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Biodegradation of Organic Pollutants in Soil

Literature Review and Preliminary Experiments

Pablo Mayorga

Publication 3:94 **Licentiate thesis**

In nature, no organic substance is synthesized unless there is provision for its degradation...

Barry Commoner

Preface

My interest in fungi began when I was a child and went mushroom hunting with my father. In my final undergraduate university years this interest was revived and, in one way or another, I tried to devote all my course projects to fungi. My ecological and environmental background was shaped in my undergraduate university years and my interest became stronger when I had a chance to work in several environmental projects like environmental impact assessments (entomological aspects) and environmental education. I even had the opportunity to be in contact and cooperate, in different ways, with the Guatemalan Environmental Protection Commission (CONAMA) and some environmentalist groups in Guatemala (NGO's).

Some of my newer passions were edible mushroom cultivation and composting. Before coming to Sweden I had indirect contact with Per Berg, who was interested in my initial project idea: Growing edible fungi in household (and other kind of urban) solid waste. Once in Sweden, the use of fungi to degrade organic pollutants in soil sounded much more promising to him. I must mention that my initial knowledge on the use of white-rot fungi to degrade organic pollutants in soil came from fax-contact with the late Professor Bengt v Hofsten (Statens Livsmedelsverk), several months before coming here (September 1990). This led me to people working with mushroom cultivation and with bioremediation using white-rot fungi. After many months of resisting to the idea of working on bioremediation (although I collected hundreds of articles on both mushroom cultivation and bioremediation), I realized the possibilities and value of this topic. I was not totally disappointed since in a stage of my work I have had to cultivate the mushrooms. I also learned a lot about other fungi I had not worked with before. I have also realized that the environmental uses of microorganisms can be very wide and fascinating.

Environmental clean up (using temporary or definitive clean up solutions) and the reduction (or total prevention) of environmental pollution from human activities often have to be achieved at the same time. Bioremediation methods have many economic advantages over traditional technologies, and are a more natural approach to the problem. The brilliant future and multidisciplinarity of bioremediation should encourage more institutions and researchers to invest and work in a serious way in this field, which still needs much research. Great care must be taken to do a good job and to know the limitations of bioremediation so the authorities, industries and common people will not be disappointed by failures like the one in Blekholmstorget in Stockholm. While several Swedish companies offer bioremediation services, only one group in Lund, one in Stockholm, one in Umeå and myself are doing academic research on this topic (to my knowledge).

Finally, it is unfortunate that obtaining research funds has been extremely difficult for me despite the urgent need for this kind of work in Sweden. A bioremediation network involving researchers and consulting companies should be created so that research funds are not spent on duplicate efforts, and to make information easily available to other researchers or consulting firms.

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Glossary of abbreviations

ACs	aromatic compounds
BOD	biological oxygen demand
BRF	brown-rot fungus or fungi
BTEX	Benzene, toluene, ethylbenzene and xylene
COD	chemical oxygen demand
CS	contaminated soil
2,4-D	2,4-dichloro-phenoxyacetic acid
2,4-DME	2,4-dichloro-phenoxyacetic acid methyl ester
DDD	1,1-*bis*(4-chlorophenyl)-2,2-dichloroethane
DDE	1,1-dichloro-2,2-bis(4-chlorophenyl)ethene
DDT	1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
GC-MS	gas chromatography-mass spectrometry
HPLC	high-performance liquid chromatography
LiP	lignin peroxidase or ligninase
MnP	manganese dependent peroxidase
PACs	polyaromatic compounds
PAHs	polycyclic aromatic hydrocarbons
PCBs	polychlorinated biphenyls
PCP	pentachlorophenol
TNT	2,4,6-trinitrotoluene
WRF	white-rot fungus or white-rot fungi
VA	veratryl alcohol
VAO	veratryl alcohol oxidase
VOCs	volatile organic compounds

Abstract

Literature review: Creosote-contaminated soils in former wood impregnation sites is an environmental problem in many industrialized countries. Biological clean up methods (bioremediation) are attractive oweing to the fact that they are less expensive than conventional physical or chemical methods and are a more natural approach.

Bioremediation requires a multidisciplinary approach so literature on several aspects of polycyclic aromatic hydrocarbons and creosote was reviewed. Among these aspects are analytical factors (sampling, extraction and analysis methods), ecotoxicological considerations, and cleanup technologies regarding soils polluted with organic compounds. The use of white-rot fungi (WRF) for bioremediation of coal-tar creosote-contaminated surface soil is the main interest of this work. These organisms are a good choice for this purpose. However, the technology for their full scale application is still being developed.

Protocols for the bioremediation of a contaminated site include characterization of the site (physico-chemical, hydrogeological and microbiological conditions), treatability tests, and implementation of the method. Each contaminated site has its own characteristic so its remediation could require a specific strategy. Perhaps more than one technology might be needed, especially at sites with mixed pollutants. Toxicological assays are a good complement to chromatographic determinations of organic contaminants in soil, before and after bioremediation.

Experimental section: During preliminary experimental work for this thesis, methodologies were developed for extracting creosote from a contaminated clay soil and analyzing the extracts by gas chromatography-mass spectrometry (GC-MS). Initial acute toxicity tests were also carried out with Microtox, but a methodology still has to be developed, especially for hydrophobic compounds.

Laboratory scale bioremediation feasibility experiments where carried out with WRF of the genus *Pleurotus*. This WRF degraded PAHs in soil but produced relatively large quantities of intermediate metabolic products under the experimental conditions used. Contaminated soil resident microorganisms, stimulated by nutrient addition (autoclaved wheat straw), proved to be equally good or better PAH degraders than this fungus. The resident microorganism treatment did not produce intermediate degradation products detectable with the GC-MS method used. The WRF showed consistent behavior in consecutive experiments, *i.e.*, degradation rates and formation of the same intermediate degradation products.

Future work will include the improvement of soil sampling and extraction techniques. It is also necessary to identify the PAH intermediate degradation products produced by *Pleurotus*. The development of acute toxicity and mutagenicity assays to determine the effect of the bioremediation processes tested on the toxic properties of the soil will also be future projects. Lastly, more species of WRF and microorganisms isolated from contaminated soil will be tested to degrade PAHs in soil.

Sammanfattning

Litteratursammanställning: Kreosotkontaminerad jord vid f.d. träimpregneringsanläggningar är ett stort miljöproblem i många industrialiserade länder. Biologisk efterbehandling (bioremediation) är ofta attraktivt eftersom sådan behandling kostar mindre än traditionella reningsmetoder (fysikaliska/kemiska) och ofta anses som en mer naturlig lösning på problemet.

Biologisk marksanering är en verksamhet på tvärvetenskaplig grund. Denna uppsats fokuserar på biologisk sanering av kreosotkontaminerad ytjord med hjälp av vitrötesvampar. Vitrötesvampar har använts med bra resultat i USA och Tyskland i olika försökskala, men de har inte testats i fullskala.

En litteraturgenomgång avseende analytiska och toxikologiska aspekter, samt tillämpade aspekter på teknik för marksanering har genomförts. Varje kontaminerad plats har sina speciella egenskaper som fordrar särskilda åtgärder. Kontaminerade jordmassor brukar innehålla blandningar av föroreningar. Detta medför att flera olika behandlingsmetoder kan behövas; och toxikologiska tester fordras för att komplettera kemiska analyser (t.ex., kromatografi) av föroreningar i jord. Jämförelser av toxikologiska effekter på jorden före och efter en behandling kan ge viktig information om toxikologisk status hos obehandlade och behandlade massor, samt en idé om behandlingens påverkan på de toxiska ämnena i jorden.

Preliminära försök: En metod för studier av vitrötesvampars nedbrytning av polyaromatiska hydrokarbonater har utveklats avseende dels analytiska aspekter dels uppställningen av nedbrytningsförsök. Bland de analytiska aspekterna finns provtagning, PAH-extraktion och kromatografi med GC-MS. HPLC har även prövats. Metoden för nedbrytningsförsökens genomförande har utvecklats mot bakgrund av vikten av att tillse att jordprovet har en viss permeabilitet, sättet på vilket svamp tillsats till jorden och den skala försöken genomförs i. Preliminära toxikologiska tester gjordes med Microtox fast-fas akut toxicitet test. Fast-fas testen medförde vissa problem som senare lösts av andra forskare. Metoden måste emellertid utvecklas för att även klara fettlösliga (PAHs) ämnen.

Försök i laboratorieskala med biologisk nedbrytning av kreosot har genomförts med vitrötesvampar av arten *Pleurotus*. Svampen bröt ner PAH i jorden, men nedbrytningsprodukter i stora mängder bildades. I en parallell uppställning stimulerades befintliga mikroorganismer i den förorenade jorden med en näringstillsats i form av autoklaverad halm. Denna uppställning visade en bättre nedbrytning av PAH än svampen. Inte heller några nedbrytningsprodukter kunde detekteras vid denna behandling. Både svamp- och halmbehandlingarna visade samma resultat i upprepade försök, dvs, svampar gav bra PAH nedbrytning och stora mängder mellanprodukter, och halmbehandling gav bättre nedbrytning och inga mellanprodukter.

Det framtida arbetet kommer att bland annat att innebära en utveckling av provbehandlingen (provtagning och extraktion). Mest nödvändigt är att utveckla ett bra sätt att blanda jorden eftersom en mycket hög variation i PAH-halten bland replikaten

varit förhanden under de genomförda försöken. Kemiska och toxikologiska (akuta och mutagena) egenskaper hos de funna nedbrytningsprodukterna skall kartläggas. Flera vitrötesvampar kommer att testas liksom jordsvampar som isolerats från den kreosotförorenade jord som använts i försöken.

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1 Presentation of the thesis

1.1 Introduction

This thesis consists of two main parts: an informative literature review and an experimental section. Both parts are related to the bioremediation of soils contaminated with organic pollutants, especially the biodegradation of polycyclic aromatic hydrocarbons (PAHs) in creosote-contaminated soils by white-rot fungi (WRF).

Bioremediation is the controlled use of biodegradation to remove toxic chemicals from soil and groundwater (Bennett and Olmstead, 1992). Bioremediation involves many disciplines such as biology, hydrology, geology and soil chemistry. Of special importance are analytical chemistry, toxicology, and applied microbiology in relation to engineering aspects of bioremediation. These topics are covered in the literature review, but applied microbiological aspects are emphasized. Given the large amount of literature on these three topics, only relevant references are included. Bioremediation also involves many decision-making levels in industry and in politics, but financial and political aspects are not discussed.

1.2 Relevance of the topic

Public health and the environment are threatened by many man-made products. Spiegel (1988) and Almström (1991) have proposed different classification criteria of hazardous wastes. Among the 65 classes of hazardous chemical compounds, 114 compounds are considered as priority pollutants by the United States Environmental Protection Agency (US EPA) (Kobayashi and Rittmann, 1982), including certain PAHs. Many PAHs are important environmental pollutants owing to their persistence, toxicity, mutagenicity and/or cancer-related effects. These compounds are formed in natural (Blumer, 1976) or anthropogenic processes, such as the incomplete combustion of organic material and are widely distributed in the environment in air, soil, water and sediments (Lee *et al.*, 1981). When airborne PAHs reach the soil or PAHs of industrial origin are spread on the soil (perhaps in higher loads than deposition of airborne PAHs), they may reach groundwater and other nearby bodies of water.

PAHs (from residues of oil, coal tar and processing of similar substances), as well as polychlorinated biphenyls (PCBs) and dioxins, are important soil pollutants (Loske *et al.*, 1990). In petroleum-processing and wood-preserving sites it is common to find PAHs; Benzene, toluene, ethylbenzene and xylenes (BTEX); Biocidal organics (pentachlorophenol (PCP), 2,4,6-trichlorophenol); and toxic metals associated with refining and wood treatment operations (arsenic, chromium, copper, lead, nickel) (Pollard *et al.*, 1994).

Contamination of soils and sediments with PAHs, associated with wood impregnation with creosote, has been reported in many industrialized countries (Sundström *et al.*, 1986). Landfilling methods have been developed to confine these soils within an area with suitable geological material (or lined with adequate materials) that will avoid leaching of pollutants to the groundwater or nearby water bodies. The pollution of

aquatic environments and sediments deserve special attention since water is the root of all food chains (Iyengar, 1989) and sediments are the ultimate recipients of a range of pollutants (Kwan and Dutka, 1990). Landfilling and incineration are the usual treatment methods for creosote-contaminated soils in Sweden (Swedish Environmental Protection Board (SNV), 1993a). Landfilling does not solve the problem permanently and, although incineration effectively destroys the organic pollutants, other environmental problems (*e.g.*, emissions to the air and ashes) may arise. A range of physical, chemical and biological methods to transform organic contaminants to innocuous products have been developed (Tedder and Pohland, 1990, 1991, 1993). Biological methods are especially attractive because they are less expensive than physico-chemical methods and are a more natural approach.

1.3 Aims and objectives

In the literature review, some principles for bioremediation of soils contaminated with organic pollutants are stated, to serve as a reference for present and future experimental work. Special attention is given to creosote- and PAH-contaminated soils in general as well as to the use of white-rot fungi as bioremediation agents for such pollutants.

The experimental section is of an exploratory character and is aimed at identifying and solving methodological problems. Examples of such problems are soil preparation, application of biological treatment to the soil, extraction of pollutants from the soil and sample extract analysis. There was no intention to go beyond laboratory scale experiments.

2 Literature review: Biodegradation of organic pollutants in soil

2.1 Introduction

Three main areas related to bioremediation of contaminated soil are reviewed. These are:

1) Chemical aspects of creosote and PAHs,

2) Toxicological and ecotoxicological considerations of PAHs and bioremediation, and

3) Remediation and reclamation technologies of soils contaminated with organic pollutants.

Sundström *et al.* (1986), Sims and Overcash (1983) and Lee *et al.*, (1981) provide good general guides to analytical methods, environmental and toxicological aspects of creosote and PAHs. The DECHEMA conference (1992) covers recent advances on most of the topics of this review, especially biological methods for soil decontamination.

In many cases, soil pollution is intimately related with water pollution (groundwater, other bodies of water and sediments). Therefore, some references on aquatic pollution, toxicology and bioremediation are cited.

2.2 Chemical aspects of creosote

2.2.1 Production and use of creosote

Creosote here refers to coal-tar creosote, obtained by the distillation of coal tar (a by-product of coal coking or gasification). It should not be confused with wood-tar creosote, which has totally different properties and contains mainly phenolic compounds. Creosote has been used mainly as a wood preservative due to its biocidal and water repellent properties. Other minor uses have been as a herbicide, fungicide, disinfectant and as an insecticide, larvicide and repellent. Owing to its toxicological and carcinogenic properties and environmental persistence, its use for purposes other than wood impregnation (railroad sleepers, poles for telephonic or electricity cables and to a lesser extent diverse sawed timber), as well as sales to the general public, have been discontinued in Sweden (Sundström *et al.*, 1986). Its use as impregnation agent in Sweden started in 1899 (Seman and Svedberg, 1990) when no environmental laws or consciousness existed. Between 1900 and 1990, 620,000 tons of creosote have been used in Sweden, and its use has tended to decrease over time. For example, between 1970 and 1979 6,800 ton per year were used, while between 1980 and 1990 4,700 ton per year were used (Nilsson, 1991).

Coal tar is one of the primary by-products of coal carbonization (coking) or coal gasification. However, they are similar processes with different economic aims, but the gas obtained from the coking and the coke obtained from the gasification can also be sold. The process diagram including products and by-products of coal gasification are summarized in a report from SNV (SNV, 1993a). Coal coking is still an important activity for the (Swedish) iron and steel industry, and the resulting tar may be used for creosote production. However, creosote is no longer distilled in Sweden (Bergman, 1994). On the other hand, the process of obtaining gas from coal was used in Sweden from the first half of the 19th century until the mid-1960's. The gas produced was used basically for city lighting and later for heating. Most of these former plants are now abandoned sites located in central parts of cities, and near water (SNV, 1993a). The soils at these sites are still contaminated with PAHs and other pollutants, and in some cases, buildings or streets have been constructed over them.

Old wood treatment facilities are often the source of creosote environmental contamination (Sundström *et al.*, 1986). Former coal gasification plant sites, where creosote was sometimes distilled from the coal tar, may contain pollutants such as PAHs, Pb, mercury, cadmium and cyanides. In Sweden there are around 50 former coal gasification plants with different degrees of contamination (SNV, 1993a), about 40 sites are contaminated with creosote from wood impregnation activities (SNV, 1993b) and 5 operating plants still use creosote (Swedish Wood Protection Institute (STSI), 1993). The source of such contamination (which ends up in the soil, groundwater and sediments of nearby bodies of water) may be the creosote storage tanks, the impregnation wells and the place where waste resulting from the impregnation process is stored (Maier, 1992). The wastes from operating creosote impregnation plants (sludges from the impregnation tanks and wood with high PAH content) are incinerated in Sweden. Although this is an effective method (it destroys the creosote), it is very expensive and the emissions to the air and the remaining ashes must be handled (Lindell, 1994).

The environmental problems associated with the use of creosote for wood preservation can be traced as far back as the coal extraction from the mines, carbonizing the coal, recovering and distilling the tar, transporting it, and as far ahead as the creosote emissions from impregnated wood used in the field. These emissions are basically to air, soil, water and sediments.

Alternative wood impregnation substances have been used (*e.g.*, water soluble salts and metals). Alternative materials to impregnated wood for poles and sleepers are aluminium and concrete. However, their use or production imply other environmental problems. According to Erlandsson (1994), creosote impregnated wood poles present the least threat to the environment if their complete life cycle is compared with chromium-copper-arsenic (CCA) impregnated wood poles, aluminium and concrete poles.

Alternative wood preservation methods have been studied extensively. Less toxic alternatives for wood treatment, like boron compounds or alkyl ammonium compounds, are often not as effective as the toxic preservatives (creosote, PCP, CCA) (Vihavainen, 1994). A promising alternative method is impregnation with a kraft lignin-copper

complex (Ohlsson and Simonson, 1992). Its performance is superior than that of CCA, but its two-step application makes it less attractive for industrial scale applications. Other new approaches to improving the biological durability of wood include chemical modification of the cell wall components, for example by acetylation with acetic anhydride (Vihavainen, 1994). Although there is a patent for the acetylation process (Simonson *et al.*, 1985) and it might be the best option developed so far (with respect to toxicity and performance), its high cost permits its use only for exclusive (luxury) wood products.

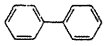
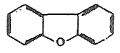
2.2.2 Chemical composition of creosote

By 1945 348 compounds resulting from the carbonization of coal had already been identified (O'Brochta and Woolridge, 1945). Creosote can be defined as the fraction of coal tar distilled between 175-450°C (Nestler, 1974) or between 200 and 350°C (Lorenz and Gjovik, 1972). Different types of creosotes have been classified according to the distillation range of compounds it contains. These contents may be varied according to where and for what purpose it will be used (poles, marine dock columns, etc.). This is achieved through variations in the distillation temperature, and depends on how much volatilization or bleeding from the wood is expected (Bergman, 1994). It could also depend on the composition of the coal and variations in the carbonization process. The physical specification of a creosote is given basically as the percentage of different distillation temperature fractions present (Seman and Svedberg, 1990). Modern creosotes tend to contain no low- (volatile and soluble) and high-boiling point compounds (Lindell, 1994).

Creosote is composed of a complex mixture of compounds including aliphatic hydrocarbons, aromatic hydrocarbons, sulfur compounds, benzoic acid, ammonium thiocyanate, O-heterocyclic compounds, phenols, N-heterocyclic compounds, amines and other benzene and naphthalene related miscellaneous compounds (Sundström *et al.*, 1986). According to Mueller *et al.* (1989) ~85% (w/w) of the chemical classes of creosote are homocyclic polynuclear aromatic hydrocarbons, ~3% heterocyclic aromatics and ~12% phenols. According to Lorenz and Gjovik (1972) 11 PAHs (including some nitrogen and oxygen heterocyclic analogues), methylated forms of naphthalene, fluorene, phenanthrene and anthracene, as well as some benzofluorenes, comprise 90% of creosote. Although most creosote components are toxic, only 16 of the PAHs contained in creosote are considered to be priority pollutants by the US EPA (US EPA, 1986). The major components of creosote and their relative abundance are listed in Table 2.2.1 together with the 16 US EPA priority PAHs, their IUPAC preferred names, some of their physico-chemical properties and some synonyms.

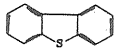
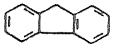
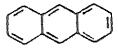
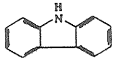
Approximately 3-4% of creosote is composed of soluble acid compounds (phenols and cresols) which may easily contaminate groundwater (Seman and Svedberg, 1990). Lower molecular weight PAHs are relatively water soluble, while those with three or more rings are bound and transported by fine particles and dissolved organic matter in the aquatic environment (Cerniglia and Heitkamp, 1989). Rostad *et al.* (1985) studied the presence of PAHs in an aquifer contaminated by coal tar wastes and found that even the PAHs considered very poorly soluble (*e.g.*, pyrene, benz[a]anthracene and indeno[1,2,3-cd]pyrene) were detected in the aqueous phase. Coal-tar and creosote are

Table 2.2.1. IUPAC preferred name, relative abundance, formula, structure, some physico-chemical properties and synonyms of creosote components[a]

PAH	relative abundance (%) in creosote[b]	formula	molecular weight	molecular structure	m.p. °C	b.p. °C	vapor pressure torr, 20°C[c]	aqueous solubility mg/l[d]	log Kp[e]	synonym(s)[f]
naphthalene[g,h]	3.0	$C_{10}H_8$	128.17		80.5	218	4.92×10^{-2}	31.7	3.37	
2-methylnaphthalene	1.2	$C_{11}H_{10}$	142.20		34.6	241	-	25.4	4.11[i]	
1-methylnaphthalene	0.8	$C_{11}H_{10}$	142.20		-22	244.6	-	-	-	
1,1-biphenyl	0.9	$C_{12}H_{10}$	154.21		71	255.9	-	7.0[i]	3.76[i]	biphenyl
dimethylnaphthalenes	2.0	$C_{12}H_{12}$	156.23	v	v	268[b]	v	v	v	
acenaphthylene[g,h]	traces[j]	$C_{12}H_8$	152.21		92	265	2.9×10^{-2}	3.93[k]	4.07	
acenaphthene[g,h]	9.0	$C_{12}H_{10}$	154.21		96.2	279	2.0×10^{-2}	3.93	4.33	naphtyleneethylene
dibenzofuran	5.0	$C_{12}H_8O$	168.19		86	287	-	10[i]	4.12[i]	

continued

Table 2.2.1. Continued

PAH	relative abundance (%) in creosote[b]	formula	molecular weight	molecular structure	m.p. °C	b.p. °C	vapor pressure torr, 20°C[c]	aqueous solubility mg/l[d]	log Kp[e]	synonym(s)[f]
dibenzothiophene[l]	traces[l]	$C_{12}H_8S$	184.24		97	332	-	-	4.38[i]	2,2'-biphenylene sulfide; dibenzo(b,d)thiophene; diphenylene sulfide; 9-thiafluorene
fluorene[g,h]	10.0	$C_{13}H_{10}$	166.22		116	293	1.3×10^{-2}	1.98	4.18	9H-fluorene[g]; 2,2'-methylenebiphenyl; 2,3-benzindene; diphenylenemethane; *o*-biphenylenemethane; diphenilene methane
methylfluorenes	3.0	$C_{14}H_{12}$	180.25	v	v	318[b]	v	v	v	
phenanthrene[g,h]	21.0	$C_{14}H_{10}$	178.23		101	340	6.8×10^{-4}	1.29	4.46	
anthracene[g,h]	2.0	$C_{14}H_{10}$	178.23		216	340	1.96×10^{-4}	0.073	4.45	anthracin; green oil; paranaphthene; tetra olive N2G
carbazole[g]	2.0	$C_{12}H_9N$	167.21		247	355	-	-	3.29[i]	9H-carbazole[g]
methylphenanthrenes	3.0	$C_{15}H_{12}$	192.26	v	v	354[b]	v	v	v	

continued

Table 2.2.2. PAHs analyzed by different standard methods in different matrices

institution:	US EPA[a]	SNV[b]	SNV[c]	SNV[d]	DIN[e]
matrix:	soil	municipal sludge	creosote	soil from Blekholmstorget	water
PAH					
naphthalene	x		x	x	
2-methylnapthalene			x	x	
1-methylnaphthalene			x	x	
acenaphthylene	x				
acenaphthene	x			x	
dibenzofuran			x	x	
fluorene	x		x	x	
phenanthrene	x		x	x	
anthracene	x		x	x	
fluoranthene	x	x	x	x	x
pyrene	x		x	x	
chrysene	x			x	
benz[a]anthracene	x				
benzo[b]fluoranthene	x	x			x
benzo[k]fluoranthene	x	x			x
benzo[a]pyrene	x	x			x
indeno[1,2,3-cd]pyrene	x	x			x
dibenz[a,h]anthracene	x				
benzo[ghi]perylene	x	x			x

[a] United States Environmental Protection Agency (US EPA, 1986)
[b, d] Swedish Environmental Protection Board (SNV, 1990, 1993c)
[c] SNV method, cited in Seman and Svedberg (1990)
[e] Deutsches Institut für Normung (DIN, 1993)

the fact that not all the compounds are equally hazardous. This may occur partly because the authorities only ask for total PAH concentration. For example, the maximum allowable total PAH concentration in Swedish soils is 200 mg/kg dry weight (Petsonk, 1992). The Swedish Institute of Environmental Medicine has suggested that the maximum PAH content in surface soils of residential areas should be 10 mg/kg dry weight (SNV, 1993c). The World Health Organization maximum allowable PAH concentration in drinking water is 0.2 μg/l (Seman and Svedberg, 1990). The US EPA considers 5,000 ppm hexane-soluble PAHs in sediments as an acceptable level for the protection of the aquatic fauna (Sundström *et al.*, 1986).

