



HAL
open science

Bioremediation and phytoremediation of industrial PAH-polluted soils

Christian Mougin

► **To cite this version:**

Christian Mougin. Bioremediation and phytoremediation of industrial PAH-polluted soils. Polycyclic Aromatic Compounds, 2002, 22 (5), pp.1011-1043. 10.1080/10406630214286 . hal-02678950

HAL Id: hal-02678950

<https://hal.inrae.fr/hal-02678950>

Submitted on 13 Jan 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - ShareAlike| 4.0 International License

Bioremediation and phytoremediation of industrial PAH-polluted soils

Christian MOUGIN

Unité de Phytopharmacie et Médiateurs Chimiques

Institut National de la Recherche Agronomique

Route de Saint-Cyr, F-78026 Versailles Cedex

Phone: 33-1-30-83-31-02 / Fax: 33-1-30-83-31-19

E-mail: mougin@versailles.inra.fr

Abstract

This article examines the ability of microorganisms and 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: higher plants, fungi, bacteria, cytochrome P450s, laccases, biotransformation, bioremediation, polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a large group of chemical compounds which are classified all over the world as priority persistent organic pollutants (POP). 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 physico-chemical 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.

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.

1 - 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 American Environmental Protection Agency 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 128.2 to 278.4 (Table 1). Physico-chemical 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 ($\log P = 3.3$ to 6.75) and tend to be strongly associated with particle surfaces in the environment ($K_{oc} = 2 \cdot 10^3$ to $3.47 \cdot 10^6$). 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 amounting in soils to 130 days for phenanthrene (a volatile and degradable compound) and 1,400 days for benzo[a]pyrene (a recalcitrant

compound). Thus, the rate of deposition of PAHs remains today 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 natural components of fossil fuels, they have been produced throughout geological time and widely distributed in the biosphere. Thus, they are commonly released into air, soil and water, and may contaminate feed and food exposed to sources of pollution, then entering the food chain through contact, inhalation or ingestion.

- PAHs 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 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 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 car exhausts.

Concern over environmental PAHs is mainly due to the fact that some of them and their transformation products are toxic to living cells, with respect to ring number and condensation degree.⁵ For example benzo[a]pyrene, chrysene and benz[a]anthracene are carcinogenic for mammals, mutagenic in bacterial and animal cells, or teratogenic. PAHs can also induce immunodepressive effects and present a high potential to biomagnification.

PAH/soil interaction

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 physico-chemical and biological factors are known to be responsible for the highly sorptive nature of PAHs resulting in a limited availability to microbial populations in the environment, and for 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 physico-chemical properties of the soils were characterized in order to estimate the factor responsible for PAH sorption. Binding to soils of non-ionic 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 proposed 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 NAPL 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 NAPL to small droplets. The latter

mechanism may be similar to that developed to metabolize natural macro-polymers (lignin...).

Sequestration

In addition, aged POP 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. Ageing 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 non-accessible 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 either by an attachment of the compound to reactive sites on the surfaces of the organic colloids, or by 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 molecular weight ranging from 2,100 to 100,000. Both chemical and enzymatic catalysis are involved.

Characteristics of polluted soils

PAH-polluted soils may be classified into 3 main types of soils, two including PAHs of pyrolytic origin (fluoranthene/pyrene ratio > 1),⁶ one with petroleum hydrocarbons (fluoranthene/pyrene ratio < 1)(Table 2). All these soils are subjected to slow PAH release.

- A first type of soil is found around wood-preserving treatment plants, where woods were 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.
- Soils from MGPs are the second main type of PAH-polluted soils. Since 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 ageing, sometimes more than one century. PAHs are the main pollutants, but cyanides, heavy metals, and sulfates are also present in these soils. The soils have an organic matter content frequently higher than 10%. They can be very recalcitrant to bioremediation.
- The last soil is contaminated by petroleum pollutants including PAHs, and can be found near oilfield batteries. Amounts of unsubstituted PAH 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.

2 – 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 macro-fauna (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 paragraphs give an overview of the two main groups of soil microorganisms actually involved in marketed soil bioremediation processes.

