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Review

Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions

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Abstract: Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds that have accumulated in the natural environment mainly as a result of anthropogenic activities such as the combustion of fossil fuels. Interest has surrounded the occurrence and distribution of PAHs for many decades due to their potentially harmful effects to human health. This concern has prompted researchers to address ways to detoxify/remove these organic compounds from the natural environment. Bioremediation is one approach that has been used to remediate contaminated land and waters, and promotes the natural attenuation of the contaminants using the *in situ* microbial community of the site. This review discusses the variety of fungi and bacteria that are capable of these transformations, describes the major aerobic and anaerobic breakdown pathways, and highlights some of the bioremediation technologies that are currently available.

Keywords: polycyclic aromatic hydrocarbons (PAHs); bioremediation; bacteria; fungi; soil; environment

INTRODUCTION

Polycyclic aromatic hydrocarbon contamination in the environment

Sources and occurrence of polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds that consist of two or more fused benzene rings and/or pentacyclic molecules that are arranged in various structural configurations. They are highly recalcitrant molecules that can persist in the environment due to their hydrophobicity and low water solubility.[1] Some representative PAHs are shown in Fig 1.

PAHs are ubiquitous in the natural environment, and originate from two main sources: these are natural (biogenic and geochemical) and anthropogenic.[2] It is the latter source of PAHs that is the major cause of environmental pollution and hence the focus of many bioremediation programmes. PAHs naturally occur in fossil fuels such as coal and petroleum, but are also formed during the incomplete combustion of organic materials such as coal, diesel, wood and vegetation.[3,4] This results in airborne PAH contamination, which is the main route for PAH transport over long distances.[5] Point sources of PAHs can originate from petroleum and diesel spills and from industrial processes such as coal liquefaction and gasification during coke production.[6] For example, creosotes and coal tar, which are by-products of coking, contain significant quantities of PAHs (eg creosote contains up to 85% PAHs[2]). More minor sources of PAHs include tobacco smoke and burnt food.

Natural processes can also provide a source of PAHs, such as volcanic eruptions and forest fires.[7] In addition, PAHs can have a geochemical origin as they are formed during pyrolysis, which involves the exposure of sediments to high temperatures during sediment diagenesis.[7]

PAHs are widely distributed in soils and sediments, groundwater and the atmosphere. They have been detected in marine sediments such as San Diego Bay, California[8,9] and the Central Pacific ocean,[10] intertidal sediments,[11] gas works site soils,[12,13] sewage sludge-contaminated soils,[14] aquifers and groundwater and in atmospheric deposits such as vehicle exhaust fumes.[4]

Point sources of PAH contamination are the most significant environmental concern. Though the areas contaminated are relatively small in size, the

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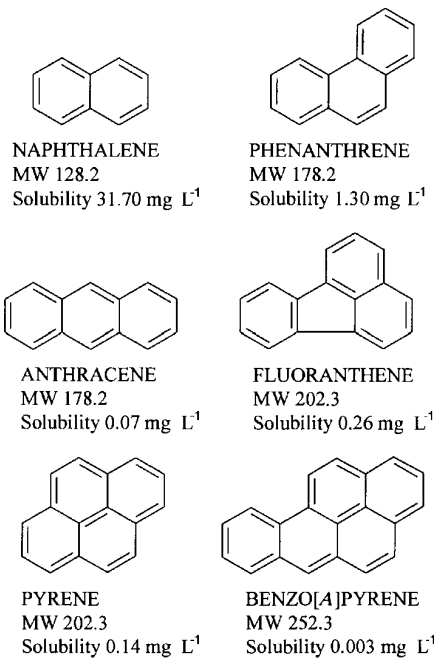

Figure 1. Chemical structures and physical characteristics of some representative polycyclic aromatic hydrocarbons (PAHs).

contaminant concentration at these sites is often high and associated with co-contaminants such as benzene, toluene, ethylene and xylene (BTEX) compounds, heavy metals and aliphatic hydrocarbons, which can hinder remediation efforts. Soils can be contaminated with between 1 $\mu g\,kg^{-1}$ and 300 $g\,kg^{-1}$ PAHs,[15] depending on the source of contamination (eg old coal gasification sites have the higher levels stated). Atmospheric levels of PAHs resulting from the incomplete combustion of materials such as coal and wood have been found to be between 60 $\mu g\,m^{-3}$ and 3 $mg\,m^{-3}$ air.[3]

Persistence of PAHs in the environment

The persistence of PAHs in the environment is dependent on a variety of factors, such as the chemical structure of the PAH, the concentration and dispersion of the PAH and the bioavailability of the contaminant. In addition, environmental factors such as soil type and structure, pH and temperature and the presence of adequate levels of oxygen, nutrients and water for the activity of the pollutant-degrading microbial community will control the time that PAHs persist in the environment[16] (see later, 'Factors affecting the bioremediation of PAHs').

In general, the higher the molecular weight of the PAH molecule, the higher the hydrophobicity and toxicity, and the longer the environmental persistence of the molecule.[1] In addition the 'age' of the contaminant in the soil/sediment matrix plays a significant role in the biodegradability of PAHs in soil.[17] A study using phenanthrene as a model PAH showed that phenanthrene mineralisation and therefore biodegradability was significantly reduced with time of ageing.[17]

The association of PAHs with co-pollutants such as hydrocarbons and heavy metals is another factor that can prolong their residence time in the environment. Aliphatic hydrocarbons and BTEX compounds are readily biodegradable by the *in situ* microbial community relative to the more complex chemical structures of the PAHs. This results in the depletion of available oxygen in the surrounding environment and the onset of anaerobicity. Though recent work has shown that there is a real potential for the biodegradation of PAHs in the absence of molecular oxygen (see 'Anaerobic metabolism of PAHs'), details regarding the efficiency and scale of PAH degradation in anaerobic environments is still limited, with rates of anaerobic organic matter oxidation up to an order of magnitude less than those under aerobic conditions.[18] In addition, it is possible that the presence of heavy metals in soil could inhibit microbial growth and hence limit the metabolism of contaminants under anaerobic conditions.

Toxicity of PAHs

It has long been known that PAHs can have serious deleterious affects to human health,[6] with the physician John Hill first recognising the link between the use of snuff and nasal cancer in 1761.[6] Following this discovery, research into the toxic effects that PAHs have upon mammalian health has continued, with many PAHs displaying acute carcinogenic, mutagenic and teratogenic properties. Benzo[*a*]pyrene is recognised as a priority pollutant by the US Environmental Protection Agency[19] as this compound is known to be one of the most potently carcinogenic of all known PAHs.[5]

When ingested, PAHs are rapidly absorbed into the gastrointestinal tract due to their high lipid solubility.[6] A major route of PAH uptake is via dermal absorption as highlighted by a study of 12 coke-oven workers.[20] An estimated 75% of the total absorbed amount of PAHs (specifically pyrene) entered the body through the skin, highlighting this as a major exposure route of PAHs. The rapid absorption of PAHs by humans results in a high potential for biomagnification in the food chain. In general, the greater the number of benzene rings, the greater the toxicity of the PAH.[1] The relative toxicity of PAHs can be measured using LD_{50} values (the lethal dose in 50% of cases). These are expressed as milligrams of toxic material per kilogram of the subject's body weight that will cause death in 50% of cases. It is important to specify the route by which the toxic material was administered to the test animal (such as oral or intraperitoneal), and the animal upon which the toxic material was tested (ie rat, mouse). See Table 1 for the LD_{50} values of some representative PAHs.