Descriptive studies of Swedish urban air quality have been made. The indicator factors considered are, for example, sulphur dioxide, nitrogen dioxide and soot (particles which may contain PAHs). However, no conclusions about trends of PAH levels in urban air can be stated with the data available (SNV, 1993d). Recommended values for good urban air quality are presented for several toxic compounds by Victorin (1991).

Maximum allowable concentrations (expressed as threshold limit values) of several toxic compounds in the air of the (Swedish) work environment have been stated (AFS, 1993). The creosote-PAHs considered in this publication are benz[a]pyrene and biphenyl. Andersson *et al.* (1983) measured the particulate and gaseous PAHs in several work environments where occupational exposure to PAHs occurs. Some of these environments are: coal processing plants, wood impregnation installations, iron and steel factories and plants where a certain process of electrolytic smelting of aluminium is carried out. Becher and Björseth (1985) developed a method for determining such exposure by analysis of body fluids (urine), identifying selected PAH metabolites.

Swedish PAH levels in foods, as well as annual intakes, have also been reported (SNV, 1993d).

Soil sampling and storage

Before taking samples from a contaminated site or contaminated material to select a remediation strategy one must know what the contaminants are, how they are distributed, in what amounts, how toxic they are, how they are associated with the soil, groundwater and particulate matter, etc. Soil is a very heterogeneous medium, owing to the variety of grain size, water content, amount of organic substances and human activities. The contaminants are also distributed in a very non-homogeneous way in soils (Liphard, 1992). Some works on sampling problems for chemical analysis of sludge, soils and plants are presented in Gómez *et al.* (1986). The lack of standards is still the biggest problem (Liphard, 1992).

Many PAH degradation experiments report very high variability between replicates in the laboratory, pilot scale or in field tests (personal experience; Davis *et al.*, 1993; SNV, 1993c). Lamé and Defize (1993) made a study on sampling error in relation to sample size and segregation of cyanide in soil. They determined that soil samples smaller than 10 g influence minimum variation, which is attributed to the difference in particle composition of each sample. In larger samples the segregation error (of the pollutant) determines the variance. Since there is no practically applicable method

available for estimating the segregation error before sampling and analysis, they developed a method to estimate the segregation error using a sampling board. This resulted in segregation plots which can be compared to the lot to be sampled. A practical problem is that some extraction devices do not have capacity for large amounts of soil.

Thomas *et al.* (1990) presented a paper on a statistical approach to screening hazardous waste sites (soil and sediments). They determined that different bioassays are suitable for different kinds of toxicants and discussed how the toxicity data can be used to map the spatial distribution patterns of pollutants in contour maps. This approach can be used as a tool for selecting contaminated site cleanup strategies.

"Fast, dark and cool" are necessary conditions for sample transportation and storage (Liphard, 1992) to avoid volatilization, photo-oxidation and other unwanted biasing effects which may alter the analytical results.

PAH extraction from soils

Sample drying is a common practice before extraction of organic contaminants from soil. However, Thibaud *et al.* (1993) showed that the water content of the soil affects the sorption and desorption of chlorobenzene and toluene from soil. Totally dry soil produced a stronger binding of the compounds to soil particles. No references have been found describing the effect of water content on the extraction of larger and more hydrophobic compounds from contaminated soils. The addition of hygroscopic substances to samples or extracts is commonplace in standard methods.

Extraction may be the most important step in this kind of study. The most common extraction techniques mentioned in the standard methods are Soxhlet (US EPA, 1986) and flask shaking (SNV, 1990). Ultrasound bath for dispersing solids during extraction is mentioned in many publications (*e.g.*, Lambert *et al.*, 1992). Supercritical fluid extraction (SFE), Soxtec and microwave extraction methods have also been used. Some extraction methods are discussed below.

Soxhlet extraction requires heating of the solvent which could cause losses of semivolatile compounds. A balance must be achieved between the longer time needed for a complete extraction and the shorter time necessary to avoid losses owing to decomposition in the boiling solvent (Brorström-Lundén, 1992). To check for the recovery rate and the effect of temperature on the integrity of the compounds a solution with known concentrations of selected PAHs (surrogate standard) can be refluxed in the extraction system for some hours and then compared with the original solution. Brorström-Lundén (1992) also mentions that overloading the extractor with sample gives poor recovery. Ten g is the recommended soil sample size for Soxhlet extraction (US EPA, 1986).

Adding pure compounds in solution to the soil sample to check recovery efficiency is also a common practice, but this is not necessarily a good indicator of the recovery efficiency because the compounds are not bound to the sample in the same way as the original contaminant compounds. Weissenfels *et al.* (1992) mention that PAHs bind

more strongly to soil particles over time. Thus spiked soil is not comparable with soil which has been contaminated for 50 or more years.

SFE is a very efficient and novel extraction method which has wide applications, *e.g.*, in the oil extraction industry and in analytical chemistry (although it is not yet used for standard analytical methods). A comprehensive review of SFE and supercritical fluid chromatography (SFC), where principles and applications are explained, is that of Chester *et al.* (1992). Hawthorne (1990) presents a more specific review of analytical-scale SFE, and Raynor *et al.* (1988) a review of the coupling of SFE, SFC and Fourier Transform Infrared Microspectrometry of polyaromatic compounds (PACs) in a coal tar pitch. Furthermore, Richards and Campbell (1991) compared the extraction efficiency of SFE, soxhlet and sonication methods for priority pollutants in soil. SFE was generally faster and more convenient, with the advantage that less dichloromethane was used than in the conventional methods.

A comparison of percentage recovery of some aromatic compounds (ACs) from soil using Soxhlet, sonication and SFE extraction methods is presented in Table 2.2.3.

Soxtec is an extraction method with the same operating principle as Soxhlet extraction, but with some differences, such as that part of the solvent is recovered after the extraction (collected after it condenses, in the upper part of the apparatus), leaving a low volume of extract and reducing the total amount of solvent used by 65%. Also, the extraction time is shorter that Soxhlet (1 hour instead of 4 or more). This method was developed by Tecator, Inc. (Herndon, Virginia) and has been adopted by the US EPA as a standard method for PAH extraction from soils (method 3541) (in the 1991 updated version of US EPA, 1986).

Microwave extraction began to be developed in 1986 (Ganzler *et al.*, 1986). CEM corporation (Matthews, North Carolina) has developed the system further and presently markets it. This method seems to be the most promising extraction technique since the extraction time is reduced to a few minutes, and only a few milliliters of solvent are needed. It is suitable for fast extractions of large sample series (Ganzler *et al.*, 1986).

Some advantages and disadvantages of different extraction methods are summarized in Table 2.2.4.

The most common solvent used for PAH extraction from soil is dichloromethane, although benzene, hexane, acetone and methanol may be also used. The US EPA standard method (US EPA, 1986) recommends a 1:1 mixture of hexane:acetone. Mixtures usually include two solvents with different dipole moments and dielectric constants. The extract may be concentrated if the extraction solvent is not suitable for chromatography and is to be replaced with a more suitable one, or if the analytes are too diluted.

Environmental samples usually contain complex mixtures of compounds. Therefore, sometimes it is necessary to separate the mixtures into different fractions. For example, aliphatic hydrocarbons and different classes of PAHs may be separated from synthetic fuel products as indicated in Figure 2.2.1. PAHs, O- and S- heterocyclics are found in

Table 2.2.3. Percentage recovery of base-neutral and acid compounds from soil, using Soxhlet, sonication and supercritical fluid extraction[a]

	Recovery (%)[b]		
Compounds	Soxhlet	sonication	SFE
phenol	69.0	60.0	70.4
2-chlorophenol	73.2	63.8	74.9
naphthalene	57.8	53.0	74.2
2,4,-dichlorophenol	81.2	73.2	76.4
2,4,6-trichlorophenol	68.8	69.0	83.1
pentachlorophenol[c]	-	-	84.3
d_6-phenol[dc]	72.4	60.6	80.4
d_5-nitrobenzene[d]	70.4	53.3	85.3
2-fluorobiphenyl[d]	74.6	60.4	88.0

[a] modified from Richards and Campbell (1991)
[b] average of 5 (Soxhlet) and 9 (sonication and SFE) replicate determinations; Spiking level was 25 μg/g; Sample size was 2 g of soil
[c] analyzed as the methylated derivatives
[d] surrogates

the neutral fraction (Sundström *et al.*, 1986). Mueller *et al.* (1991) present other step separation methods of components from different fractions of creosote.

PAH analysis

Although it is a relatively old publication, Lee *et al.* (1981) mention different methods that have been used for PAC analysis. The analytical methods used are basically:

1) Column and thin layer chromatography (liquid phase);

2) HPLC with fluorescence and UV detectors;

3) GC, with electron capture detector and flame ionization detector (ECD and FID);

4) Mass spectrometry (MS) (which may be coupled to a HPLC or GC);

5) Ultraviolet absorption and luminescence spectroscopy (for individual compounds or for appreciation of total concentration of a mixture);

Table 2.2.4. Some advantages, disadvantages and limitations of different extraction methods[a]

Parameter	Extraction method					
	Soxhlet	sonication	SFE	separatory funnel	mixing/ shaking/ tumbling	microwave closed vessel
standard method	yes	some	no	yes	yes	new technique
sample throughput[b]	-	-	low	-	-	high
extraction time (hours or minutes)	4-24 h	30-60 m	10-60 m	-	long	15-30m
solvent volume (ml)	100-500	300	-	large	large	25-50

[a] CEM Corporation/Nord Lab Plus (1994)
[b] sample throughput refers to how many extraction units may be operated simultaneously

6) Nuclear magnetic resonance (especially useful to identify individual compounds or its metabolites);

7) Infrared spectroscopy (for individual compounds).

Lee *et al.* (1981) explain which methods are most adequate for different situations and different compounds. Most methods reported in the literature (including the standard methods) use GC or HPLC. Both GC and HPLC have disadvantages which could be overcome by using both methods as a complement to each other (Brorström-Lundén, 1992). Using GC-MS permits the analyst to detect compounds present in trace amounts, with the possibility of identifying them.

Novel analytical methods

New methods are constantly being developed and published. For example, an improved method (*e.g.*, large sample throughput, faster by direct coupling between extraction and analysis and more efficient analysis) for PAH extraction from soil coupled to HPLC analysis has recently been published (Dong, 1993). Supercritical fluid chromatography is also a recently developed method (*e.g.*, Chester *et al.*, 1992) which has not yet been standardized. Perhaps the newest trend which might reduce the cost of analytical procedures is the use of field analytical methods. One example is the field mass spectrometry (McDonald *et al.*, 1994). An emerging technology which could permit making fast on-site (qualitative and quantitative) determinations is Environmental Immunochemistry (see Van Emon and Mumma, 1990 and Vanderlaan *et al.*, 1991). Such methods have been developed to detect even trace concentrations of

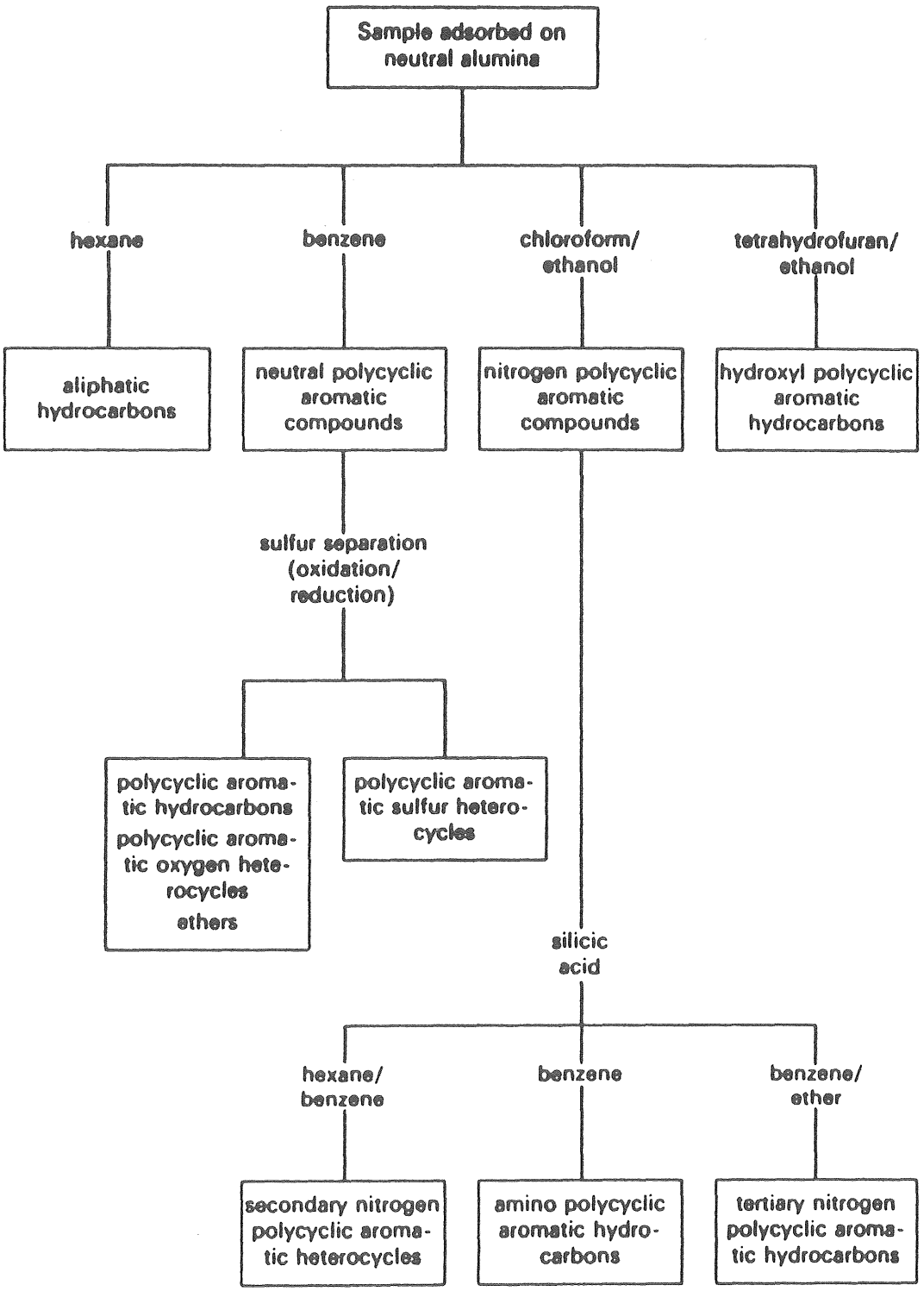


Figure 2.2.1. Chemical class separation for synthetic fuel products (Sundström *et al.*, 1986)

benzo[a]pyrene and atrazine in environmental samples, and for analysis of body fluids to assess exposure to chemicals.

2.3 Toxicological considerations

Ecotoxicology is the study of the effects of toxic substances on communities of populations in ecosystems, while toxicology is the study of the effects of toxicants on individual organisms. However, it is unlikely that toxicity assays can predict the ecological effects of pollutants (Moriarty, 1991). Animal models may also be unsuitable for predicting, for example, the carcinogenic dose of a compound in humans (Ames, 1991).

The toxic effects may be acute (*e.g.*, death), sublethal or chronic. Among the sublethal and chronic effects are genetic disorders which impair, for example, the reproductive cycle of a species. These and other organic malfunctions may or may not result in death. Some genetic (DNA) related effects of toxicants are: clastogenicity (chromosome breakage), mutagenicity (mutation of genes), genotoxicity (general DNA damage), teratogenicity (production of physical defects in offspring *in utero*) and carcinogenicity (production of malignant neoplastic tumors in the exposed tissue).

The toxic effects discussed in detail in this thesis are acute toxicity and mutagenicity/genotoxicity, especially in toxicity assays using microorganisms. Acute toxicity refers to the instant or short term effect of the toxicants to the test organisms, either inhibiting or blocking important vital metabolic activities, and resulting in death. Mutagenicity and genotoxicity are longer term effects in the genetic code, affecting the synthesis of specific proteins or impairing the reproductive capacity of the test organism. These effects are easily seen in microorganisms like bacteria since they transport and assimilate the toxicants faster than eukaryotic cells and show the effects faster, due to their short reproductive time.

Toxicity tests using different types of organisms, often representing different trophic levels (producers, primary consumers, predators and decomposers), have been developed to assess the potential hazard (mainly to humans) of many substances, mixtures or contaminated materials. Despite their limitations with respect to reflecting the human case reality, these toxicity tests provide a tool for comparing the effects of substances in the selected species. In general, the toxic effects are manifested after a certain kind of exposure to a certain dose (*e.g.*, by ingestion, inhalation or skin contact), in a certain medium or environment (*e.g.*, in aquatic environments, in the air, or with contaminated solid materials) and with a certain time of exposure.

Some points to be considered in order to avoid making erroneous generalizations based on results from toxicity tests are:

- Organisms living in different environments are susceptible in many ways to numerous compounds. It is possible to determine which organisms are the most susceptible to a given toxicant.

- Toxicology tests use very high doses, probably higher than those the organisms would be in contact with in reality.

- In laboratory toxicity tests using the maximum tolerated dose, half of all chemicals (natural and synthetic) are carcinogens, one-third are teratogens and about half are clastogens. Human beings are more exposed to natural toxic compounds than to man made compounds, so the risk of exposure to synthetic compounds is lower than assumed (Ames, 1991).

2.3.1 Toxicity assays

Methods have been developed in different countries to judge whether or not a chemical substance is hazardous. One is the ESTHER method (Landner, 1989, in Landner, 1990). This method collects exposure and effect-related information on the substance in question, assigns points to each parameter according to the parameter limits, and then integrates the information. These are presented in Table 2.3.1.

A Nordic working group selected 3 parameters from the ESTHER method and constructed a simplified method to judge the potential hazard of a chemical (Lundgren, 1989 in Landner, 1990). These parameters are: biodegradability (or recalcitrance, determined in terms of the biological and chemical oxygen demand - BOD/COD -); bioaccumulation (determined in terms of the octanol-water partition coefficient, K_{ow}); and toxicity to aquatic and terrestrial organisms (determined in terms of the concentration which kills half of the experimental population, usually in mg/l (LC50) for aquatic tests, and the dose in mg/kg body weight (LD50) for terrestrial organisms. LD50 may be determined orally and LC50 by inhalation).

Acute toxicity tests

Organisms from diverse aquatic and terrestrial taxonomic groups have been used to determine the toxic concentration and toxic dose of pure chemical compounds or environmental samples. These include: bacteria, yeasts, protists, algae, vascular plants, coelenterates, rotifers, earthworms, mollusks, crustaceans, insects, sea urchins, fish, frogs and mammals (*e.g.*, rats and mice) (Kaiser and Palabrica, 1991). Each test is conducted under standard conditions (*e.g.*, temperature, pH, salinity). The battery-of-tests approach is used in some instances, and by combining acute toxicity tests with mutagenicity assays, the scope of the approach is widened (Kwan *et al.*, 1990). In the battery-of-tests approach organisms representing different trophic levels (*i.e.*, producers, primary consumers, predators and decomposers) have been used (Hund *et al.*, 1992).

It takes a great deal of time and energy to work with "higher" organisms, owing to their relatively long and complex life cycles and nutritional needs. Perhaps the biggest obstacle is having a stock of organisms ready to use in any moment. For these reasons, toxicity kits for contaminated waters and sediments have been developed with bacteria, rotifers, crustaceans and others. Some examples are presented below.

Table 2.3.1. Parameters and limits used in the ESTHER model to judge the environmental hazard potential of a chemical substance (modified from Landner, 1990)

Information	Units	Abbreviation	Parameter values and limits[a]
Exposure-related information			
use	ton/year	A	< 10/ -1000/ > 1000
emission	%	U	< 5/ -20/ -80/ > 80
primary recipient	%	I	
air		I_A	
soil		I_S	< 50/ > 50
water		I_W	
treatment plant		I_{Sew}	
expected distribution in the environment	%	C	
soil		C_S	
water		C_W	< 10/ -50/ > 50
soil + water		C_{SW}	
transformability, degradability	%	P	> 70/ -50/ -20/ < 20
bioaccumulation	log K_{ow}	B	< 1/ -3/ -5/ > 5
bioavailability	MW	T	> 1000/ < 1000
Effect-related information			
acute aquatic toxicity	mg/l	AAT	> 1000/ -100/ -10/ -1/ -0.1/ < 0.1
chronic aquatic toxicity	mg/l	CAT	> 10/ -1/ -0.1/ -0.01/ -0-001/ < 0.001
effect on terrestrial plants		TPT	not yet established
effect on soil organisms		SOT	not yet established
acute toxicity in mammals	mg/kg body weight	AMT	> 2000/ -250/ -25/ < 250
chronic toxicity in mammals	mg/kg/day	SMT	> 200/ -25/ -2.5/ < 2.5
mutagenicity	(screening)	M	2 neg/ unclear/ 2 pos
inhibition of activated sludge (microorganisms)	mg/l	SST	> 1000/ -100/ -10/ -1/ < 1

[a] to integrate the information and obtain the exposure and effect indices, the values/limits of parameters are assigned points. These are used in equations for each index which relate to exposure or effects on the aquatic ecosystem, the terrestrial ecosystem, top consumers and biological treatment system. Penalty points may be added when data is entered.

Microtox

The Microtox test is a simple and fast acute toxicity test routinely used at our laboratory (Department of Sanitary Engineering), and it is accepted as an alternative to conventional toxicity tests (Carlson and Morrison, 1994). This test correlates very well with other toxicity tests involving "higher" organisms like the rat, fishes (like *Alburnus alburnus)* and crustaceans (like *Nitocra spinipes*, a brackish water crustacean) (Kaiser and Palabrica, 1991; Sundström *et al.*, 1986; Tarkpea *et al.*, 1986). Symons and Sims (1988) found that Microtox correlated well to trout bioassays and that it was more sensitive than sludge microorganism inhibition.

In the Microtox test, the toxicant(s) is (are) put into contact with the light-emitting marine bacterium, *Photobacterium phosphoreum* (synonym = *Vibrio fisheri*). The effect is measured as effective concentration, the concentration of the substance which causes a 50% reduction in the light emission by the bacteria (EC50), measured using a special photometer. It is usually determined at 5, 15 and/or 30 minutes.

Kaiser and Palabrica (1991) compiled reported EC50 values for more than 2000 pure compounds tested with Microtox. Papers containing information on toxicity assays for PAHs and/or creosote are Eisman *et al.* (1991), Reteuna *et al.* (1989), Sundström *et al.* (1986), Tarkpea *et al.* (1986) and Sims and Overcash (1983).

The Microtox test can be used as a monitoring tool to detect changes in toxicity of contaminated materials after bioremediation. This and other toxicity assays complement (but perhaps do not totally replace) quantitative chemical analyses. Symons and Sims (1988) detected a reduction in toxicity with this assay after biological degradation of toxic organic contaminants in soil.

