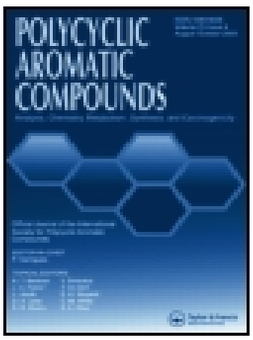

Polycyclic Aromatic Compounds

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BIOREMEDIATION AND PHYTOREMEDIATION OF INDUSTRIAL PAH-POLLUTED SOILS

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This article examines the ability of microorganisms and higher plants to transform polycyclic aromatic hydrocarbons, and their importance in the remediation of polluted soils. Emerging areas of research intended to improve the available treatment processes are discussed.

Keywords bacteria, bioremediation, biotransformation, cytochrome P-450, fungi, higher plants, laccases, polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a large group of chemical compounds which are classified worldwide as priority persistent organic pollutants (POPs). In addition, they induce some adverse effects on living organisms. Consequently, there is an obvious need to develop practical remediation strategies for heavily impacted sites, in order to decrease their hazardous potential for living organisms. Bio- and phytoremediation, because of their cost-effectiveness and their ability to offer complete destruction of the pollutants without soil deterioration, may be an interesting alternative to more drastic physicochemical removal processes. *Bioremediation* is the use of organisms, most commonly microorganisms (bacteria and fungi), to degrade and detoxify environmental pollutants. A similar result could be achieved by *phytoremediation*, which involves higher plants or trees. Nevertheless, one main disadvantage of all biological processes is the difficulty to control the key parameters for optimal pollutant degradation.

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This review presents: (1) the hazardous potential of PAHs; (2) the potential of bacteria, fungi, and higher plants for PAH transformation; (3) the main bioremediation processes used to reduce the environmental hazard of PAHs; and (4) prospects for future research, including phytoremediation. It also provides links to key research or review articles.

PAHs: A FAMILY OF ENVIRONMENTAL POLLUTANTS WITH A HAZARDOUS POTENTIAL

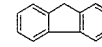
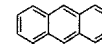
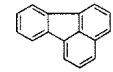
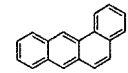
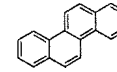
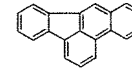
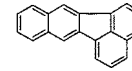
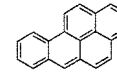
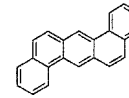
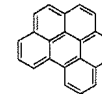
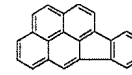
Molecular Structure, Properties, and Sources of PAHs in the Environment

The 16 PAHs selected as priority pollutants by the U.S. Environmental Protection Agency (U.S. EPA) are made up of two or more fused benzene rings and/or pentacyclic moieties, arranged in linear, angular, and cluster arrangements. Their molecular weights range from 128 to 278 (Table 1). Physicochemical properties of PAHs have been reviewed (1–5). These chemicals are thermodynamically stable because of their large resonance energy. Moreover, these neutral compounds have very low aqueous solubility (lower than 1 mg L^{-1}), they are highly hydrophobic ($\log P = 3.30$ to 6.75), and tend to be strongly associated with particle surfaces in the environment ($K_{oc} = 2.00\ 10^3$ to $3.47\ 10^6$). In addition, most of them present a low sensitivity to volatilization and photolysis. As a result, they are generally persistent under many natural conditions, with half-lives in soils amounting to 26 days for phenathrene (a volatile and degradable compound) and 6,250 days for benz[*a*]anthracene (a recalcitrant compound) (5). Thus, the rate of deposition of PAHs today remains higher than their rate of degradation, inducing an accumulation of the chemicals in the environment.

Environmental sources of PAHs are multiple. Formed during almost all combustion reactions as well as being natural components of fossil fuels, PAHs have been produced throughout geological time and widely distributed in the biosphere. They are commonly released into air, soil, and water, and may contaminate feed and food exposed to sources of pollution, thus entering the human body through contact, inhalation, or ingestion.

Some PAHs may have a natural origin. As biogenic compounds, they are components of surface waxes of leaves, plant oils, cuticles of insects, and lipids of microorganisms. They are also formed from biosynthetic pathways in plants, including carotenoids, lignin, alkaloids, terpenes, and flavonoids, which are continuously cycled through the biosphere. PAHs of geochemical origin also occur in the environment. They are

TABLE 1. Physicochemical Properties and Structure of the 16 Polycyclic Aromatic Hydrocarbons Selected as Priority Pollutants by the U.S. Environmental Protection Agency

Name	Molecular weight	Water solubility (mg L^{-1})	log P	K_{oc}	Structure
Naphthalene $C_{10}H_8$	128	31.00	3.30	$2.00\ 10^3$	
Acenaphthylene $C_{12}H_8$	152	16.10	3.94		
Acenaphthene $C_{12}H_{10}$	154	3.90	3.92	$7.08\ 10^3$	
Fluorine $C_{13}H_{10}$	166	1.90	4.18	$1.38\ 10^4$	
Phenanthrene $C_{14}H_{10}$	178	1.15	4.46		
Anthracene $C_{14}H_{10}$	178	0.045	4.54	$2.95\ 10^4$	
Fluoranthene $C_{16}H_{10}$	202	0.26	5.20	$1.07\ 10^5$	
Pyrene $C_{16}H_{10}$	202	0.132	5.18	$1.05\ 10^5$	
Benz[*a*]anthracene $C_{18}H_{10}$	228	0.0094	5.76	$3.98\ 10^5$	
Chrysene $C_{18}H_{12}$	228	0.002	5.81	$3.98\ 10^5$	
Benzo[*b*]fluoranthene $C_{20}H_{12}$	252	0.0015	5.80	$1.23\ 10^6$	
Benzo[*k*]fluoranthene $C_{20}H_{12}$	252	0.0008	6.00	$1.23\ 10^6$	
Benzo[*a*]pyrene $C_{20}H_{12}$	252	0.0016	6.13		
Dibenz[*ah*]-anthracene $C_{22}H_{14}$	278	0.0006	6.75	$3.80\ 10^6$	
Benzo[*ghi*]perylene $C_{22}H_{12}$	276	0.00026	6.63		
Indeno[1,2,3-*cd*]-pyrene $C_{22}H_{12}$	276	0.0002	6.70	$3.47\ 10^6$	

Data are compiled from Refs. (1–5).

formed when organic substances are exposed to high temperature (pyrolysis), and by the aromatization of biological compounds during humification. During this process the aromatic compounds formed comprise mainly alkylated benzene rings; those alkyl groups of sufficient length allow cyclization and aromatization with time. Petroleum, coal (all fossil fuels), and ancient sediments provide the largest source of petrogenic polynuclear aromatic compounds. Finally, PAHs of pyrogenic origin are released into the environment during vegetation fires.

PAHs also have anthropogenic origins in many industrially developed countries where point source contamination is common as a result of spills or mismanaged industrial operations. The contaminated areas are generally small, but the PAH concentrations are high and often associated with many other types of xenobiotics and heavy metals. Industrial pollution includes steelworks, manufactured gas plants (MGPs), and wood preservation treatment plants. Among low-level inputs (non-point sources) are atmospheric depositions due to waste incineration exhausts and deposits of airborne particles from automobile exhausts.

Concern over PAHs is due mainly to the fact that some PAHs and their transformation products are toxic to living cells, with respect to ring number and condensation degree. PAH toxicity has been reported in several reviews (5–8). For example, benzo[*a*]pyrene, chrysene, and benz[*a*]anthracene are carcinogenic for mammals, mutagenic in bacterial and animal cells, or teratogenic. PAHs also can induce immunodepressive effects and present a high potential for biomagnification.

PAH-Soil Interactions

PAH-soil interactions determine the fate, transport, and bioavailability of the hydrocarbons for degrading microorganisms in aquatic and terrestrial environments. Bioremediation technologies have been developed and improved due to the ability of degrading organisms to grow and multiply in polluted soils. Nevertheless, the direct contact between PAHs and microorganisms, governed by chemical bioavailability, is essential for an efficient treatment. Several complex physicochemical and biological factors are known to be responsible for the highly sorptive nature of PAHs, resulting in their limited availability to microbial populations in the environment and in their increasing recalcitrance with time.

