology for Water Use and Conservation Workshop, eds (Cocoyoc, Mexico: Organization for Economic Co-Operation and Development), 305-321.

Meagher, R.B., Rugh, C.L., Kandasamy, M.K., Gragson, G., and Wang, N.J. (2000). Chapter 11. Engineered phytoremediation of mercury pollution in soil and water using bacterial genes. In: Phytoremediation of Contaminated Soil and Water, N Terry, and G. Banuelos, eds (Boca Raton: Lewis Publishers), pp. 203-221.

Rugh, C.L., Bizily, S.P., and Meagher, R.B. (2000). Phytoremediation of environmental mercury pollution. In: Phytoremediation of toxic metals: Using plants to clean-up the environment, B. Ensley, and I. Raskin, eds (New York: Wiley and Sons), pp. 151-169.

Rugh, C.L., Senecoff, J.F., Meagher, R.B., and Merkle, S.A. (1998). Development of transgenic yellow-poplar for mercury phytoremediation. Nature Biotech. 16, 925-928.

Rugh, C.L., Wilde, D., Stack, N.M., Thompson, D.M., Summers, A.O., and Meagher, R.B. (1996). Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana*. plants expressing a modified bacterial merA gene. Proc. Natl. Acad. Sci. USA 93, 3182-3187.

Field release of *P. fluorescens* HK44:. Long term persistence and field performance of a bioremediation bioluminescent bioreporter

Professor Gary S Sayler, Center for Environmental Biotechnology, University of Tennesse, Knoxville, Tennesse, USA

Under support of the US Department of Energy (DOE), the University of Tennessee received the first US Environmental Protection Agency (EPA) Consent Order (March 1986) for environmental test release of genetically modified bacteria for applications in bioremediation. In October 1986, under this Consent Order, *Pseudomonas fluorescens* HK44 containing an introduced stable catabolic plasmid (NAH7-like) for polyaromatic hydrocarbon biodegradation with a bioluminescent (*lux*) bioreporter gene transcriptional fusion was inoculated into replicated test and control subsurface soil lysimeters at DOE's Oak Ridge National Laboratory. Both donor and recipient *P. fluorescens* strains used to construct the GMO were isolated from manufactured gas plant contaminated soils. Origins of the *lux* genes and antibiotic resistance markers delivered by transposon Tn4431, to create the bioreporter fusion were *Vibrio fisheri* and *E. coli*, respectively.

The focus of these field studies was to determine under what conditions the GMO could be maintained in the subsurface soil to promote PAH bioremediation. A fundamental hypothesis tested was that light emission from the bioreporter could be used as a real-time bioremediation process-monitoring tool to provide on-line control strategies for system optimization. These objectives were pursued over a two-year intensive investigation after which time additional intermittent monitoring has been conducted to the present.

Organismal and process monitoring was conducted in a multi parametric format, including selective cultivation, colony hybridization, PCR amplification of target genes, bioluminescent MPN, on-line and *in-situ* bioluminescence, hydrocarbon decay, temperature, humidity and respiration. Experimental manipulations, included water table and moisture adjustments, exogenous micronutrients, re-contamination with PAH in transformer oil, and soil gas phase aeration.

Population dynamics of the GMO followed a predicted long-term decay, but HK44 was readily detectable and recoverable at densities of $10^2 g^{-1}$ after two years and $<10^2 g^{-1}$ after 1100 days. Population decay could be interrupted and regrowth of the GMO to densities as high as $10^9 g^{-1}$ could be achieved with aeration and hydrocarbon replacement. *In-situ* bioluminescent in subsurface soil was measured in real-time and corresponded to experimental manipulations and hydrocarbon bioremediation. Virtually all naphthalene components of the PAH mixture were removed during the field test, but discrimination of abiotic and natural attenuation

influences vs. GMO bioremediation was not clear; thus limiting absolute quantitation of the bioremediated component.