Microtox solid phase toxicity assay (contact toxicity)

The toxicity of contaminated water, effluents or other liquid samples (including soil extracts) containing dissolved (and perhaps suspended) toxicants may be tested directly and more easily than toxicants associated with solid phase materials, such as sludge and soil. The Microtox solid phase assay was designed for contact toxicity tests for solid samples. However, some problems and limitations of the solid phase method suggested by the manufacturers of Microtox were found by Carlson and Morrison (1994) when determining the toxicity of heavy metals in sludge. Some solutions for these problems and limitations are presented below.

The osmotic surrogate: The Microtox osmotic adjustment solution (2% w/v NaCl) may give misleading toxicity values of solid samples because the Cl^- ions extract the metals from the solid sample and form chloro-complexes in the liquid phase. Therefore, the metal concentration in the solution may increase after extraction from the solid matrix and the chloro-complexes formed after extraction have different (lower or higher) toxicities than the hydrated ions. Most of the cadmium, manganese, nickel and zinc is found as hydrated free ions in natural soil solutions, and copper is found mostly as organic complexes. Sodium perchlorate (8.1% w/v $NaClO_4$) is a good substitute for NaCl, especially if lead is present. This compound is non-toxic, dissolves readily in

water, is inert in water. Additionally, it does not form complexes with environmentally important metals. However, it may not be used together with phosphate buffers containing potassium because $KClO_4$ is formed. Sodium sulphate may also be used as an osmotic surrogate and it has the advantage that it gives a more stable blank value. If lead is present it precipitates as a sulphate salt, but this surrogate may be used if no lead is present in the sample. The most interesting aspect is that parallel toxicity tests of solid samples in sodium sulphate and sodium perchlorate can be used to differentiate toxicity of lead from that of other metals.

Na^+ is necessary to maintain a stable light emission of the blank during 30 min, which is the time necessary for solid phase contact toxicity tests. Therefore, there should be enough sodium in the solution. The physiological process involved in this phenomenon is explained by Carlson and Morrison (1994). Sucrose is a non-extracting osmotic surrogate that has been used, but it acts as a nutrient, stimulating growth and altering the light emission pattern of the bacteria. It also alters the metal active transport across the bacterial membranes.

Increasing ionic strength decreases the sensitivity of the Microtox bacterium, at least for metal toxicants. The toxicity of non-metallic toxicants does not seem to be affected by ionic strength.

Apparent toxicity: Light emission inhibition may result from extreme pH, beyond the range of 6-8. The pH also affects the sludge/water partitioning, and the osmotic adjuster of the Microtox test does not contain a buffer. Additionally, the pH may not be controlled in the Microtox cuvettes. 1mM phosphate buffer at pH 7 is recommended for metal toxicity assays for solid samples. The buffer must not interact with the osmotic surrogate (which is the case of potassium-containing buffers and sodium perchlorate).

Bacterial sorption to surfaces: Bacteria may be sorbed to fine sludge or soil particles and not go through the solid phase separation sieve, resulting in a false light emission reduction. This has yet to be studied and improved. Sorption of the bacteria to other Microtox equipment surfaces or to suspended particles, and how they affect the light emission should be investigated.

These findings could be applied to determine the acute toxicity of soils from mixed hazardous waste sites. Soils from coal tar contaminated sites may also contain metals like lead, mercury and cadmium, and the soil treated in the experimental part of this thesis contained considerable amounts of lead and some copper.

Toxicity assays and hydrophobic toxicants

PAHs and many organic toxicants are hydrophobic, so their contact and uptake by the test organisms in aqueous media would be expected to be quite low. Dissolving the toxicants in an organic solvent may help to solve this problem. Tarkpea *et al.* (1986) used acetone, dimethyl sulfoxide (DMSO) and propylen glycol as solvents to test fairly water-insoluble compounds ($<$ 1000 mg/l) with the Microtox [aqueous phase] test. The most convenient solvent was acetone, with a concentration (in the Microtox test tubes) of 500 μg/ml. All these solvents had high EC50 values, and for acetone, the 5 min

EC50 = 16,000 mg/l. Other publications presenting methodologies to solubilize hydrophobic compounds for toxicity assays are Reteuna *et al.* (1989) and Eisman *et al.* (1991). One disadvantage of using a solvent (for the Microtox assay) is that the substance may precipitate in the aqueous phase and disturb light measurements (Tarkpea *et al.*, 1986).

Both aqueous and organic solvent extractions of environmental samples have been used by several researchers (*e.g.*, Kwan and Dutka, 1990). Donnely *et al.* (1991) suggested that aqueous extracts are adequate to test contaminated site leachate toxicities and solvent extracts to evaluate the relative total hazard of the sample. However, Kwan and Dutka (1993) say that the concentration of toxicants in these extracts is usually low, yielding low if any toxicity values. Other papers in which contaminants are extracted for toxicity assays in liquid medium are Kwan *et al.* (1990), Dutka *et al.* (1991) and Ho and Quinn (1993).

The extraction procedure may alter the original characteristics of the contaminated material. Extraction is not required in toxicity assays like the Toxi-Chromotest Kit. This test determines the toxicity of the readily available toxicants in solid samples like sediments, suspended solids, soils and other solid wastes directly. The time-work of sample extraction is also saved (Kwan, 1993). This test can be used even in the field and can provide quantitative or semiquantitative results.

Calleja and Persoone (1993) mention that the effects of solvents used to test toxicity of lipophilic substances are usually assessed separately from the toxicant. This is called the maximum allowable concentration of the solvent in the assay (Kwan and Dutka, 1990). However, synergistic or antagonistic effects between toxicants and solvents may affect the results of a toxicity bioassay (Kwan and Dutka, 1993). Solvents may also affect the molecular structure of the toxicant (Ariëns, 1971 cited by Calleja and Persoone, 1993), affecting the transport mechanisms through the cellular membrane into the cell. The solvents themselves may also inhibit enzymes or alter metabolic processes. Despite this investigation, the synergistic and antagonistic mechanisms are not yet well understood. Therefore, the conclusions drawn from solvent based bioassays should be carefully considered, especially when comparing data from various researchers. Since the solvent of choice may vary with the compounds to be tested, and since it may have different effects on different aquatic test species, the selected solvent should have the least influence on the toxicity of the hydrophobic chemical in that particular assay. Methods like ultrasound should be used, if possible, to increase the solubility of lipophilic compound as well as their availability for the test species (Calleja and Persoone, 1993).

Since contaminated materials usually contain mixed toxicants (*e.g.*, metals and organics), they could be tested separately through selective extraction of the unwanted toxicant. Wei and Morrison (1994) present a methodology in which volatile compounds were removed by air, ethylenediaminetetraacetic acid (EDTA) was used to remove metals, and different organic solvents to remove organics with different properties. However, if acid digestion was used to remove organics, the availability of metals increased since metals could be bound to organic substances.

Mutagenicity/genotoxicity assays

90% of mutagenic (DNA damaging) compounds have proven to be carcinogenic (Sims, 1990). Therefore, mutagenicity assays should be used (together with acute toxicity assays) before, during and after bioremediation of PAH-contaminated soil or other materials where suspected carcinogenic pollutants are involved. Some examples of mutagenicity assays are: the Ames test (bacterial cultures), Mutatox (photometric), and the Toxi-Chromotest (colorimetric).

Promutagens are compounds which are not mutagenic until after they have been modified by some metabolic process. In mutagenicity assays these compounds are activated by the addition of the S9 supernatant fraction of rat liver cell mitochondria.

Mutatox is a test based on a "dark" mutant strain of *Photobacterium phosphoreum*, which emits light if mutagenic compounds restore its "lights on" inverted gene. It is sensitive to chemicals which are DNA damaging agents, DNA intercalating agents, DNA synthesis inhibitors, and direct mutagens (Kwan *et al.*, 1990). Details of this test are presented by Kwan *et al.* (1990) and Kwan and Dutka (1990). Johnson (1992) mentions that the Mutatox test compares favorably in sensitivity with the Ames test, and it is also easier and more rapid (no need to work with aseptic bacterial cultures), and therefore less expensive than the Ames test. The sensitivity of the Mutatox assay, its specificity and predictability makes it a valuable screening tool to monitor complex environmental samples for genotoxins. Mutatox adds valuable information when used in the "battery of tests" approach (Kwan *et al.*, 1990).

When working with complex environmental samples, the Mutatox test may be used to determine which fractions of solvent extracted contaminated sediments (separated by chromatography) contain mutagenic compounds. The identification of compounds in each fraction was done with GC-MS (Ho *et al.*, 1993).

Bos *et al.* (1985, 1987) detected mutagenic activity in vapors arising from creosote at 37°C, despite its high distillation temperature. They found that fluoranthene (which boils at 375°C) was the main cause of this mutagenicity. This was determined by the Ames test (taped plate assay) with S9 activation. This test may be used for the routine screening of volatile mutagens from industrial fluids, solid products, etc.

Factors affecting toxicity assays results

It is known that PAHs may be bound to particles in the air and in aquatic environments (Lee *et al.*, 1981; Cerniglia and Heitkamp, 1989). Weissenfels *et al.* (1992) mention that PAHs bind more strongly to soil particles with time. The same has been observed in sediments (Landrum, 1989; Landrum *et al.*, 1992) and in water containing dissolved humic material or small organic particles (Landrum, 1983; McCarthy and Jiménez, 1985). This reduces the bioavailability of PAHs, and there will be a difference between the bioavailability (and consequent toxicity and biodegradability) of environmental samples and laboratory spiked samples. Standardized ageing of spiked soils or sediments is suggested by Landrum *et al.* (1992) in order to be able to make interlaboratory comparisons.

2.3.2 Toxic properties of PAHs and creosote

Some toxic properties of PAHs (determined through toxicity tests) are reviewed by Sims and Overcash (1983). Sundström *et al.* (1986) present data for some of these PAHs and for several types of creosote. The latter authors mention that the toxicity of creosote

> ... seems to be the result of synergistic effects or the effect of a few specific components of very high toxicity.

For example, phenanthrene showed similar sublethal toxic effects in *Daphnia pulex* compared to those of creosote (Sundström *et al.*, 1986). Some chronic and acute toxic effects of the 16 priority PAHs and other creosote PAHs to several types of organisms are presented in Table 2.3.2. Toxic doses of creosote in different toxicity tests are given in Table 2.3.3.

Some low molecular weight PAHs are acutely toxic, while most PAHs with higher molecular weight are genotoxic (Heitkamp *et al.*, 1988). Lee *et al.* (1981) mention that some major components of creosote (*i.e.*, carbazole and dibenzofuran) had not been proven to be carcinogenic (13 years ago). No reports about cancer induction relating to these compounds have been made to date. There is also inconclusive evidence that biphenyl could induce tumor formation (data from Sigma-Aldrich Corporation material safety data sheet).

Toxicity assessment and bioremediation

Athey *et al.* (1989) and Thomas *et al.* (1990) state that chemical analyses alone are insufficient to guide cleanup decisions, as well as inaccurate predictors of toxicity. Assays for acute toxicity and mutagenicity, combined with chemical analyses provide adequate data for making satisfactory risk assessments of contaminated sites. This approach is recommended to assess the initial and final (after bioremediation) toxicity of compounds in contaminated soils (Donnelly *et al.*, 1991). However, Thomas *et al.* (1990) mention that chemical analysis (infrared measurements) did not predict toxicity levels for different standard toxicity assays. They also found that different toxicity assays showed different sensitivities to creosote.

Several papers have been published on the use of toxicity assays to evaluate the effect of bioremediation on the toxic properties of different contaminants in soil. In some cases they are used as a complement to chemical analyses. Some examples are described below.

Several standard phytotoxicity assays are used in toxicity assessment. Baud-Grasset *et al.* (1993a) determined the overall toxicity before and after bioremediation of creosote-contaminated soil with WRF. They used seed germination and root elongation tests with higher plants and found that reduction in toxicity correlated well with disappearance of pollutants, as determined by chemical analysis. The first assay was done directly on the soil and the second with soil eluates. Seed germination in soil proved to be a more sensitive test, suggesting that aqueous extractions did not remove all of the

Table 2.3.2. Some toxic effects of the 16 priority PAH and other creosote components[a]

PAH	Acute toxicity		Cancer-related effects		Other reported effects on terrestrial and/or aquatic organisms (LC50 in mg/l[e])
	LD50 mg/kg[b]	Microtox EC50 mg/l[c]	carcinogenicity in animals mg/kg[d]	mutagenicity Ames test	
naphthalene					
	1780	0.929	-	-	48h-LC50 *Daphnia magna* (crustacean), 20°C static (s)= 24.1; 96h-LC50 *Pimephales promelas* (fish), 25°C flow through (ft)= 6.08
acenaphthylene					
	?	0.283	0[f]	?	
acenaphthene					
	?	?	0[f]	?	96h-LC50 *Cyprinodon variegatus* (fish), 25°C s= 2.2
fluorene					
	?	?	0[f]	?	
phenanthrene					
	700	0.073	-	-	inhibits growth of *Escherichia coli*; 48h-LC50 *D. magna*, 20°C s= 1.0
anthracene					
	430[g]	?	3300	-	inhibits growth of *E. coli*; 48h-LC50 *D. magna*, 20°C s= saturated solution was non toxic
fluoranthene					
	2000	?	0[f]	+	stimulates growth of *Calluna vulgaris*, *Scenedesmus obliquus* and *A. oranunii* at 0.01-0.02 ppm (vol); 96h-LC50 *C. variegatus* 25°C s> 560

continued

Table 2.3.2. Continued

PAH	Acute toxicity: LD50 mg/kg[b]	Acute toxicity: Microtox EC50 mg/l[c]	Cancer-related effects: carcinogenicity in animals mg/kg[d]	Cancer-related effects: mutagenicity Ames test	Other reported effects on terrestrial and/or aquatic organisms (LC50 in mg/l[c])
pyrene					
	514-678	?	-	+[g]	lethal in 9-13 h to chinook salmon (1 mg/l)
chrysene					
	320[g]	?	99	+	
benz[a]anthracene					
	?	?	2	+	stimulates growth of *E. coli*
benz[e]acephenanthrylene					
	?	?	40	?	
benzo[k]fluoranthene					
	?	?	72	?	
benzo[a]pyrene					
	50	?	0.002	+	tumor induction in newt and clawed toad (*Xenopus laevis*), stimulates growth of *C. vulgaris*, *S. obliquus* and *A. oranunii* at 0.01-0.02 ppm (vol)
indeno[1,2,3-cd]pyrene					
	?	?	72	+[g]	stimulates growth of *C. vulgaris*, *S. obliquus* and *A. oranunii* at 0.01-0.02 ppm (vol)
dibenz[a,h]anthracene					
	?	?	0.006	+	stimulates growth of *E. coli*, inhibits respiration of sludge microorganisms
benzo[ghi]perylene					
	?	?	+[f]	?	

continued

Table 2.3.2. Continued

PAH	Acute toxicity: LD50 mg/kg[b]	Acute toxicity: Microtox EC50 mg/l[c]	Cancer-related effects: carcinogenicity in animals mg/kg[d]	Cancer-related effects: mutagenicity Ames test	Other reported effects on terrestrial and/or aquatic organisms (LC50 in mg/l[e])
Other creosote components					
1-methylnaphthalene					
	?	?	?	?	96h-LC50 *Nitocra spinipes* (crustacean), 21°C s= 13
dibenzofuran					
	?	?	?	?	96h-LC50 *C.variegatus*, 25°C s= 1.8
dibenzothiophene					
	470[h]	?	?	?	48h-LC50 *D. magna*, 19°C s= 0.466

[a] Modified from Sims and Overcash (1983)
[b] mg/kg body weight, orally administered to mice or rats
[c] At 30 minutes (Kaiser and Palabrica, 1991)
[d] It is not indicated whether this is the minimum dose at which cancer is induced
[e] Sundström *et al.* (1986)
[f] Lee *et al.* (1981); This classification system is based on the reported percentage of animals that developed tumors: 0 = none, noncarcinogenic, + = up to 33%, weakly carcinogenic, ++ = above 33%, strongly carcinogenic; The mode of administration is not indicated.
[g] From Cerniglia and Heitkamp (1989); LD50 determined in mice; Some values from this paper differ somewhat from corresponding values presented in the rest of references used in this table
[h] From Sigma-Aldrich Corporation material safety data sheet obtained with purchased chemicals
- = negative, + = positive, ? = unknown data for this compound or data not found

contaminants. Similarly, Baud-Grasset *et al.* (1993b) determined that the mutagenicity of PAH contaminated soils extracts decreased after bioremediation with WRF. They used the *Tradescantia*-micronucleus test. In a 4-day root-elongation test, garlic plants were found to be more sensitive to herbicides like phenoxyacetic acids than to creosote (Sundström *et al.*, 1986).

In contrast to the above, Belkin *et al.* (1993) studied the genotoxicity, with the Mutatox assay, after PAH contaminated soil bioremediation with natural bacterial population in soil columns. Despite the decrease of the original contaminants, an increase in genotoxicity was detected. If surfactants were added to the percolating fluids, the genotoxicity practically disappeared. This assay did not always correlate with the Microtox test since, in some cases, changes in genotoxicity were parallel with acute toxicity, and in other cases the opposite occurred. They attribute the increased

Table 2.3.3. Some toxic effects of creosote to aquatic organisms (modified from Sundström *et al.*, 1986)

Organism	Conditions, temperature (°C) and solvent	Effect/dose (mg/l)
Estuarine organisms		**96h-LC50**
Crassostrea virginica (eastern oyster)	flow through (ft), 21.4, acetone (a)	0.71
Mysidiopsis bahia	static (s), 25.5, triethylene glycol (teg)	0.018
Panaeus duorarum (pink shrimp)	ft, 24.2, a	0.24
Cyprinodon variegatus (sheepshead minnow)	s, 25, teg ft, 24, a	0.72 3.5
Crustaceans		
Homarus americanus	larvae, 20 adult, 10	0.02 1.76
Crangon septemspinosa	10 20	0.13 0.11
Fish		
Lepomis machrochirus		0.99
Salmo gairdneri		0.88
Carassius auratus[a]		2.62
Comparison of three tests[b]	a (500 mg/l)	**96h-LC50**
Alburnus alburnus (bleak)		10.52
Nitocra spinipes		0.89
		15min-EC50
Microtox		0.43

[a] Marine creosote
[b] Tested with the same kind of creosote in the three tests

genotoxicity to incomplete degradation. Since the acute toxicity assay did not predict the actual hazard of the incompletely degraded products, the determination of genotoxicity is a necessary complementary routine. However, Wang *et al.* (1990) found that after bioremediation of diesel oil PAHs by stimulating soil resident microorganisms, the mutagenicity (Ames test) and acute toxicity (Microtox) decreased, corresponding with chromatographic analysis of solvent extracts of the soil.

Metabolism of activated sludge microorganisms has also been used as a toxicological test. Carberry and Kovach (1992) determined the decrease in toxicity of pentachlorophenol and toluene to growth of selected microbial consortia and activated sludge by pretreatment with the WRF *Phanerochaete chrysosporium*.

It seems that more research is needed to evaluate alternative toxicity and mutagenicity assays after the different bioremediation methods to corroborate the data presented above.

2.3.3 Biological effects of PAHs

Creosote is highly toxic against many aquatic organisms, with 95h-LC50 values often below 1 mg/l. However, the oral toxicity levels of creosote (in rats, dogs, cats, rabbits and even birds) are much higher than those in aquatic organisms (Sundström et al., 1986).

As mentioned above, PAHs are bound to different kinds of particles in the air, aquatic environments and in soils, and the binding force increases over time. This phenomenon may make chemical extraction more difficult and reduce bioavailability for biodegradation and toxicity assays, and it also reduces bioavailability to the biota. More hydrophobic PAHs have higher affinities for dissolved humic material in aquatic environments. This decreases their bioavailability, the biological impact of bioaccumulation (in fish and in *Daphnia magna*) and transfer to humans via food chains (McCarthy and Jiménez, 1985; McCarthy *et al.*, 1985). A good review on the bioavailability of PAHs in the aquatic environment is that of McElroy *et al.* (1989).

Bioaccumulation of PAHs is influenced by the feeding strategies of the organisms. For example, sediment filtrators and suspension filtrators exhibit differences in the uptake and depuration of PAHs. Although PAH bioaccumulation has been observed in aquatic organisms, no biomagnification has been found (McElroy *et al.*, 1989). Some works cited by Heitkamp *et al.* (1988) mention that there is potential for the bioaccumulation of PAHs into food chains. Broman *et al.* (1990) studied the trophic transfer of PAHs between a species of mussel and a species of duck. They found that the PAH composition varied from one organism to the other, probably owing to the increased metabolic activity with increasing trophic level. The latter may be due to a selective biotransformation capacity for different PAHs. Fish from contaminated areas have a higher incidence of tumor development than those from non-contaminated areas (Sims and Overcash, 1983). Many other aspects of PAH toxicity in aquatic ecosystems are covered in Varanasi (1989).

In soil-plant ecosystems, variations in effects have been seen according to the plant species. Little PAH uptake occurs in selected plant species. However, growth stimulation, depression and even tumor-like tissue development have been seen in some plants exposed to PAHs (Sims and Overcash, 1983).

Exposure to air-dispersed PAHs from inhalation by a duck species was not significant compared with exposure from ingestion (Broman *et al.*, 1990). Some studies of airborne PAHs indicate that rat model assays underestimate the carcinogenic potential of particle-bound airborne PAHs for humans (Gerde *et al.*, 1991). This could be significant in working environments with high loads of PAHs in the air. However, the high doses of chemicals used in tests with laboratory rodents do produce cancer and could be comparable to the worst case of human exposure (Ames, 1991). Such high-dose exposure is not likely to occur under common circumstances, and it is very difficult to determine the normal exposure doses since they are very small.

2.3.4 Human health effects of PAHs

The health effects of creosote have been reviewed by the US EPA with a view to restricting its use, and by the International Agency for Research on Cancer (IARC) (Sundström *et al.*, 1986). IARC has classified creosote as a human carcinogen belonging to group 2A because there is sufficient evidence that it is carcinogenic and limited evidence from epidemiologic studies that creosote is carcinogenic in humans (Sundström *et al.*, 1986). PAHs are carcinogenic and may cause birth defects in laboratory animals at high doses. Creosote is a recognized carcinogen in humans (skin, forearm, scrotum, face, neck, penis and experimentally in lungs). The most important means of exposure for humans is oral ingestion (Sundström *et al.*, 1986; SNV, 1993a). Risk estimations in Sweden suggest that approximately 100 cancer cases/year may occur owing to exposure to air borne PAHs, and that about 300 cancer cases/year may occur owing to ingestion of PAHs with food (Törnqvist and Ehrenberg, 1994).

Maclaren and Hurley (1987) and Karlehagen (1992) studied mortality in tar distillation workers and cancer incidence among creosote-exposed workers. In the former, lung and bladder cancer were associated to the work, while in the latter no valid conclusions were postulated. However, the second investigation indicated that the risk of developing skin cancer was higher in the past (1950s). The lack of exposure data makes this kind of study difficult.

The spreading of the PAHs in the soil is very slow, but they may reach the groundwater and come into buildings constructed on contaminated sites. A bad smell is one of the inconveniences of creosote-contaminated sites (SNV, 1993a). So far, no references have been found on cancer cases in persons living near creosote contaminated sites. However, intoxication from phenolic compounds contaminating the drinking water of Vansbro (Sweden) was reported in 1992. The drinking water network was contaminated with phenolic compounds which probably came from a former creosote impregnation plant (TT, 1992a, b = Tidningarnas Telegrambyrå, AB, Swedish News Agency). Simple ACs like benzene are carcinogenic and may cause serious problems to the central nervous system (SNV, 1993a). It is unclear if (and how) PAHs from creosote-contaminated sites may reach human beings.

2.4 Contaminated soil remediation and reclamation technologies

2.4.1 Introduction

Many methods have been developed for treating hazardous liquid, gaseous or solid emissions from industries. Soils, underlying groundwater and sediments have been contaminated with a variety of substances, owing to accidental spills, inadequate (production or disposal) practices and a lack of environmental consciousness and laws. The most common solutions for contaminated soils or sediments clean-up are: removal and landfilling in an area with suitable geological material to avoid migration of pollutants to groundwater; or removal and incineration (SNV, 1993b). A range of physical, chemical, biological and combinations of these methods have been developed (Tedder and Poland, 1993, 1991, 1990). Some hazardous waste treatment technologies discussed in Jackman and Powell (1991) are: biological treatment, wet air oxidation, chemical fixation and solidification, and chemical oxidation. A review of several aspects of bioremediation technologies was made by Maier (1992). Sims (1990) presents a critical review of the factors to be considered when selecting contaminated soil remediation technology and Sims *et al.* (1990) present a review on the approach to on-site bioremediation of contaminated soil. The most basic elements in selection of a biological remediation method are:

1) A thorough site/soil/waste characterization;

2) Treatability studies (at laboratory, bench and pilot scale); and

3) Design and implementation of the bioremediation plan.