Sorption-Desorption Kinetics

Several mechanisms condition the partitioning of the pollutants between water, dissolved humic matter, and soil or sediment humic matter. Humic acids are a major fraction of soil natural organic matter (NOM),

which is the dominant sorbent of hydrophobic compounds in soil. Sorption of pollutants to humic acids associated with the soils usually inhibits transport and bioavailability, whereas binding to dissolved humic material will facilitate desorption from the solid phase. Several physicochemical properties of the soils were characterized in order to estimate the factor responsible for PAH sorption. Binding to soils of nonionic compounds such as PAHs depends on the organic carbon concentration of the soil. The affinity for association of a contaminant with the organic material of soil (expressed as the organic carbon partition coefficient K_{oc}) is a function of the hydrophobicity of the compound (expressed as the octanol-water partition coefficient K_{ow} or P). Then, naturally occurring organic matter is an excellent sorbent for PAHs. Moreover, the molecular size appears to be an important factor determining the binding of solids to organic matter. PAHs exist in soil not only sorbed to solids but also in liquids immiscible with water, called non-aqueous phase liquids (NAPLs). As such, the availability of the contaminants to biodegradation may be drastically reduced.

Two kinetically distinct processes were found to be associated with PAH binding onto soil material, a "fast" and a "slow" process. The initial fast adsorption process is thought to reflect rapid adsorption of the PAHs onto hydrophobic areas of soil surfaces, whereas the following slow adsorption process is thought to be based on migration of the PAHs to less accessible sites within the soil matrix. Thus, longer incubation times result in migration of an increasingly large fraction of the pollutant into the organic material.

NOM and NAPLs represent a long-term source of pollution with a slow but continuous release of PAHs, being governed by desorption kinetics. Nevertheless, utilization of NAPLs by microorganisms may involve extracellular enzymes (peroxidases, laccases) that cleave the substrate to small, soluble low-molecular fragments easily assimilated by the cells. Microorganisms may also produce surface active agents, which enhance the desorption kinetics of pollutants from NOM, and convert the NAPLs to small droplets. The latter mechanism may be similar to the developed to metabolize natural macropolymers (e.g., lignin).

Sequestration

In addition, aged POPs become increasingly less available because of a time-dependent sequestration in soils. The mechanisms are not fully understood. PAHs become physically unavailable to microorganisms. Pollutant concentration is one of the variables, but the presence of numerous pollutants in polluted soils may affect the sequestration of a specific chemical. Aging of the pollutant is the result of its entrapment

through a tortuous pathway to places where degrading organisms are present. The site of the retention is within the micropores of soil humic materials. In short-term contaminated soil, PAH biodegradation competes with migration of PAHs into nonaccessible soil compartments.

Bound Residues

Many xenobiotics are partly converted to bound residues in soils. They may represent new molecular species formed from the parent compound or transformation products, often as complexes with organic constituents of soils or living organisms. These complexes are formed by an attachment of the compound to reactive sites on the surfaces of the organic colloids, leading to the incorporation of the compound into the structure of humic and fulvic acids. Amines, phenols, and quinones of both xenobiotics and humic materials are highly reactive. The complexes have a high molecular weight. Both chemical and enzymatic catalysis are involved. The residues are stable, often considered as releasing the xenobiotics in concentrations too low to represent a hazard. Nevertheless, it is not clear today whether bound residues present a future problem of toxicological significance.

Characteristics of Polluted Soils

PAH-polluted soils may be classified into three main types of soils, two including PAHs of pyrolitic origin (fluoranthene:pyrene ratio >1) (9) and one with petroleum hydrocarbons (fluoranthene:pyrene ratio <1) (Table 2). All these soils are subjected to slow PAH release.

1. A first type of soil is found around wood-preserving treatment plants, where woods are impregnated with inorganic and organic compounds (creosotes). PAH pollution is often less than 5,000 mg/kg. It comprises mainly 3-ring PAHs (phenanthrene and anthracene) and 4-ring compounds (fluoranthene, pyrene). PAHs are also associated with cresols, phenols, chromate, or copper arsenate. Soil organic carbon content is generally low (less than 2%) and pollutants are moderately aged. Their potential for bioremediation is high.
2. Soils from MGPs are the second main type of PAH-polluted soils. From the early 1800s to about 1970, coal was carbonized to produce gas. High-residue content (up to 25,000 mg/kg) is commonly found on the sites after plant demolition. PAHs are mainly 4-ring (fluoranthene, pyrene, benzo[*a*]anthracene) and 5-ring compounds (benzo[*b*]fluoranthene, benzo[*a*]pyrene). Residues are subjected to long-term aging, sometimes more than one century. PAHs are the main pollutants, but cyanides, heavy metals, and sulfates are also

TABLE 2. Characteristics of the Main Types of PAH-Polluted Soils

	Wood-preserving facility	Manufactured gas plant	Oil field battery
PAH contamination (mg kg^{-1})	<5,000	<25,000	<5,000
Fluoranthene:pyrene ratio	>1	>1	<1
3-ring (%)	50–60	10–30	30–45
4-ring	35–45	40–50	35–45
5-ring	3–5	15–30	10
6-ring	<5	5–15	5–15
Additional chemicals	Cresols, phenols, chromated copper arsenate	Cyanides, heavy metals, sulfates	Methylated PAHs, BTEX
Soil organic carbon content	Low	High (>10%)	±High
Aging	30 years	100 years	±
Potential for bioremediation	++	—	+

BTEX: benzene, toluene and xylene.

present in these soils. The soils frequently have an organic matter content higher than 10%. They can be very recalcitrant to bioremediation.

3. The last soil is contaminated by petroleum pollutants including PAHs, and can be found near oil field batteries. Amounts of unsubstituted PAHs are generally moderate, and the pollutants are associated with low-molecular-weight hydrocarbons (benzene, toluene, and xylenes) and methylated forms of PAHs. The main PAHs encountered are 3-ring (phenanthrene) but above all 4-ring (pyrene, chrysene) chemicals. Petroleum-polluted soils in the vicinity of refineries are closely related by their characteristics to soils from MGPs.

SOIL BACTERIA, FUNGI, AND PLANTS: THEIR HIGH POTENTIAL FOR PAH BIOTRANSFORMATION

Principles of Xenobiotic Transformation

All living organisms, including microorganisms, higher plants, and animals catalyze biotransformation reactions. In the soil, prokaryotic organisms include bacteria and their subdivision of actinomycetes, whereas fungi, algae, and macrofauna (arthropods and earthworms) are the main eukaryotic organisms. All of these organisms have specific ecological niches and functions, and each contributes to the overall biotic activity. The degradation of xenobiotics through microbial metabolic processes is considered to be the primary mechanism of biological transformation. The different groups of microorganisms can mediate an almost infinite number of biochemical transformations. The most numerous organisms in soil are bacteria, whereas fungi form the largest biomass. The soil microorganisms represent 2% to 4% of soil carbon, and 5% to 8% of nitrogen content. They are involved in numerous functions, such as mineralization and humification of soil organic matter, biogeochemical cycles, production of toxins and compounds of interest (antibiotics), and degradation of xenobiotics.

The following gives an overview of the two main groups of soil microorganisms actually involved in marketed soil bioremediation processes.

1. First are bacteria, which can be rod-shaped, coccoidal, helical, or pleomorphic, with a size ranging from 0.1 to 2 μm. Characterized by a complex cell envelope which contains cytoplasm and a few cell organelles, they can be aerobic or anaerobic. Their deoxyribonucleic acid (DNA) is circular in cytoplasm, but plasmidic DNA is frequent. Bacteria are capable of rapid growth and reproduction which occurs by binary

fission. Genetic exchange is predominant by conjugation (cell-to-cell contact), transduction (exchange via viruses) or transformation (transfer of naked DNA). They are present in all soils with a competitive pH near neutrality. The most common genera are *Arthrobacter, Pseudomonas*, and *Bacillus*.

Actinomycetes are classified as bacteria. Nevertheless, despite some characteristics in common with bacteria, they are also similar to fungi. Mostly aerobic they grow as elongated single cells, branched into filaments or hyphae, which are similar to those of fungi although with a smaller diameter (0.5–2.0 μm). These gram-positive bacteria produce asexual spores called conidia. Their cellular organization and DNA material are similar to these of other bacteria. They are more tolerant to alkaline soil pH and low moisture content than bacteria, thus actinomycete populations tend to be high in desert soils. *Streptomyces* genera dominate the populations.