PAHs are also suspected carcinogens but are not thought to be genotoxic unless they are 'activated' by mammalian enzymes to reactive epoxides and quinones. This occurs via a cytochrome P_{450} monooxygenase enzyme-mediated reaction that

Table 1. LD_{50} values of some representative PAHs

Material	Number of carbon rings	LD_{50} value (mg kg^{-1})	Test subject	Exposure route
Naphthalene	2	533–710	Male/female mice respectively	Oral
Phenanthrene	3	750	Mice	Oral
Anthracene	3	>430	Mice	Intraperitoneal
Fluoranthene	4	100	Mice	Intravenous
Pyrene	4	514	Mice	Intraperitoneal
Benzo[*a*]pyrene	5	232	Mice	Intraperitoneal

Generally, toxicity increases with an increase in number of benzene rings, but data should be examined using careful consideration of the exposure route, etc (data taken from the Risk Assessment Information System (RAIS) http://risk.lsd.ornl.gov).

oxidises the aromatic ring to form epoxide and diol–epoxide reactive intermediates. It is reported that these intermediates may undergo one of at least four different mechanisms of oxidation and/or hydrolysis before the intermediates combine with and/or attack DNA to form covalent adducts with DNA. DNA adducts can lead to mutations of the DNA, resulting in tumours.[21]

The solution — bioremediation?

Bioremediation, which is also referred to as bioreclamation and biorestoration, can be described as 'the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state'.[22] The main principle of this technique is to remove pollutants from the natural environment and/or convert the pollutants to a less harmful product using the indigenous microbiological community of the contaminated environment. Bioremediation strategies are developed to promote the microbial metabolism of contaminants, by adjusting the water, air and nutrient supply. This is accomplished by the biostimulation (the addition of a bulking agent such as wood chips and/or nutrients such as N/P/K) and bioaugmentation (often an inoculum of microorganisms with known pollutant transformation abilities) of the contaminated environment.

Bioremediation of PAH-contaminated soils, sediments, and water can be accomplished in a variety of ways, eg *in situ* treatment or *ex-situ* methods such as bio-piling and composting. Specific details of bioremediation in relation to PAH treatment are covered later, see 'Approaches to the bioremediation of PAH-contaminated environments'. Waste can also be treated in bioreactors, though this can be more costly than *in situ* technologies. It is important for bioremediation to be comparable in cost and success to physical and chemical treatments of contaminated land, such as landfilling, incineration and soil washing. The applicability of bioremediation can be variable, but this is generally due to unfavourable site conditions (see 'Factors affecting the bioremediation of PAHs'), therefore a thorough understanding of site conditions will allow optimisation of bioremediation and subsequently more effective results. In commercial situations bioremediation of PAH-contaminated soils is not typically carried out when the site contains significant amounts of PAHs that have more than four rings as the low percentage removal of PAHs of this molecular weight and the time taken for successful reduction in PAH concentrations is not economically viable (Bio-Logic, personal communication). The method used is normally nutrient addition (see 'Nutrient availability') and aeration by frequent turning of contaminated soil. Total PAH levels during a bioremediation trial are generally reduced from approximately 3000mg to 1000 mg total PAHs, per kg.

MICROBIAL METABOLISM OF PAHS

There are three fundamentally different mechanisms in the aerobic metabolism of PAHs by microorganisms (Fig 2) and specific details of bacterial and fungal (ligninolytic and non-ligninolytic) PAH metabolism are discussed below. The basis of these mechanisms is the oxidation of the aromatic ring, followed by the systematic breakdown of the compound to PAH metabolites and/or carbon dioxide. Anaerobic metabolism of PAHs is thought to occur via the hydrogenation of the aromatic ring with details of these processes given in the section 'Anaerobic metabolism of PAHs'.

PAH-degrading microorganisms are ubiquitously distributed in the natural environment, such as in soils (bacteria and non-ligninolytic fungi) and woody materials (ligninolytic fungi). Many PAH-contaminated soils and sediments host active populations of PAH-degrading bacteria. For example, phenanthrene-degrading bacteria were isolated from PAH-contaminated mangrove sediments in Hong Kong.[11] These isolates were able to degrade phenanthrene under a range of salinities both in pure and mixed cultures. Anaerobic environments, eg municipal sewage sludges[23] and marine sediments,[9] can also host a diverse array of PAH-degrading bacteria. Unlike non-ligninolytic fungi, the ligninolytic fungi, such as *Phanerochaete chrysosporium*, are commonly associated with woody materials and are not commonly found in soils. However, these fungi can be enriched in a soil by the addition of straw, wood chips and other lignin-rich substrates. A thorough listing of microorganisms capable of PAH degradation is provided by Mueller *et al*.[2]