In addition to a review of the process capabilities, this selection requires also a rational review of limitations and site-specific constraints in order to use the resources allocated for the clean-up efficiently. Although expensive analytical procedures are necessary to determine the initial status and to monitor the performance of the treatment process, the presence of solvent-extractable hydrocarbons is insufficient to propose a bioremediation technology (Pollard *et al.*, 1994). Some ways of monitoring biodegradation are: CO_2 evolution, chloride production (if the pollutant is chlorinated compounds), and changes in total organic carbon and chemical oxygen demand (Bennett and Olmstead, 1992). Toxicity assays may complement chemical analyses when choosing a remediation method or when evaluating its performance.

Bioremediation is an attractive way to clean up contaminated soils since, in many cases, it has been shown to be effective, economical and environmentally compatible (Bumpus *et al.*, 1993; Pollard *et al.*, 1994). Other remediation techniques are inadequate (*e.g.*, pump-and-treat for contaminated groundwater treatment), do not solve the problem permanently (*e.g.*, landfilling) or may be very expensive (*e.g.*, incineration) (Bennett and Olmstead, 1992).

Different definitions have been given of specific bioremediation methods in the US and in Europe, and remediation methods have been classified in several ways. Sims (1990) puta groups the general remediation systems into: *in situ*, prepared bed and in-tank reactor. They may also be grouped according to the place where the treatment is carried out: in the contaminated site (on site) or away from the contaminated site (off site). The term *in situ* may create confusion. It is used in this work when talking about a specific soil bioremediation method which involves the pumping of groundwater. The term *ex situ* covers off site methods in general. General information about on site and off site bioremediation are presented by Rosén (1990) and Bennett and Olmstead (1992). Some general remediation and reclamation technologies for contaminated soils are described below.

2.4.2 On site methods

General on site treatment methods are:

- Doing nothing and letting nature take its course. The problem about this "method" is that the contaminants might reach the groundwater before the subsurface microorganisms degrade them (Bennett and Olmstead, 1992). This method is sometimes referred to as hazardous waste land treatment (*e.g.*, Brown *et al.*, 1983) or natural attenuation (US EPA and US Army, 1993).

- *In situ* soil bioremediation can be used for cleaning contaminated groundwater, saturated soil below the water table and unsaturated soil above the water table by addition of nutrients, sometimes microorganisms, and oxygenation by adding air, oxygen or hydrogen peroxide. It can be also used for decontamination of soil above the water table with forced aeration with the help of vacuum ventilation (bioventing). Several ways of providing oxygen for bioremediation methods are presented by Maier (1992).

- Aqueous, air or steam on site extraction.

- Pump-and-treat method for contaminated groundwater treatment. (This may be considered an off site method since the actual treatment is done above ground).

Details on *in situ* bioremediation and the pump-and-treat methods are presented below.

In situ bioremediation

In situ bioremediation is achieved by stimulating the resident microbial population by adding and managing nutrients and oxygen in a controlled closed-loop system. Soluble transformation products are collected, treated and re-injected at the site (Pollard *et al.*, 1994). Before adding nutrients and oxygen, it must be known how much is already present in the soil and groundwater. Nutrients added may be, for example, N, P and K, and the organic pollutants may be considered as the sole source of carbon and energy. Oxygen is provided by aerating the circulating water, by creating a vacuum, which makes air circulate, or by adding hydrogen peroxide (Maier, 1992).

Some advantages of *in situ* bioremediation are:

- It is the least expensive method of cleaning up sites contaminated with organic compounds;

- It treats groundwater and soil (saturated and vadose zones) in one process;

- It treats dissolved compounds and compounds sorbed to soil particles (if they are bioavailable) at the same time;

- It requires no excavation or transportation of soil, and thus does not affect the physical structure of the site (Maier, 1992). This is advantageous if it is not possible to excavate a site, for example, in the case of leaking tanks under buildings or highways (Bennett and Olmstead, 1992);

- It might not require the addition of externally cultivated (foreign) microorganisms if competent microorganisms are already present. If competent microorganisms are present (*i.e.*, if they can mineralize the pollutants), only inorganic nutrients and a source of molecular oxygen (delivered as hydrogen peroxide) are added. However, if the acclimated microorganisms do not degrade the waste in laboratory experiments, enrichment cultures of externally cultivated microorganisms must be added (= bioaugmentation). These organisms are cultivated in above-ground bioreactors (Maier, 1992; Bennett and Olmstead, 1992);

Some disadvantages/limitations of *in situ* bioremediation are:

- By maintaining the amendment circulation and the water regime in the system, a concentration gradient of pollutants and degradation products is created. This may avoid, to a certain extent, their spreading outside the treatment area. Otherwise, physical barriers (dikes) must be built.

- The method requires the most detailed site description (of the hydrogeological, geological and geochemical conditions) and inventory (of the chemical and microbiological conditions) of all the biosanitation methods. The hydrogeological, geological and geochemical conditions that need to be known include: groundwater conditions, soil porosity, groundwater gradient, soil inhomogeneities, layering of the soil and presence of metals or metal ions. The most important factors are the groundwater flow and soil porosity (Maier, 1992).

- The method is not applicable if the subsurface saturated horizontal conductivity is less than 10^{-6} m/s. Otherwise the delivery of nutrients, cosubstrate, a source of oxygen, and recovery of metabolic products and insoluble compounds does not take place effectively and "clogging" of the soil pores by biomass accumulation may occur (Pollard *et al.*, 1994). This clogging is called biofouling (Maier, 1992). The microorganisms may move easily within the subsurface but it usually happens that the population "thins out", owing to sorption to soil particles or natural decay. This is avoided by using multiple injection points.

- With the definition of *in situ* bioremediation used here, its use is limited by the presence of groundwater. However, this is not to be generalized to all on site methods.

- It requires constant monitoring of process parameters (oxygen content, pH, pollutant concentration and spreading out of the site groundwater). This monitoring may be automated.

- It is very difficult to determine the location and segregation of contaminants, as well as the transport of nutrients and oxygen through the soil and groundwater.

- The process is affected by climatic and other abiotic conditions which are difficult to manage from the surface (*e.g.*, low temperature, anoxic soils, precipitation).

- The degradation rates of the pollutants may be limited by the properties of the pollutants (if the pollutants may be easily degraded and used as sole source of carbon and energy), their concentration (too low or too high concentrations of pollutants may not induce or inhibit the degradation) (Maier, 1992).

- The process may take a long time, and the long term effects of addition of microorganisms and/or nutrients to the subsurface are not well understood (Bennett and Olmstead, 1992).

- Heterogeneities in the distribution (and diversity) of microbial populations in subsurface environments may influence the results of on-site stimulation of resident microbiota for bioremediation purposes. Hazardous wastes were once discharged directly on the soil or subsoils (land treatment), and yet the biogeochemical interactions between the wastes and the vadose zone microorganisms (bacteria and fungi) are essentially unknown (Fredrickson, 1992).

Many bacteria have been proven to degrade a wide range of organic compounds. The bacterial genera capable of degrading PAHs include the following:

Pseudomonas, Flavobacterium, Alcaligenes, Aeromonas, Vibrio, Beijerenckia, Bacillus, Nocardia, Corynebacterium and *Micrococcus* (Cerniglia and Heitkamp, 1989). Bacteria of the genus *Pseudomonas* are the most used bacteria, and are commercially used in bioremediation.

The **pump-and-treat method for contaminated groundwater** may be used in connection with *in situ* soil bioremediation or as an on site method for contaminated groundwater (biological or physico-chemical) treatment. This method, however, does not meet the clean-up criteria (Bennett and Olmstead, 1992). The process parameters (stirring, pH, nutrient and oxygen concentration) are totally controlled since it is a closed system. This fact is advantageous if volatile compounds are present, because they will also be removed and degraded. After the water has been treated it is pumped back to the aquifer or to a sewer, in which case it receives further biological and chemical treatment at a sewage treatment plant. One problem with this method is that the concentration of pollutants may vary greatly during the treatment period. One advantage

is that the treatment units may be mobile and transported to where they are needed (Maier, 1992).

Bioventing is an on site technology limited to volatile organic compounds (VOCs) amenable to aerobic biotransformation (Pollard *et al.* (1994). It may be an option to provide aeration for the *in situ* bioremediation method.

2.4.3 *Ex situ* or off site methods

Some off site methods for reclamation or remediation of contaminated soils are:

- Beneficial use (asphalt, road building);
- Thermal treatment (low temperature (< 300°C), high temperature);
- Solvent extraction (aqueous, organic solvent, supercritical); and
- Soil bioremediation through: land farming, composting, or slurry reactor (Maier, 1992; Pollard *et al.*, 1994). Slurry reactor treatment may be also operated on site. Land treatment, enhanced land treatment and land treatment with forced ventilation may be defined as on site or off site methods, as discussed below.
- Soil washing, by treating the soil in reactors with detergents (Bennett and Olmstead, 1992).

A brief description of some off site biological remediation methods, as well as some advantages and disadvantages/limitations are presented below.

Two possible advantages of *ex situ* methods are:

- Additional microorganisms may be also added;
- The distribution of contaminants and matrix composition is easier to control, but still difficult to predict (*e.g.*, the soil may be mixed to homogenize the pollutant distribution and the soil types).

Disadvantages/limitations of *ex situ* methods are:

- Digging and transportation of the soil is expensive and polluting,
- Large areas are needed for such treatments.

A general description of some off site bioremediation methods is given below.

Land treatment may easily be confused with land farming. In the context described, land treatment may be considered an on site treatment similar to natural attenuation. Hazardous waste land treatment is a technology which has been used to treat waste from wood impregnation with preservatives such as creosote, oil wastes and other types of

contaminants (Brown *et al.*, 1983) by applying them to the soil. Symons and Sims (1988) describe it as a

> ... technology that utilizes the natural physical, chemical, and biological processes in the soil to degrade, immobilize, and transform wastes without detrimental effects to the environment. Wastes are mixed into the upper soil layer... The soil, waste, and site characteristics are managed and altered to optimize degradation, immobilization, and transformation of waste constituents.

This technology was banned in the USA unless non-migration of the toxic compounds (as long as they remain hazardous) was guaranteed (McFarland *et al.*, 1992). Clays are very good soils for this purpose because they retard the mobility of contaminants (Anderson *et al.*, 1983). But today, contaminated sites (*i.e.*, waste dumps, closed wood impregnation plants, coal gasification plants, petroleum industry waste sites) where toxic compounds were placed on soil intentionally or by accident must be cleaned up.

The term **land farming** is used when contaminated soil is spread in a thin layer on the ground (US EPA and US Army, 1993). This method has been used for decades by the petroleum industry, and is the most widely used bioremediation method. It can be done outdoors or indoors in a greenhouse-like tent. The excavated soil is spread in a thin layer (a few decimeters thick) over a large area and the aeration and mixing is done with conventional agricultural equipment. Nutrients and water are added using an irrigation system and the leachate is collected, treated and recirculated. A tight sealing or lining may (or may not) be used on the ground of the treatment site to prevent the spread of the leachate (Maier, 1992). A source of oxygen, lime for pH adjustment and primary substrate may also be added. The excavated soil may be stored while the treatment site is prepared; then the soil is returned the land treatment unit (Sims, 1990). The process must be monitored with a mass balance approach and with toxicity assays to control the disappearance of contaminants and changes in toxicity of the soil contaminants (Pollard *et al.*, 1994).

In this method, the microorganisms may be resident or externally cultivated and added, as in the *in situ* method. The requirements of oxygen and nutrients are the same as in the *in situ* method. It is also necessary to control the humidity level, pH and nutrient transport. Additional control of precipitation is necessary if the process is carried out outdoors (Maier, 1992).

This technology (called enhanced land treatment by Pollard *et al.*, 1994) may present the same problems as *in situ* treatment with respect to operation temperature, anoxic soil and low or variable hydraulic conductivity (Pollard *et al.*, 1994).

Some advantages of land farming are:

- If it is a closed treatment facility, the temperature, water regime and other parameters may be controlled,

- Tilling and adding bulk material (straw, wood chips, etc.) ease aeration and the latter may serve as substrate for cometabolic degradation,

- Microorganisms may be inoculated to enhance the initial PAH degradation, but these organisms must compete with and survive together with the resident microbiota (Pollard *et al.*, 1994),

- The method only requires low technology and standard methods may be used for contaminated soils, sands, and solids.

Some disadvantages of land farming are:

- It is limited to surface soil contamination,

- It requires large tracts of land,

- It is relatively slow (Bennett and Olmstead, 1992),

- This method is not suitable for volatile contaminants and mixtures, since they will evaporate and the microorganisms will not have a chance to degrade them. This is not good if the volatiles are hazardous. On the other hand, it is difficult to make chemical analyses of volatile compounds (Maier, 1992). If it is an indoors facility, volatiles may be controlled and collected using, for example, activated carbon filters. However, this increases costs (Bennett and Olmstead, 1992).

Borazjani *et al.* (1991) obtained significant degradation of pentachlorophenol and creosote (apparently even high molecular weight PAHs) in soil and sludge from several wood preserving sites. They used what they call a land treatment technique, which consists of adding chicken manure (4% dw) to the contaminated soil (as source of organic and inorganic nutrients). They conclude that this could be a simple, low cost and safe technology to use in different types of soils and sites.

The US EPA and US Army (1993) include methods like composting and biopiles in what they call controlled solid phase biological treatment. Sims (1990) classifies closed composting and slurry phase treatment as in-tank methods.

Composting of hazardous materials (understood as the aerobic and thermophilic degradation of organic materials) is achieved by adding vegetable waste such as straw, bark or wood chips, or manure as nutrient and bulking agents (to increase porosity and facilitate aeration). The filling material composes 70% to 90% of the total volume (Maier, 1992).

The process is very similar to composting of sewage sludge or household waste. It may be done in open windrows, static windrows or in closed reactors. In the first mode, long and thick compost rows (stacks) are made. A draining system is required, and water and nutrients may be applied with an irrigation system. In static windrows, aeration through the compost mass is achieved using a pump for sucking or blowing air by plastic tubes under the windrows. The exhaust air is passed through an activated coal

filter. The compost heaps may also be aerated by turning them over. Nutrients and microorganisms may be added from another tube system. In a closed reactor, the treatment time can be greatly reduced thanks to better control of biotic and abiotic parameters (Maier, 1992).

Organic contaminants including pesticides, hydrocarbons and explosive wastes in soil have been successfully degraded by composting processes, especially at thermophilic temperatures (50-60°C) (Ziegenfuss *et al.*, 1991; Williams and Myler, 1990).

In a full scale composting trial (Seman and Rydergren, 1991) to degrade creosote PAHs in contaminated sediment (from the river Vanån in Vansbro, Sweden), the best results were obtained by adding 30-day-old household waste compost at different times during the experiment. 90% reduction in total PAHs in the first 4 months and 99% after 10 months was observed. At the end of the experiment, phenanthrene was degraded by 99%, fluoranthene by 97% and chrysene by 92%. One question arising from this particular case is whether the compost provides only nutrients to the sediment microorganisms or whether the compost microorganisms also participate in the xenobiotic degradation. Since the contaminated soil or sediment is "diluted" with compost, the pollutant content in the soil is also diluted. This may make the interpretation of analytical results more difficult.

Some disadvantages of composting are:

- The method requires more oxygen than other methods, in order to degrade the filling material (Maier, 1992),

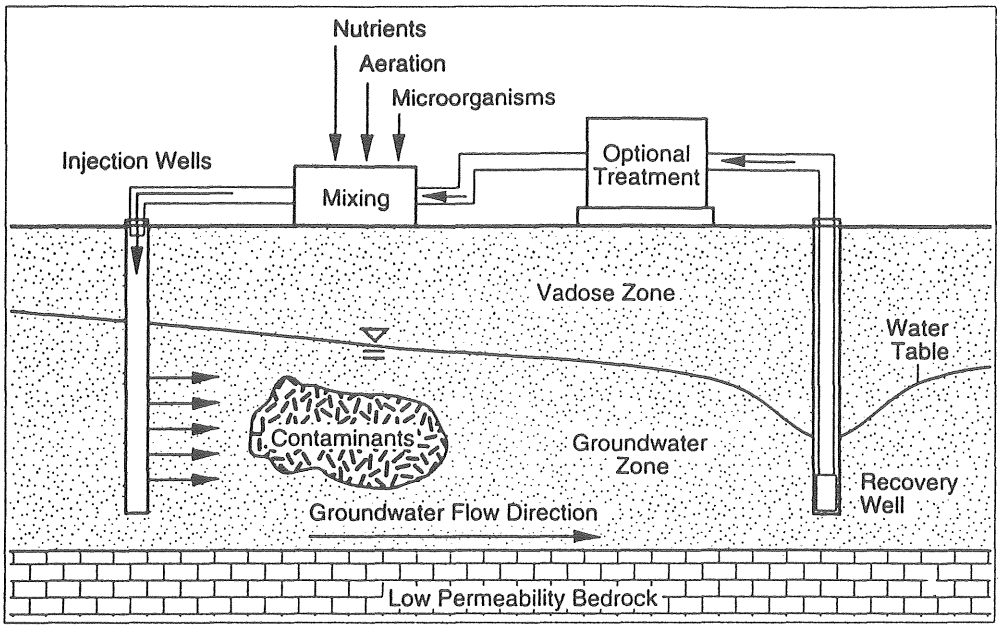
- Regulatory concerns around the containment of the composting material, formation and migration of leachates, emissions of volatilized chemicals and ultimate disposal of the compost residue (Ziegenfuss *et al.*, 1991).

When using WRF for bioremediation of contaminated soils, the technique has been referred to as composting (Qiu and McFarland, 1991) or land treatment. These techniques have been tested successfully even in slurry bioreactors (Stroo *et al.*, 1989). The use of WRF for bioremediation is discussed below.

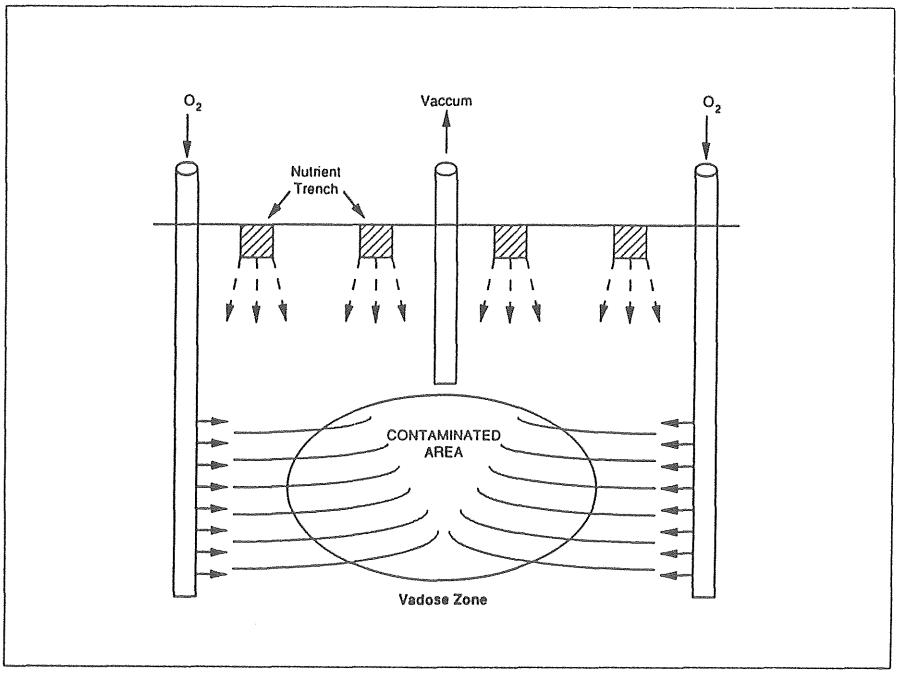
The principles of *in situ* bioremediation, bioventing, land farming and composting are illustrated in Figures 2.4.1 a,b,c and d.

The case of Blekholmstorget: an example of *in situ* and *ex situ* full-scale treatments

In Blekholmstorget, Stockholm, a coal gasification plant operated from 1853 to 1922, and creosote was distilled from the coal tar. As is true at all gas works and creosote distillation sites, the soil was contaminated with heavy metals, cyanides, phenols, and PAHs. The sediments of a nearby river also contained large amounts of PAHs, but it was not included in the clean up program. It was decided that the site should be cleaned up and then houses, offices and a street built there. Some offices had already been built in the area. The authorities decided to use physical barriers to avoid further spreading

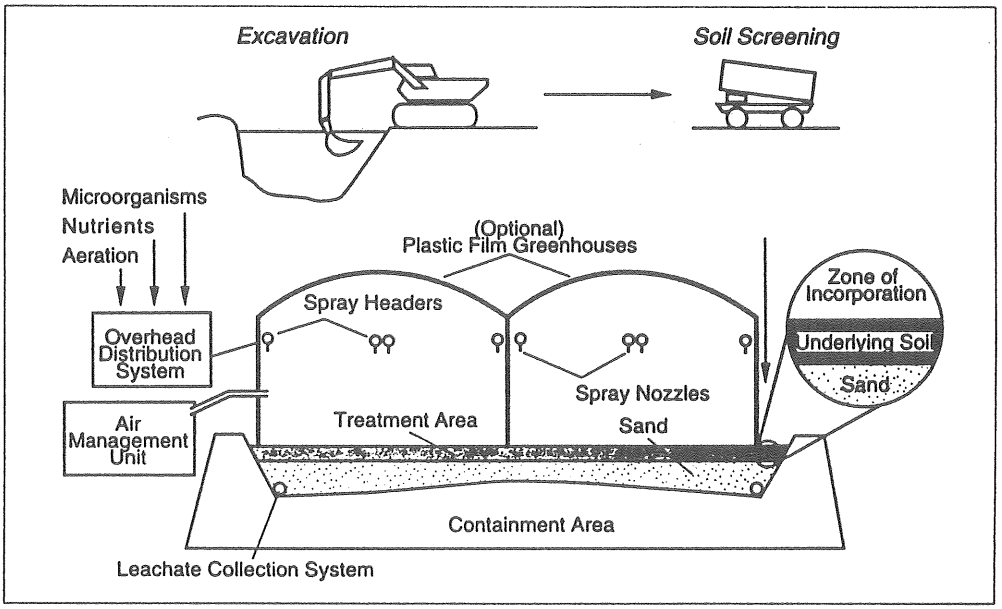


a)



b)

Figure 2.4.1. Principle of some bioremediation methods: a) *in situ* treatment of the groundwater zone; b) *in situ* oxygenation aided by forced ventilation (bioventing) (from Maier, 1992) (continued)



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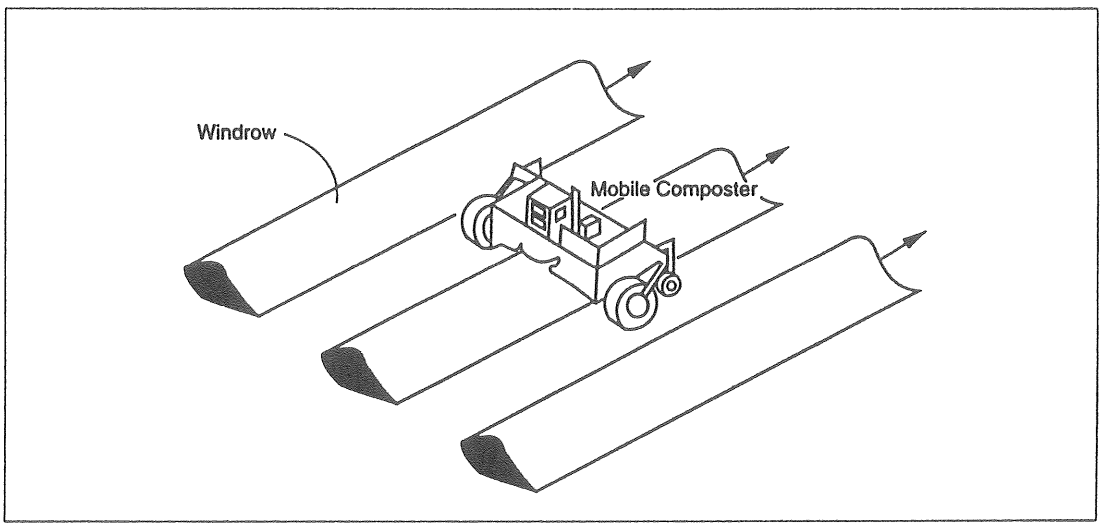

d)

Figure 2.4.1. Principle of bioremediation methods, continued: c) land farming; and d) composting (from Maier, 1992)

of the contaminants, and bioremediation to get rid of the PAHs (SNV, 1993c). The feasibility studies (Ellis *et al.*, 1991) concluded that it was possible to use bioremediation in the site. PAHs (as heavy as chrysene) were found to be best degraded by using additional microorganisms and surfactants.