Observations on future successful biotechnology risk assessment review and process application include:

- Provide detailed taxonomy including 16s rRNA phylogeny of the proposed GMOs
- Provide absolute ancestry of all genetic elements introduced
- Insure the GMO phenotype does not exhibit potential pathogenic properties, i.e growth at 37°C, enzymes or polymers
- Avoid, if possible, mobile genetic elements and antibiotic resistance markers
- Provide sufficient efficacy data from lab or microcosm simulation on detection, monitoring and process effectiveness
- Anticipate natural population decay kinetics and contamination attenuation by indigenous and abiotic processes
- Strive for $>10^8$ active organisms g^{-1} to achieve maximal treatment efficiency
- Soil gas phase 0_2 concentration is a key rate limiting process for aerobic bioremediation in the vadose

Prospects and challenges for bioremediation with GMMs

Professor Kenneth N Timmis, National Research Centre for Biotechnology, Braunschweig, Germany.

Microbial life on planet Earth is thought to have originated some 3,800m years ago, and by about 3,500m years ago microbes had evolved most of the central biochemical processes we currently know, some 2,000m years before eukaryotes appeared. Microbes have evolved a phylogenetic and metabolic diversity that greatly exceeds all other forms of life, and that enables them to colonise a vast spectrum of diverse environments, ranging e.g from polar ice to thermal vents; microbial habitats thus define the biosphere. The diverse range of nutrients and sources of energy that can be used by microbes, coupled with the rapid growth rates of some, make them major players in the biogeochemical cycling of elements, and in the turnover of polluting wastes in the environment. Many toxic xenobiotics represent a nutritional opportunity to the microbial flora, which is often able to eliminate such pollutants from the environment. However, in contrast to the millions of years of exposure and evolution of microbes to natural polluting compounds, they have only been exposed to xenobiotics for a few decades, a very brief period in evolutionary time. It is therefore to be expected that metabolic activities towards such compounds are not optimal.

Modern genetic methods allow accelerated evolution in the laboratory of optimised catabolic functions, and of important accessory functions, and represent a powerful option to accelerate the elimination of toxic chemicals from the environment, when the rate-limiting factor is sub-optimal catabolic potential. The use of such designed biocatalysts is reasonably straightforward in end-of-pipe applications and will not be further discussed. *In-situ/ex situ* applications are, however, more ecological in nature and less subject to operator control. Since polluted sites are often characterised by rather extreme physico-chemical parameters that are unfavourable to colonisation by an introduced GMM, the use of genetic modules to optimise selected site-adapted members of the habitat to be treated will become increasingly important. In any case, effective application of GMMs will require a much better understanding of the ecological parameters regulating the biodegradation of xenobiotics in the environment. In particular, microbes designed to better degrade a pollutant need to function as integrated members of the natural microbial communities colonising the polluted habitat. Since the vast majority of the microbes active in natural habitats cannot be cultivated and studied by traditional means, interactions of GMMs with other community members, with the pollutants, and with the abiotic components of the habitat, must be investigated by culture-independent methods.

Important underlying ecological issues that need to be better understood are:

- The significance of the high diversity of degraders in natural microbial communities

- The structural and functional organisation of catabolic communities in biofilms

- The population dynamics of community members during catabolism of the pollutant and the parameters regulating the population changes and community activities

- The strategies whereby microbes access hydrophobic substrates ('bioavailability' with regard to biodegrading microbes may not be the same as 'bioavailability' with regard to risk assessment), or: what is the face of the bug? What is the face of the pollutant?

- The metabolic routes and networks followed during catabolism of the pollutant, and relevant kinetic data and metabolic fluxes of the community

- The sharing of the available nutritional resources by the community, and underlying metabolic interactions

The understanding gained from such studies needs to be underpinned by a theoretical framework of microbial community structure and function, to be obtained by modelling the community and its metabolic, physiologic and genetic potential and interactions. Such modelling will identify parameters that directly or indirectly limit or regulate key community or GMM activities, and suggest interventions that can reduce limitations.

Metal accumulation by plants

Professor J.A.C Smith, Department of Plant Sciences, University of Oxford, Oxford

Metals are present in many soils at concentrations inimical to plant growth. Naturally metalliferous soils have arisen from the weathering of parent geological materials, and these substrates often support highly distinctive vegetation (which has proved a spur to fields such as geochemical prospecting). But through the exploitation of this natural resource there are now many anthropogenic sources of toxic metals contaminating today's environment. These include atmospheric discharges from industrial processes such as metal processing and burning of fossil fuels, deposition onto the soil in the form of mine waste, sewage sludge, animal manures, fertilisers and agrochemicals, as well as transfer to the soil through irrigation water or run-off, and accidental discharges from the nuclear industry.