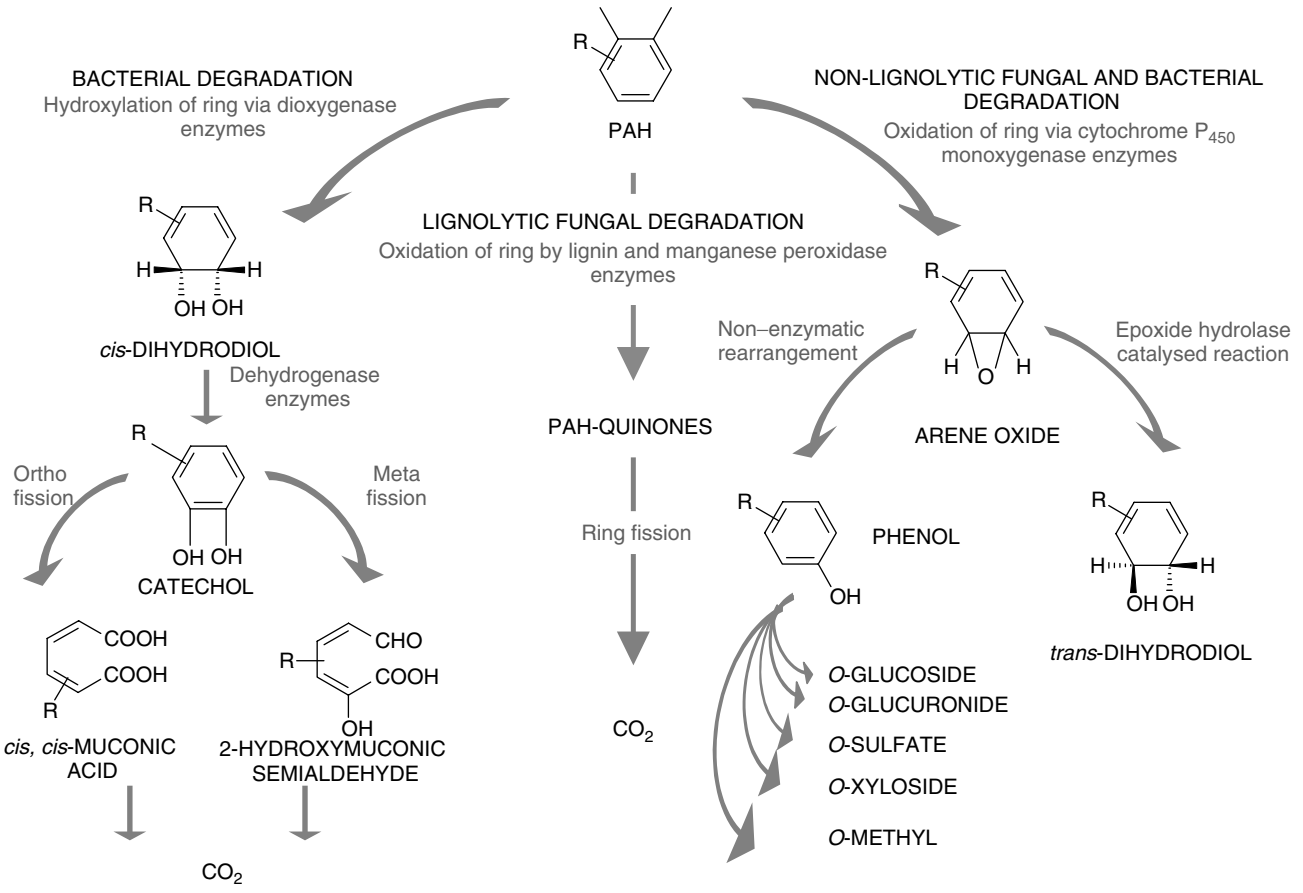

Figure 2. The three main pathways for polycyclic aromatic hydrocarbon degradation by fungi and bacteria.[1].

Bacterial metabolism of PAHs

The principal mechanism for the aerobic bacterial metabolism of PAHs is the initial oxidation of the benzene ring by the action of dioxygenase enzymes to form *cis*-dihydrodiols. These dihydrodiols are dehydrogenated to form dihydroxylated intermediates, which can then be further metabolised via catechols to carbon dioxide and water. The metabolic pathways and enzymatic reactions involved in the microbial degradation of naphthalene have been studied in detail with an example pathway of naphthalene transformation given in Fig 3.

There is a large diversity of bacteria that are able to oxidise naphthalene using dioxygenase enzymes, including organisms from the genus *Pseudomonas* and *Rhodococcus* (see Refs 1 and 2 for a full listing). A few bacteria are also capable of oxidising PAHs by the action of the cytochrome P_{450} monoxygenase enzyme to form *trans*-dihydrodiols such as *Mycobacterium* sp.[24] Rockne and colleagues reported the ability of marine methanotrophs in degrading PAHs via the action of the methane monoxygenase gene.[25] It is thought however that these are minor mechanisms compared with the activity of the dioxygenase enzymes.[26] The mechanisms involved in this pathway are detailed in the next section.

The toxicity of naphthalene metabolites generated during bacterial degradation has been little studied. The metabolites of naphthalene, such as naphthalene dihydrodiols, have a higher water solubility than naphthalene and are therefore potentially more bioavailable, and could pose a greater toxicity than the naphthalene precursor. Naphthalene 1,2 dihydrodiols show minimal toxicity to human liver cells relative to control samples, whereas the metabolites of 1-naphthol, 1,2-naphthoquinone and 1,4-naphthoquinone, generated during the human cytochrome P_{450}-mediated oxidation reactions, showed a significant toxicity to human liver cells and mononuclear leucocytes.[27] These metabolites were considerably more toxic than the naphthalene precursor.[27] In comparison to the naphthalene metabolites produced by humans and some fungi, naphthalene intermediates generated during the cytochrome P_{450}-mediated oxidation by *Mycobacterium* sp generate *trans*-dihydrodiols that could reasonably be expected to show minimal toxicity to humans.

Fungal metabolism of PAHs

There are two main types of fungal metabolism of PAHs; these are mediated by the non-ligninolytic and ligninolytic fungi (also known as the white-rot fungi). The majority of fungi are non-ligninolytic, as they do not grow on wood, and therefore have no need for the lignin peroxidase enzymes that are produced by the ligninolytic fungi. However, many ligninolytic fungi such as *Phanerochaete*

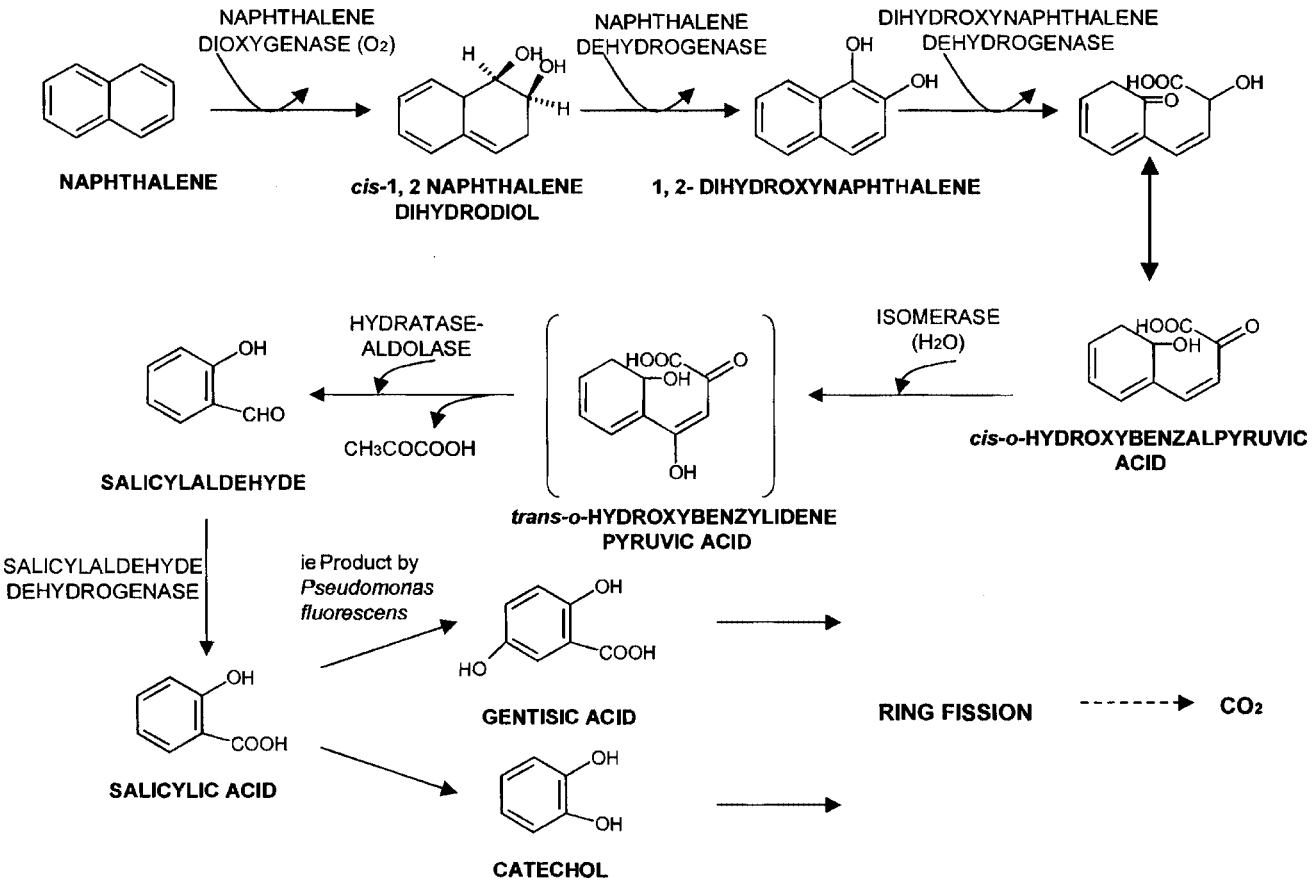

Figure 3. The main pathways in the aerobic degradation of naphthalene by bacteria.[85].

chrysosporium[28] and *Pleurotus ostreatus*[29] can produce both non-ligninolytic and ligninolytic type enzymes, but it is unclear to what degree each enzyme contributes to the breakdown of the PAH molecule.