2. Fungi are the second major group of soil organisms. These eukaryotic organisms comprise molds, mildews, rusts, and mushrooms (all aerobic), as well as yeasts (fermenting organisms). Filamentous fungi are characterized by extensive branching and mycelial growth, as well as by the production of sexual (for asco- and basidiomycetes) and asexual spores. Deuteromycetes (*Fungi imperfectii*) lack sexual reproduction, but a sexual stage is quite often discovered in which case these organisms are reclassified into other classes. Fungi are more tolerant to acidic soils and low moisture than bacteria. They can be pathogenic for plants and animals, or associated with plants in forming mycorrhizae. Most common genera are *Penicillium, Aspergillus, Fusarium, Rhizoctonia, Alternaria*, and *Rhizopus*.

The biotransformation of xenobiotics through bacterial and fungal processes can be due to direct metabolism or to an indirect effect of organisms on the environment (10). The three processes involved in direct metabolism, namely biodegradation, cometabolism, and synthesis, are discussed below.

1. During biodegradation, one or several interacting organisms metabolize a given xenobiotic into carbon dioxide and other inorganic components. In this way, the organisms obtain their requirements for growth and energy by mineralizing the molecule. From an environmental point of view, biodegradation is the most interesting and valuable process because it leads to the complete breakdown of a molecule without generation of accumulating intermediates.

2. The prevalent form of xenobiotic metabolism in the environment is cometabolism, in which organisms grow at the expense of a cosubstrate to transform the xenobiotic without deriving any nutrient or energy

for growth from the process. Cometabolism is a partial and fortuitous metabolism and enzymes involved in the initial reaction lack substrate specificity. Generally cometabolism results only in minor modifications of the structure of the xenobiotic, but different organisms can transform a molecule by sequential cometabolic attacks or one organism can use cometabolic products of another as a growth substrate. Minor modifications resulting from cometabolism could greatly influence pollutant bioavailability and mobility in soil. In addition intermediate products with their own bio- and physicochemical properties can accumulate, thus causing some adverse effects on the environment.

3. The last process, synthesis, includes conjugation and oligomerization. Xenobiotics are transformed into compounds with chemical structures more complex than those of the parent compounds. During conjugation a xenobiotic (or one of its transformation products) is linked to hydrophilic endogenous substrates, resulting in the formation of methylated, acetylated, or alkylated compounds; glycosides; or amino acid conjugates. These compounds can be excreted from the living cells or stored. During oligomerization, a xenobiotic combines with itself or with other xenobiotic residues (proteins, soil organic residues). Consequently they give high-molecular-weight compounds, which are stable and often incorporated into cellular components (e.g., cell walls) or soil constituents (soil organic matter). This biochemical process not only affects the activity and the biodegradability of a compound in limiting its bioavailability, but also raises concern about the environmental impact of the bound residues.

Typically, in pharmacology, the direct metabolism of xenobiotics involves enzymatic reactions grouped together in three phases (Figure 1).

1. Phase 1 metabolism is probably the most important phase of xenobiotic metabolism. It comprises functionalization reactions, namely oxidation, reduction, and hydrolysis of the parent compound. By the introduction of additional functional groups such as OH, NH_2, SH, and COOH, these processes often result in the formation (activation) of metabolites with modified physiological and biological properties, and a predisposition for further metabolism in the secondary phase (11). The biochemical reactions can be catalyzed either by enzymatic systems or nonenzymatic processes.
2. Phase 2 metabolism is a synthetic process known as conjugation that results in the formation of final metabolites by linkage of the activated metabolite with cell constituents. These metabolites are diversely distributed and sequestered by the organisms (e.g., in plant vacuoles) or excreted. Conjugation reaction are mediated by enzymatic systems.

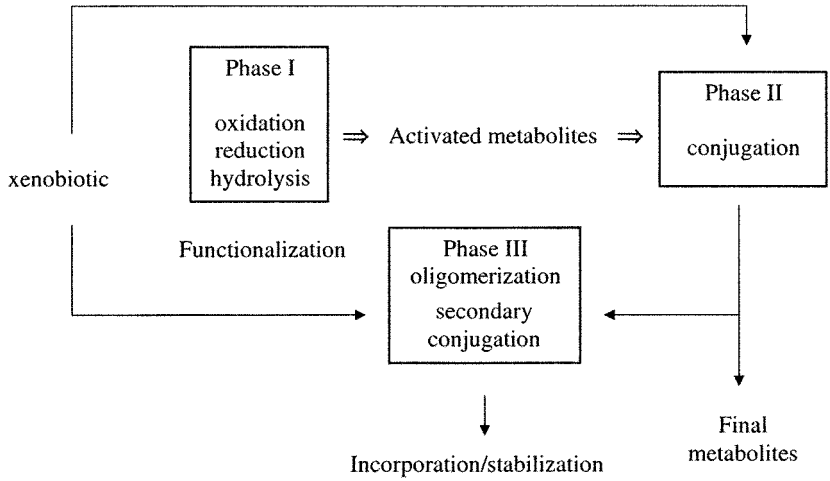


FIGURE 1. The three phases involved in the metabolism of xenobiotics.

3. Phase 3 metabolism involves synthetic reactions leading to the oligomerization of several units of the parent compound or secondary conjugation of the parent compound with cellular components of the cells. They contribute to the formation of high-molecular-weight compounds or bound compounds, which are incorporated and stabilized within the cells. Phase 3 reactions can be catalyzed either by enzymatic systems or nonenzymatic processes.

Compounds resulting from these three types of metabolic reactions exhibit distinct physicochemical and biological properties. In moving from their initial state to phase 2 or 3 products, they generally become more hydrophilic, except in the case of insoluble polymers. Their initial mobility is also reduced as well as their toxic or hazardous power.

PAH Transformation Pathways

PAH metabolism has been an area of research since the beginning of the 1970s. First centered on the mammalian metabolism because of the metabolic activation of PAHs to electrophilic species able to bind with nucleophilic groups of DNA, ribonucleic acid (RNA), and proteins, studies were then extended to microbial and fungal transformations (12, 13). Consequently, the biochemical pathways of PAH tranformation have been described for many years and extensively reviewed (7, 12, 13). One objective of these studies was to determine whether bioremediation of PAH-contaminated soils could generate potentially toxic intermediates.

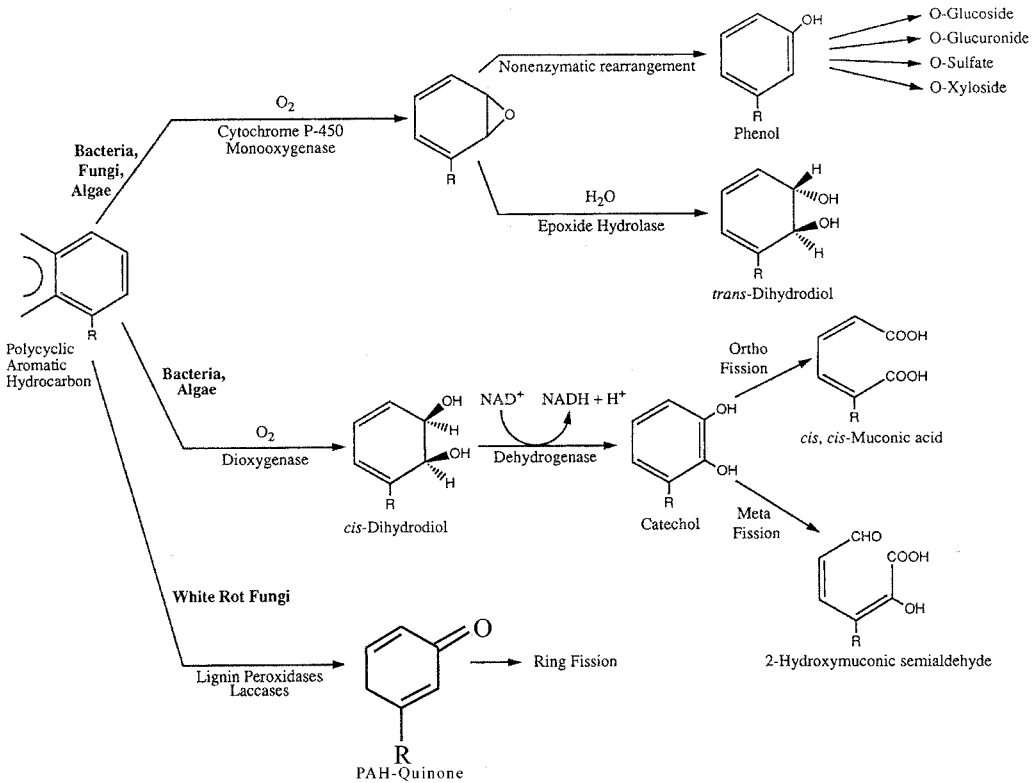


FIGURE 2. The different mechanisms of PAH oxidation by living organisms. Adapted from Ref. (14).