The full scale project consisted of *in situ* treatment of the site and an *ex situ* treatment of 3500 m^3 of removed soil (land farming method). Salts (N and P), surfactants, a simple source of carbon (syrup) and additional bacteria isolated from the site were added in both cases. Both treatments were considered unsuccessful by the authorities (SNV, 1993c) and were criticized by the Swedish media (*e.g.*, Falk, 1992), since:

- Only low molecular weight PAHs, which are easily degraded or transported to groundwater, and not the heavier ones (5- and 6-ring PAHs) were degraded (despite the fact that an additional source of carbon was added). Only the bad smell of the soil disappeared; and

- The target PAH concentration was not achieved.

Some concrete difficulties were:

- There was not a clear trend in the reduction of PAH concentration due to very high variation in PAH values even between samples taken 1 meter apart,

- It was found that PAH heavier than chrysene were present in the soil, so the analyses including only up to chrysene were considered inappropriate,

- The off site treatment should have been done indoors, since during the rain periods the soil was saturated with water, probably spoiling the oxygen transport and the drainge conditions,

- The on site part had operation problems from the beginning, and more chemical analyses would have been necessary to better understand what happened (SNV, 1993c),

- Bacterial treatment was used despite the fact that it is reported in the literature that bacteria may use, as their sole source of carbon and energy PAHs, of up to 4 rings (Cerniglia, 1981, 1984). However, co-oxidation was intended from the addition of syrup as a source of carbon,

- Building construction took place during the on site treatment, and an offices building was already there, thus making the treatment and sampling difficult;

The housing project was postponed until all the underlying soil had been removed (SNV, 1993c).

In **slurry bioreactors** the soil is treated as an aqueous slurry in a closed reactor using a well characterized added microbial population (Pollard *et al.*, 1994).

Some advantages of slurry bioreactors are:

- Process control reduces treatment times, compared with methods described above. A better mixing (in completely stirred tank reactors, a well established technology) can be obtained, resulting in higher microbial growth and concomitant faster degradation,

- The method is ideal for clayey soils and for cases where temperature is adverse to biotransformation,

- It may be operated aerobically or anaerobically.

Disadvantages/limitations of slurry bioreactors are:

- The anaerobic microorganisms are less flexible in adapting to changes in substrate availability and are less tolerant to inhibitory toxic metals,

- If the process is not done on the site, the costs may be high (Pollard *et al.*, 1994; Bennett and Olmstead, 1992). It may be carried out either on site or off site.

Anaerobic biodegradation of hazardous waste has received a great deal of attention lately, and may occur in anoxic spots in the subsurface during *in situ* bioremediation or be carried out in anaerobic digestors. Most of the bioremediation experiments at different scales are based on aerobic processes since microbial growth and activity, as well as the highest free energy yield from oxidation of organic compounds, occurs under aerobic conditions. Significant anaerobic activity occurs in the subsurface. This is also true in soils with high clay contents forming aggregates larger than 3 mm where anaerobic centers exist owing to the water adsorption properties and the fine mesoporous structure (Pollard *et al.*, 1994).

Compounds which can be degraded in anaerobic systems are:

- Chlorinated compounds that readily undergo reductive dechlorination,

- Aromatic hydrocarbons (toluene and xylene isomers) through methanogenic route or under sulphate reducing conditions,

- Toluene, phenol and p-cresol under ferric iron-reducing conditions,

- Low molecular weight PAHs (naphthalene) under denitrification conditions, although desorption (bioavailability) still remains a major problem. These findings could, however, be the basis for full-scale pre-treatment of soils contaminated with low molecular weight PAHs (Pollard *et al.*, 1994).

Other aspects of bioremediation

Mixed cultures, both in aerobic and anaerobic conditions, have proven very successful, since the presence of many non-competing species assures that what is not degraded by

one organism is degraded by another. It is possible that synergistic microbial communities are important for biodegradation to take place. Lappin *et al.* (1985) observed that the initial degradation of the herbicide mecoprop [2-(2-methyl-4-chlorophenoxy)propionic acid] was accomplished by a group of bacteria isolated from wheat root system and not by individual species. The anaerobic degradation of chlorinated compounds has proven to be very efficient (Bhatnagar and Fathepure, 1991). Another study on anaerobic degradation of organic pollutants is that of Battersby and Wilson (1989). The anaerobic catabolism of PAHs could be an important pathway of removal of PAHs in anaerobic ecosystems like sediments (Cerniglia and Heitkamp, 1989). The anaerobic degradation of organic pollutants may also be carried out by a consortium of microorganisms. Abramowitz *et al.* (1993) showed that anaerobic bacteria in river sediments dechlorinated PCBs to monochlorobiphenyls, but aerobic bacteria were required to degrade these compounds to carbon dioxide.

Since most contaminated sites contain more than one kind of contaminant and each site presents particular characteristics, Sims *et al.* (1990) mention that bioremediation is especially promising if it is part of an integrated site clean up approach. This means that a plan using a "treatment train" of physical, chemical and/or biological processes is involved to clean all sources of contamination in a site.

Combined physicochemical/biological treatments may be used when:

1) The compounds are extremely resistant to biodegradation,

2) The proper conditions may not be obtained (*e.g.*, complete anaerobic conditions),

3) There is too high or too low contaminant concentration, or the presence of other toxic contaminants.

Some combined technologies which have been used are, for example, photolytic and biological methods (*e.g.*, Matsumara and Katamaya, 1992 and Portier *et al.*, 1989). These researchers investigated combined microbiological and photolytic degradation approaches for the detoxification of dioxin-contaminated sludges and groundwaters. The Bio Trol soil washing process is an integrated train of treatments including soil washing, biological water-phase treatment and slurry phase bioreactor. It has proved to be very efficient and fast to remove pentachlorophenol and to degrade PAHs in a sandy soil from a wood-preserving site (Stinson *et al.*, 1992).

Examples of **pretreatment operations** which may assist in accomplishing effective removal of the target contaminants are mentioned below.

Air stripping removes VOCs from the water phase to the gas phase by circulating water down a packed bed reactor column and air up the column. The separated VOCs may be sorbed to activated carbon, incinerated or released to the atmosphere if they are not hazardous;

Activated carbon adsorption may completely remove even trace amounts of a range of organic contaminants from water and waste water. After adsorption to the activated

carbon, the process may be supplemented with biological processes. Powdered activated carbon may be added to sewage sludge or biologically activated carbon may be used. In the latter, microorganisms degrade the sorbed pollutants, extending the life of the filter before regeneration is required; and

Metals removal can be achieved by ion exchange or coagulation/flocculation. Groundwater with large amounts of iron can produce iron precipitates when large amounts of substances like peroxide or phosphate are added. These precipitates may block the interstitial soil particle spaces, impeding the proper transfer of microorganisms, nutrients or water through the contaminated site (Bennett and Olmstead, 1992).

2.4.4 Some limiting factors of bioremediation processes

Sims (1990) reviewed general limiting factors for all sorts of contaminated soil remediation methods. Pollard *et al.* (1994) have reviewed the constraints on bioremediation of petroleum and creosote-contaminated soils. The selection and effectiveness of the bioremediation method depends on the waste/site/soil characteristics and the interaction among them. Limiting factors include waste composition, temperature, substrate, bioavailability, accompanying pollutants and soil structure.

Some general factors which may affect the effectiveness of a bioremediation process are:

- The occurrence of contamination in more than one medium (soil, soil vapor, groundwater, distinct hydrocarbons phase),
- The presence of a complex mixture of organic and inorganic pollutants with a wide range of environmental and toxic properties,
- Heterogeneous and difficult to characterize subsurface conditions, and
- Sub-optimal environmental conditions [for field bioremediation] (Pollard *et al.*, 1994).

Some specific problems are mentioned below. For a complete overview, see Pollard *et al.* (1994).

Waste composition

The chemical composition of hydrocarbon wastes varies greatly depending on the nature, composition, degree of processing of the source material and the extent of weathering undergone by the exposed waste product.

It has been explained that coal-tar creosote is a very complex mixture of chemicals. In creosote-contaminated sites, other processes and materials contribute to making the waste found in these sites even more complex. These are: the waste stream from auxiliary unit operations, the residues of secondary process chemicals, carrier oils

associated with wood treating solutions, biodegradation products of hydrocarbons, wood fragments, alternative wood preservatives and process chemicals. Weathered hydrocarbon waste is more recalcitrant since the most readily degradable compounds are degraded, leaving behind the most recalcitrant ones. These compounds are preferentially partitioned to the residual oil phase, soil organic material and soil solid surfaces, reducing their bioavailability. Asphaltenes are highly refractory compounds found in weathered oil wastes. Asphaltic-naphthenic oils are key components in Bunker C residual fuel oil, which is widely used for the delivery of creosote to untreated timber in wood-treatment facilities (Pollard *et al.*, 1994).

The sequence in which creosote components and pentachlorophenol are degraded during land treatment is: phenols, low molecular weight heterocycles, high molecular weight heterocylces and PCP. Older creosote-contaminated soils or sediments are more difficult to treat (Pollard *et al.*, 1994).

Temperature-climatic considerations

Extreme temperatures and wide fluctuations of temperature on diurnal or seasonal cycles do not favor stable and active PAH-degrading microbial populations. The partition and kinetic rates of the compounds may also be affected, as well as their viscosity, solubility and other physical characteristics (Pollard *et al.*, 1994). However, there are psychrophilic and thermophilic organisms which degrade hydrocarbons at high and low temperatures. For example, the WRF *Phanerochaete chrysosporium* has an optimum temperature of 39°C (Stroo *et al.*, 1989), and some researchers are testing hydrocarbon-degrading bacteria at temperatures as low as 10°C (Persson and Hahn-Hägerdal, 1992).

Irrigation (during land treatment) increases the soil heat capacity, and the addition of mulches also helps to keep down the temperature fluctuation. Year-round treatment costs increase if heating and temperature control are to be provided (Pollard *et al.*, 1994).

Bioavailability and multiphase partitioning

Transformation of the organic pollutants occurs most readily in the aqueous phase. This is restricted by oil-phase partitioning, sorption and rate-limiting diffusion processes that limit the aqueous phase concentration of the substrate. Mass transfer limitations may be overcome by using slurry bioreactors and bioemulsifiers. Ageing of the contaminated soil and agglomeration of soil particles may also reduce the bioavailability of PAHs to the active microbial population (Pollard *et al.*, 1994) as well as their toxicity (Weissenfels *et al.*, 1992b).

Fine-grain soils (clays and silts) also have a high affinity for PAHs. Mineral surfaces may attract organic compounds, but this is negligible compared with the effect of residual oil and soil organic matter in the contaminated soil. Variations in reported half-lives of PAHs in soil may be explained by their biostabilization (*i.e.*, tight and irreversible bonding) in the soil matrix. It might take these compounds the same amount of time they have been in contact with the soil to desorb and diffuse before becoming

bioavailable. The more hydrophobic compounds take longer to diffuse into the soil particles. However, the use of surfactants is a plausible solution for mobilization of these compounds (Pollard *et al.*, 1994).

Microbial toxicity assays of synthetic and natural surfactants revealed that the latter were generally less inhibitory than the former. Natural surfactants showed positive effects in the enzyme activity of a hydrocarbon-degrading bacterium while synthetic ones showed slightly negative effects (Münstermann *et al.*, 1992). Some nonionic surfactants have proven to inhibit the mineralization of some PAHs (Laha and Luthy, 1991). One alternative is to use surfactant-producing microorganisms. However, in a model oil biodegradation experiment, the purified surfactants (rhamnolipids) were found to be more effective (temporarily improving the degradation) than surfactants produced by added microorganisms (Czeschka *et al.*, 1992). With respect to WRF, surfactants like Tween 80 have only been used to stimulate ligninolytic enzyme production (Jäger *et al.*, 1985).

Waste toxicity

Microbial populations exposed to hydrocarbons become able to utilize them. However, high loads or interfering waste components (short chain alkanes, BTEX, high molecular weight PAHs) may attenuate this response. Therefore, there must be a treatability concentration range, above which the contact toxicity of the compounds inhibits metabolic activity and below which the microorganisms may switch to an alternative substrate. Co-oxidation may be used in the latter case to reduce the contaminant levels (Pollard *et al.*, 1994). The difference between the terms co-oxidation and cometabolism will be discussed later.

Wood protection through impregnation with PCP or creosote has been and is still considered as a way to avoid wood rotting by WRF, among other organisms (Stroo *et al.*, 1989). Many species of fungi have been used to degrade toxic chemicals, but very few species have been tested for their resistance to toxic effects by these chemicals. Alleman *et al.* (1993) developed a method to test this. Cellulose antibiotic assay disks, impregnated with the chemical to be tested, were placed on agar where fungi were inoculated. The non-growth area indicated toxicity and correlated well with other reported techniques.

In a treatability assessment experiment of different kinds of waste (including oil and wood treatment waste with PCP and creosote) (Sims *et al.*, 1987, cited by Pollard *et al.*, 1994) it was observed that the wood preserving wastes were more toxic (aqueous toxicity in the Microtox assay) than the oil wastes. The toxicity of degradation products may also be a limiting factor for bioremediation processes like land treatment. This was observed for the creosote waste in the above-mentioned experiment. However, the acute toxicity increased in the beginning and decreased over time (in both sandy and clay loams). The mutagenicity (Ames test), however, persisted throughout the experiment (400 days) in the clay loam. Toxic metabolic intermediates may also be removed, by physico-chemical techniques like air stripping or activated carbon adsorption, to enhance biodegradation processes (Bennett and Olmstead, 1992).

The salinity of soil may also interfere with the biodegradation of xenobiotics. Ward and Brock (1978, in Pollard *et al.*, 1994) found that the rates of hydrocarbon metabolism decreased with increasing levels of salinity.

As mentioned above, refinery and wood-preserving wastes often contain appreciable quantities of metals, including lead, cadmium, mercury, zinc, copper, arsenic, chromium, nickel and vanadium. Lead (II) oxide was used to remove sulphur from crude oils in an old process. Chromium, copper and arsenic may be found in wood-treatment sites, since they are alternative treatment chemicals. These metals are not degraded, although their chemical state may be altered. Metals in these kinds of wastes exist as soluble or soil-bound forms (Pollard *et al.*, 1994), and it is known that their chemical state (speciation) determines their bioavailability and toxicity (Morrison, 1989).

Said and Lewis (1991) report that some heavy metals, at concentrations lower than previously expected, may inhibit the biodegradation of 2,4-dichloro-phenoxyacetic acid methyl ester (2,4-DME) by aquatic microorganisms. However, some microorganisms have developed tolerances to high concentrations of heavy metals (Wood and Wang, 1983, in Pollard *et al.*, 1994). The presence of toxic metals in contaminated soils should be considered when using WRF for bioremediation. The growth of the WRF *Pleurotus* can be retarded or inhibited by high concentrations of certain heavy metals (Jain *et al.*, 1989, 1989), particularly cadmium and mercury (Favero *et al.*, 1990; Bressa *et al.*, 1988). These metals are absorbed (translocated) from the substrate to the fungus, and are selectively accumulated by the mycelium and fruit bodies.

Soil texture and structure

Soil texture influences the water regime (infiltration, retention, yield), aeration status, soil temperature and tilth (workability). Soils with higher surface areas facilitate the aerobic utilization of organic compounds such as n-hexadecane up to a point, after which decreasing grain size may produce other problems. When dealing with fine-grained soils, their moisture, nutrient and oxygen limiting capacities are the most important factors to be taken into account (Pollard *et al.*, 1994).

Soils with more than 12% (w/w) clay content may form aggregates which entrap microorganisms and substrate. Aggregates larger than 3 mm may have anaerobic centers, and as aggregate size decreases, transformation rate increases. However, grinding of waste-soil matrices has little effect, possibly because oil does not disperse well once applied to the soil. Therefore, the aggregate size effect must be considered before applying the waste to the soil. Tilling only to expose undegraded contaminants may be unprofitable (Pollard *et al.*, 1994).

Most land treatment studies have been performed on sandy loams. There is only limited data for clay loams, which are the most potentially suited soils for land treatment (Pollard *et al.*, 1994). Song and co-workers (1990, as cited in Pollard *et al.*, 1994) showed that the half-lives of different degradable fuels were longest in sands (which has poor water retention and low microbial diversity) but only slightly shorter in clay loam or loamy soil.

Competing microorganisms

Foreign microorganisms might be added during a bioremediation process if they degrade compounds the resident microorganisms do not degrade. In this case, competition amongst these microorganisms must be considered, since it could be a limiting factor. The foreign microorganisms must both compete and cohabit with the resident ones. This seems not to be a problem for certain WRF growing in soil, since their oxidative enzymes act like antimicrobial agents (Aust, 1990). However, the WRF *Phanerochaete chrysosporium* is not a very good competitor in the soil environment (Stroo *et al.*, 1989).

Non-competing microorganisms (bacteria, other fungi and actinomycete and some of their metabolic products) associated with the lignocellulosic substrate are also part of the fungal (*Agaricus bisporus*, the champignon) nutrition (Fermor *et al.*, 1991; Fermor and Grant, 1985).

2.4.5 Microbiological aspects of bioremediation

Despite the multidisciplinarity of bioremediation, microbiology plays the central role in it (Fox, 1992). Microorganisms may attack hazardous organic compounds in one of three ways:

1) Mineralizing the compounds directly to harmless inorganic molecules like carbon dioxide, water and salts,

2) Mineralizing the compound only as a cometabolite, *i.e.*, the microorganisms needs another organic compound for growth or to induce the formation of enzymes which will degrade the target compound, and

3) Converting the compound to another compound (biotransformation) which may also be toxic and resistant to further degradation (Alexander, 1981, as cited in Bennet and Olmstead, 1992). This may be solved by using a consortium of microorganisms (Bennet and Olmstead, 1992) which co-oxidize the different compounds or transformation products.

Some microorganisms may also accumulate organic pollutants (Kobayshi and Rittmann, 1982).

PAH-degrading organisms

Since the 1940's, it has been known that certain bacteria, yeasts and molds have the capacity to degrade PAHs (Sisler and ZoBell, 1947). Today, more groups of microorganisms with this capacity have been recognized. Among them are species of blue-green algae, bacteria, diatoms and eukaryotic microalgae, and additional species of yeasts and filamentous fungi. These organisms have the enzymatic capacity to oxidize PAHs ranging in size from naphthalene (2 rings) to benzo[a]pyrene (4 rings) (Cerniglia, 1981, 1984). Microorganisms acclimated to PAH-contaminated sediments have the capacity to (easily) use up to 3-ring PAHs as the sole source of carbon and energy

(Herbes and Schwall, 1978; Herbes, 1981). A bacterium of the genus *Mycobacterium* capable of utilizing pyrene (4-ring PAH) as a sole source of carbon and energy was isolated (from sediments located near a point source for petrogenic chemicals) and characterized by Heitkamp *et al.* (1988). Other organisms, such as certain fungi, may degrade these [and larger] PAHs when grown on an alternative substrate (Cerniglia, 1984) (*i.e.*, cometabolically).

Co-oxidation may occur in soils contaminated with complex (mixed) organic wastes, in such a way that compounds or their degradation products which may not be utilized by one organism will be used by another one. Cometabolism refers to the oxidation of any non-growth substrate, regardless of whether or not a growth substrate is present (Keck *et al.*, 1989).

PAHs are very persistent in nature, and may undergo photolysis or volatilization. However, the most significant process affecting their persistence is biodegradation (Sims and Overcash, 1983). The stability of PAHs in nature (resistance to biodegradation by natural populations of microorganisms) depends on their ring arrangement and on the sorption of PAHs to soil particles. The least stable PAHs are those which have all their rings in line (*e.g.*, anthracene); cluster-PAHs have intermediate stability (*e.g.*, pyrene and benzo[a]pyrene); and the most stable PAHs are those which have their rings in steps (*e.g.*, phenanthrene, chrysene) (Blumer, 1976). Dioxins and PCBs are more resistant to biodegradation than PAHs, owing to their structures and their high degrees of chlorination (Loske *et al.*, 1990).

PAH bioavailability decreases when the PAHs are bound to soil particles and to existing hydrophobic organic compounds (*e.g.*, humic compounds in natural soils, coal and coke particles in coking plants, tar refineries and gas works sites) (Weissenfels *et al.*, 1992a). In mineral soils, most humic materials are associated with clay (Qiu and McFarland, 1991). Additionally, the binding strength of PAHs to soil particles increases with time (Weissenfels *et al.*, 1992a).

There are many reviews on degradation of organic contaminants by microorganisms (metabolic pathways, engineering applications, etc.), including Kobayashi and Rittmann (1982), Bennett and Olmstead (1992), Morgan and Watkins (1989) and Nandan and Raisuddin (1992). Reviews on WRF include Hammel (1989) and Higson (1991). An excellent review on the mechanisms used by WRF to degrade pollutants is that of Barr and Aust (1994), which is highly recommended although it is not discussed in detail here. Metabolic aspects of PAH degradation are discussed below.

White-rot fungi

Lignin is a very complex tridimensional polymer formed by non-repeating phenylpropane subunits (Figure 2.4.2) connected by different types of carbon-carbon and ether bonds. The polymer shows stereo irregularity and contains chiral carbons in L and D configurations. The molecule is very large and insoluble. WRF are responsible for most of the lignin degradation in nature (Kirk and Farrell, 1987) since they have evolved a non-specific extracellular enzyme system, adequate for degrading a molecule with these characteristics. Kirk and Farrell (1987), Boominathan and Reddy (1992) and

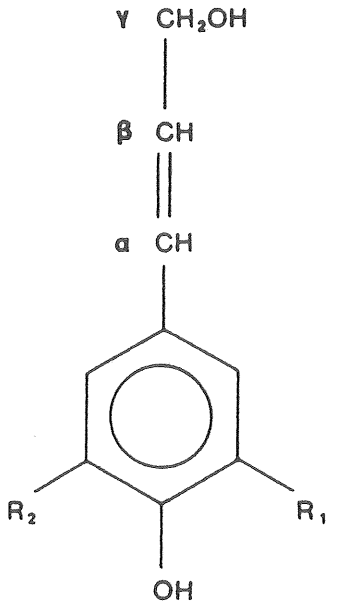

Figure 2.4.2. Phenyl propane monomer subunit of the lignin polymer (the polymer is composed of three phenolic acids: *p*-coumaryl alcohol when R_1 and R_2 = H; sinapyl alcohol when R_1 and R_2 = OCH_3; and coniferyl alcohol when R_1 = OCH_3 and R_2 = H) (Lynch, 1987)

Härtig and Lorbeer (1993) have reviewed lignin degradation. Since the degradation of lignin is non-selective (at the molecular level, because of the random distribution of the monomeric units) and the WRF do not need to be acclimatized, their ligninolytic enzymes also have the capability of degrading PAHs and a wide variety of organic pollutants, making WRF an attractive option for bioremediation purposes.

Lyr (1963) noted that *Trametes versicolor* was resistant to pentachlorophenol (PCP) and that the extracellular oxidase of that fungus dechlorinated PCP. This was the study that gave the early clues that WRF could be used to degrade chlorinated compounds. Other studies on fungal depletion of pentachlorophenol include Unligil (1968) and Duncan and Deverall (1964). However, the approach in those papers was directed towards the protection of wood, not towards the degradation of wood preserving agents.