Non-ligninolytic fungi

The first step in the metabolism of PAHs by non-ligninolytic fungi is to oxidise the aromatic ring in a cytochrome P_{450} monoxygenase enzyme catalysed reaction to produce an arene oxide.[16] This route is similar to the mammalian metabolism of PAHs. In comparison to the oxidation of the aromatic ring by dioxygenase enzymes to form *cis*-dihydrodiols, the monoxygenase enzyme incorporates only one oxygen atom onto the ring to form an arene oxide. This is subsequently hydrated via an epoxide-hydrolase catalysed reaction to form a *trans*-dihydrodiol.[30] In addition, phenol derivatives may be produced from arene oxides by the non-enzymatic rearrangement of the compound, which can act as substrates for subsequent sulfation or methylation, or conjugation with glucose, xylose, or glucuronic acid.[2] Although most non-ligninolytic fungi are not capable of the complete mineralisation of PAHs, these PAH-conjugates are generally less toxic and more soluble than their respective parent compounds. For example, Pothuluri and colleagues[31] demonstrated this with the degradation of fluoranthene by the non-ligninolytic fungal species *Cunninghamella elegans*. The metabolites 3-fluoranthene-β-glucopyranoside, 3-(8-hydroxy-fluoranthene)-β-glucopyranoside, fluoranthene *trans*-2,3-dihydrodiol and 8-hydroxy-fluoranthene-*trans*-2,3-dihydrodiol showed no mutagenic effects to a rat liver homogenate fraction, and 9-hydroxy-fluoranthene-*trans*-2,3-dihydrodiol was considerably less toxic than fluoranthene.

Chrysosporium pannorum, Cunninghamella elegans and *Aspergillus niger* are examples of non-ligninolytic fungi that use a P_{450} monoxygenase enzyme-mediated oxidative pathway for PAH degradation. An example pathway of the cytochrome P_{450}-mediated oxidation of phenanthrene is detailed in steps 1 to 4 of Fig 4. The latter steps (5 and 6) are thought to be mediated by lignin peroxidase enzymes[29] (detailed in the next section).

Ligninolytic fungi

White-rot fungi are a group of fungi that produce ligninolytic enzymes involved in the oxidation of lignin present in wood and other organic matter. There are two types of ligninolytic enzymes; these being peroxidases and laccases.[32] These enzymes are secreted extracellularly, and oxidise organic matter via a non-specific radical based reaction.[33] There are two main types of peroxidase enzyme depending on their reducing substrate type, lignin peroxidase (LP) and manganese peroxidase (MnP), both of which are capable of oxidising PAHs.[32] Laccases, which are

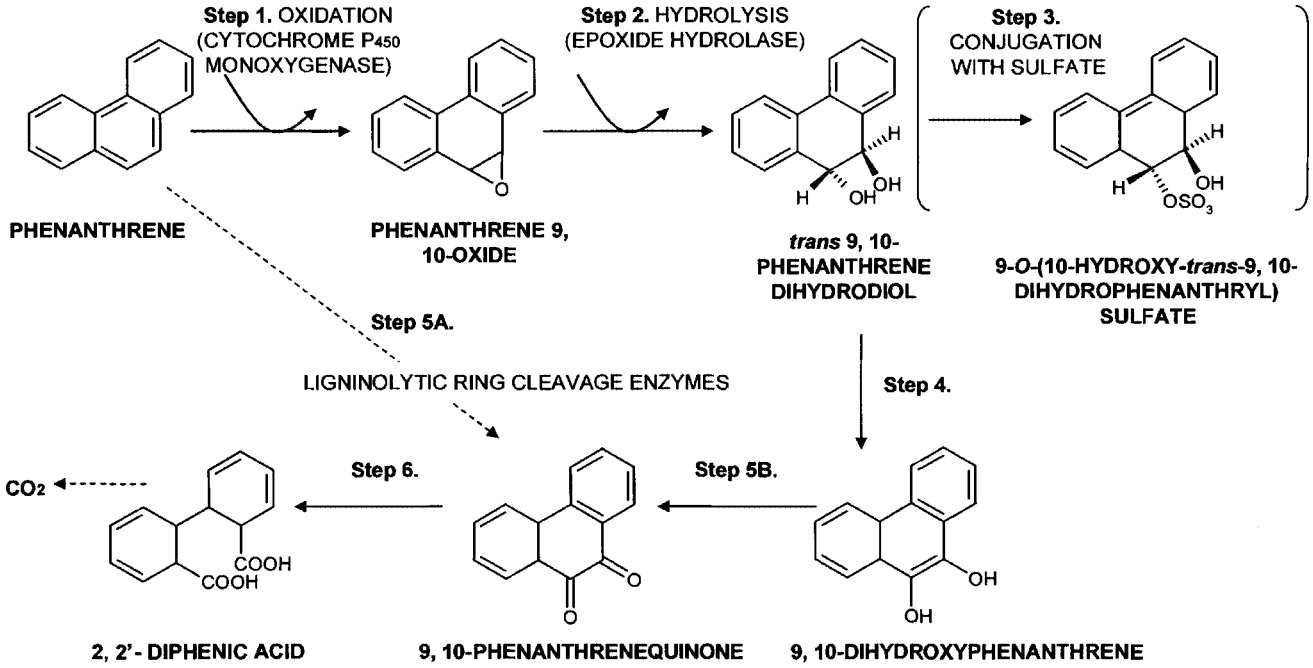

Figure 4. Proposed pathway for the degradation of phenanthrene by the ligninolytic fungus *Pleurotus ostreatus*.[29].

phenol oxidase enzymes, are also capable of oxidising PAHs.

Under ligninolytic conditions, white-rot fungi can oxidise PAHs by generating free radicals (ie hydroxyl free radicals) by the donation of one electron,[16] which oxidises the PAH ring. This generates a selection of PAH-quinones and acids rather than dihydrodiols (see steps 5 and 6, Fig 4). There is significant interest surrounding the use of ligninolytic fungi to degrade PAHs, as they have low substrate specificity and are therefore able to degrade even the most recalcitrant of compounds. Also, the enzymes involved are extracellular, and are theoretically able to diffuse into the soil/sediment matrix and potentially oxidise PAHs with low bioavailability (see 'Bioavailability' section).