It is understood that the initial step in the aerobic catabolism of PAHs occurs via insertion of one or two atoms of oxygen by several enzymatic systems, according to the organism involved (Figure 2) (14). In soil, the main PAH degraders are bacteria and fungi. Nevertheless, almost all the data have been obtained during degradation experiments performed in liquid cultures, then extrapolated to environmental media.

Bacterial Metabolism of PAHs

A wide variety of bacteria metabolize PAHs and use them as their sole source of carbon and energy (8, 15). The general principles of bacterial metabolism of PAHs, under aerobic conditions, have been clearly demonstrated since 1968 by David T. Gibson and coworkers. Usually, the first reaction (often rate limiting) involves the attack of a single terminal ring by a dioxygenase, leading to the incorporation of both atoms of molecular oxygen into the aromatic ring to form a *cis*-dihydrodiol. The dihydrodiol is further rearomatized through a dehydrogenase to yield a dihydroxylated metabolite with a catechol-like structure. Then follows an oxygenolytic ring cleavage of the catechol by the action of another dioxygenase. An *ortho* pathway involves the cleavage of the bond located between the carbon atoms carrying the 2-hydroxyl groups, to yield *cis-cis* muconic acid. In addition, a *meta* pathway involves cleavage of the bond between a carbon atom with a hydroxyl group and one of its adjacent carbon atoms to yield a semialdehyde. These ring fission pathways lead to metabolically usable substrates. Bacteria have been shown to transform a wide range of low- and high-molecular-weight PAHs.

A minor PAH bacterial transformation pathway involves cytochrome P-450 monooxygenases, described below for fungi.

Fungal Metabolism of PAHs

Numerous fungi among zygomycetes, deuteromycetes, ascomycetes, and basidiomycetes metabolize PAHs (16–18). Fungal metabolism of PAHs is being extensively studied at present because of the increasing development of remediation processes using filamentous fungi. Fungal metabolism of PAHs is highly regio- and stereoselective, and is in some cases similar to the mammalian metabolism. J. P. Ferris and coworkers initiated the studies on fungal metabolism of PAHs in the 1970s. Then, Carl E. Cerniglia and coworkers retained the zygomycetes *Cunninghamella bainieri* and *Cunninghamella elegans* as models. In 1985, John A. Bumpus extended the studies to white rot (ligninolytic) fungi. In contrast to bacteria, nonligninolytic fungi produce metabolites that include *trans*-dihydrodiols, phenols, quinones, tetralones, and

dihydrodiol epoxides, whereas ligninolytic basidiomycetes are able in addition to cleave the aromatic rings and mineralize them.

All of these fungal metabolites are first produced by phase 1 reactions. Phase 2 metabolism, in which some of them are conjugated with sulfate, glucuronic acid, or other moieties, also occurs. Most of the metabolites produced from PAHs by fungi are less toxic than the parent compounds and result in detoxification. Nevertheless, small amounts of mutagenic and carcinogenic compounds can be formed during fungal metabolism of unsubstituted and methyl-substituted PAHs. Fungal metabolites can be retained within the cells, or released in their environment. Metabolic pathways have been described:

- The first step of unsubstituted PAH metabolism is ring epoxidation by a cytochrome P-450 monooxygenase, leading to an unstable arene oxide. Arene oxides are immediately hydrated to *trans*-dihydrodiols by epoxide hydrolase, or rearranged nonenzymatically to phenols. The toxicity of arene oxides is less than that of the parent compound.
- Epoxide hydrolase catalyzes the addition of a water molecule to an arene oxide to form a *trans*-dihydrodiol. The reaction is known concerning naphthalene, anthracene, phenanthrene, fluoranthene, benz[*a*]anthracene, and benzo[*a*]pyrene. The carcinogenicity of *trans*-dihydrodiols can be lower than that of the parent compound (benzo[*a*]-pyrene *trans*-9,10- and 4,5-dihydrodiols) or higher (benzo[*a*]pyrene *trans*-7,8-dihydrodiols). These reactions appear to be similar to those reported for mammalian enzyme systems.
- The nonenzymatic rearrangement of a PAH arene oxide in solution produces phenols. Phenolic metabolites have been detected from naphthalene, anthracene, phenanthrene, fluoranthene, pyrene, and benzo[*a*]pyrene. In general, according to the target organism, phenols are then conjugated during phase 2 metabolism. Consequently, their toxicity is suppressed.
- Several strains of fungi produce quinones. In nonligninolitic fungi, quinones may be formed from *trans*-dihydrodiols. White-rot fungi produce nonspecific extracellular enzymes (mainly lignin peroxidase, manganese-dependent peroxidase, and laccase) that oxidize PAHs during growth on various carbohydrates. The strains most studied are *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Pycnoporus cinnabarinus*. They partly metabolize phenanthrene, fluorene, pyrene, benzo[*a*]pyrene to CO_2 and unidentified minor products. Moreover, lignin peroxidase and laccases purified from these strains have been shown to be responsible for the initial steps of PAH oxidation, leading to the formation of quinones. In most cases, such a reaction requires

the presence of a redox mediator, which is either a xenobiotic or a natural compound produced by the fungus.

- When a monooxygenase catalyzes the second oxidation of a PAH *trans*-dihydrodiol, the result is a dihydrodiol-epoxide. Benzo[*a*]-pyrene *trans*-7,8-dihydrodiol 9,10-oxide, produced by *C. elegans*, is the ultimate carcinogenic and mutagenic metabolite of benzo[*a*]pyrene in mammals. The fungus also produces benzo[*a*]pyrene *trans*-9,10-dihydrodiol 7,8-oxide, which is less mutagenic. Dihydrodiol-epoxides can be metabolized further by epoxide hydrolase to tetrahydrotetraols, which are less mutagenic than the dihydrodiol-epoxides (16).
- Among a large number of methylated PAHs, 3-methylcholanthrene and 7,12-dimethylbenz[*a*]anthracene are known for their extremely high mutagenicity and carcinogenicity. *C. elegans* transform the former to secondary alcohol and ketones, including 1- and 2-hydroxy-3-methylcholanthrene and 1- and 2-keto-3-methylcholanthrene. The second compound is hydroxylated in either or both of the methyl groups. Fungi also metabolize other methylated, nitrated, and fluorinated PAHs.
- Phenols and *trans*-dihydrodiols derived from PAHs are detoxified during phase 2 reactions by alkylation or conjugation with another molecule, including sulfate, glucosides, glucuronides, and xylosides. Phenanthrene and pyrene have been shown to be converted into methoxylated compounds by *Aspergillus niger*. Sulfate conjugation, a common mammalian detoxification reaction, is also performed by fungi such as *C. elegans*, which produce sulfate conjugates of naphthalene, anthracene, benz[*a*]anthracene, and benzo[*a*]pyrene. Glucuronic acid conjugates of PAHs are detoxification products of both mammals and fungi. A soluble UDP-glucosyltransferase from *C. elegans* catalyses the conjugation of naphthalene, benz[*a*]anthracene, and 3-hydroxybenzo[*a*]pyrene. At least two fungi, *C. elegans* and *P. chrysosporium*, produce glucose conjugates from phenanthrene, fluoranthene, and pyrene. Finally, xylose conjugates have been found among the metabolites of *Rhizoctonia solani* grown with anthracene.

In general, fungal metabolites have high water solubility and enhanced chemical reactivity. Forming bound residues during humification processes also detoxifies PAHs in soils.