- 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 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 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.

- 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.⁷ The three

processes involved in direct metabolism, namely biodegradation, cometabolism and synthesis, will be discussed here.

- 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 from 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.
- 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 another can use cometabolic products of one organism as a growth substrate. Intermediate products with their own bio- and physico-chemical properties can accumulate, thus causing some adverse effects on the environment.
- 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 (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 3 phases (Figure 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, NH₂, 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. The biochemical reactions can be catalyzed either by enzymatic systems and non-enzymatic processes.
- 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 (for example in plant vacuoles) or excreted. Conjugation reactions are mediated by enzymatic systems.
- 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 and non-enzymatic processes.

Compounds resulting from these 3 types of metabolic reactions exhibit distinct physico-chemical and biological properties. In moving from their initial state to phase 2 or 3 products, they become generally 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 1970's. First centered on the mammalian metabolism because of the metabolic activation of PAHs to electrophilic species able to bind with nucleophilic groups of DNA, RNA and proteins, studies were then extended to microbial and fungal transformations.^{8,9} Consequently, the biochemical pathways of PAH transformation have been described for many years, and extensively reviewed. One objective of these studies was to determine whether bioremediation of PAH-contaminated soils could generate potentially toxic intermediates.

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).¹⁰ 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.^{11,12} The general principles of bacterial metabolism of PAHs, under aerobic conditions, have been clearly demonstrated since 1968 by David T. Gibson and co-workers. 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 two-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. In general, all the metabolites formed from these pathways remain held in the inside of the cells, as a prelude to assimilation.

A minor PAH bacterial transformation pathway involves cytochrome P450 monooxygenases, described below for fungi.

Fungal metabolism of PAHs

Numerous fungi among zygomycetes, ascomycetes and basidiomycetes metabolize PAHs.¹³⁻¹⁵ Fungal metabolism of PAHs is 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 co-workers initiated the studies on fungal metabolism of PAHs in the 1970's. Then, Carl E. Cerniglia and co-workers retained the zygomycetes *Cunninghamella bainieri* and *elegans* as models. In 1985, John A. Bumpus extended the studies to white rot (ligninolytic) fungi. In contrast to bacteria, non-ligninolytic fungi produce metabolites that include *trans*-dihydrodiols, phenols, quinones, tetralones and dihydrodiol epoxides, whereas ligninolytic basidiomycetes are in addition able 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.

- The first step of unsubstituted PAH metabolism is ring epoxidation by a cytochrome P450 monooxygenase, leading to an unstable arene oxide. Arene oxides are immediately hydrated to *trans*-dihydrodiols by epoxide hydrolase, or rearranged non-enzymatically 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 non-enzymatic 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. According to the target organism, phenols can be less or more toxic than the parent compounds. Phenols are then conjugated during phase 2 metabolism.
- Several strains of fungi produce quinones. In non-ligninolytic 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 CO₂ 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.

- 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 tetrahydrodiols, which are less mutagenic than the dihydrodiol-epoxides.
- 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-hydroxy-benzo[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

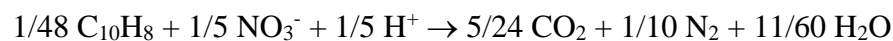
Our present knowledge of higher plant metabolism of PAHs is scarce, but it is admitted that plants may offer a great potential.^{5,16} By studying PAH metabolism in whole plants, it has been found that hydrocarbon adsorption onto roots, as well as uptake, are significant. On the contrary, translocation of the PAHs with 4 rings or more from roots to leaves is negligible. Background contents of PAHs in plant tissues in North America and Europe are very low, at the $\mu\text{g kg}^{-1}$ level.⁵ Consequently, data concerning their metabolism are lacking, even if incorporation of PAHs into cellular components has been reported. For similar reasons, plant germination and growth are strongly inhibited by PAHs with fewer than 3 rings. Plant cell suspensions are efficient tools to study PAH transformation. In these systems, they are oxidized to phenols or quinones, then conjugated with sugars.