Degradation studies of the ligninolytic fungi have shown that PAHs may be degraded by a combination of ligninolytic enzymes, cytochrome P_{450} monooxygenases, and epoxide hydrolases that can result in the complete mineralisation of the compound.[29] Degradation studies of high molecular weight PAHs such as pyrene and benzo[*a*]pyrene by ligninolytic fungi (ie *Phanerochaete chrysosporium* and *Pleurotus ostreatus*) have suggested that a combination of ligninolytic and non-ligninolytic enzymes may be the key to the complete mineralisation of these recalcitrant compounds.[29]

Substantial research has focused upon the potential of this group of fungi to remediate PAH-contaminated materials. Bioremediation trials that used ligninolytic fungi to remediate PAH-contaminated soils and sediments have shown mixed results. For example, Canet and colleagues[34] used four white-rot fungi species to degrade a coal-tar-contaminated soil. Although the soil in this study was supplemented with straw (as a substrate for the fungi), the indigenous soil microorganisms were more successful in PAH-degradation than the introduced fungal species. In another study that monitored the potential of white-rot and brown-rot fungi to degrade a PAH-contaminated soil, *Pleurotus ostreatus* and *Antrodia vaillantii* were used to inoculate an artificially-contaminated soil to degrade a range of PAHs.[35] The *P ostreatus* fungal inoculum significantly increased the degradation of PAHs relative to the un-amended soils, but resulted in the accumulation of potentially toxic PAH metabolites. As this white-rot fungus also inhibited the *in situ* microbial populations within the soil, this may have prevented the complete mineralisation of the PAHs, resulting in the accumulation of PAH metabolites. The authors suggest that a fungal–bacterial consortium would be beneficial to the decontamination of this soil. As the brown-rot fungus *A vaillantii* showed equal if not better PAH degradation than *P ostreatus*, and did not generate dead-end PAH metabolites, it was suggested that this fungus could be exploited as a valuable inoculum in bioremediation trials.

In order to increase the efficiency of white-rot fungi in the remediation of PAH-contaminated soil, May and colleagues[36] designed a two-stage pilot-scale reactor that initially extracted PAHs from a contaminated soil and subsequently treated the extracted PAHs in a fungal bioreactor. This fungal bioreactor utilised *P chrysosporium*, and was successful in degrading high molecular weight PAHs such as benzo[*a*]pyrene.

Anaerobic metabolism of PAHs

PAHs are a common contaminant of anaerobic environments such as aquifers[37–39] and marine

sediments.[8–10,40] Even aerobic environments such as contaminated soils, sediments and groundwater can develop anaerobic zones.[41] This is due to the organic contaminant stimulating the *in situ* microbial community, resulting in the depletion of molecular oxygen during aerobic respiration. This oxygen is not replenished at the same rate as its depletion, which results in the formation of anaerobic zones proximal to the contaminant source.

It was not until recently that the potential of microorganisms to degrade PAHs in the absence of molecular oxygen has been recognised. Previous studies have tended to focus upon the thermodynamically more favourable aerobic processes of bioremediation of recalcitrant organic compounds such as PAHs, whereby molecular oxygen is incorporated into the aromatic ring prior to the dehydrogenation and subsequent PAH ring cleavage (see earlier for details of the mechanisms of aerobic degradation of PAHs). In the absence of molecular oxygen, alternative electron acceptors such as nitrate, ferrous iron and sulfate are necessary to oxidise these aromatic compounds, with recent research clearly demonstrating that PAH degradation will occur under both denitrifying[18,42] and sulfate-reducing[8,9,39,43] anaerobic conditions.

The mechanisms of anaerobic PAH degradation are still tentative, though recent studies have proposed a mechanism for the anaerobic degradation of naphthalene,[39,43] which is summarised in Fig 5. The first step is the carboxylation of the aromatic ring to 2-naphthoic acid, which may activate the aromatic ring prior to hydrolysis. Stepwise reduction of 2-naphthoic acid via a series of hydrogenation reactions results in decaclin-2-carboxylic acid which is subsequently converted to decahydro-2-naphthoic acid. There may be other mechanisms for anaerobic naphthalene degradation, however these have not yet been elucidated. For example, it is proposed that the initial step in anaerobic naphthalene degradation under sulfate-reducing conditions occurs via a hydroxylation reaction to form a naphthol intermediate.[44]

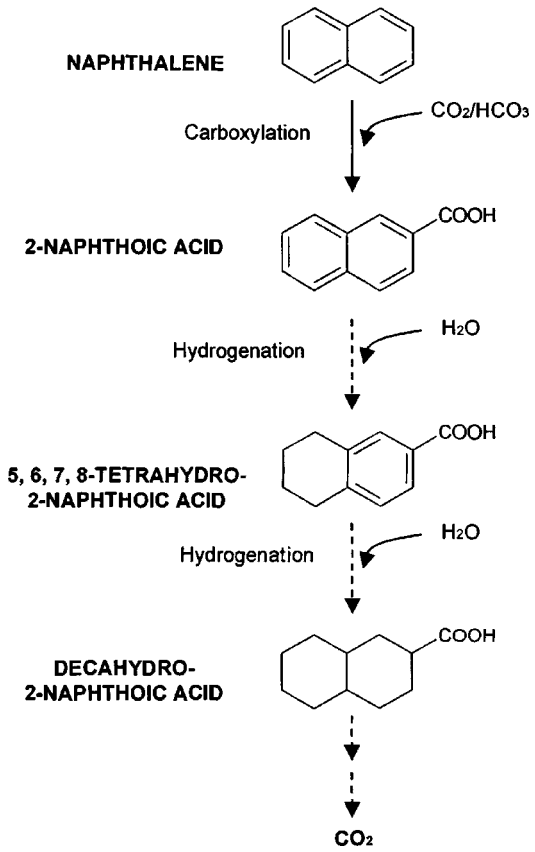

Figure 5. Simplified proposed pathway for the anaerobic metabolism of naphthalene under sulfate-reducing conditions.[39,43].

FACTORS AFFECTING THE BIOREMEDIATION OF PAHS

There are many examples of the successful application of bioremediation technologies to contaminated sites using approaches such as biopiling and composting. Many published studies have investigated the efficacy of bioremediation on a bench scale and under ideal laboratory conditions, such as a circum-neutral pH and mesophilic temperatures. However, it is apparent that environmental factors that vary from site to site (such as soil pH, nutrient availability and the bioavailability of the contaminant) can influence the process of bioremediation by inhibiting growth of the pollutant-degrading microorganisms. The main environmental factors that could affect the feasibility of bioremediation are summarised in the following five sections, whilst the final section addresses the concern of the toxicity of the metabolites formed during the biodegradation of PAHs.

Temperature

Temperature has a considerable effect on the ability of the *in situ* microorganisms to degrade PAHs and, in general, most contaminated sites will not be at the optimum temperature for bioremediation during every season of the year. The solubility of PAHs increases with an increase in temperature,[45] which increases the bioavailability of the PAH molecules. In addition, oxygen solubility decreases with increasing temperature, which will reduce the metabolic activity of aerobic microorganisms.