Certain plants have, however, succeeded in evolving tolerance to relatively high concentrations of soil metals. In fact, some of these have become classic examples of 'microevolution' in plants in response to a particularly strong selective pressure (Antonovics *et al.*, 1971), e.g the occurrence of zinc-tolerant grasses under galvanised electricity pylons, or copper-tolerant ecotypes in the vicinity of metal smelters. However, it is now clear that different plants may show fundamentally different mechanisms of metal tolerance. At one extreme are the *metal excluders*, in which metal tolerance is inversely related to metal concentrations within the plant biomass. At the other extreme are the so-called *metal hyperaccumulators*, which are the most metal-tolerant plants known and which can accumulate certain metals (such as Zn, Mn, Ni, Co, or Cd) to over 1 % of shoot dry biomass (Baker *et al.*, 1999). These plants pose many interesting questions as to how such metals can be accumulated and sequestered in biological tissues without toxic effects, as well as raising broader issues concerning the ecological significance of metal hyperaccumulation.

For a number of years, it has been clear that hyperaccumulator plants represent a potentially valuable resource for the remediation of contaminated soils (Salt *et al.*, 1995; Chaney *et al.*, 1997). These plants possess a number of useful attributes for this type of 'phytoremediation' technology. For example, they extract metals very effectively out of the soil; they translocate most of the metal out of the root system to the above-ground parts of the plant in the shoot; and the high shoot concentrations themselves tend to deter grazing herbivores, helping to minimise further dispersal into the food chain. It is even feasible to harvest the above-ground parts as part of a true recycling process to recover metals from the dry biomass. Estimates suggest that this type of phytoremediation technology could be up to 100 times cheaper than conventional methods such as excavation and removal to landfill, as well as being less environmentally damaging.

On the other hand, there are a number of significant disadvantages to a phytoremediation approach based on naturally occurring hyperaccumulator plants (Baker *et al.*, 1999). First, they are without exception rare plants, often with very restricted population sizes: only about 430 species are known worldwide, representing less than 0.2 % of all flowering plants. Second, most hyperaccumulator species are rather small, slow-growing plants, so successive harvests would be needed over an extended period of time (perhaps 10 to 15 years at minimum) to decontaminate the soil to acceptable levels. Third, most hyperaccumulators are rather specific to particular metals (for example, the Ni hyperaccumulators can tolerate Co, but are very sensitive to Zn and Cu: Krämer *et al.*, 1996), so this would not allow treatment of mixed metal wastes with these plants. And fourth, many are adapted to special climatic or edaphic conditions that would preclude their cultivation in particular environments.

An obvious route to the realization of an effective phytoremediation technology would be to introduce the metal-hyperaccumulation trait into a high-biomass plant with favourable growth characteristics and harvestability (Chaney *et al.*, 1997; Salt *et al.*, 1998). Theoretically, this could be achieved by introgression using closely related genotypes, or by techniques such as protoplast fusion. The more targeted route, however, will be to identify the essential genes involved in metal hyperaccumulation and to introduce these specifically into the appropriate genetic backgrounds. This will necessitate more detailed understanding of the hyperaccumulation mechanism than achieved hitherto. Certain key components have been identified, such as the need for appropriate metal-binding ligands to detoxify the metal ions, and specific transporters to allow movement of metals through the plant (Krämer *et al.*, 1996; Meagher, 2000). But it is not yet understood at the genetic level how these processes are uniquely regulated in hyperaccumulator plants. Production of transgenic plants exhibiting a metal-hyperaccumulation trait has not yet been reported, but this is an area of intense activity that will very likely produce notable advances in the near future.

References

Antonovics, J., Bradshaw, A.D and Turner, R.G. (1971) Heavy metal tolerance in plants. *Adv. Ecol. Res.* **7,** 1-85.

Baker, A.J.M., McGrath, S.P., Reeves, R.D and Smith, J.A.C (1999) Metal hyperaccumulator plants: a review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. In *Phytoremediation of Contaminated Soil and Water*, eds N Terry and G. Bañuelos, pp 85-107 Lewis Publishers, Boca Raton, Florida.