One of the earliest environmental biosanitation techniques using WRF was developed to decolorize kraft pulp liquid effluents which contain large amounts of chlorinated phenols (Huynh *et al.*, 1985, cited in Bennett and Olmstead, 1992). Other early works reported the capacity of WRF to degrade recalcitrant organic pollutants like PAHs, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), tetrachlorodibenzodioxins

(TCDDs), lindane and certain PCBs (Bumpus *et al.*, 1985; Eaton, 1985). Compounds that may be degraded by WRF (especially *Phanerochaete chrysosporium*, the most widely studied WRF) include ACs and PACs, chlorinated aromatics and polyaromatics, non-aromatic chlorinated compounds (pesticides like lindane and DDT), biopolymers and lignin models, triphenylmethane dyes (Bumpus and Aust, 1987; Bumpus *et al.*, 1989). Synthetic azo-dyes (Paszczynski *et al.*, 1991), cyanides (Shah and Aust, 1993) and munitions (like 2,4,6-trinitrotoluene, TNT) (Fernando *et al.*, 1990) are examples of other environmentally important compounds which have been degraded by WRF. A partial list of specific representative compounds degraded by this fungus is presented in Table 2.4.1.

Degradation of DDT deserves special mention. Köhler *et al.* (1988) stated that ligninase or lignin peroxidase (LiP) did not play a role in DDT degradation, because DDT disappeared from the culture medium under non-ligninolytic conditions. However, these researchers only investigated the initial degradation of DDT and not the degradation (mineralization) of the initial metabolic products. Some DDT metabolites formed by *Phanerochaete chrysosporium* are 1,1-*bis*(4-chlorophenyl)-2,2-dichloroethane (DDD, which is a major dechlorination product of the fungus), 1,1-dichloro-2,2-bis(4-chlorophenyl)ethene (DDE, which is both produced and degraded by the fungus) and dicofol. It was thought that DDE was (but is not) a dead-end product of microbial DDT metabolism. In fact, it has not been shown that neither LiP nor manganese dependent peroxidase (MnP) play a role in DDT or DDE metabolism. The fungus has the constitutive capacity of degrading DDT to DDD (Bumpus *et al.*, 1993). It is now known that the reductive dechlorination (but not the mineralization) of DDT occurs under non-ligninolytic conditions of the fungus (Bumpus and Aust, 1987, cited by Barr and Aust, 1994).

Ligninolytic enzymes

Several families of basidiomycetes (*e.g.* Corticiaceae, Polyporaceae) classified as WRF, some ascomycetes and some actinomycetes have the enzymatic capacity of degrading lignin (Guillén *et al.*, 1990). Orth *et al.* (1993) concluded that lignin degrading peroxidases are present in nearly all ligninolytic fungi but may be expressed differently in different species. Also, there is substantial variability in the levels and types of ligninolytic enzymes produced by different WRF. It was believed that the initial lignin fragmentation was done by extracellular enzymes (Glaser, 1990). However, a recent review by Evans *et al.* (1994) states that electron microscope and biochemical studies of lignocellulose degradation by wood rotting fungi showed that:

1) ligninolytic enzymes are too large to penetrate undegraded secondary wood cell walls, and

2) the initiation of decay at a distance from the fungal hyphae must involve diffusible low-molecular mass agents. Possible examples are hydrogen peroxide, veratryl alcohol (3,4-dimethoxybenzyl alcohol, VA), oxalate, Fe^{2+}-Fe^{3+} and Mn^{2+}-Mn^{3+}.

The enzymes known to be involved in lignin degradation are:

Table 2.4.1. Environmental pollutants degraded by the WRF *Phanerochaete chrysosporium*[a]

polycyclic aromatic compounds
benz[a]pyrene
pyrene
anthracene
chrysene
chlorinated aromatic compounds
pentachlorophenol
4-chloroaniline
2,4,5-trichlorophenoxyacetic acid
polychlorinated biphenyls
dioxin
pesticides
DDT
lindane
chlordane
toxaphene
atrazine[b]
munitions
TNT
RDX (cyclotrimethylenetrinitroamine)
HMX (cyclotetramethylenetetranitroamine)
others
cyanides
azide
aminotriazole
carbon tetrachloride

[a] Barr and Aust (1994)
[b] Mougin *et al.* (1994)

- **Extracellular enzymes**: Peroxidases that use H_2O_2 as their electron acceptor (LiP and MnP (Rogalski *et al.*, 1991)); H_2O_2 producing enzymes (Datta *et al.*, 1991; Glaser, 1990); and Oxidases like laccase (= benzenediol:oxygen oxidoreductase, EC 1.10.3.2) (Rogalski *et al.*, 1991) which uses O_2 as its electron acceptor.

- **Intracellular enzymes**: The fragmented lignin is then assimilated by the fungi and intracellular enzymes complete the degradation to CO_2 and H_2O (Glaser, 1990). Tyrosinase (= monophenol, dihydroxyphenylalanine:oxygen oxido-reductase, EC 1.14.18.1) (Andrawis and Kahn, 1985) is an intracellular enzyme which serves this purpose.

Recently, Muheim *et al.* (1991) described another intracellular enzyme which plays a key role in lignin degradation. It is an aryl-alcohol dehydrogenase from *Phanerochaete chrysosporium*. De Jong *et al.* (1992) have also described a new extracellular enzyme produced by *Bjerkandera adusta*. It is a manganese inhibited peroxidase (MIP) which may also have oxidase activity.

It has been determined that most of the oxidative reactions involved in lignin biodegradation are one-electron oxidations of:

- Phenols (by laccase or MnP) yielding phenoxy-radicals, and

- Non-phenolic compounds (by LiP) yielding radical-cation intermediates (Muheim *et al.*, 1991). These radical cations may undergo further non-enzymatic reactions (Sannia *et al.*, 1991). Alpha and beta carbon bond cleavage, demethoxylation and aromatic ring cleavage are examples of spontaneous reactions triggered by the above-mentioned radicals (Muheim *et al.*, 1991).

The presence of different isoenzymes of ligninolytic enzymes (polymorphism) in different species and the expression of different isoenzymes in the same fungal species may depend on the growth conditions, the substrate and the presence of certain compounds, but primarily on genetic factors in the particular fungal culture.

The ligninolytic rates and patterns differ profoundly among a range of WRF. This correlates with different wood colonization strategies and developmental characteristics, as seen in ultrastructural studies of wood degradation by fungi (Rayner and Boddy, 1988). This could be attributable to the following factors:

- Softwoods and hardwoods have different degrees of compactness and their lignins contain different proportions of the three different types of phenylpropane monomer units which compose them. These lignins also differ in their types of intermonomeric linkages (Karlsson, 1992). This could be a reason for the preference of certain fungi for the different types of wood.

- Different ligninolytic enzymes and isoenzymes are present in different fungal species (*e.g.*, Linko, 1988; Guillén *et al.*, 1990).

- The presence of some isoenzymes in one species may be influenced by the growth substrate. Some fungi, such as *Phlebia radiata*, present the whole spectrum of enzymes (Rogalzki *et al.*, 1991). Others, like several species of *Pleurotus*, growing on a synthetic medium, do not express LiP (Guillén *et al.*, 1990). However, Linko (1988) reports 6 LiP isoenzymes for the same species also grown in a synthetic medium. García *et al.* (1987, cited by Daniel *et al.*, 1991) did not

detect LiP in wood degraded by this species. The major enzyme expressed by *Phanerochaete chrysosporium* growing on a lignocellulosic medium (aspen wood from a mechanical pulp) is an isozyme of MnP. LiP and glyoxal oxidase were also present but in lower quantities (Datta *et al.*, 1991). No reports on laccase production by this species have been found.

- The difference in wood degradation strategies may also be attributable to differences in the mechanisms by which each ligninolytic enzyme group acts. For example, chelated Mn^{+3} [formed from Mn^{+2} by MnP] is a ligninolytic agent. Its molecular weight is less than 500 daltons, while LiP has a molecular weight of more than 40,000. This permits the Mn^{+3} to penetrate into the lignocellulose matrix more deeply than LiP (Forrester *et al.*, 1990).

VA is a metabolite of lignin degradation. Since LiP oxidizes it to veratraldehyde, this aldehyde has been used to monitor the activity of this enzyme. Aryl alcohol oxidase (AAO = aromatic alcohol oxidase, E.C. 1.1.3.7, or veratryl alcohol oxidase = VAO) performs the same function on this substrate but via another mechanism. Therefore, additional tests must be performed to distinguish between oxidation by LiP and VAO. Guillén *et al.* (1990) found that *Pleurotus eryngii* can oxidize VA in the absence of H_2O_2. Forrester *et al.* (1990) report that Mn^{+3} from *Lentinula edodes* also oxidizes VA. VAO has been found in some fungi like *Trametes versicolor*, *Pleurotus sajor-caju*, *Bjerkandera adusta* (Muheim *et al.*, 1990) and *Fomes lignosus* (Guillén *et al.*, 1990). *Phanerochaete chrysosporium* does not produce this enzyme. However, its LiP is very similar to that of *Bjerkandera adusta* (Muheim *et al.*, 1990).

Some studies have shown that *in vitro* polymerization occurs when isolated lignins react with laccases or peroxidases. Therefore, Muheim and co-workers (1991) suggest that during lignin catabolism by WRF the balance of peroxidase-induced polymerization and depolymerization is shifted towards the latter by the fungal uptake of the smaller fragments. They also state that this stresses the importance of reductive reactions. Hydrogen peroxide produced by LiP may also play a role in this balance (see Cai and Tien, 1991). Tonon and Odier (1988) mention that LiP is inactivated by hydrogen peroxide. This inactivation is protected by VA.

Glaser (1990) explains some details on the mechanisms of specific LiP and MnP isozymes from *Phanerochaete chrysosporium* involved in lignin (and xenobiotics) oxidation. Enzymatic and non-enzymatic mechanisms involved in xenobiotics degradation and mineralization by WRF are discussed in depth by Barr and Aust (1994).

Enzyme activity assays

Detecting the presence of ligninolytic enzymes and monitoring their activity during degradation tests of lignin or xenobiotics are very important. Ligninolytic enzyme activity tests have proven to be useful in screening fungi with potential xenobiotic degradation capacities (Sack and Günther, 1992).

Different methods have been developed to detect the presence of and/or to determine the activity of ligninolytic enzymes. Many methods are presented in Wood and Kellogg (1988). Some general examples are:

- Colorimetric methods (*e.g.*, Marr, 1979; Niku-Paavola *et al.*, 1990),
- Chromatographic methods (*e.g.*, Felici *et al.*, 1985),
- Electroblotting methods (*e.g.*, Hruskocy and Flurkey, 1986),
- Immunological methods (*e.g.*, Daniel *et al.*, 1991).

PAH metabolic pathways

Cerniglia and Heitkamp (1989) compiled some general statements on our present knowledge of microbial degradation of PAHs (mainly in aquatic environments). These are:

1) A wide variety of bacteria, fungi and algae have the ability to metabolize PAHs.

2) Hydroxylation of unsubstituted PAHs always involves the incorporation of molecular oxygen.

3) Prokaryotic microorganisms metabolize PAHs by an initial dioxygenase attack to *cis*-dihydrodiols which are further oxidized to dihydroxy products.

4) Eukaryotic microorganisms use monooxygenases to initially attack PAHs to form arene oxides followed by the enzymatic addition of water to yield *trans*-dihydrodiols.

5) PAHs with more than three condensed benzene rings do not serve as substrates for microbial growth (with the exception of the pyrene utilizing bacterium mentioned in the section of PAH-degrading microorganisms), although they may be subject to cometabolic transformations.

6) Fungi hydroxylate PAHs as a prelude to detoxification, whereas bacteria oxidize PAHs as a prelude to ring fission and assimilation.

7) Many of the (bacterial) genes coding for PAH degradation are plasmid associated.

8) Lower weight PAHs such as naphthalene degrade rapidly, while higher weight PAHs like benz[a]anthracene and benzo[a]pyrene are quite resistant to microbial attack.

9) Most rapid biodegradation of PAHs occurs at the water/sediment interface and degradation rates can be influenced by environmental factors.

10) Microbial adaptations can occur from chronic exposure to PAHs.

11) There are higher biodegradation rates in PAH-contaminated sediments than in pristine sediments.

Mammalian cells initiate PAH metabolism similarly to some fungi (Sims and Overcash, 1983), except that fungi do not attack the K-region of PAHs and the stereochemistry of fungal metabolism is the opposite of that of mammalian metabolism (Cerniglia, 1981, 1984). The metabolic pathways and enzymes involved in PAH oxidation by prokaryotic and eukaryotic microorganisms are illustrated in Figure 2.4.3.

Cerniglia *et al.* (1992) reviewed our present knowledge of the different fungal enzymes involved in xenobiotics degradation. Two major groups of cytochrome P-450 enzymes are present in fungi (yeasts and filamentous fungi). These enzymes are involved in the degradation of many aliphatic and aromatic hydrocarbons. Laccase from soil fungi and from WRF is involved in hydroxy-naphthalenes degradation. LiP is also one of the fungal ligninolytic enzymes involved in organic pollutants (*e.g.*, PAHs) degradation. Figure 2.4.4 shows some of the enzyme systems, from different groups of fungi, involved in the degradation of organic pollutants.

Grimmer *et al.* (1992) studied the degradation of several PAHs by soil fungi and WRF (*Pleurotus ostreatus*). They identified the sites of initial attack by each group of fungi (Figure 2.4.5). This might vary from species to species since not all fungi present the same range of ligninolytic enzymes. Additionally, Bergbauer (1991) mentions that the substrate affinity and enzyme regulation among WRF may be different.

Fungi like *Cunninghamella elegans* initially oxidize PAHs to metabolites, which are mainly detoxification products, while WRF like *Phanerochaete chrysosporium* can produce carbon dioxide from certain PAHs in pure culture (Cerniglia *et al.*, 1992). Further metabolic steps by different groups of microorganisms, and fungi in particular, are reviewed by Cerniglia and Heitkamp (1989) and Sims and Overcash (1983), and Cerniglia *et al.* (1992).

Mechanisms WRF use to degrade PAHs

Some mechanisms used by *Phanerochaete chrysosporium* to degrade organic pollutants were reviewed by Barr and Aust (1994). They are briefly summarized below.

- One-electron oxidation of chemicals to free radicals by LiP and MnP using hydrogen peroxide. The peroxidases catalyze the free radical reactions directly (through simple electron transfer) or indirectly. After directly reacting with LiP, the chemicals may undergo carbon-carbon bond cleavages, benzylic alcohol oxidations, demethylations, hydroxylations or dimerizations. Veratryl alcohol (VA) is a compound produced by the fungus (from glucose or lignin), and it takes part in the indirect mechanism. It is oxidized by LiP to a cation radical, which can then oxidize other chemicals. It is a low molecular weight and freely diffusible oxidant resulting in an increased ability of the fungus to degrade lignin.

- Highly oxidized compounds (such PCBs, chlorinated phenols and nitroaromatic munitions) must first be reduced to undergo further oxidation. One mechanism

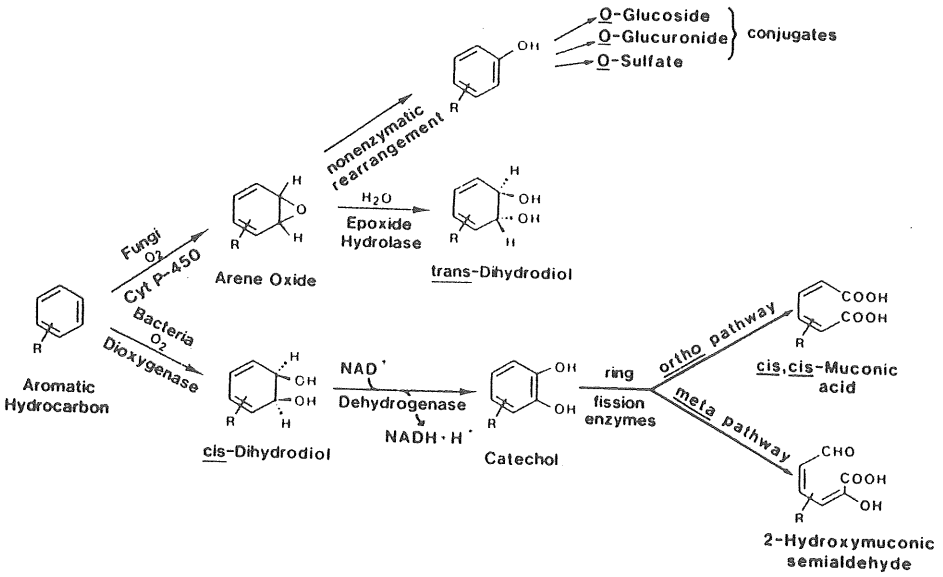

Figure 2.4.3. Metabolic pathways utilized by prokaryotic and eukaryotic microorganisms for the oxidation of PAHs (Cerniglia, 1984)

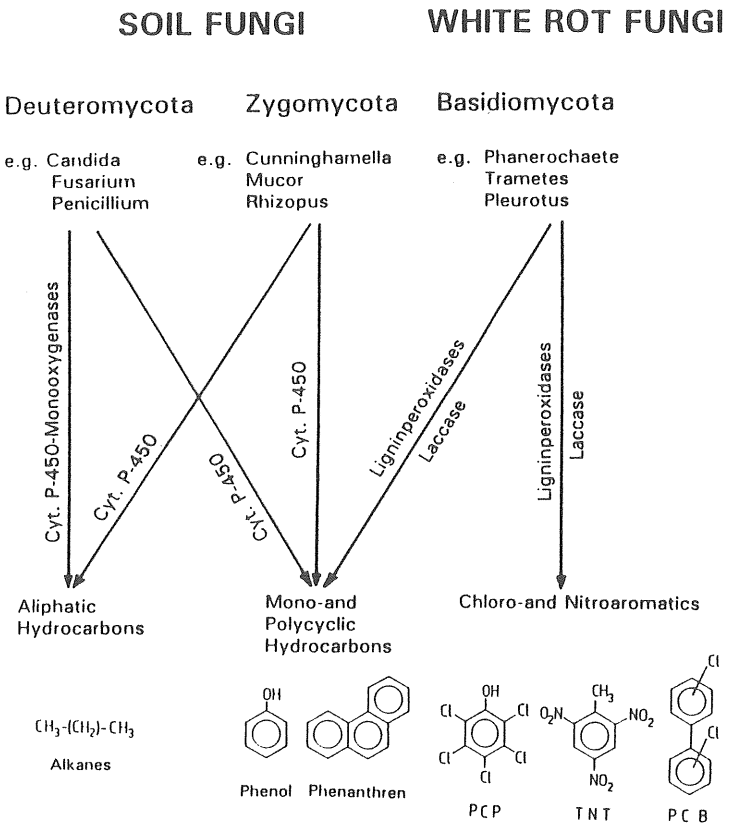

Figure 2.4.4. Examples of compounds degraded by enzymes produced by different groups of fungi (Fritsche, 1992)

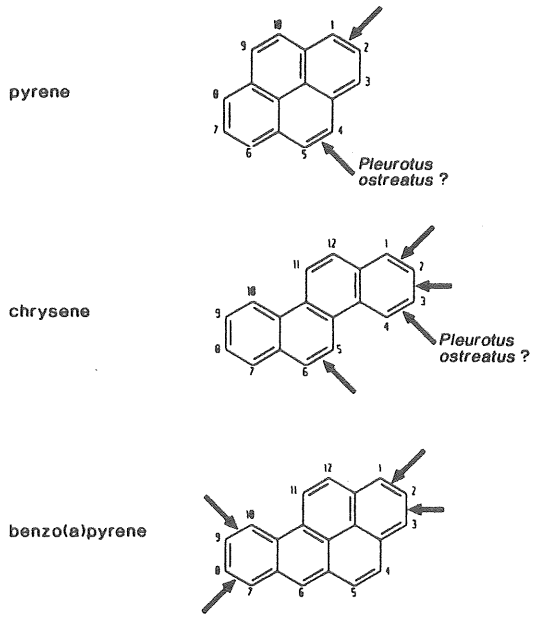

Figure 2.4.5. Site of attack of selected PAHs by soil fungi (*Cunninghamella elegans* and *Mucor circinelloides*) and the WRF *Pleurotus ostreatus* (Grimmer *et al.*, 1992)

involves VA, in which radicals formed by this oxidizing agent may result in reduction. Oxalic acid, also produced by this fungus, may be an electron donor in the LiP-dependent reductive pathway as well. MnP may also present reductive mechanisms using hydroquinones as electron donors. The hydroquinones are formed by extracellular quinone reductase, also produced by *Phanerochaete chrysosporium*. The plasma membrane potential mechanism may also play a role in the reductive mechanisms. The maintenance of a proton gradient is used by the fungus, for example, to control the extracellular medium pH or to reduce organic pollutants by excreting protons.

- Production of active oxygen species such as superoxide, hydroxyl radical and hydrogen peroxide. Veratryl alcohol may produce molecular oxygen from hydrogen peroxide. Hydrogen peroxide may also be oxidized to the superoxide anion radical by the veratryl alcohol cation radical, by reacting with molecular oxygen. Dismutation of the superoxide anion results in the evolution of oxygen. This regulation of the oxygen concentration in the environment around the fungus is important because the degradation of chemicals is more efficient in an oxygen rich environment. LiPs also seem to be involved in the production by the fungus

of hydroxyl radicals. This molecule reacts through electrophilic addition with different compounds such as halogenated highly oxidized compounds (similarly to Fenton's reagent). Hydroxylation of compounds including PCBs and chlorinated phenols by Fenton's reagent releases chloride. It is possible that the hydroxyl radical is involved in the degradation of several pollutants by this fungus.

As might be expected, microbial PAH degradation products are more soluble than the parent PAHs. It has been observed that most PAH intermediate metabolites are less mutagenic than the original forms, although small amounts of carcinogenic derivatives have been detected (Cerniglia *et al.*, 1985). This change in solubility also affects their mobility in soil and migration to groundwater, as well as their uptake, accumulation and further metabolism by aquatic organisms.

Fungal PAH degradation products

Many reviews have been published on the metabolism of PAHs by different groups of fungi (*e.g.*, Cerniglia and Heitkamp, 1989; Cerniglia *et al.*, 1992; Sutherland, 1992). Sutherland (1992) reviewed the formation and toxicity of fungal PAH metabolic products. Most of the knowledge in this field comes from research on mold-like fungi such as *Cunninghamella elegans*, and to a lesser extent from others such as *Aspergillus* spp., *Rhizotocnia solani* and several species of yeasts and macrofungi. Many fungi oxidize unsubstituted PAHs to arene oxides, *trans*-dihydrodiols, quinones, tetralones and phenols. Phenols and trans-dihydrodiols may be further metabolized (detoxified) by conjugation with sulfate, glucuronic acid, glucose or xylose. Only a few fungi appear to mineralize PAHs completely to CO_2 (Cerniglia *et al.*, 1992; Sutherland, 1992). One disadvantage of obtaining conjugates (detoxification products) is that some microorganisms in the environment may have enzymes that remove the conjugative groups, restoring the toxicity of the compound. Therefore, ring cleavage is a more desirable result than detoxification (Sutherland, 1992). The most studied WRF is *Phanerochaete chrysosporium* and quinones, glucose conjugates and unidentified products are among its PAH degradation products (Sutherland, 1992). PAH methoxy derivatives formed by *Pleurotus* have been also identified (Grimmer *et al.*, 1992).

Most compounds formed after fungal degradation of PAHs are usually less mutagenic (Sutherland, 1992) and more polar than the parent compound (Bumpus, 1989). However, some of the minor metabolites from unsubstituted or certain methylated PAHs may be mutagenic (Sutherland, 1992). Loske *et al.* (1990) and Hansson (1991) do not report the formation of degradation products by *Pleurotus* in field and small scale experiments to degrade PAHs in soil.

Much of the knowledge on biodegradation of organic pollutants comes from experiments with individual or mixed radio-labelled compounds (*i.e.*, radiorespirometry) in liquid culture. Although the physical and chemical conditions are different from those in the environment, valuable information has been obtained (degradation pathways, metabolite formation, degree of mineralization, etc.). Some differences are also observed if the degradation takes place in a liquid medium or in soil.

Liquid medium experiments

In general, liquid culture experiments have shown fast PAH degradation rates and provided useful information such as degree of mineralization and metabolic product formation. The remaining amount of certain monoaromatic and PAHs (representing groups of compounds with different solubility, complexity and recalcitrance) after degradation by *Phanerochaete chrysosporium* in liquid culture during 60 days were: p-cresol, 47.6%; 2-methylnaphthalene, 8.8%; phenanthrene, 14.7%; benzo[a]pyrene, 13.8% (Aust and Bumpus, 1987).