Plant Metabolism of PAHs

Our present knowledge of higher plant metabolism of PAHs has been mainly provided by H. Harms and coworkers. It is admitted that plants may offer a great potential (5, 6, 19), although their germination and

growth can be strongly inhibited by PAHs with fewer than three rings, which remain bioavailable in soils (20). By studying PAH metabolism in whole plants, it has been found that hydrocarbon adsorption onto roots, as well as uptake, are significant but highly variable. Volatile PAHs may also penetrate in plants via the leaf cuticle (6). As a consequence, different accumulation or translocation of PAHs occur accordingly to the plant species (21). Nevertheless, background contents of PAHs in plant tissues in North America and Europe are very low (at the μg kg^{-1} level), but the values set down a question concerning the possible accumulation of toxic hydrocarbons in plants, thus entering the food chain (5). Data concerning their metabolism in whole plants growing on contaminated media are poor, and plant cell suspensions seem to be better tools to study PAH transformation. In these systems the hydrocarbons are oxidized to phenols or quinones, and then conjugated with carbohydrates (22, 23). Bound residues are also formed within the cells.

One present area of research concerns plant interactions with the rhizosphere or arbuscular mycorrhizal fungi. In these cases bacterial and fungal metabolisms are involved in PAH degradation, respectively (6).

Anaerobic Degradation of PAHs

Unsubstituted polyaromatic compounds can be degraded in anoxic environments under nitrate-reducing, iron-reducing, sulfate-reducing, and methanogenic conditions. As a consequence, it may be possible to stimulate biodegradation of PAHs through the addition of anaerobic electron acceptors when molecular oxygen is lacking. Anaerobic degradation concerns mainly the light-molecular-weight PAHs, such as naphthalene, acenaphthene, fluorene, phenanthrene, and fluoranthene. An example of theoretical equations has been published concerning naphthalene (24):

under nitrate-reducing conditions:

$$1/48\ C_{10}H_8 + 1/5\ NO_3^- + 1/5\ H^+ \rightarrow 5/24\ CO_2 + 1/10\ N_2 + 11/60\ H_2O$$

under sulfate-reducing conditions:

$$1/48\ C_{10}H_8 + 1/8\ SO_4^{2-} + 3/16\ H^+ \rightarrow 5/24\ CO_2 + 1/16\ H_2S + 1/160\ HS^- + 1/12\ H_2O$$

Enzymatic Systems Involved in PAH Biotransformation

The main enzymatic systems involved in PAH bacterial, fungal, and plant metabolism include dioxygenases and cytochrome P-450

monooxygenases. For about 10 years, exocellular enzymes, such as peroxidases but particularly laccases (that are produced mainly by white-rot fungi to degrade lignin), have been extensively studied.

Dioxygenases

Dioxygenases are enzymes produced by bacteria which catalyze the following equation:

$$R + O_2 + NADH + H^+ \rightarrow R(OH)_2 + NAD^+$$

They are involved in both the physiological substrate and xenobiotic metabolism. One well-known enzyme is naphthalene dioxygenase (13). It comprises a multicomponent system of three proteins, including a flavoprotein. The terminal dioxygenase is an iron-sulfur protein of 158 kDa with two subunits of 20 and 55 kDa. Little is known about its regulation, but naphthalene dioxygenase seems to be induced by PAHs.

Cytochrome P-450 Monooxygenases

Microbial, fungal, and plant P-450s have been described elsewhere (25, 26). P-450s constitute a large family of heme-thiolate proteins widely distributed among living organisms. In most cases, they function as monooxygenases by binding and activating molecular oxygen, incorporating one of its atoms into an organic substrate, and reducing the second atom to form water, according to the following reaction:

$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NA(D)P^+$$

The result of catalysis, depending on the P-450 protein and its substrate, leads in most cases to hydroxylation, epoxidation, or heteroatom dealkylation.

For most eucaryotic P-450s, a FAD/FMN-dependent NADPH-P-450 reductase is needed to transfer the electrons used for oxygen activation from cytosolic NADPH. In plants and filamentous fungi, P-450s and reductases are usually microsomal membrane-bound proteins exposed to the cytosol. A second type of P-450s has been identified in prokaryotes and animal mitochondria. In addition to a flavoprotein, it needs a small redox iron-sulfur protein (ferredoxin) to transfer the electrons from NAD(P)H to the terminal P-450 component (26). Such P-450s are usually soluble or associated to inner mitochondrial membranes.

P-450s are encoded by a superfamily of genes (27). The sequences of more than 500 have already been recorded in all living organisms.

They are named and classified in more than 150 families, according to the identity in amino acid sequences of the deduced proteins. With a few exceptions, based on phylogenetic considerations, proteins with a sequence identity of 40% or less are considered to define a new family. When two P-450s are more than 55% identical, they are designated as members of the same subfamily. A number designates families and a letter refers to subfamilies, both following the prefix CYP. Plant P-450s correspond to the families CYP71 to CYP99, then CYP701 and above. Fungal P-450s comprise families CYP51 to CYP66. More than 200, 40, and 90 genes have been registered in plants, fungi (including yeast), and prokaryotes, respectively. The apoprotein sequences are highly variable among P-450s, with M_r ranging from 45 to 65 kDa. Identity in their amino acid sequences (400 to 525 residues) is sometimes less than 20%, but their three-dimensional structure seems to be highly conserved. The most highly conserved structure in all P-450s is the core surrounding the haem prosthetic group. The haem most proximal side is formed of a loop including the cysteine residue serving as sixth ligand to the iron, and the 11 most highly conserved residues of the protein. In microsomal enzymes, an anchor signal is located on the N-terminal segment.

Knowledge concerning regulation of plant and fungal P-450s is only starting to accumulate. In plants, expression seems to follow a developmentally regulated tissue-specific pattern in most cases. Physicochemical (light, osmotic stress, wounding) factors, physiological (infection, aging, hormones) factors, or xenobiotics (agrochemicals, ethanol, or drugs like phenobarbital or aminopyrine) were reported to induce plant P-450s. In many cases, expression is very low or undetectable in the absence of induction. Mechanism-based inactivators as well as heterocyclic molecules or methylenedioxy compounds can inhibit plant, microbial, and fungal P-450 enzymes. Carl E. Cerniglia studied extensively the P-450 monooxygenase system from the fungi *Cunninghamella* sp. involved in PAH metabolism. The transformation of PAHs to more hydrophilic metabolites by fungi is closely linked to the activity of intracellular enzymes such as P-450s.

Fungal Peroxidases

Wood-decaying fungi belonging to the white-rot group of basidiomycetes have been attracting scientific attention for almost two decades. These organisms, able to depolymerize lignin, secrete two families of peroxidases, namely lignin-peroxidase (LiP, EC 1.11.1.14) and Mn-peroxidase (MnP, EC 1.11.1.13) and in some cases a family of laccases (see below). The idea of applying the ligninolitic system to the

degradation of xenobiotics is widespread. Peroxidases are monomeric glycoproteins with an iron-protoporphyrin structure. They are been described as catalyzing C─C bond breakage, and reported since 1986 to degrade PAHs (28). These exocellular enzymes (LiPs, MnPs) are responsible for the mineralization of PAHs, which has never been reported in nonligninolytic fungi. The degradative mechanism is an unspecific, oxidative, free radical process that is initiated by carbon, nitrogen, or sulfur limitation. The enzymes are oxidized by peroxide and then reduced back to the ground state in two steps, each of which lead to the formation of a low-molecular-mass intermediate oxidant. In the case of LiPs, the intermediate oxidant (redox mediator) is a nonphenolic compound such as veratryl alcohol. In the case of MnPs, the redox mediator is MnII. When oxidized, redox mediators are highly unspecific and oxidize a large number of aromatic and aliphatic compounds. In addition direct attacks by peroxidases have been reported, although with a lower efficiency. H_2O_2 levels, generated by fungal enzymatic systems, regulate the activity of the peroxidases. This constraint may appear as a limit during in situ treatments.

Fungal peroxidases are stable enzymes, whose isoforms have a molecular weight ranging from 38 to 50 kDa. Redox potentials of the peroxidases are comprised between 1.1 and 1.5 V. Fungal exocellular oxidases have acidic pH optima, which for LiPs are 2.5–3.0 and for MnPs are 4.0–4.5.