Chaney, R.L., Malik, M., Li, Y.-M., Brown, S.L., Brewer, E.P., Angle, J.S and Baker, A.J.M. (1997) Phytoremediation of soil metals *Curr. Opin Biotechnol.* **8,** 279-284.

Krämer, U., Cotter-Howells, J.D., Charnock, J.M., Baker, A.J.M. and Smith, J.A.C (1996) Free histidine as a metal chelator in plants that accumulate nickel. *Nature* **379,** 635-638.

Meagher, R.B (2000) Phytoremediation of toxic elemental and organic pollutants *Curr Opin Plant Biol* **3,** 153-162.

Salt, D.E., Blaylock, M., Kumar, P.B.A., Dushenkov, V., Ensley, B.D., Chet, I. and Raskin, I (1995) Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants *Bio/Technology* **13,** 468-474.

Salt, D.E., Smith, R.D and Raskin, I (1998) Phytoremediation. *Annu Rev Plant Physiol Plant Mol. Biol.* **49,** 643-668.

Defusing the environment: Engineering plants to degrade explosives

Dr Neil C Bruce, Institute of Biotechnology, University of Cambridge

Over the last twenty years there has been major international concern over the contamination of land and ground water with persistent organic pollutants. Remediation has been attempted mainly through the economically and environmentally costly large-scale removal of soil and water; however, large areas of contaminated land still exist and such demanding and expensive remediation procedures are not an option for the developing world. The lack of affordable and effective cleanup technologies demands the development of novel processes. Much work has focused on microbial biodegradation, but results to date have been disappointing. The effectiveness of such processes has been inhibited by factors such as poor biomass, the requirement of an additional substrate for cometabolism and the lack of induction of the relevant metabolic genes. These problems may be overcome through the use of genetically modified bacteria with altered regulation of degradative genes; however, it is not clear that such modified organisms will thrive in the environment, and furthermore, current legislation severely restricts the release of genetically modified microorganisms into the environment. Recent attention has, therefore, focused on phytoremediation, which is the use of plants to remediate environmental toxicity.

Plants have potentially impressive economic benefits as a robust and renewable resource. They produce large amounts of biomass and have a remarkable ability to extract compounds from the surrounding environment. Their root systems are dense and extensive and promote increased microbial activity in their rhizosphere. Plants also have a high degree of public acceptance with an appreciation of their aesthetic quality and environmental benefits. The reluctance by regulatory authorities to approve the release of genetically modified to approve the release of genetically modified bacteria is partly because of the mobility of bacteria and the ease of horizontal gene transfer among dissimilar prokaryotes. However, these problems can be addressed by the use of transgenic plants, as by virtue of their size, plants are more easily controlled than microorganisms. Furthermore, by selecting sterile plants and controlling propagation by harvesting the plants prior to flowering, uncontrolled genetic release can be prevented. A major disadvantage of plants is that their innate biodegradative abilities are limited and often rates of uptake and metabolism can be slow. Recently, transgenic plants were shown to germinate and grow in the presence of normally toxic levels of ionic mercury[1,2]. This raised the question of whether the impressive biodegradative abilities of certain bacteria could be combined with the high biomass and stability of plants to yield an optimal system for *in-situ* bioremediation of organic pollutants in soil. We therefore decided to combine the biodegradative capabilities of soil bacteria with the high biomass and stability of plants to yield an optimal system for *in-situ* bioremediation of explosives in soil.