Bumpus (1989) studied the degradation of 22 radio-labelled PAHs in liquid culture by *Phanerochaete chrysosporium*. He observed a range of 70-100% degradation in 27 days. In the case of phenanthrene, which was almost completely metabolized, 7.7% was transformed to CO_2, 25.2% was found in the aqueous fraction, 11% was found in the particulate fraction (fungal mycelium), and 56.1% was recovered in the solvent fraction. In the latter, 91.9% was composed of polar metabolites.

Loske *et al.* (1990) studied the degradation of radio-labelled anthracene by *Trametes versicolor*, *Bjerkandera adusta* and *Pleurotus ostreatus*. The mass balance is not clearly shown, but it is mentioned or represented graphically that most of the radio-labelled carbon was found in the water soluble phase (*ca.* 40-50%), between 4-10% was transformed to carbon dioxide, 10% remained in the organic phase of the media and some was incorporated into the mycelium.

WRF have also been tested for degrading organic pollutants in slurry reactors (Stroo *et al.*, 1989). Different kinds of reactors with immobilized fungal mycelium have been designed and tested to treat polluted liquids (*e.g.*, Lewandowski *et al.*, 1990; Venkatadri and Irvine, 1993).

Solid state experiments

The behavior of PAHs in soils has already been discussed, and should be considered in biodegradation experiments in soil matrices.

The fate of ^{14}C in radio-labelled benzo[a]pyrene, a 5-ring PAH, was determined after a small scale degradation experiment with *Phanerochaete chrysosporium* in two types of soil (Qiu and McFarland, 1991). The major fate of the contaminant carbon was humification (*i.e.*, polymerization to soil humic components) rather than conversion to CO_2. They also found that the presence of clay-metal-SOM (soil organic material) favors the bound residue formation and limits the fungal PAH mineralization. This could be explained by the fact that when isolated lignins react with peroxidases and laccases *in vitro* polymerization occurs (Haemmerli *et al.*, 1986, cited in Muheim *et al.*, 1991). This could happen with PAHs and humus during soil bioremediation with WRF. After binding irreversibly to humus, the pollutants may continue the natural course of humus metabolism without endangering or damaging the biota. However, this irreversibility (mobility in the soil) needs further study (Qiu and McFarland, 1991). Additionally, Bartha (1981) (cited in Loske *et al.*, 1990) mentions that aromatic and

chlorinated compounds added to soil with normal microbiota end up in the high molecular weight humic acid fraction.

Contaminated soil bioremediation using WRF

The first research groups to study the degradation of recalcitrant organic compounds by WRF in the USA and Germany are Chang *et al.* (1985), Aust and co-workers (1986, 1987) and Hüttermann *et al.* (1988). They filed national and international patents on the processes. Many publications have followed their patents, refining the evidence, finding limitations and new applications. Full scale treatment using WRF has not yet been carried out.

WRF have been used to degrade organic pollutants in soil (solid phase). Some authors call the technique simulated land treatment (Stroo *et al.*, 1989) or composting (Qiu and McFarland, 1991). Factors that could affect the performance of WRF in polluted soils are: temperature, soil moisture, pH, toxicity of the chemicals and inoculum density (Stroo *et al.*, 1989; Lamar and Dietrich, 1990).

Some advantages, disadvantages and limitations of using WRF for this purposes are discussed in detail by Aust (1990). Some relevant points are (Aust, 1990; Loske *et al.* 1990, Stroo *et al*, 1989):

- Lignin is composed of very large molecules, so it must be initially degraded by extracellular enzymes before it can be metabolized intracellularly to carbon dioxide. Something similar may happen with large PAH molecules,

- The persistence of many environmental pollutants is attributable to their insolubility or binding to soil, which makes them poorly available for soil microorganisms. WRF have evolved an enzyme system capable of degrading lignin, which is also insoluble. It will probably be more a question of whether or not the enzymes reach the target contaminants through the soil/water matrix,

- Ligninolytic enzymes are non-specific, so a wide variety of compounds may be degraded by this free radical generating system,

- The degradation of the target compounds (pollutants) is independent of their concentration, because they are degraded cometabolically. Since there is no need of specific inducers for these enzymes, lignocellulose or other substrate may be supplied as the source of carbon and energy, activating the ligninolytic enzymes secretion by the WRF,

- No new organisms are introduced to the environment, since WRF occur in nature,

- The toxic substances are degraded by the fungus and the its growth substrate is converted to humus, after which the resident microorganisms are reactivated,

- WRF are generally good competitors in occupying a habitat. When introduced in soils pregrown in a substrate, they have the advantage of using the substrate

selectively, owing to its lignocellulosic character. Evidence exists suggesting that the ligninolytic degrading system has antimicrobial properties. However, *Phanerochaete chrysosporium* is not a good competitor in the soil environment,

- When the WRF substrate is consumed, the degradation stops and the WRF dies since it cannot compete with the resident microorganisms,

- The restored soils may safely be used for any purpose,

- Agricultural wastes used as substrate for the WRF are recycled,

- WRF technology is promising both in soil and liquid reactor systems. Soil may also be treated *in situ*, (but only) for surface contamination (Barr and Aust, 1994).

Some practical obstacles of this technology, especially if it is considered for use in large scale treatment are: keeping the fungus with an adequate amount of nutrients and at an optimum temperature. *Phanerochaete chrysosporium* has a quite high optimum temperature (39°C) (Stroo *et al.*, 1989). The water content of the soil is easy to control and the problem of temperature may be reduced if the warm months are selected. Another problem is the supply of oxygen to the soil for the growing fungus (Glaser, 1990). Some solutions to this problem have been discussed before, as well as the problem of the tridimensional distribution of the pollutants in the field.

A reason for using WRF for PAH-contaminated soil clean up is that they are capable of degrading PAHs with 5 or more rings. This has been achieved in aqueous conditions (Bumpus *et al.*, 1985) and in some solid phase (contaminated soil) (Stroo *et al.*, 1989; Loske *et al.*, 1990) and slurry bioreactors (Stroo *et al*, 1989). However, Davis *et al.* (1993) found in a solid phase field test that the fungus *Phanerochaete sordida* failed to degrade these PAHs. They attribute this to the scavenging for ligninolytic enzymes by the most abundant PAHs (which had fewer than 5 rings) and to a too low fungus to soil (1:10) ratio. Good results have been obtained by Stroo and co-workers (1989) at 25 and 50% fungus inoculum addition and 25% by Morgan *et al.* (1993). Despite all this, WRF can be used to improve the on site bioremediation of wood-preserving wastes (Stroo *et al.*, 1989).

Fungi screening for PAH degradation may be an expensive and time-consuming task. Sack and Günther (1992) found a good correlation between *in vitro* ligninolitic enzymes activity and *in vitro* PAH degradation. They suggest that the screening of efficient PAH-degrading fungi may be done through simple ligninolytic enzyme activity assays. This approach could probably also be used to monitor PAH degradation.

Immobilized enzymes

The production, purification and immobilization of pure ligninolytic enzymes would be an option for bioremediation methods, with the advantages that no fungi have to be cultured and kept viable, and that the immobilized enzymes could be used several times. Georgiou (1989) mentions that, through genetic engineering it could be possible to produce large amounts of these enzymes, which would be an improvement, since

present methods are very expensive. Phenanthrene was metabolized by cultures of *Phanerochaete chrysosporium* but not by the purified lignin peroxidase H8 isozyme alone. Other enzymes like monooxygenases are probably involved in the metabolism of some PAHs by WRF (Sutherland, 1992).

Other fungi and organic pollutant degradation

Bacteria and fungi are among the organisms usually isolated from creosote-contaminated soils (Line, 1977). Both groups of organisms play an important role in the decontamination of terrestrial environments. There is 5 to 10 times as much fungal biomass in soils than bacteria, but the fungal activity is one order of magnitude less. Also, the aerobic degradation of xenobiotics by bacteria is well studied, but not that of fungi (Fritsche, 1992).

Fungi from diverse taxonomic groups have been tested for degradation of organic pollutants. The most widely studied fungi are the zygomycete *Cunninghamella elegans* and the basidiomycete *Phanerochaete chrysosporium*. The first fungus is a common decomposer of organic matter, and has been found in soil, air, nuts, dung and decaying vegetation (Hesseltine and Ellis, 1973). The second was first isolated from stored conifer wood chips (Burdsall, 1985). *Sporotrichum pulverulentum* is the name of the imperfect state of the latter fungus. It has not been found if their properties differ, but they should be considered as the same species.

Despite the success achieved using WRF in xenobiotics degradation, it has been shown that other groups of fungi isolated from diverse environments have comparable or better ability than WRF to degrade organopollutants. There is increasing evidence that soil fungi from polluted and agricultural soils may have PAH-degrading capacities comparable to those of WRF. Many papers at the DECHEMA conference (1992) deal with contaminated soil resident fungi. Despite this, some authors (Davis *et al.*, 1993) consider the performance of competent contaminated-soil resident microorganisms unpredictable. The success of the resident microorganisms could be due to site-specific conditions and to the particular microbiota of the site. Their failure when tested as controls might be attributable to the need for conditions different from those needed by the WRF.

Some findings in support of WRF as more efficient degraders of xenobiotcs than controls (stimulation of the resident microorganisms) are:

- Stroo *et al.* (1989) observed that the WRF treatment was significantly better than the control (stimulation of resident microorganisms) in a beaker scale experiment to degrade PAHs in soil. However, some degradation did occur in the control, even of the 6-ring PAHs. Unfortunately the statistical procedure by which they reached this conclusion is not clearly shown.

- Hansson (1991) obtained better PAH degradation from *Pleurotus* sp. than in the control (straw + pasteurized soil). In this experiment he used 5 kg pasteurized gas-works soil mixed with fungus-colonized straw in plastic bags. The degradation percentages for individual PAHs obtained after 27 weeks are presented in Table

2.4.2. 3 and 4-ring PAHs were degraded even by the controls, despite the soil was previously pasteurized; this could have weakened or inactivated some of the resident microorganisms. The heavier PAHs were more difficult to degrade (even by the WRF). For 5 and 6-ring PAHs, he obtained 23-78% degradation with the fungal treatment and 0% degradation with the control.

- Loske *et al.* (1990) obtained 80% total PAH degradation (also including 5- and 6-ring compounds) after 16 weeks with *Pleurotus ostreatus*. However, the PAHs are not analyzed individually and no results are presented regarding the controls.

These authors do not mention formation or detection of intermediate degradation products.

The degradation of monoaromatic systems by soil fungi has been well studied. Although there is only limited information on the degradation of PAH by soil fungi (Sack *et al.*, 1992), some researchers have found that certain soil fungi may be as efficient as WRF to degrade PAHs. Some of these reports are:

- During wood decomposition, an ecological succession occurs. Certain molds start the decay, followed by WRF and later by other molds (Rayner and Boddy, 1988). Bergbauer (1991) tested many ecological groups for syringic acid degradation. This compound is released during angiosperm wood lignin degradation. He found that some molds degraded syringic acid totally and that WRF oligomerized it to 1,3-dimethyl-pyrogallol and to other methylated and hydroxylated derivatives. The implications of this study are that while WRF are responsible for the primary attack of wood and are the most successful lignin degraders, other microorganisms (present during or at later stages of the process) are adapted to using certain monomeric lignin degradation products. These ecological differences should be taken into account when selecting adequate fungi for specific biotechnological uses.

- The fungus *Cladosporium* sp. isolated from a wood-treatment plant sludge pond, was tested by Borazjani *et al.* (1989) *in vitro* to degrade PAHs and PCP. Its growth was not inhibited at concentrations as high as 5500 μg/ml of PAHs but PCP inhibited it at 10 μg/ml. However, it is a potential fungus for use in the bioremediation of wood-treating wastes. The fungus *Cladosporium resinae* (formerly of the genus *Amorphotheca*) is known as the kerosene fungus (Line, 1977) and has been isolated from jet fuel.

- Two species of WRF, 3 species of brown-rot fungi (BRF), one species of litter decaying fungi and a soil mold isolated from a PAH-contaminated site, were tested *in vitro* for their individual cometabolic PAH-degrading ability by Sack and Günther (1993). The extracellular fungal enzyme activity correlated well with PAH disappearance from the media, so this procedure could be used to screen PAH-degrading fungi. The WRF were good PAH degraders but good PAH-degrading abilities, comparable to WRF, were also observed in one of the BRF and in the mold. The BRF showed no extracellular enzymes activities, although it degraded most PAHs at high rates. It is known that some BRF excrete laccase

Table 2.4.2. Percentage degradation of some PAHs by *Pleurotus* sp. and by the control after 27 weeks (Hansson, 1991)

compound	% degradation after 27 weeks of	
	fungal treatment	control
phenanthrene	92	90
anthracene	94	72
fluoranthene	71	27
pyrene	76	8
benz[a]anthracene	79	5
chrysene	63	51

but this enzyme was not detected when the PAH-degrading BRF was tested. Probably monooxygenase activity was induced by the PAHs (and other unknown enzyme systems probably also took part). The authors also suggest that soil molds isolated from contaminated sites deserve further research, both regarding their PAH-degrading ability and their metabolic product identification.

- Although BRF play a limited role in lignin degradation, the BRF *Lentinus lepideus* was able to use some ACs as sole source of carbon. Collett (1992) implied that this fungus could be used as a bioremediation agent for such compounds.

- WRF are claimed to be the only organisms to degrade PAHs with 5 or more rings. However Grimmer *et al.* (1992) showed that the zygomycetous fungi *Cunninghamella elegans* and *Mucor circinelloides* (the latter isolated from a PAH contaminated soil) degraded PAHs such as benzo[ghi]perylene (6 rings) and coronene (7 rings) 60% and 40%, respectively, after 30 days in submerged culture (cometabolically).

- Martens and Zadrazil (1992), screened several species of WRF and BRF for PAH (radio-labelled benz[a]anthracene and pyrene) degradation in PAH-spiked agricultural soil. They noticed that one group of fungi colonized the soil and another did not. The interesting result was that PAH degradation was higher in the non-colonizing group. They suggested that the non-colonizing fungi, which degraded the substrate virtually to disintegration provided nutrients (straw degradation products) and/or enzymes from lysing mycelium growing in the straw, stimulating the resident microorganisms. In contrast, the colonizing fungi

may have depressed these organisms, owing the limited degradation only to the colonizing fungi. For benz[a]anthracene 15-25% and 40-60% $^{14}CO_2$ evolution was reported (for colonizing and non-colonizing groups respectively) and for pyrene <20% and 60-70%. With respect to pyrene, even the uninoculated controls liberated more $^{14}CO_2$ (32%) than the colonizing fungi.

Other approaches to xenobiotic biodegradation or soil bioremediation, basically using fungi

- Many yeast species (*e.g.*, *Saccharomyces* and *Candida*) have proven to degrade organic pollutants (Cerniglia *et al.*, 1992).

- Insect fungal simbionts are a promising source of detoxifying enzymes for treatment of agricultural materials and environmental contaminants (Shen and Dowd, 1989; Dowd, 1992).

- Donnelly and Fletcher (1993) propose the use of certain ectomycorrhizal fungi as on-site bioremediation agents of 2,4-dichloro-phenoxyacetic acid (2,4-D), atrazine and PCBs. The xenobiotics degradation capacity of the plant roots and its associated microorganisms (rhizosphere) is a related topic. Anderson *et al.* (1993) present a critical review on this topic. Compounds such as pesticides, oil residues, VOCs and PAHs have been degraded using this method.

- Some marine fungi have the capacity to use atrazine and certain hydrocarbons as their sole source of carbon and energy (Schocken *et al.*, 1982; Kirk and Gordon, 1988). Aquatic fungi which metabolize petroleum hydrocarbons (Cerniglia *et al.*, 1992) could be useful for remediation of oil spills in marine environments. Other aquatic lignin-degrading (saprobe) fungi have been isolated from streams (Shearer and Bodman, 1983; Rossi *et al.*, 1983), groundwater environments (Kuhen and Kohen, 1988) and marine environments (Newell and Fell, 1982). These could probably also degrade some aromatic hydrocarbons. Park (1972) presents some methods for isolating fungi from aquatic environments.

- In countries situated in extreme latitudes, where there is cold weather for a large part of the year, it could be ideal to find efficient PAH-degrading microorganisms which tolerate low temperatures. One group is investigating this with hydrocarbon-degrading bacteria (Persson and Hahn-Hägerdal, 1992).

- Some actinomycetes (*Streptomyces*) degrade lignin quite efficiently (Borgmeyer and Crawford, 1985) and have been used to degrade azo-dyes by Paszczynski *et al.* (1991).

Other environmental biotechnology applications of WRF and other fungi

The papers in Arora *et al.* (1992) review several environmental biotechnology applications of fungi. Some examples are presented below.

Several groups of fungi have been used in the cellulose and paper industry. The work of Walden *et al.* (1986) explains the cellulose production processes in detail: the different pulp preparation processes, products and wastes obtained, toxicity and other characteristics of the waste, some waste treatment methods, etc. Verma *et al.* (1988) made a complete ecological characterization of paper mill effluents. WRF have been used for:

- Biological pulping and bleaching (Balasubramanya *et al.*, 1989; Reid and Pace, 1991, cited in Ziomek *et al.*, 1991).

- Treatment of the chlorinated bleaching effluents (even using other microorganisms) (Bergbauer *et al.*, 1991; Eriksson and Kolar, 1985; Kolankaya *et al.*, 1989; Lundquist *et al.*, 1977; Milstein *et al.*, 1988; Prouty, 1990). Immobilization of fungal mycelium has been used by several researchers to degrade liquid wastes. An example is the MyCOR method (patented by Chang *et al.*, 1985), used to decolorize Kraft pulp liquor.

- Degradation of the solid waste from the pulping process, producing single cell protein (SCP) and/or animal feed (Kannan *et al.*, 1990; Sandhya *et al.*, 1990; Swaminathan *et al.*, 1989), and even edible mushrooms safe for human consumption (Kannan *et al.*, 1990; Mueller *et al.*, 1985, 1984; Mueller and Gawley, 1983).

Degradation of hazardous organic compounds in contaminated waters:

- In waters contaminated by explosives manufacturing effluents (Tsai, 1991; Fernando and Aust, 1991; Fernando *et al.*, 1990).

Other applications:

- To test the biodegradability of newly synthesized products as a requirement for considering their production (*e.g.*, azo dyes, Paszczynski *et al.*, 1991).

- Production of feed for ruminants and food (edible mushrooms, single cell protein) for humans from agricultural wastes (Ortega-Cerrilla *et al.*, 1986; Calzada *et al.*, 1987; Cahal, 1989; Zadrazil and Kamra, 1989; Royse *et al.*, 1991).

2.5 Additional discussion

Analytical aspects of PAHs

It should be noted that not only the 16 PAHs belonging to the US EPA list of priority pollutants or the 11 Swedish Environmental Protection Board (SNV) listed PAHs mentioned here are important environmentally, or from the health point of view. Additional creosote components which should be considered in bioremediation and analytical protocols are biphenyl, dibenzofuran, carbazole, benzofluorenes, certain methylnaphthalenes, methylfluorenes, methylphenanthrenes and methylanthracenes,

which account for about 24% of the composition of creosote (Lorenz and Gjovik, 1972).

Some S- (sulphur-containing) analogues of PAHs have been identified as carcinogens and more toxic to *Daphnia magna* than the corresponding PAHs. Additionally, some minor components which have been also identified as potent mutagens and carcinogens are certain aromatic amines and azaarenes (Sundström *et al.*, 1986).

Not only total PAHs but also individual PAHs should be included in analytical reports for bioremediation purposes.

In the laboratory, the soil may be cleaned and mixed near to homogenization, but this is not probable in the field. It would be nearly impossible and too expensive to homogenize the soil at a contaminated site. This implies that very high variations in pollutant concentrations may be found. The development of sampling and analytical methods, as well as an adequate statistical approach, are needed to solve this problem commonly reported by researchers.

Remediation technologies

Results from treatability studies must be carefully analyzed and interpreted, since their success does not always indicate or predict success in the field. One example is the degradability study made by Ellis *et al.* (1991) before the full scale *in situ* bioremediation project at Blekholmstorget.

It cannot be said that one particular technology is the best or the worst, or that bioremediation should totally replace physical or chemical methods. The remediation technique of choice for contaminated soils is site specific, depends on the nature of the pollutant(s) and the characteristics of the soil.

Through gaining an understanding of the complicated degrading mechanisms used by WRF we will be able to manipulate their environments and their metabolisms. Then we will be able to use them and to apply this technology successfully (Barr and Aust, 1994).

There seem to be two divergent bioremediation trends. The first supports the use and superiority of WRF. The second supports the use of resident microorganisms. Despite the fact that these organisms may show unpredictable performance, they deserve further thorough research because they represent a simpler technology which could cost less and be more "natural" than the WRF approach.

Concluding remarks

- The complex multidisciplinary context of bioremediation needs the participation of a team, so as to avoid obtaining incomplete views.

- Toxicity assays with aqueous extracts of contaminated and biologically treated soils might be more significant than assays with organic solvent extracts, since in

real sites rain leachates, and not organic solvent leachates, reach groundwater. It is always useful to complement these assays with chemical analyses.

- The remediation treatment of choice is site specific.

- WRF are efficient PAH and organopollutant degrading agents of potential use for bioremediation. However, other fungi from contaminated soils, and other biological methods like composting, merit further study.

- WRF are capable of degrading a range of environmental pollutants, so their general use may be tested almost anywhere where organic pollutants are a problem. The results of most laboratory and pilot scale experiments are promising, but full scale projects still have to be approved by regulatory agencies like the US EPA.

- It cannot be said that one method is better than another. Combinations of different processes might be the best choice.

3 Feasibility studies

3.1 Introduction

Preliminary experiments have been carried out to test the ability of white-rot fungi of the genus *Pleurotus* to degrade PAHs in creosote-contaminated clayey soil, and to develop a methodology. Problems dealt with were, for example, making an adequate soil homogenization, applying the fungus to the soil and developing adequate analytical (chromatographic and toxicological) methods. Replicate sample variation (PAH concentration) is a major problem that remains to be solved.

Some experiments were done before the literature review was completed (and new information is still being obtained). This review may help to explain some of the results obtained in this experimental section. However, many hypotheses and suggestions arise in the discussion of each experiment. Some of these questions may be answered in future work.

In the first experiment, *Pleurotus pulmonarius* was tested. PAH degradation was evaluated after 3 months by GC and HPLC analyses, with changes in peak areas observed. Trials for toxicological analysis with Microtox were also done. Despite the limited resources and the exploratory nature of the experiment, the results obtained were positive and further development was considered worthwhile.

In the second experiment, *Pleurotus ostreatus* was used for the same purpose, but in "beaker" scale. This time, duplicate samples were analyzed every month for 4 months and degradation dynamics were considered. Some compounds were identified and quantified using GC-MS. PAHs were degraded, but large amounts of intermediate degradation products were formed by the fungal treatment. Although it is not known if these degradation products are intermediate or final, they are referred to here as intermediate degradation products. A single beaker containing soil and autoclaved straw gave higher degradation than the fungal treatment, and generated no detectable intermediate degradation products.

In the third experiment, the PAH degradation ability of *Pleurotus pulmonarius* was compared with that of resident microorganisms stimulated by nutrient supplementation with wheat straw. The better degradation rates from the straw treatment was confirmed, as well as the generation of intermediate degradation products in the fungal treatment.

Three additional fungi were used for experiment 3, but owing to the unavailability of analytical equipment, the PAH degrading efficiencies of these fungi are not yet known. These fungi were *Pleurotus ostreatus* (the same one used in experiment 2), and 2 strains of *Phanerochaete chrysosporium* (a cellulase-less mutant which is a very good lignin degrader, and a wild strain. Both strains were tested by Johnsrud and Eriksson, 1985).

3.2 Experiment 1

3.2.1 Methodology

Fungus

Pleurotus pulmonarius INRA 3300 (National Institute of Agricultural Research, Bordeaux, France) was used. It was obtained, pregrown on 20 kg wheat straw without N supplementation (usually soya flour) and with $CaCO_3$ as buffer, from a commercial mushroom grower (Frillesås Champignon AB, Frillesås, Sweden). The culture was 40 days old when it was applied to the contaminated soil.

Contaminated soil

100 kg of creosote-contaminated clayey soil (CS) were obtained from a hazardous waste landfill (owned by Reci Industri AB, Göteborg, Sweden). It originated from a mixed waste contaminated site where creosote cistern train cars had been washed during a period of several years. Some areas of this site contained creosote in concentrations greater than 200 mg/kg dry weight, which exceeds the Swedish maximum allowable PAH concentration in soil.