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.

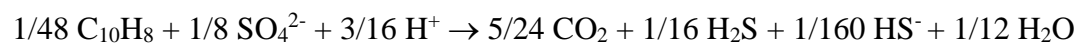
Anaerobic degradation of PAHs

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:¹⁷

-under nitrate-reducing conditions:



-under sulfate-reducing conditions:



Enzymatic systems involved in PAH biotransformation

The main enzymatic systems involved in PAH bacterial, fungal and plant metabolism include dioxygenases and cytochrome P450 monooxygenases. For about ten 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

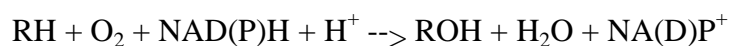
Dioxygenases are enzymes produced by bacteria, which catalyze the following equation:



They are involved in both the physiological substrate and xenobiotic metabolism. One well-known enzyme is naphthalene dioxygenase.⁹ It comprises a multi-component system of 3 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 induced by PAHs.

Cytochrome P450 monooxygenases

Microbial, fungal and plant P450s have been described elsewhere.^{18,19} P450s 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:



The result of catalysis, depending on the P450 protein and its substrate, leads in most cases to hydroxylation, epoxidation or heteroatom dealkylation.

For most eucaryotic P450s, a FAD/FMN-dependent NADPH-P450 reductase is needed to transfer the electrons used for oxygen activation from cytosolic NADPH. In plants and filamentous fungi, P450s and reductases are usually microsomal membrane-bound proteins, exposed to the cytosol. A second type of P450s 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 P450 component. Such P450s are usually soluble or associated to inner mitochondrial membranes.

P450s are encoded by a superfamily of genes.²⁰ The sequences of more than 500 of them 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 P450s 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 P450s correspond to the families CYP71 to CYP99, then CYP701 and above. Fungal P450s comprise families CYP 51 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 P450s, 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 P450s 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 eleven 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 P450s is only starting to accumulate. In plants, expression seems to follow in most cases a developmentally regulated tissue-specific pattern. Physicochemical (light, osmotic stress, wounding), physiological (infection, ageing, hormones) factors, or xenobiotics (agrochemicals, ethanol, or drugs like phenobarbital or aminopyrine) were reported to induce plant P450s. 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 P450 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 P450s.

Fungal peroxidases

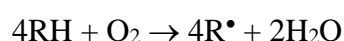
Wood-decaying fungi belonging to the white-rot group of basidiomycetes have been attracting scientific attention for almost two decades. These organisms, able to depolymerize lignin, secrete two families of peroxidases, namely lignin-peroxidase (LiP, EC 1.11.1.14) and Mn-peroxidase (MnP, EC 1.11.1.13) and in some cases a family of laccases (see the following paragraph). The idea of applying the ligninolytic 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.²¹ 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 non-phenolic 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. H₂O₂ 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, those 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 are for LiPs 2.5-3.0 and 4.0-4.5 for MnPs.

Fungal laccases

Recently, Gianfreda *et al.* reviewed our present knowledge on laccases (Lac, EC 1.10.3.2).²² 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 host. 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 or textile industry, as well as the degradation of various xenobiotics, which are recognised as environmental pollutants. For that reason, numerous data are published concerning laccases for a few years.

These multi-copper proteins catalyse the one-electron oxidation of various aromatic compounds (specifically phenols, arylamines or aminophenols) while concomitantly reducing molecular oxygen to water, according to the equation:



The formed radical R° 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 or forest litter. The laccase molecule is a monomeric, dimeric or tetrameric glycoprotein usually containing 4 atoms of copper per monomer, bound to three redox sites. Redox potentials of fungal laccases range from 0.24 to 0.54 V (versus SCE). Formed from 520 to 550 aminoacids, 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 1996.²³ They 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-sulphonic acid (ABTS) or 1-hydroxybenzotriazole (HBT). Collins *et al.* confirmed these results and also demonstrated the oxidation of benzo[a]pyrene by the laccase.²⁴ 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.^{21,25,26} Laccases

from other fungal strains (*P. cinnabarinus*, *Corioloropsis gallica*) were also able to catalyze PAH oxidation in the presence of exogenous mediators.^{27,28} Natural mediators (methionine, cysteine...) have also been identified.²⁹ The mechanisms involved in the laccase mediator system have recently been elucidated.³⁰ PAHs were converted to radical cations (PAH^{•+}) after abstraction of one electron by oxidized species of the mediators (ABTS^{•+} or HBT[•]). Then, they undergo a nucleophilic attack by water 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 versus SCE) was increased following enzymatic oxidation of ABTS to ABTS^{•+} (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.³¹ 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 towards the hydrocarbons is also due to the presence of interfering compounds co-extracted from the soil, such as metals and organics with oxidation potentials lower than these of PAHs. For example, the results suggested that the dication ABTS²⁺, 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 organisms 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.

3 – 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.³² Two types of soil treatment techniques are being developed. Firstly, they include *in situ* processes where the soil is directly treated without excavation. They 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 soil treatments can be performed *ex situ*, and are 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 re-introduced in the polluted material (bioaugmentation). *Ex situ* processes are being extensively developed world-wide, because

they make it possible to easily control the key parameters required for optimal pollutant degradation, in reducing also the heterogeneous structure of the soil.

Among the remediation methods available, several parameters indicate that bioremediation is an interesting technology, in contrast with physico-chemical treatments. The first parameter is related to the pollutant. When bioavailable, common PAHs are chemical compounds well degraded 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, ageing 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 re-used if the target pollutant levels are reached. Their 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, which are bioslurry, biopile and landfarming (Table 3). The US Federal Remediation Technologies Roundtable provides numerous case studies.³³

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, the 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. US EPA presents this emerging technology.³⁴

The biopile technology

Biopiles, also known as biocells, bioheaps, biomounds and compost piles, involves soil excavation, sifting and heaping into piles (0.9 to 3 meters 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 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 a MGP soil at a pilot scale.³⁵

Additional information is provided by US EPA.³⁶ A closely related method is composting with addition of fertilizers such as manure.

Landfarming

Landfarms are similar to biopiles in that they are aboveground, engineered systems that use oxygen from air to degrade pollutants. By 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. Sometimes bacteria, which have been selected for their effectiveness in breaking down hydrocarbons, are added. The efficiency for PAH degradation is 90% for treatment periods of 6 to 24 months. Landfarming is also a cheap method: 30 to 60 \$ ton⁻¹ soil.

4 - 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 on 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. A large number of studies, reported in the literature, showed that a variety of ionic and nonionic 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, degree and age of contamination. Different levels of study may be used.

Synthetic classical surfactants (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 them and hydrocarbon. In some cases, emulsifier production is induced by growth of microorganisms on hydrocarbon. β -Cyclodextrins are also a family of emulsifiers presently studied.

We investigated the dissolution of solid deposits of high molecular PAH 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 (non-ionic) 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 non-adsorbed fraction is able to solubilize PAHs.³⁷ 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 BaP 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 towards degrading microorganisms. Bacteria and fungi appear to be relatively tolerant to non-ionic 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 associated 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.

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. There 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 world wide 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.^{14,38}

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 adsorbed through the roots and translocated into the leaves, before elimination by phytovolatilization. Other chemicals can be adsorbed 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. In these systems, a PAH present in the soil induces the plant response, which increases or changes its exudation, thus modifying favourably 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. The impact of each process in the dissipation of PAHs has not been elucidated. The main plant species are festue (*Festuca*), alfalfa (*Medicago*), sudangrass (*Sorghum*), ryegrass (*Lolium*), corn (*Zea*), soybean (*Glycine*)... 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 (sometimes toxic) or from enhanced formation of pollutants to bound residues, which could be released in the long term.