Toxic explosive residues are major environmental contaminants due to the manufacture, testing and disposal of munitions. Explosives can be broadly classified into three groups: nitroaromatics (e.g trinitrotoluene, TNT, is probably the most persistent pollutant in military sites), nitramines (e.g hexahydro-1,3,5-trinitro-1,3,5-triazine, RDX) and nitrate esters (e.g glycerol trinitrate, nitroglyerin, GTN and pentaerythritol tetranitrate, PETN). We have isolated bacteria that degrade all the major classes of explosives [3,4,5,6]. Our recent studies of the biodegradation of energetic compounds by bacteria resulted in the isolation of a strain of Enterobacter cloacae, termed PB2, which is capable of utilising the nitrate ester explosives PETN and GTN as the sole source of nitrogen. Liberation of nitrite is mediated by an NADPH-dependent PETN reductase (PETNR) which has FMN bound as prosthetic group[4]. The gene for PETNR, onr, has been cloned, sequenced and overexpressed in E coli and the crystallographic structure of this enzyme has recently been determined[5,7]. Interestingly, this enzyme is related in sequence and structure to Old Yellow Enzyme from Saccharomyces carlsbergenesis. All the members of this family have Tim-barrel structures, FMN as a prosthetic group and display various chemistries with electrophilic substrates. Activity towards α,β-unsaturated carbonyl structure is a common property of this family. PETNR is, however, unusual, in that it displays activity towards the highly recalcitrant aromatic explosive TNT via a reductive pathway resulting in nitrogen liberation[6]. Furthermore, we have recently demonstrated that PETNR can reductively liberate nitrite from the nitramine explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). PETNR is therefore remarkable in that it is able to degrade all the major classes of explosives by distinct mechanisms. Current work is focused on relating structure and function within this growing family of enzymes with a view to engineering novel enzymes exhibiting useful characteristics towards explosives for bioremediation purposes.

Our work has been targeted on the use of genetic engineering for broadening and improving the degradative capabilities of plants for phytoremediation of the major classes of explosives. We have engineered transgenic tobacco plants that express PETNR to degrade nitrate ester explosives and TNT[7]. Importantly, we have shown that seeds from transgenic tobacco plants which express PETNR were capable of germinating and growing in media containing levels of GTN and TNT that are toxic to the wild-type plant. Furthermore, we have shown that transgenic seedlings are able to denitrate GTN at a much faster rate than wild-type seedlings. We have also recently constructed transgenic plants that express the gene encoding the aromatic nitroreductase gene from *E. cloacae* PB2. This small monomeric flavoprotein reduces aromatic nitro groups to nitroso, hydroxylamino and amino groups, with oxidation of NAD(P)H. Partially reduced nitroaromatic compounds are highly reactive, and when produced intracellularly are known to react with cell constituents to form covalent bonds, thus being removed from the environment. Our initial studies with nitroreductase plant lines look very promising, with the transgenic plants detoxifying high concentrations of TNT. Since the bacterial degradative pathways for many classes of pollutants have been elucidated,

this may be a generally applicable method of achieving bioremediation of contaminated soil in the environment.

References

Rugh, C.L., Wilde, D., Stack, N.M., Thompson, D.M., Summers, A.O., and Meagher, R.B (1996) Proc Natl Acad Sci USA 93: 3182-3187.

Rugh, C L., Senecoff, J F., Meagher, R B and Merkle, S A (1998) *Nature Biotech* 16: 925-928.

Binks, P R., Nicklin, S and Bruce, N C (1995) *Appl Eviron Microbiol* 61: 1318-1322.

Binks, P R., French, C E., Nicklin, S and Bruce, N C (1996) *Appl Environ Microbiol* 62:1214-1219.

French, C E., Nicklin, S and Bruce, N C (1997) *J Bacteriol.*178:6623-6627.

French, C E., Nicklin, S and Bruce, N C (1998) *Appl Environ Microbiol* 64: 2864-2868

Barna ,T., Khan, H., Bruce, N C., Scrutton, N S and Moody, P.C.E (2000) (submitted for publication).

French, C E., Rosser, S J., Davies, G J., Nicklin, S and Bruce, N C (1999) *Nature Biotech* 17: 491494.