Fungal Laccases

Recently, Gianfreda et al. reviewed our present knowledge on laccases (Lac, EC 1.10.3.2) (29). The two main biological functions ascribed to fungal laccases are first their involvement in the lignin degradation process, together with other ligninolytic enzymes such as peroxidases, and second their role in fungal virulence as a key agent in pathogenesis against plant hosts. In addition, laccases exhibit in vivo other functions that can be the basis for some industrial applications. For example, in Aspergillus nidulans, laccases are known to act on pigment formation. Then, some fungi also secrete laccases to remove potentially toxic phenols released during lignin degradation or toxins produced by other organisms. As a consequence, the enzyme is an efficient tool in several potential applications in the dye, paper, and textile industries as well as the degradation of various xenobiotics which are recognized as environmental pollutants. For that reason, numerous data have been published concerning laccases (29). These multicopper proteins catalyze the 1-electron oxidation of various aromatic compounds (specifically phenols, arylamines, or aminophenols) while concomitantly reducing

molecular oxygen to water, according to the equation:

$$4RH + O_2 \rightarrow 4R^{\bullet} + 2H_2O$$

The formed radical $R^{\bullet}$ is then subjected to molecular arrangement, oxygen binding, or oxidative coupling reaction.

Laccase activity has been extensively demonstrated in more than 60 fungal strains. The enzyme also exists in plants, bacteria, and insects as well as in soil and forest litter. The laccase molecule is a monomeric, dimeric, or tetrameric glycoprotein usually containing four atoms of copper per monomer, bound to three redox sites. Redox potentials of fungal laccases range from 0.24 to 0.54 V (vs. SCE). Formed from 520 to 550 amino acids, their molecular weights vary from 60 to 80 kD. Only the exocellular forms are known to be active. Fungal laccases are very stable enzymes, thermoresistant, with an optimal pH comprised between 3 and 5. The main role of fungal laccases is to depolymerize lignin, and to break down this polyphenolic macromolecule into small components. Fungal laccases are also involved in morphogenesis and plant pathogenesis. Laccases are inhibited by small anions, metals, and fatty acids. About 30 forms of fungal laccases have been characterized and 20 genes cloned and sequenced.

First evidence of the in vitro involvement of fungal laccases in PAH metabolism was reported in 1996 by Johannes and coworkers (30). These authors showed that a purified enzyme from *T. versicolor* cultures was able to transform anthracene into 9,10-anthraquinone. Nevertheless the reaction rate, which is slow, increased in the presence of redox mediators such as 2,2′-azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) or 1-hydroxybenzotriazole (HBT). Collins et al. confirmed these results and also demonstrated the oxidation of benzo[*a*]pyrene by the laccase (31). Efficient oxidation of PAHs was achieved in the presence of ABTS or a putative mediator present in the filtered culture fluid. Then in the presence of ABTS or HBT, laccase from *Trametes* sp. was shown to transform several individual compounds: acenaphthylene, acenaphthene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and perylene, mainly to their corresponding diones (28, 32, 33). Laccases from other fungal strains (*P. cinnabarinus, Coriolopsis gallica*) were also able to catalyze PAH oxidation in the presence of exogenous mediators (34, 35). Natural mediators (e.g., methionine, cysteine) have also been identified (36). The mechanisms involved in the laccase mediator system have recently been elucidated (37). PAHs were converted to radical cations ($PAH^{+\bullet}$) after abstraction of one electron by oxidized species of the mediators ($ABTS^{++}$ or $HBT^{\bullet}$). Then they undergo a nucleophilic attack by water

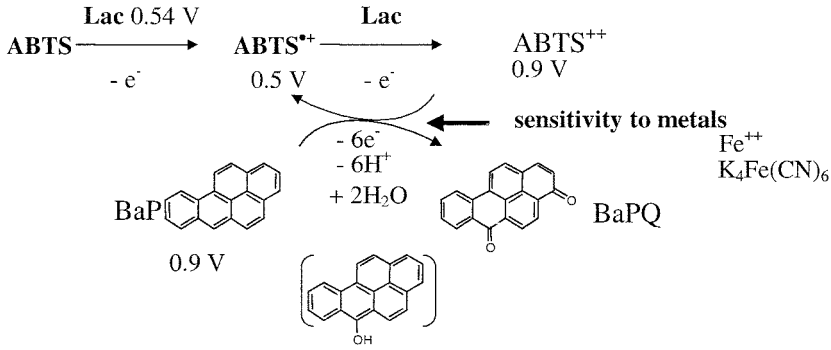


FIGURE 3. Mechanism of laccase-mediated oxidation of benzo[*a*]pyrene.

and the resulting radical easily undergoes further oxidation to the hydroxy PAH, readily oxidized to the final quinone. Consequently, the redox potential of the laccase (initially 0.54 V vs. SCE) was increased following enzymatic oxidation of ABTS to $ABTS^{\bullet+}$ (0.5 V) and to $ABTS^{++}$ (0.9 V), thus allowing oxidation of PAHs (benzo[*a*]pyrene = 0.9 V; Figure 3).

Our team investigated the transformation of complex mixtures of PAH in liquid phase in the presence of laccases of *P. cinnabarinus* associated to ABTS. Several hydrocarbons from a synthetic mixture, such as anthracene and benzo[*a*]pyrene, were converted up to 80% into quinones, whereas others also belonging to 3- and 5-ring chemicals were less transformed (38). Chrysene and benzo[*k*]fluoranthene were not oxidized by the laccase mediator system. Moreover, hydrocarbons extracted from an industrial soil were all recalcitrant to enzymatic attack. This lack of transformation can be attributed partly to the limited bioavailability of soil-extracted pollutants for the enzymes. In this case, PAHs are adsorbed onto organic matter present in the soil extracts. On the other hand, the lack of reactivity of the laccases toward the hydrocarbons is also due to the presence of interfering compounds coextracted from the soil, such as metals and organics with oxidation potentials lower than those of PAHs. For example, the results suggested that the dication $ABTS^{2+}$, the reactive form responsible for PAH oxidation, preferentially oxidized metals such as Fe present in the soil extract instead of PAHs (Figure 3). This result appears as a limitation for the efficient use of laccase-producing fungi for soil remediation.

Multiple mechanisms can be used by soil organism for PAH degradation. Although the biochemical mechanisms have been elucidated, the most advantageous organisms for detoxifying specific PAH compounds need additional properties, such as resistance to pollutant toxicity, easy

production, handling, and inoculation into polluted media. Another important property of an advantageous organism is its ability to compete with natural strains in soil for bioremediation.

BIOREMEDIATION PROCESSES: A WAY TO REDUCE THE ENVIRONMENTAL HAZARD OF PAHs

Bioremediation is the use of the natural or engineered capacity of microorganisms to transform pollutants, thus offering a permanent and destructive solution. Principles of the bioremediation of PAH-polluted soils have been reviewed (39). Two types of soil treatment techniques are being developed. First, they include in situ processes where the soil is directly treated without excavation. These are useful in the presence of a deep contamination of the soil by pollutants, which remains scarce in the case of PAHs, because of their low mobility. The second type of soil treatment can be performed ex situ, and is well adapted to soil PAH-contamination. Ex situ processes begin by the excavation or scraping of the polluted soil, which can be moved into a treatment plant (off-site treatment) or treated on site. Only ex situ processes allow an efficient optimization of incubation parameters, including pH, aeration, agitation, moistening, and addition of suitable electron acceptors, nutrients, solvents, or surfactants to enhance the rate of PAH desorption and to increase the activity of microorganisms (biostimulation). Refinements to the process also include isolation and/or production of degradative organisms which are then reintroduced in the polluted material (bioaugmentation). Ex situ processes are being developed extensively worldwide, because they make it possible to easily control the key parameters required for optimal pollutant degradation in also reducing the heterogeneous structure of the soil.

Among the remediation methods available, several parameters indicate that bioremediation is an interesting technology, in contrast with physicochemical treatments. The first parameter is related to the pollutant. When bioavailable, common PAHs are chemical compounds degraded well by most microorganisms, which develop numerous degradation pathways. As the extent of the polluted zone can be considerable, the soil is easily scraped and piled before treatment. On the contrary, aging of the pollutant appears to limit biodegradation as pollutants become less available for degradative enzymes. Bioremediation technologies can be applied to all types of soils, whatever their texture or permeability. They are partially governed by local constraints, such as space, noise, smell, and dust. In other terms, off-site methods are useful in the case of urban areas. The advantages of bioremediation processes are that they

are economically and environmentally acceptable solutions. They induce low costs and the treated soil can be reused if the target pollutant levels are reached. The disadvantages are that they require a long time to start to work, associated with a pollutant concentration threshold which can be achieved through bioremediation.