Biodegradation of PAHs can occur over a wide temperature range, however most studies tend to focus on mesophilic temperatures rather than the efficiency of transformations at very low or high temperatures. However, it is apparent that microorganisms have adapted to metabolise PAHs at extreme temperatures, for example naphthalene and phenanthrene degradation was reported from crude oil in seawater at temperatures as low as 0 °C.[46] In comparison, the laccase and manganese peroxidase enzymes of ligninolytic fungi were reported to have a temperature optimum of ~50 °C and >75 °C respectively in spent-mushroom compost during the degradation of PAHs,[47] with over 90% degradation of the contaminating PAHs occurring at these temperatures.

pH

Many sites contaminated with PAHs are not at the optimal pH for bioremediation. For example, retired gasworks sites often contain significant quantities of demolition waste such as concrete and brick. Leaching of this material will increase the pH of the native soil and/or made ground of the site, resulting in less favourable conditions for microbial metabolism. In addition, the oxidation and leaching of coal spoil will create an acidic environment by the release and oxidation of sulfides. As the pH of contaminated sites can often be linked to the pollutant, the indigenous microorganisms at the sites will not have the capacity to transform PAHs under acidic or alkaline conditions. Therefore, it is common practice to adjust the pH at these sites, for example by the addition of lime.[48]

Phenanthrene degradation in liquid culture has been investigated at a range of pH values (pH 5.5–7.5) with *Burkholderia cocovenenas*, an organism isolated from a petroleum-contaminated soil.[49] Although bacterial growth was not significantly affected by the pH, phenanthrene removal was only 40% at pH 5.5 after 16 days, whereas at circum-neutral pH values, phenanthrene removal was $\geqslant$ 80%. *Sphingomonas paucimobilis* (strain BA 2) was however more sensitive to the pH of growth media, with the degradation of the PAHs phenanthrene and anthracene significantly inhibited at pH 5.2 relative to pH 7.[50]

PAH degradation has been recorded in an acidic soil (pH 2) contaminated by coal spoil by the indigenous microorganisms, with the concentrations of naphthalene, phenanthrene and anthracene reduced over a 28-day period.[51] Naphthalene concentrations were reduced by 50% in soils downstream of a nearby coal pile, with phenanthrene and anthracene reduced by between 10 and 20%. The authors showed that a consortium of fungi and bacteria accomplished this, and suggest the presence and activity of PAH-degrading acidophilic bacteria. These results suggest that future research would benefit from the isolation and characterisation of PAH-degrading microorganisms from both acidic and alkaline environments.

Recent unpublished research from the authors' laboratory, which investigated the presence, activity and diversity of *Pseudomonas* species from a PAH-contaminated concrete highlighted the possibility that microorganisms isolated from alkalophilic environments may be able to degrade PAHs at an elevated pH.[52] The potential of these *Pseudomonas* to degrade naphthalene in liquid culture was compared with a selection of characterised PAH-degrading *Pseudomonas* species. It was found that some of the environmental isolates were able to both reduce the pH of the liquid media from 9 to 6.5 within 24 h, and also utilise naphthalene as a sole source of carbon. In contrast, the naphthalene-degrading microorganisms *Pseudomonas fredrikbergensis* (DSM 13 022) and *Pseudomonas fluorescens* (DSM 6506), were severely inhibited by the elevated pH. This suggests that the *in situ* microorganisms at a contaminated site may be not only tolerant of the site conditions, but may have the potential to metabolise PAHs in sub-optimal conditions (in this case, high pH).

Oxygen

Though it is now well established that bioremediation of organic contaminants such as PAHs can proceed under both aerobic and anaerobic conditions (see earlier section, 'Anaerobic metabolism of PAHs'), most work has tended to concentrate upon the dynamics of aerobic metabolism of PAHs. This is in part due to the ease of study and culture of aerobic microorganisms relative to anaerobic microorganisms. During aerobic PAH metabolism, oxygen is integral to the action of mono- and dioxygenase enzymes in the initial oxidation of the aromatic ring.[53] Ways in which to maintain adequate oxygen levels for aerobic metabolism for *in situ* treatments are discussed below and include hydrogen peroxide for sub-surface contamination. For surface contamination, simple soil tilling and/or mixing, for example using compost turners, can aerate contaminated material well enough to allow PAH transformation to proceed.

There is still debate as to whether the benefits of anaerobic bioremediation are outweighed by the negatives, with the aeration of contaminated anaerobic aquifers successfully used to stimulate aerobic microbial communities resulting in significant reductions in PAH concentrations in groundwater. This has been accomplished using hydrogen peroxide,[54] sodium nitrate[38] and perchlorate.[55] In addition, the aerobic biodegradation of hydrocarbons has been reported to be up to an order of magnitude higher relative to anaerobic biodegradation.[18] However, it has also been reported that rates of anaerobic PAH degradation under denitrifying conditions were comparable to those under aerobic conditions.[56]

Though it appears that the future of anaerobic bioremediation is promising, there are several drawbacks to the promotion of anaerobic bioremediation. Not all environments contain an active population of anaerobes that are able to degrade PAHs. This has been shown in a creosote-contaminated sediment, where limited biodegradation of PAHs was seen under denitrifying, sulfate-reducing and methanogenic conditions,[40] even though there was an actively respiring anaerobic community present in the sediment. Similar results were found when investigating the potential for PAH degradation in sediment samples from San Diego Bay, California.[9] Metabolism of PAHs in these sediments that had had low levels of previous exposure to PAHs only occurred after a long lag period, and was promoted when they were 'spiked' with PAH-contaminated sediments that contained an active community of PAH-degraders. This suggests that the dominant *in situ* microbial community did not consist of PAH-degrading microorganisms and that bioremediation was limited by low numbers of

PAH-degrading microorganisms rather than adverse environmental conditions.[9]

Another potential disadvantage of the promotion of *in situ* anaerobic bioremediation is that the geochemistry of the subsurface will be altered by the imposition of reducing conditions. As an environment is driven anaerobic, all residual oxygen is depleted, and electron acceptors such as nitrate, ferric iron and sulfate are reduced during respiration.[57] This results in the mobilisation of ferrous iron, and therefore the release of phosphate from iron(III)–phosphate complexes. Both of these are toxic to the environment; iron(II) is rapidly oxidised when exposed to oxygen, causing an orange precipitate in freshwater frequently associated with acid mine drainage[58] and excess phosphate in freshwaters can cause eutrophication. In addition, there is often a concomitant increase in pH, which can result in the solubilisation of carbonate minerals and the release of trace metals.[59] Respiration will also produce potentially potent greenhouse gases such as H_2S, CH_4 and N_2O.[60]

It is clear that more research is needed to fully understand the implications associated with the promotion of anaerobic bioremediation. The discovery of a wide diversity of pollutant-transforming anaerobes is a significant step forward in understanding the processes involved in bioremediation, and the design and application of anaerobic remediation both *in situ* and *ex situ* to the contaminated site.

Nutrient availability

In addition to a readily degradable carbon source, microorganisms require mineral nutrients such as nitrogen, phosphate and potassium (N, P and K) for cellular metabolism and therefore successful growth. In contaminated sites, where organic carbon levels are often high due to the nature of the pollutant, available nutrients can become rapidly depleted during microbial metabolism.[61] Therefore it is common practice to supplement contaminated land with nutrients, generally nitrogen and phosphates to stimulate the *in situ* microbial community and therefore enhance bioremediation.[62,63]

The amounts of N and P required for optimal microbial growth and hence bioremediation have been previously estimated from the ratio of C:N:P in microbial biomass (between 100:15:3[64] and 120:10:1[65]). However, a recent study has shown that optimal microbial growth and creosote biodegradation occurred in soil with a much higher C:N ratio (25:1) than those predicted from the ratio in microbial biomass, with lower C:N ratios (5:1) causing no enhancement in microbial growth.[63] The level of nutrients required for PAH transformation are generally thought to be similar to those required for other organic pollutants such as petroleum compounds. However, little work has been done regarding the most favourable nutrient levels required for the optimal degradation of PAHs, and further work in this area would benefit future bioremediation trials.