APPENDIX B - LIST OF WORKSHOP DELEGATES

Dr Colin Cartwright	WS Atkins Environment
Dr Ian Thompson	CEH, Oxford
Dr Michelle Barclay	CEH, Oxford
Prof Mark Bailey	CEH, Oxford
Prof Andrew Smith	Dept of Plant Sciences, University of Oxford
Dr Neil Bruce	Institute of Biotechnology, University of Cambridge
Prof Ken Killham	Dept of Plant & Soil Science, University of Aberdeen
Prof Rich Meagher	Dept of Genetics, University of Georgia
Prof Gary Sayler	Center for Environmental Biotechnology, University of Tennessee
Prof Ken Timmis	National Research Centre for Biotechnology, Braunschweig
Prof Chris Leaver	Dept of Plant Sciences, University of Oxford
Prof Chris Knowles	Oxford Centre for Environmental Biotechnology, University of Oxford
Dr Martin Broadley	Horticulture Research International, Wellesborne
Dr Ken Bruce	Division of Life Sciences, Kings College London
Dr Adrian Butt	DEFRA
Miss Alexandra Christopher	Dept of Chemistry, University of Oxford
Dr Gillian Davis	University of Oxford
Dr Richard Ellis	NERC Centre for Population Biology, Imperial College at Silwood Park
Dr Dawn Field	CEH, Oxford
Dr Anne Glover	Dept of Molecular and Cell Biology, University of Aberdeen, Remedios
Dr Rosie Hails	CEH, Oxford
Ms Judith Hann	
Dr Frances Harper	School of Life Sciences, University of Dundee
Dr Penny Hirsch	IACR Rothamsted
Dr David Hopper	Institute of Biological Sciences, University of Wales, Aberystwyth
Mr Robert Ingle	Dept of Plant Sciences, Oxford
Dr Simon Jackman	CEH, Oxford
Dr Theresa Kearney	Environment Agency
Mr Anthony Keeling	Elsoms Seeds Ltd
Ms Renatta Krasowaik	School of Biosciences, University of Birmingham
Dr. Mike Larkin	Questor Centre, Queen's University of Belfast
Ms Rachel Leighton	Dept of Plant Sciences, Oxford
Dr Andy Lilley	CEH, Oxford
Prof Jim Lynch	School of Biological Sciences, University of Surrey
Dr Lynne Macaskie	School of Biosciences, University of Birmingham
Dr Sarah MacNaughton	AEA
Dr Steve McGrath	IACR Rothamsted
Prof Andy Meharg	Dept of Plant & Soil Science, University of Aberdeen
Dr Andreas Meyer	Institut fuer Forstbotanik und Baumphysiologie, Freiburg
Dr Kate Millar	School of Biosciences, University of Nottingham
Ms Lucy Moore	Dept of Plant Sciences, Oxford
Dr Martin Naylor	CEH, Oxford
Dr Marion Rawlins	DEFRA
Dr Brian Reid	School of Environmental Science, University of East Anglia
Dr Jonathan Rees	Dept of Plant Sciences, Oxford
Dr Gary Robinson	Dept of Biosciences, University of Kent at Canterbury

Dr Natasha Smith	ADAS
Mr Ben Sykes	BBSRC
Dr Phillip White	Horticulture Research International, Wellesborne
Dr Andy Whitely	CEH, Oxford
Dr Neil Willey	Centre for Research in Plant Science, University of the West of England
Dr Peter Williams	School of Biological Sciences, University of Wales, Bangor
Dr Luet Wong	Dept of Chemistry, University of Oxford

APPENDIX C - WORKSHOP PROGRAMME

9.45	Registration
10.20	Introduction to the workshop from project consortium
10.30	Presentation from Ken Timmis (National Research Centre for Biotechnology, Braunschweig, Germany) **Prospects and challenges for bioremediation with GMMs**
11.10	Presentation from Ken Killham (Department of Plant and Soil Science, University of Aberdeen) **GM biosensors for monitoring contaminant bioavailability and assessing the potential for bioremediation of contaminated land**
11.50	Presentation from Gary Sayler (Center for Environmental Biotechnology, University of Tennesse, USA) **Field release of *P. fluroescens* HK44: Long term persistence and field performance of a bioremediation bioluminescent bioreporter**
12.30	summary of morning session
12.45	buffet lunch
13.30	Presentation from Andrew Smith (Department of Plant Sciences, University of Oxford) **Metal accumulation by plants**
14.10	Presentation from Rich Meagher (Genetics Department, University of Georgia, USA) **Phytoremediation of toxic chemicals in our environment**
14.50	Presentation from Neil Bruce (Institute of Biotechnology, University of Cambridge) **Defusing the environment: engineering transgenic plants to degrade explosives**
15.30	summary of afternoon session
15.45	general discussion on issues raised
16.30	end of meeting