Three main methods are commonly used for bioremediation of PAH-polluted soils: bioslurry, biopile, and landfarming (Table 3). The U.S. Federal Remediation Technologies Roundtable provides numerous case studies (40).

1. *The bioslurry remediation system.* Water is mixed with the sieved polluted soil (10% to 50% of water weight) to produce a slurry treated in a bioreactor. The use of reactors provides a rapid degradation of PAHs due to enhanced mass transfer rates and increased contaminant-to-microorganism contact. The system can be supplemented with nutrients, electron acceptors, surfactants, and degrading organisms (native or exogenous). The treatment units, fixed or rotative, make it possible to treat high concentrations of PAHs in the sludge, resulting from high soil contamination ranging from 2,500 to 250,000 mg kg^{-1}. Heavy soils with high clay content are easily treated by bioslurry. Operating pH ranges from 4.5 to 8.8, with a temperature maintained from 15° to 35°C. The duration of the treatment is shorter than 6 months, and the removal reaches 98–99% for 3-ring, 85–95% for 4-ring, and 55–85% for PAHs with up to 4 rings. Costs, including the treatment of the slurry, separation of the liquid, gaseous, and solid phase, and the specific treatment of each phase, was between 50 and 250 \$ m^{-3}. Present research concerns the continuous addition of Fenton's reagent to oxidize heavy PAHs in the bioreactors. The combined bioslurry treatment and chemical oxidation for the treatment of PAH-polluted soils may be highly effective. The U.S. EPA has presented this emerging technology (41).

2. *The biopile technology*. Biopiles, also known as biocells, bioheaps, biomounds, and compost piles, involve soil excavation, sifting, and heaping into piles (0.9 to 3 m high). The soil is packed on a protective layer formed by a bottom HPDE liner. Slotted or perforated piping placed throughout the pile collects leachates and forces air to move by injection or extraction (static biopiles). The soil is periodically reversed in the dynamic biopile to ensure aeration. Nevertheless, the soil needs to be turned or tilled at certain times during the operational life of all biopiles to promote continued biodegradation. In addition, the watering system at the top of the pile brings water (40% to 75% of the moisture holding capacity), surfactants, and nutrients. All the plants may be covered with a greenhouse or a gore-tex cover to regulate temperature and limit water evaporation. The system is effective for permeable soils with

TABLE 3. Main Characteristics of Ex Situ Bioremediation Processes Applicable to PAH-Polluted Soils

Processes	Benefits	Limitations	Key parameters to control
Bioslurry	Treatment of all types of soils, with high clay content Optimization of physicochemical and biological parameters Effective use of surfactants Most efficient mass transfer rate Increase contaminant-to-microorganism contact Most rapid biodegradation Use of native or engineered strains	High operating and capital costs Separation of liquid/soil phases after the slurry treatment Specific treatment of each phase	Solid particle ratio, pH, temperature, agitation, nutrients, amendments, surfactants Toxicity of amendments and possible contaminants
Biopile	Low operating costs Use of native or exogenous strains	Permeable soils only Ambient temperature Low mass transfer Low efficiency against HMW Slow biodegradation rates Collection of leachates and volatile compounds Space and time requirements No use of engineered strains	Moisture, airing, nutrients, amendments, surfactants Toxicity of amendments and possible contaminants
Landfarming	Treatment of all types of soils Very low operating costs Use of native or exogenous strains	Ambient temperature Collection of leachates Space and time requirements No use of engineered strains	Moisture, airing, nutrients, amendments Toxicity of amendments and possible contaminants

HMW: high molecular weight PAHs.

less than 50,000 mg kg^{-1} of organic pollutants. Presence of significant heavy metal concentrations (>2,500 mg kg^{-1}) may inhibit microbial growth. Volatile constituents tend to evaporate rather than biodegrade during treatment. Vapor generation during aeration can be controlled and treated. Treatment speed is 6 to 24 months. During that period, PAH transformation efficiency is near 80%, and the cost is 40 to 220 \$ ton^{-1} soil. We performed a study using *T. versicolor* for the bioremediation of an MGP soil at a pilot scale (42). Additional information has been provided by the U.S. EPA (43). A closely related method is composting with addition of fertilizers such as manure.

3. *Landfarming*. Landfarms are similar to biopiles in that they are above-ground, engineered systems that use oxygen from air to degrade pollutants. In contrast to biopiles, excavated soil is spread on the ground and landfarms are periodically aerated by tilling or plowing to encourage microorganism growth. In some cases, polluted soil is incorporated in the top layer of an agricultural soil. Nutrients and moisture may be added and collection of leachates may be necessary. Landfarming concerns all types of soil, with PAH and heavy metals lower than 50,000 and 2,500 mg kg^{-1}, respectively. The efficiency of PAH degradation is 90% for treatment periods of 6 to 24 months. Landfarming is also an economical method: 30 to 60 \$ ton^{-1} soil.

PROSPECTS FOR FUTURE RESEARCH

Four main areas of research need to be developed in the future in order to optimize the biological transformation of PAHs. They are: (1) methods to improve their bioavailability for degrading organisms, (2) methods for improving the ability of organisms to transform PAHs, (3) phytoremediation systems, and (4) a better knowledge about the fate and effects of transformation products in the treated media.

Improving the Bioavailability of PAHs

Emulsifiers and surfactants that facilitate the partitioning of PAHs from the solid phase of the soil or from NAPL to the water phase have been studied for many years in numerous laboratories as tools to increase solubility/bioavailability of the hydrocarbons (44). A large number of studies reported in the literature showed that a variety of ionic and non-ionic surfactants enhance the solubilization of PAHs and interfere with pollutant metabolism. As a general rule, solubility enhancement varies with surfactant structure, concentration in solution, hydrophobicity of

the PAHs, and degree and age of contamination. Different levels of study may be used.

Synthetic classical surfactant (namely Triton X-100 or Tweens) and biosurfactants have been extensively taken into account. Thus, a number of hydrocarbon-degrading microorganisms produce extracellular emulsifying agents which enhance contact between themselves and hydrocarbons. In some cases, emulsifier production in induced by growth of microorganisms on hydrocarbon. β-Cyclodextrins are also a family of emulsifiers presently studied (44, 45).

We investigated the dissolution of solid deposits of high-molecular PAHs from a solid surface (glass). In our case, the efficiency of the surfactants concerning the solubilization in the micellar phase is neutral $<$ anionic $<$ cationic. The washing of polluted soils by surfactant solutions shows higher hydrocarbon solubilization by the cationic compound benzyl dodecyl dimethyl ammonium (BDDA) at concentrations near their critical micelle concentrations (cmc). For other cationic or neutral (nonionic) surface-active agents, a comparable efficiency is reached for more than 30-fold the cmc. Anionic agents are inefficient in soils. Moreover, surfactants behave differently in soil suspensions. The cationic surfactant is adsorbed on soil and only the nonadsorbed fraction is able to solubilize PAHs (46). The neutral compounds behave as dispersing agents and break down the soil. When BDDA (cationic) and Triton X-100 (neutral) are sequentially applied to soils, an antagonist effect has been evidenced between the two surfactants. The adsorption of cationic surfactant on soil reduces the availability of benzo[*a*]pyrene for the Triton X-100.

Inhibition of PAH transformation has been reported in the presence of surfactants. Proposed mechanisms for inhibition of microbial degradation, mostly at supra-cmc levels, include surfactant toxicity or preferential use of the surfactant as a growth substrate. A major advantage of surfactants must be their lack of toxicity toward degrading microorganisms. Bacteria and fungi appear to be relatively tolerant to nonionic surfactants, even at high doses, but the use of selected tolerant organisms is being investigated. In addition several surfactants are used by bacteria or fungi as nutrients, or may induce the production of degrading enzymatic systems whose activity is not inhibited by the presence of the surfactants.