It is worth noting that fungi are able to effectively recycle nutrients (specifically nitrogen), and that excessively high nutrient loadings may in fact inhibit microbial metabolism. In addition, the high molecular weight PAH-oxidising ligninolytic enzymes of the white-rot fungi are produced under nutrient deficient (often low nitrogen) conditions.[66]

It therefore appears imperative that the nutrient status of the site is established prior to the supplementation of the site with additional nutrients. Even though microbial metabolism may be temporarily increased, the long-term inhibition of functionally important organisms may result in the failure of the bioremediation of high molecular weight PAHs (such as benzo[*a*]pyrene).

Bioavailability

Bioavailability can be defined as the effect of physicochemical and microbiological factors on the rate and extent of biodegradation[2] and is believed to be one of the most important factors in bioremediation. PAH compounds have a low bioavailability, and are classed as hydrophobic organic contaminants.[67] These are chemicals with low water solubility that are resistant to biological, chemical and photolytic breakdown.[67] The larger the molecular weight of the PAH, the lower its solubility (see Fig 1), which in turn reduces the accessibility of the PAH for metabolism by the microbial cell.[68,69] In addition, PAHs can undergo rapid sorption to mineral surfaces (ie clays) and organic matter (ie humic and fulvic acids) in the soil matrix. The longer that the PAH is in contact with soil, the more irreversible the sorption, and the lower is the chemical and biological extractability of the contaminant.[17] This phenomenon is known as 'ageing' of the contaminant. Therefore the bioavailability of a pollutant is linked to its persistence in a given environment.

Release of PAHs from the surface of minerals and organic matter can be achieved by the use of surface-active agents (also known as surfactants or detergents). These are compounds that contain both a hydrophobic and hydrophilic moiety, thus providing a 'bridge' between the hydrophobic PAH molecule and the hydrophilic microbial cell. Some microorganisms can produce surfactants (biosurfactants), that can enhance the desorption of PAHs from the soil matrix.[70,71] These are potentially more effective than using synthetic surfactants, as they are thought to be less toxic to the *in situ* microbial community and do not produce micelles, which can encapsulate contaminant PAHs and prevent microbial access.[71]

The bioavailability of PAHs in soil can be assessed using both chemical and biological methods, though it is questionable which type of test(s) are most representative of the bioavailability of hydrophobic organic contaminants in soil, as both approaches have inherent limitations.[72] There are many biological techniques to assess the bioavailability of PAHs in soil. These can be based upon the monitoring of

biological function, such as microbial respiration rates (mineralisation) of ^{14}C-labelled contaminants, the bioluminescence of microorganisms such as *lux* microorganisms and/or *lux*-tagged pollutants that are in contact with a contaminated material[73] and by assessing the degree of dermal diffusion and gastrointestinal sorption of PAHs in earthworms and hence the bioavailability and ecotoxicity of PAHs in the soil.[74] In addition, bioavailability can be measured by monitoring changes in the expression of genes that code for PAH degradation using molecular probes,[51] and also by the extraction of soil pollutants using a simulated mouth and gut digestive fluid such as saliva, in order to demonstrate the risk that the contaminant will pose if ingested.[75]

Biological assays are often supported by performing a chemical assay of the bioavailability of the contaminant in the soil, which physically extracts the contaminant from the soil matrix using a chemical solvent. Organic solvents have been traditionally used to extract organic contaminants during harsh extraction processes (such as Soxhlet extraction), although this does not demonstrate the true bioavailability of the contaminant, but the total contaminant concentration in the soil. However, a more representative approach was used by Hatzinger and Alexander,[17] who extracted the contaminants with mild organic solvents (such as methanol) to represent the bioavailable proportion in the soil. As microorganisms can mostly only access those contaminants that are in the aqueous phase, water-based solvents are also being used to more accurately predict the bioavailable fraction of organic contamination in soil. One such compound is hydroxypropyl-β-cyclodextrin[72] (HPCD), which can encapsulate hydrophobic contaminants. In addition, HPCD does not appear to inhibit *lux*-type microorganisms, allowing for a combined biological and chemical assessment of bioavailability.

Toxicity of end-products

The principle of bioremediation is to remove or detoxify a contaminant from a given environment using microorganisms. Most commercial bioremediation trials tend to monitor the success of the treatment by the degree of removal of the parent contaminant and do not consider the possibility of the biological production of more toxic breakdown metabolites. However, it is important to ensure that the contaminated material is suitably detoxified at the end of the treatment.[12,76] A recent study using a bioreactor to treat PAH-contaminated gasworks soil monitored both the removal of PAHs and the accumulation of oxy-PAHs, such as PAH-ketones, quinones and coumarins.[12] These compounds are formed during the microbial metabolism of PAHs (see earlier 'Microbial metabolism of PAHs'), and can also be formed from chemical oxidation and phototransformation of PAHs.[77] Such transformation products can be equally toxic, if not more toxic, to human health when compared with the parent PAH,[21] with many of the oxy-PAHs formed during the treatment of PAH-contaminated soils more persistent than the parent compounds.[12] In this study, Lundstedt and colleagues showed that although there were no new oxy-PAHs formed during the bioremediation of an aged gasworks soil, the concentrations of 1-acenaphthenone and 4-oxapyrene-5-one increased in the soil by 30% and 60% respectively over 30 days of bioslurry treatment. In addition, they showed that some oxy-PAHs actually increased in concentration during treatment, and were subsequently more persistent to microbial degradation than their corresponding parent PAH compound. As oxy-PAHs are more toxic than the parent PAHs, this study highlights the importance of monitoring the metabolites of bioremediation, specifically for toxic dead-end products, and assessing the toxicity of the material both before and after treatment.

The Microtox® bioassay has been used to perform an assessment of the success of composting of a garden soil spiked with a range of high molecular weight PAHs.[47] In this study, the authors found that the degradation products were far less toxic than the parent PAHs, and concluded that composting this soil with spent mushroom compost was successful. In addition, a multitude of eco-toxicological tests, such as bacterial (Microtox®), algal and Daphnia-based tests were performed to ensure that the toxicity of a coke oven soil was suitably reduced using landfarming.[76] They concluded that landfarming resulted in a significant reduction in toxicity of the coke works soil.

However, it is important to understand the relevance of ecotoxicity tests to the overall toxicity of the remediated land. Many of these tests monitor for 'acute' toxicity of compounds (via organism death), whereas it would be more representative, particularly when assessing the carcinogenic and mutagenic PAHs, to consider the 'chronic' toxicity of these soils, such as monitoring for organism DNA damage and the occurrence of DNA adducts[21] (see earlier, 'Toxicity of PAHs').