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 end-products, which have their own bio- and physico-chemical 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 is commonly found following bioremediation that a great part of the pollutants is removed from the soil. By contrast, there is no correlation between PAH removal and decrease in toxicity measured by several types of assays (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.³⁹ This is the case for 9-fluorenone, which is formed by the fungus *Trametes versicolor* useful for soil and water remediation. The metabolite is a potent inducer of fungal enzymes, whereas the parent compound fluorene was less active (Figure 4). 9-Fluorenone was more potent than xylydine, 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 can cause adverse effects on soil microorganisms. For this reason, it is important to determine if bioremediation has

achieved the desired goals, either in terms of pollutant concentration or toxicity decrease. Finally, even if toxicity is reduced, remaining PAHs or transformation products could enter compartments of the ecosystem via ingestion of soil/sediment particles by the macrofauna (earthworms) or by uptake into plants.

Conclusions

This paper clearly evidences 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 is the main limitation for their biodegradation, by preventing the direct contact between pollutants and degrading organisms. Consequently, bioremediation remains difficult to control in order to obtain a high efficiency in the shortest period of treatment. For several years, physico-chemical processes for soil treatment, for example thermal desorption, have been rapidly developing with low costs and high efficiency, and may prove to be good competitors of bioremediation.

Acknowledgements

I am grateful to Christine Young for its attentive reading of the manuscript.

References

1. Dabestani, R. and Ivanov, I.N., *Photochem. Photobiol.* **70**, 10 (1999).
2. <http://www.epa.gov/superfund/ressources/soil/attachc.pdf>
3. <http://chrom.tutms.tut.ac.jp/JINNO/DATABASE/00alphabet.html>
4. <http://esc.syrres.com/interkow/physdemo.htm>
5. Sims, R.C. and Overcash, M.R., *Residue Reviews* **88**, 2 (1983).
6. Baumard, P., Budzinski, H. and Garrigues, P., *Environ. Toxicol. Chem.* **17**, 765 (1998)
7. Bollag, J.-M. and Liu, S.-Y., *Pesticides in the Soil Environment* (Soil Science Society of America, Madison, 1990), Chap. 6, pp. 169-211.
8. Cerniglia, C.E., *Rev. Biochem. Toxicol.* **3**, 321 (1981).
9. Cerniglia, C.E., *Adv. Appl. Microbiol.* **30**, 31 (1984).
10. Ronald M.A., Cerniglia, C.E. *BioScience* **45**, 332 (1995).
11. Kanaly, R.A. and Harayama, S., *J. Bacteriol.* **182**, 2059 (2000).
12. Bressler, D.C. and Fedorak, P.M., *Can J. Microbiol.* **46**, 397 (2000).
13. Sutherland, J.B., *J. Ind. Microbiol.* **9**, 53 (1992).
14. Cerniglia, C.E., *J. Ind. Microbiol. Biotechnol.* **19**, 324 (1997).
15. Canet, R., Lopez-Real, J.M. and Beck, A.J., *Land Contamin. Reclam.* **7**, 191 (1999).
16. Salt, D.E., Smith, R.D. and Raskin, I., *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 643 (1998).
17. Rockne, K.J. and Strand, S.E., *Environ. Sci. Technol.* **32**, 3962 (1998).
18. Chapple C., *Annu. Rev. Plant Mol. Biol.* **49**, 311 (1998)
19. Mougín C.P., Corio-Costet, M.-F. and Werck-Reichhart, D. 2001. *Pesticide Biotransformation in Plants and Microorganisms: Similarities and Divergences*. (ACS Book n°777, Washington, 1999), Chap. 9, pp. 166-181.
20. <http://drnelson.utm.edu/homepage.html>
21. Günther T., Sack U., Hofrichter M. and Lätz M., *J. Basic Microbiol.* **38**, 113 (1998).