In conclusion, the use of neutral surfactants as a tool to increase PAH solubilization in soil is well developed. However, the amounts of surfactant are generally high in comparison with the low amounts of PAHs being solubilized. The most successful use of surfactants to accelerate desorption and enhance biodegradation is clearly asociated with bioslurry

reactors, because a high water content of the soil is necessary for surfactant activity. Nevertheless we cannot make generalizations about the results, and only a few of them can be used for industrial processes. Future research may concern combinations of surfactants to enhance contaminant removal as well as the use of organisms naturally producing surface-active agents. We must also keep in mind that the use of surfactants might lead to the leaching of PAHs. The remediation processes must be planned to prevent losses of pollutants out of the systems.

Improving the Ability of Organisms to Transform PAHs

In some cases, toxic and recalcitrant pollutants accumulate in the environment. Indeed the complete breakdown of a pollutant, which often includes unusual chemical bonds or substitutions, requires a sequence of metabolic reactions. Consequently, microorganisms do not have enough time to develop appropriate degradation pathways. They are two kinds of solution to this problem: the development of a microbial community capable of specifically degrading the pollutant, or the development of the capabilities of given strains by genetic construction leading to genetically modified organisms (GMOs) or genetically engineered microorganisms (GEMs). Such an approach has its limits. There is an ecological problem in using these kinds of organisms in open systems, because genes can ramble and contaminate wild strains or species. For this reason, the use of GMOs is regulated worldwide by legislation.

Another promising line of research involves improving the metabolic properties of given organisms. First, this strategy requires knowledge of structural and catalytic properties of key enzymes involved in PAH metabolism as a basis of their directed evolution to obtain the most effective isoforms. For example, one of our goals is to obtain laccases directly acting on PAHs without the presence of a redox mediator. The genes encoding for these engineered enzymes should be expressed in filamentous fungi well known for their advantages in bioremediation (17, 47).

In parallel, another line of research should focus on the development of suicidal elements for biological containment of GMOs to limit their ecological and environmental risks.

Phytodecontamination *Ex Planta*

Phytoremediation is the use of plants or trees, their associated microflora, and agronomic techniques to lower the amounts of pollutants and toxicity in soils. It is an efficient and low-cost remediation technique.

Phytodecontamination of organic pollutants involves several processes. Compounds are first absorbed through the roots and translocated into the leaves before elimination by phytovolatilization. Other chemicals can be absorbed by roots and then accumulate in aerial parts of the plant. These parts are harvested and destroyed (burned); this process is called phytoextraction. Another process is phytodegradation, which means pollutant degradation by the plant metabolism. These three processes are minor pathways involved in the phytoremediation of soils polluted by PAHs.

The most interesting and promising processes in the case of PAHs include rhizo- and mycorrhizo-spheric degradation (6). In these systems, a PAH present in the soil induces the plant response, which increases or changes its exudation, thus modifying favorably the rhizospheric microflora habitat, composition (biodiversity), or activity. In addition both physical and chemical soil conditions may be improved, and humification should be increased. Roots also increase the contact surface between microorganisms, soil, and pollutants, and transport oxygen in soil. This is of great importance to sustain PAH oxidation by microorganisms. The impact of each process in the dissipation of PAHs has not been elucidated. Plant species are, for example, slender oat (*Avena*), alfalfa (*Medicago*), soybean (*Glycine*) (6). Recent studies based on the decrease of the parent PAHs show that the concentration of pollutants in soils can be reduced by vegetation. Nevertheless, the molecular mechanisms are unclear. Dissipation should result either from transformation of PAHs into metabolites (sometime toxic) or form enhanced formation of pollutants to bound residues. Some of them, entrapped within humic complexes, could be released in the long term during wetting and drying of soils. At the opposite, residues bound to soil components by covalent bonds are stable.

Better Knowledge on the Fate and Effects of Transformation Products in the Treated Media: Ecotoxicity Assays

One main disadvantage of biological processes for environmental bioremediation is the production of intermediate products or endproducts, which have their own bio- and physicochemical properties. These metabolites can accumulate in the environment, thus causing adverse effects on living organisms. Laboratory experiments may provide information on the structure of the compounds possibly generated through enzymatic reactions. In the case of PAHs, even if most of the degradation products have been identified, it remains difficult to predict the residual toxicity of the treated soil. For this reason, it is essential to

incorporate ecotoxicity assays into the biodegradation studies. It has commonly been found following bioremediation that a great part of the pollutants has been removed from the soil. By contrast there is no correlation between PAH removal and decrease in toxicity measured by several types of assays (e.g., Microtox, teratogenicity, genotoxicity), several treated soils being more toxic than untreated ones. For example, we have shown that xenobiotics are able to induce laccase production in fungi (48). This is the case for 9-fluorenone, which is formed by the fungus *T. versicolor* useful for soil and water remediation. The metabolite is a potent inducer of fungal enzymes, whereas the parent compound fluorene is less active (Figure 4). 9-Fluorenone is more potent that xylidine, used for many years in laboratory experiments to achieve high production of laccase. Phenanthrene dione also exhibited a noticeable stimulating effect. These results clearly establish that metabolites formed through PAH biodegradation can exert an impact on soil

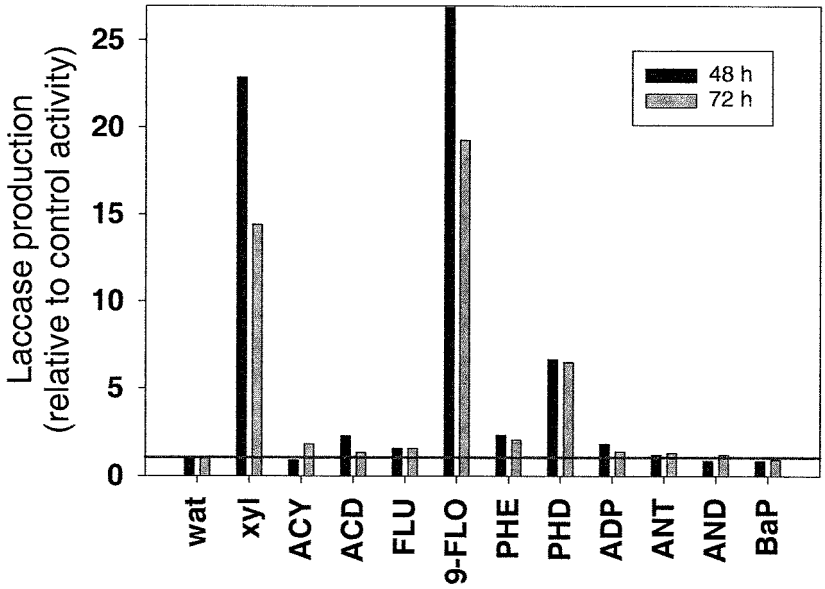


FIGURE 4. Laccase production in liquid cultures of *Trametes versicolor* supplemented with polycyclic aromatic compounds. Constitutive laccase activity, used as a control, was 0.13 ± 0.02 unit ml^{-1} and 0.16 ± 0.02 unit ml^{-1} 2 and 3 days. Abbreviations: wat, water; xyl, xylidine (positive control); ACY, acenaphthylene; ACD, 1,2-acenaphthene dione; FLU, fluorene; 9-FLO, 9-fluorenone; PHE, phenanthrene; PHD, phenanthrene dione; ADP, diphenic acid; ANT, anthracene; AND, 9,10-anthracene dione; B*a*P, benzo[*a*]pyrene.

microorganisms. For that reason, it is important to check the toxicity of the treated soil and to identify the degradation pathways. Another point to consider is the ability of PAHs and their transformation products in modifying the overall activity of enzymatic systems involved in their own metabolisation.

CONCLUSIONS

This article clearly shows the great potential of soil organisms, including bacteria, fungi, and higher plants, to transform PAHs. In liquid media, most of the PAHs are well transformed and several pathways can be involved. On the contrary in soils, the low bioavailability of the hydrocarbons, by reducing the direct contact between pollutants and degrading organisms, is the main limitation for their biodegradation. Consequently, bioremediation processes remain difficult to control in order to obtain a high efficiency in the shortest period of treatment. Because physicochemical processes for soil treatment (e.g., thermal desorption) have been developing with low costs and high efficiency, extensive research is needed to improve the more pertinent factors governing PAH bio- and phytoremediation solutions, allowing them to become effective and economically competitive technologies.

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