APPROACHES TO THE BIOREMEDIATION OF PAH-CONTAMINATED ENVIRONMENTS

Treatment of soils and sediments

Soils and sediments can be treated for PAH contamination both by *in situ* and *ex situ* methods.[48] Landfarming is an *in situ* treatment for soils, which focusses upon stimulating the indigenous microorganisms in the soil by providing nutrients, water and oxygen. For example, a pilot-scale landfarming treatment of PAH-contaminated soil from a wood-treatment facility was achieved by biostimulation of the soil with water, ground rice hulls (as a bulking agent), and pelletised dried blood (as a nitrogen source) and bioaugmentation of the microbial community with an inoculum of *Pseudomonas aeruginosa* (strain 64).[78] Aeration was provided by tilling of the soil. The workers found that 86% of total PAHs

were removed from the soil over 1 year, including a reduction in high molecular weight PAHs such as benzo[*a*]anthracene and benzo[*a*]pyrene (79.5% and 11.3% respectively). Biopiling of soil[48] and the treatment of soil in bioreactors[12] are *ex situ* treatments that are less cost effective than *in situ* treatment, however *ex situ* treatment benefits from being more subject to monitoring and control.

Composting, which is also an *ex situ* treatment for PAH-contaminated soil, is an aerobic process whereby microorganisms degrade organic materials which results in thermogenesis and the generation of organic and inorganic compounds.[79] The process of composting can be divided into four main stages according to the temperature of the material; these are mesophilic, thermophilic, cooling and maturation.[80] These four stages are driven by changes in the microbial community, with an increase in the metabolic activity of the *in situ* microorganisms creating heat. This allows for the establishment of thermophilic microorganisms which displace the mesophilic organisms in the decomposition of organic matter. Temperatures decrease as organic matter is depleted, upon which the composting process enters the cooling and maturation stages.

The success of bioremediation of PAHs by composting has been reported in several studies.[47,81–84] Composting in bioreactors is a popular option because it is possible to exert greater control over parameters such as temperature and oxygen supply during the composting process, and a variety of organic materials as bulking agents can be used. For example, the concentrations of PAHs (anthracene, phenanthrene and pyrene) were successfully reduced during a 60-day period of composting (30 days thermophilic composting, 30 days cooling and maturation) in laboratory-scale bioreactors using a variety of municipal wastes such as paper, grass and food as a carbon source.[82]

Treatment of waters

As with soils and sediments, contaminated groundwater can be remediated both *in situ* and *ex situ* to the contaminated site. However, it is often not feasible to remediate contaminated groundwater *ex situ* due to the costs involved with abstraction and shipping of the contaminated water, and the fact that much of the contamination will be sorbed within the aquifer. Therefore, *in situ* treatment of aquifers can be accomplished by the biostimulation, and possibly the bioaugmentation of the indigenous aquifer community.

The *in situ* treatment of an aquifer contaminated with a range of organic pollutants, including phenols, BTEX compounds and PAHs was carried out on a site that produces flooring, damp proofing and roofing materials manufactured from bitumen and synthetic resins.[38] In addition, records suggest that the site was formerly an oil works and used for coal gasification. This has resulted in the aquifer below the site containing a multitude of organic pollutants, with a mean concentration of $11\,\mu g\,L^{-1}$ PAHs in the groundwater.

The site was remediated using a combination of bioaugmentation and biostimulation over a $2^1/_2$-year period. Nutrients (Purisol 100, supplies nitrogen and phosphate; ICI Chance and Hunt), a commercially available bacterial inoculum (PHENOBAC, Microbac Ltd, Durham) and oxygen (supplied by the reduction of sodium nitrate to gaseous oxides of nitrogen) were circulated through the aquifer by means of a series of injection and abstraction wells (see Fig 6). The *in situ* remediation of this site was successful, even though the contaminants had undergone significant ageing in the aquifer. PAHs were reduced to a concentration of $0.7\,\mu g\,L^{-1}$, and co-contaminants such as phenols reduced from $1100\,\mu g\,L^{-1}$ to $12\,\mu g\,L^{-1}$.

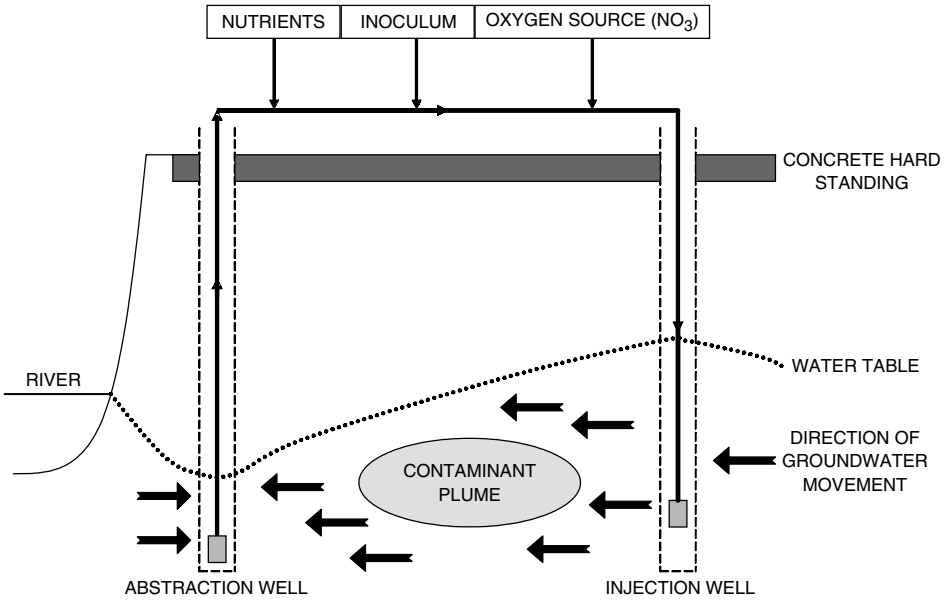

Figure 6. Cross-section of the *in situ* remediation of contaminated groundwater using an injection and abstraction well to circulate an oxygen source, inoculum and nutrients through a hydrocarbon contaminated aquifer.[38].

CONCLUSIONS

The persistence and toxicity problems associated with PAHs in the environment have resulted in a large amount of laboratory-based work that has concentrated on the ability of a variety of microbes (fungi and bacteria) to transform these complex aromatic molecules. The pathways of aerobic PAH transformation have been established and it is known that many environments contain microbes capable of reducing PAH concentrations. These factors have led to an interest in the potential use of microbes to remediate PAH-contaminated soils and more recent work has established that it is possible to use microbial-based processes to remediate PAH-contaminated soil. These processes, eg land-farming and biopiling, are effective on shallow contamination but when PAH contamination is at depth then the use of bioremediation becomes more problematical. However, a recent field study has shown that bioremediation of contaminated aquifers is possible by the introduction of aeration to the subsurface. In addition, the potential of the biodegradation of PAHs under anaerobic conditions is promising, allowing further advances for the *in situ* treatment of the contaminated subsurface. Overall, we feel that the bioremediation of PAH-contaminated sites is feasible given the breadth of our current knowledge. Although the inherent limitations of the bioremediation of PAH-contaminated environments are known, further research is required to test these limitations, and exploit the potential of the *in situ* microbial communities to metabolise PAHs (particularly the larger molecular weight PAHs) in those sites with sub-optimal conditions, such as extreme pH and/or temperatures. In addition, further research is required to develop potential anaerobic remediation technologies that can be applied to remediate the numerous subsurface sites that are contaminated with PAHs.

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