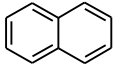
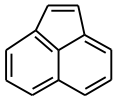
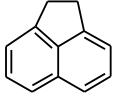
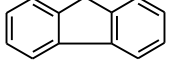
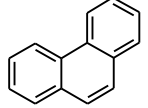
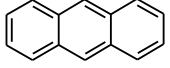
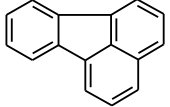
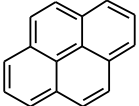
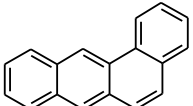
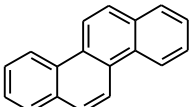
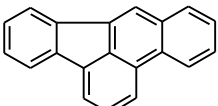
22. Gianfreda L., Feng X. and Bollag J.-M., *Bioremed. J.* **3**, 1 (1999).
23. Johannes, C., Majcherczyk, A. and Hüttermann, A., *Appl. Microbiol. Biotechnol.* **46**, 313 (1996).
24. Collins, P.J., Kotterman, M.J.J., Field, J.A. and Dobson, A.D.W., *Appl. Environ. Microbiol.* **62**, 4563 (1996).
25. Böhmer, S., Messner, K. and Srebotnik, E., *Biochem. Biophys. Res. Commun.* **244**, 233 (1998).
26. Johannes, C., Majcherczyk, A., Hüttermann, A., *J. Biotechnol.* **61**, 151 (1998).
27. Majcherczyk, A., Johannes, C. and Hüttermann A., *Enzyme Microb. Technol.* **22**, 335 (1998).
28. Rama, R., Mougín, C., Boyer, F.-D., Kollmann, A., Malosse, C. and Sigoillot, J.-C., *Biotechnol. Letters* **20**, 1101 (1998).
29. Pickard, M.A., Roman, R., Tinoco, R. and Vazquez-Duhalt, R., *Appl. Environ. Microbiol.* **65**, 3805 (1999).
30. Johannes, C. and Majcherczyk, A., *Appl. Environ. Microbiol.* **66**, 524 (2000).
31. Macherczyk, A. and Johannes, C., *Biochim; Biophys. Acta* **1474**, 157 (2000).
32. Mougín, C., Jolival, C., Malosse, C., Sigoillot, J.-C., Asther, M. and Chaplain, V., *Polycycl. Aromat. Comp.* (submitted).
33. Mueller, J.G., Cerniglia, C.E. and Pritchard, P.H., *Bioremediation "Principles and Applications"* (Cambridge University Press, 1996), pp. 125-194.
34. <http://www.frtr.gov/cost/>
35. <http://epa.gov/ORD/SITE/reports>
36. Rama, R., Sigoillot, J.-C., Chaplain, V., Asther, M. Jolival, C. and Mougín, C., *Polycycl. Aromat. Comp.* **18**, 397 (2001).
- <http://epa.gov/OUST/cat/biopiles.htm>

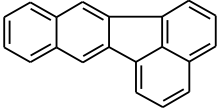
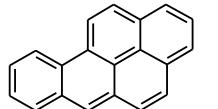
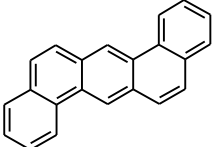
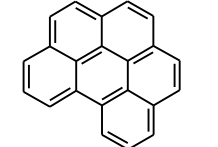
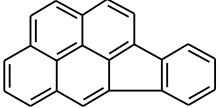
37. Dur, J.-C., Rama, R., Mougin, C. and Chaplain V., *Polycycl. Aromat. Comp.* **20**, 143 (2000).

38. Stahl, J.D. and Aust, S.D., *Rev. Toxicol.* **2**, 189 (1998).

39. Mougin, C., Kollmann, A. and Jolival, C. *Biotechnol Lett.* (*in press*).

Table 1. Physicochemical properties and structure of the 16 PAHs selected as priority pollutants by the US Environmental Protection Agency.

Names	M.W.	Water solubility (mg L ⁻¹)	Log P	K _{oc}	Structure
Naphtalene C ₁₀ H ₈	128	31.00	3.30	2 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	
Fluorene 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[a,h]- anthracene C ₂₂ H ₁₄	278	0.0006	6.75	3.80 10 ⁶	
Benzo[ghi]perilene 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 ^[1-5]

Table 2. Characteristics of the main types of PAH-polluted soils.

	Wood-preserving facility	Manufactured gas plant	Oilfield battery
PAH contamination (mg kg ⁻¹)	<5000	<25000	<5000
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	++	--	+

Table 3. Main characteristics of *ex situ* bioremediation processes applicable to PAH-polluted soils.

Processes	Benefits	Limitations	Key parameters to control
Bioslurry	<ul style="list-style-type: none"> • Treatment of all types of soils, with high clay content • Optimization of physico-chemical and biological parameters • Effective use of surfactants • Most efficient mass transfer rate • Increase contaminant-to-micro-organism contact • Most rapid biodegradation • Use of native or engineered strains 	<ul style="list-style-type: none"> • High operating and capital costs • Separation of liquid/soil phases after the slurry treatment • Specific treatment of each phase 	<ul style="list-style-type: none"> • Solid particle ratio, pH, temperature, agitation, nutrients, amendments, surfactants • Toxicity of amendments and possible contaminants
Biopile	<ul style="list-style-type: none"> • Low operating costs • Use of native or exogenous strains 	<ul style="list-style-type: none"> • 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 	<ul style="list-style-type: none"> • Moisture, airing, nutrients, amendments, surfactants • Toxicity of amendments and possible contaminants
Landfarming	<ul style="list-style-type: none"> • Treatment of all types of soils • Very low operating costs • Use of native or exogenous strains 	<ul style="list-style-type: none"> • Ambient temperature • Collection of leachates • Space and time requirements • No use of engineered strains 	<ul style="list-style-type: none"> • Moisture, airing, nutrients, amendments • Toxicity of amendments and possible contaminants

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

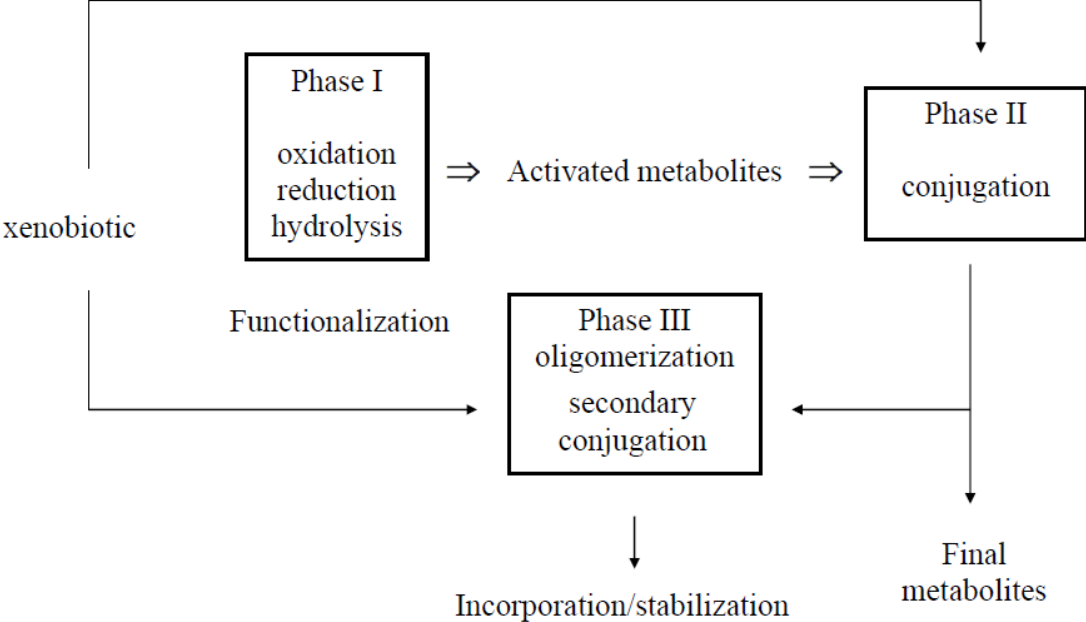


Figure 2. The different mechanisms of PAH oxidation by living organisms. Adapted from Ref

10.

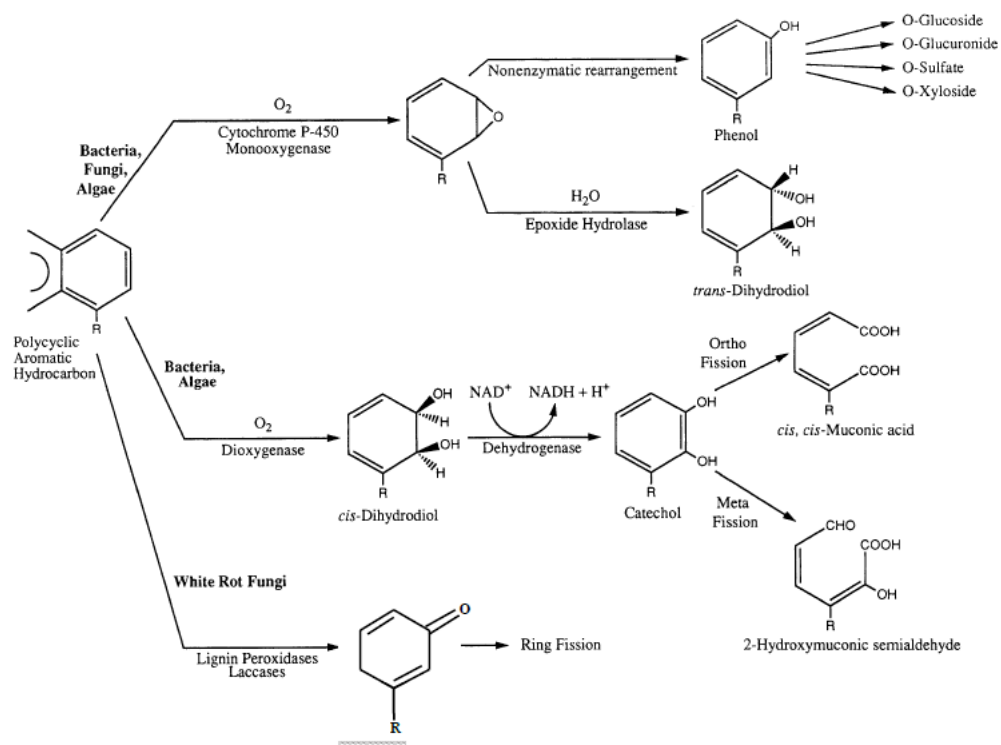


Figure 3. Mechanism of laccase-mediated oxidation of benzo[a]pyrene.

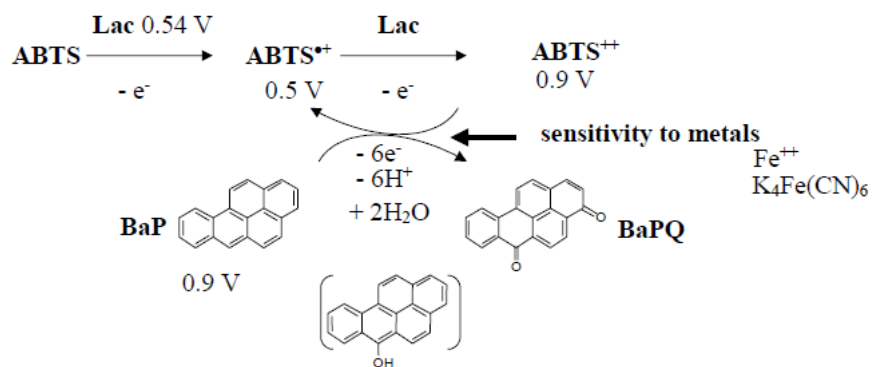


Figure 4. Laccase production in liquid cultures of *T. versicolor* supplemented with polycyclic aromatic compounds. Constitutive laccase activity, used as a control, was 0.13 ± 0.02 unit ml⁻¹ and 0.16 ± 0.02 unit ml⁻¹ 2 and 3 days. Abbreviations are: wat, water; xyl, xylinine (positive control); ACY, 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; BaP, benzo[a]pyrene; BaPD, mixtures of 1,6- 3,6- and 6,12-benzo[a]pyrene diones.