Sample processing and analysis

Coarse material (nails, rocks, glass) were removed from 10 kg of contaminated clay soil. The soil was then homogenized in a construction materials testing machine (Tecnotest, SNC di Coughi & C., Italy). All but 500 g was used for the degradation test. The 500 g were stored in a plastic bag at 8°C in the dark for extraction trials and for physico-chemical analyses. The unprocessed soil was kept in a closed plastic container at room temperature (18-23°C) under an air extracting hood.

Physicochemical characteristics of the soil

The following data was obtained as indicated:

- Water content: drying overnight at 105°C, weight loss determined with a semianalytical balance.

- Hydrolytic pH (in duplicate): suspending 10 g of CS in 100 ml ultrapure water stirring for 1 hour (Jackson, 1958).

- Organic matter content (in triplicate): ashing dried samples at 550°C for 3 hours in a furnace. Weight losses were determined with an analytical balance.

- Lead and copper determination: 0.1020 g of ashed CS were digested in 4 ml of *aqua regia* (1:3 mixture of concentrated nitric and hydrochloric acids) on a hot plate for 5 min. After cooling it was brought to volume (50 ml) with ultrapure water. The Pb and Cu contents were determined, using a Perkin Elmer 603

atomic absorption spectrophotometer with a graphite oven.

- Dry sieving and particle size distribution: As indicated in, *e.g.*, Marshall and Holmes (1979).

Experiment setup

Fungus-colonized straw was placed above and under several CS layers ("cakes" about 3 cm thick and 20 cm in diameter) in a closed plastic bag and then placed inside a 12-liter plastic container (Figure 3.2.1). It was kept in a room with constant temperature and air humidity (20°C and 60%) for 90 days. No additional irrigation was provided.

PAH extraction and analysis

Very little creosote could be extracted from the CS in its original form, so it was mixed with sand (1:1 wt) in a mortar before extraction, to make it more permeable, resulting in better creosote extraction.

10 g of mixed untreated CS and 20 g of mixed treated CS were extracted with 250 ml dichlromethane (Merck, *pro analysi*) for 4 hours (this is the same sample size and extraction time, but 2.5 times the volume of solvent used by Mueller *et al.*, 1991) in Soxhlet extractors. Samples of the treated CS were taken from the surface and from the middle of the soil "cake". The extracts were transferred to 250 ml volumetric flasks, brought to volume with dichloromethane after the extracts reached room temperature, and stored in the dark at -18°C. The extracts were analyzed without further treatment (*i.e.*, separation, purification, dewatering, concentration).

Soil samples from day 0 and day 90 (surface and middle of the soil cake) were analyzed using GC-FID, and samples from day 0 and day 90 (middle of the soil cake) were analyzed using HPLC. These sampling sites were selected on the basis of color differences of the soil at each place.

The sand and some extraction thimbles were extracted with dichloromethane, and the extracts analyzed using GC. No disturbances were detected, nor did the solvent have any interfering impurities. Some fungus-colonized straw which had been in contact with the CS for 90 days was also extracted, and only traces of the most abundant PAHs were detected. It has been determined by other researchers (*e.g.*, Davis *et al.*, 1993) that wood chips mixed with creosote-contaminated soil may absorb insignificant amounts (not more than 1%) of the analytes in the soil.

The chromatographic equipment and conditions used were the following:

GC

Varian 3300 with FID; Shimadzu RC 5A integrator; SE 30 capillary column (non-polar stationary phase), 30 m x 0.32 mm ID, 0.25 μm film thickness; 0.4 μl injection, split/splitless injector (closed during injection); relay closed between 0.01 and 1 minute; 20 ml/min split; and 6 psi carrier gas (He) pressure. The temperatures were: injector

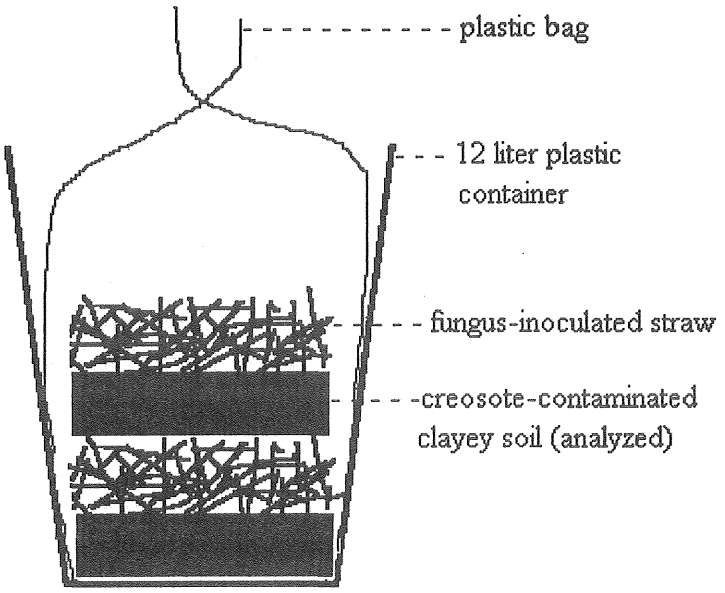

Figure 3.2.1. Setting of the creosote-contaminated clayey soil and fungus-inoculated straw for experiment 1

temperature, 200°C; detector temperature, 270°C; column temperature program: 35°C for 1 minute then 30°/minute to 120°C, then 10°/minute to 270°C, maintain until no more peaks come out. The FID was set with attenuation 1 and range 11.

HPLC

Waters-Millipore model 600E System Controller; model 700 satellite WISP (autoinjector); Waters 400 Scanning Fluorescence Detector; LC-PAH (Supelcosil) column, 25 cm x 4.6 mm ID, stationary phase particle size, 5μm; flow rate, 1.3 ml/sec (= 2 mm/sec); initial solvent ratio 60% water to 40% acetonitrile, during 5 minutes; then to 100% acetonitrile in 25 minutes, and maintain for 40 minutes. Fluorescence detector wavelength, 243 nm; injection volume, 5 μl.

Selected GC peaks and all the HPLC peaks with similar retention times were compared. The data was normalized to peak area units per gram of soil.

Acute toxicity of the CS

As a preparatory exercise, the acute toxicity of the untreated CS was determined using the Microtox solid phase standard test (with a Microtox M500 system, Microbics Corporation). To determine the toxicity attributable to heavy metals or organic compounds, a small amount of CS was extracted with a 1:1 mixture of benzene:pentane (BDH Chemicals Ltd., and Riedel-de Haën AG, both analytical grade, respectively) to

extract the organics, and another with EDTA (10^{-4}M) to extract the metals. As a control, unextracted CS was processed and tested.

For each extraction, small amounts of CS were placed in 50 ml glass tubes with enough extractant to cover the sample, and then shaken with a vortex for 3 minutes. The CS which adhered to the walls of the tubes was scraped off, dried for 24 h in an oven at 65 °C and weighed. Then the preparations were tested with the Microtox solid phase test following the manufacturer's instructions. The control CS was also shaken in a tube with the vortex, but without extractant. The soil adhering to the walls of the tube was used. Soil dilutions were prepared in 4 tubes, diluting by half each time. The EC50 is expressed in mg soil/ml Microtox solution. It was obtained by plotting the log gamma value (y axis) versus log concentration (x axis); the EC50 is 10^x where x = log concentration and y = 0. The gamma value, the ratio of the amount of light lost to the amount of remaining light (Tarkpea *et al.*, 1986), is determined as follows:

$$\Gamma = \frac{\text{blank reading - test reading}}{\text{test reading}}$$

3.2.2 Results and discussion

Soil characteristics

The CS used in this experiment was identified as a sandy-silty clay. It was very sticky and had a very strong and irritating smell, owing to the creosote. The brownish clay turned grey after homogenization.

The CS had the following physicochemical characteristics:

- water content: 32%
- hydrolytic pH: 6.7
- organic matter content: 11%
- heavy metals: Cu, *ca.* 100 μg/g ashed sample; Pb, *ca.* 1500 μg/g ashed sample.

It should be noted that the organic matter content value includes the creosote.

Fungal growth on the CS

The fungus grew on the surface of the CS cake, surrounding it completely. The CS cake was cut in half and no fungal mycelium was observed inside it. The color of the top 2-3 mm of the CS layer was lighter than the inner part of the layer, which was very similar to the original soil with respect to color and odor.

Reisolation of the fungus from the soil could be done in future experiments, to determine whether it is still viable after several months of degradation in the CS.

Enzyme activity assays (*e.g.*, Sack and Günter, 1993) could also be used to check if the fungus is still excreting enzymes. It is difficult to do these assays in the organic medium, since most tests are colorimetric. Perhaps aqueous extracts of the substrate and soil could recover some enzymes which then could be tested in liquid medium after removing suspended particles. Changes in pH (due to fungal activity) should also be determined at different times during the experiment.

Although it has been shown that a range of heavy metals taken up (translocated) by fungi from the substrate are toxic to fungi like *Pleurotus* (*e.g.*, Jain *et al.*, 1989, 1988), the metals present in the soil seemed not to affect the fungus used in the present experiment. This is probable since the fungus covered the soil "cakes", and fruit body primordia began to develop near the end of the experiment. However, a more detailed examination of the fungal physiology and full development of fruit bodies would be necessary, in order to confirm whether or not the heavy metals caused toxic effects to the fungus.

PAH degradation determination

No standards were available at the time the chromatographic analyses were made, so the compounds were not identified but were roughly quantified. The area of chromatographic peaks (GC and HPLC) from untreated CS extracts (day 0) were compared with those of treated CS extracts (day 90) (Figures 3.2.2 and 3.2.3). A reduction in the peak area of most compounds from fungus-treated soil extracts was observed with both chromatographic methods. The degradation was higher on the surface of the CS cake than in the middle.

Since neither autoinjector nor internal standard was used, the peak areas may vary more than 10% in repeated GC analysis. However, the HPLC system used had an autoinjector. In this way, the injection volumes are more reliable, more constant and more readily comparable than manual injection volumes. It was determined in later experiments that an automated injector yielded variations in concentration of no more than 1%. A number was assigned to peaks with similar retention times. After running some PAH solutions in later work, the tentative identity of some of the 7 selected GC compounds could be:

1) 2-methylnaphthalene, 2) dibenzofuran, 3) fluorene, 4) (unidentified compound), 5) phenanthrene, 6) anthracene and 7) carbazole.

The 16 priority PAHs (and probably other creosote components) elute in different order in GC than in HPLC (US EPA, 1986) so no tentative identity was done with HPLC data.

Some peaks detected by GC at day 0 and at day 90 (middle soil) were not detected in the day 90 surface soil chromatograms (not shown). Degradation took place to a lesser extent in the middle of the soil cake than in the surface soil. This could be explained by limited penetration of the mycelium or fungal enzymes in the clay, or less oxygen availability than on the surface.

Considering that clays generally have a hydraulic conductivity of 10^{-9} m/s, the enzymes in the aqueous phase could travel only 0.8 cm in 90 days (if the CS was saturated, and with water circulation). The structure of contaminated clays must be altered by mixing with sand or other bulky material to increase permeability, obtain better extraction, better penetration of the mycelium and better degradation rates. The formation of small soil aggregates could give a larger contact area for the growth of fungal mycelium and reduce the distance the enzymes or mycelium have to travel.

GC peaks 1 and 4 showed higher concentrations at day 90 surface soil. This could also be attributable to variations in the distribution of the creosote in the soil. Note that the HPLC peak 7 increased significantly after fungal treatment (Figure 3.2.3). It could be an intermediate degradation product, but was already present at day 0. The percentage reduction of selected and total peak areas and the range of percentage reduction of selected peaks is presented below (Table 3.2.1).

Although the GC column and temperature conditions were not optimal, good peak resolution was obtained. After raising the final column temperature to 320°C (far above the maximum tolerable temperature) more compounds were eluted (data not shown).

The HPLC system had an autoinjector, so the retention times were more reproducible in repeated injections with the HPLC than with the manual injections in the GC. Small variations in the retention times in the HPLC determinations could have occurred, owing to changes in the pressure of the system. The HPLC provided more information than the GC, since more compounds were eluted with the HPLC method used. The HPLC analysis shows that all the peaks (except peaks 2, 7 and 13) decreased after fungal treatment. The later peaks could be 5- and 6-ring PAHs, meaning that even these compounds were degraded by the fungal treatment.

One limitation with the HPLC method used is that non-fluorescent compounds are not detected. In preliminary HPLC tests with the fluorescence detector (not shown) fluorene was hardly detected, despite the fact that it was present in a concentration in which other PAHs were clearly detected. This is not in accordance with Dong (1993), who mentions that acenaphthene is the only US EPA priority PAH which is not fluorescent. Perhaps a different wavelength should have been used.

It could be useful to run GC analyses as a complement to HPLC analyses, since some compounds not detected by the fluorescence detector may be detected by FID or MS, or vice versa. A UV detector could also be used with the HPLC system to detect non-fluorescent compounds. Ideally, a GC with a mass spectrometer (MS) should be used since very small concentrations may be detected. An additional advantage is that compounds may be identified by their mass spectra.

Microtox

The EC50 (mg soil/ml Microtox solution) of the different untreated CS preparations were:

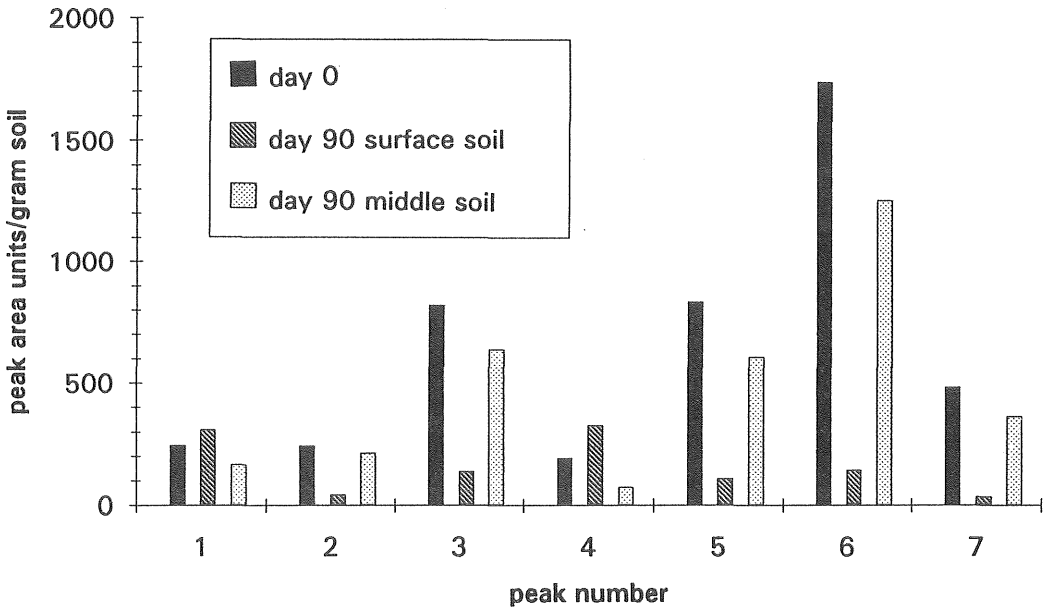

Figure 3.2.2. Change in selected GC peak areas after 90 days of fungal treatment (experiment 1)

- unextracted soil: 0.5;
- benzene-pentane extracted soil: 0.6;
- EDTA extracted soil: 5.6

Despite the EC50 value of the EDTA-extracted soil was obtained by extrapolation (since the EC50 value was situated above the maximum soil concentration tested), it is clear that the acute toxicity of the soil was attributable to the heavy metals, under the conditionsof determination. Removal of these metals with EDTA greatly decreased the toxicity of the CS, reflected by an increase in the EC50. After organic solvent extraction and drying, some PAHs might have been extracted and others volatilized, leaving in the soil the metals. That is probably why the organic solvent extracted sample still showed an EC50 similar to that of the original soil. It could be worth extracting a sample with both EDTA and organic solvent and testing its toxicity.

Some problems and limitations of the Microtox solid phase test used in this experiment were later found by Carlson and Morrison (1994). Therefore, it would be worthwhile to adopt the changes suggested by these authors in future Microtox solid phase tests.

The PAHs in the contaminated soil may not have been available for the bacteria in the aqueous medium, owing to their hydrophobicity and owing to the fact that they bind to

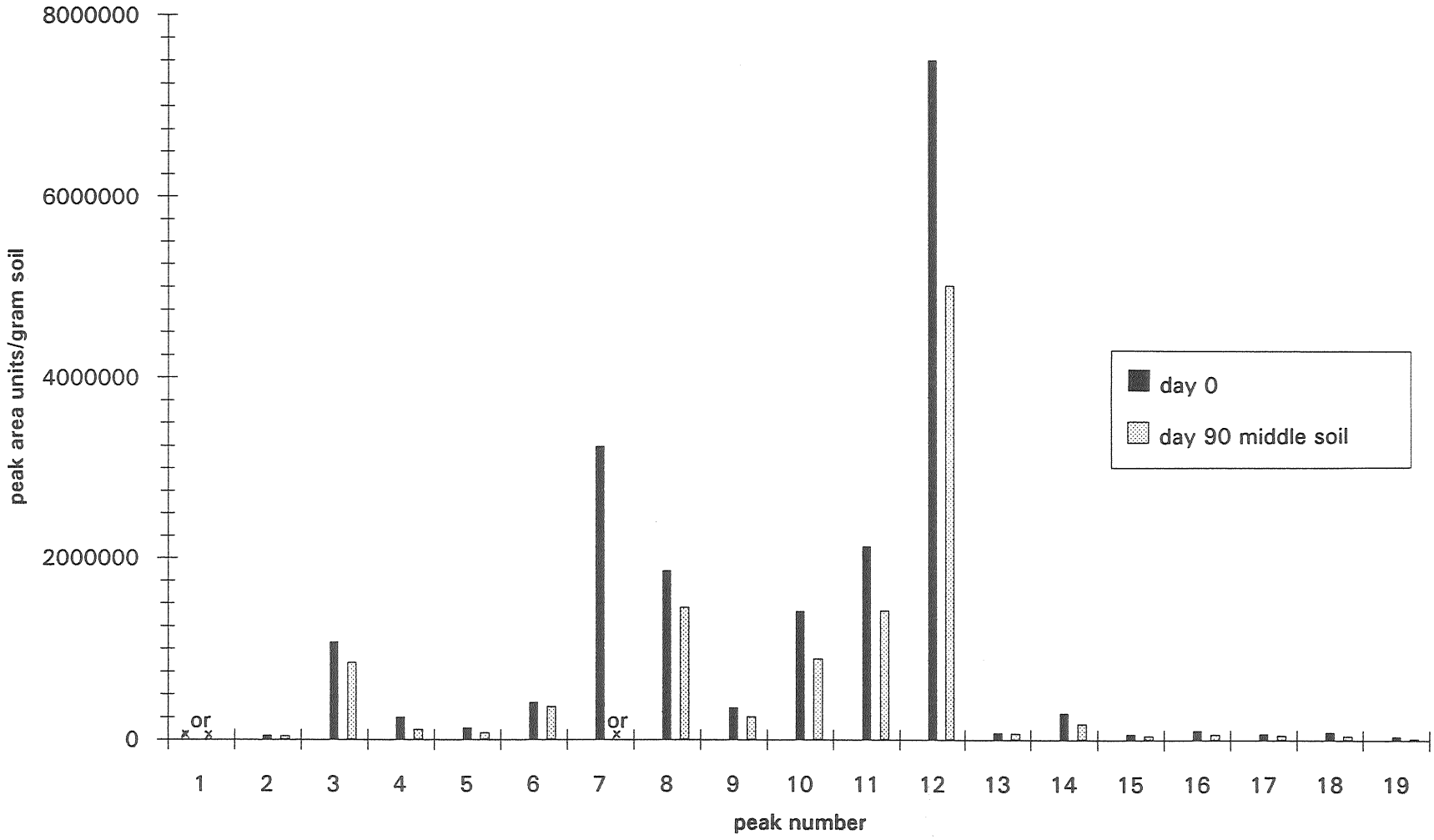

Figure 3.2.3. Changes in selected HPLC peak areas after 90 days of fungal treatment (middle soil, experiment 1) (or= peak(s) over range)

Table 3.2.1. Percentage reduction and range of percentage reduction of the sum of relevant peak areas, and percentage reduction of total peak areas (experiment 1)

	reduction (%) in peak area of:		
	relevant peaks		all peaks
	sum	range	sum
GC			
surface soil	76	83-93	46
middle soil	27	12-62	24
HPLC			
middle soil	31	4-62	30

soil particles (Weissenfels *et al.*, 1992b). It has been mentioned before that such compounds should be tested as an extract in organic solvent. Acetone could be suitable for use in the liquid phase Microtox test (*e.g.*, Tarkpea *et al.*, 1986). Although some low molecular weight PAHs are acutely toxic, mutagenicity tests should also be considered, owing to the genotoxic nature of many PAHs (especially the ones with high molecular weight) (Heitkamp *et al.*, 1988) and of some of their intermediate or final degradation products.

3.2.3 Conclusions and future work

- The structure of the contaminated clay must be altered to increase its permeability,
- It seems that no major amounts of PAHs are uptaken by the fungal mycelium or its substrate after being in contact with PAH contaminated soil for 3 months,
- Only acute toxicity attributed to the presence of heavy metals in the CS could be determined by the Microtox method used,
- The chromatographic analyses suggest that some creosote PAHs are degraded by *Pleurotus pulmonarius* in the contaminated soil.

In future experiments:

- GC will be used as the analytical method, since access to an MS is possible,

- Standards should be used to make some kind of quantification,

- It is necessary to adopt the modifications proposed by Carlson and Morrison (1994) to improve the Microtox solid phase test,

- A Microtox method must be developed to determine the acute toxicity of hydrophobic PAHs,

- Smaller scale experiments should be carried out to increase the probabilities of evener degradation, and not only on the surface of the samples.

3.3 Experiment 2

3.3.1 Introduction

The PAH degradation experiment was repeated on a smaller scale, with monthly extracts of duplicates to observe the degradation dynamics of the PAHs. The intention was to confirm that the fungus degrades PAHs in soil, and to test possible controls and alternative treatments. A GC-MS and internal standard were used this time, so compounds could be identified and quantified in some way.

3.3.2 Methodology

Fungal strain and inoculum preparation

The fungus used was *Pleurotus ostreatus* (Somycel 3200) and was obtained from a professional mushroom grower (Frillesås Champignon AB, Frillesås, Sweden). The inoculum was prepared as follows:

The fungus was propagated on 2% malt extract agar plates (DIFCO). Next, 2 cm^2 young mycelium + underlying agar were subdivided into 8 pieces and transferred to 100 ml of 2% malt extract liquid medium in 250 ml Erlenmeyer flasks with gauze and cotton plugs. After 4 weeks of static incubation, in the dark and at room temperature (20-23°C), the developed mycelium was homogenized with a tissue homogenizer (Ultra Turrax TP 18/10, IKA Werk, Germany) at 10,000 rpm for 30 seconds in an ice bath. The contents of the Erlenmeyer flasks were mixed after homogenization and kept under continuous stirring. 1 ml mycelium homogenate was transferred under aseptic conditions with a pipette (from the same depth) to 9 ml of 2% malt extract liquid medium in test tubes. After 2 weeks, the developed mycelium and growth medium were emptied onto 10 g (dry weight) autoclaved (120°C for 20 minutes) chopped wheat straw (1-2 cm long), which had been placed in clean plastic bags after autoclaving. Two weeks later, the contents of the bags were applied to the soil which had previously been placed in autoclaved beakers.

Contaminated soil preparation

Ten kg of soil, from which coarse material (rocks, nails, wood) was previously removed, was thoroughly mixed with sand (10% coarse, 57% medium and 37% fine

particles) (1:1 by weight, dry sand to wet soil ratio) in a construction material mixing machine (Tecnotest, S.N.C. di Coughi & C., Italy). The mixture was kept in a plastic bag in the dark at 4°C until used. The soil was mixed with sand to make it more porous, because in preliminary tests the fungal mycelium did not penetrate and creosote PAHs were not easily extracted from the unmodified clay. In large scale bioremediation this mixing could be very expensive, since thousands of cubic meters of soil are involved.

Degradation test

The "reactors" were 100 ml beakers with 20 g of fungus-colonized straw added to 20 g of soil-sand mixture (Figure 3.3.1). This is referred to below as fungal treatment. The reactors were placed in a humidity chamber (Figure 3.3.2), together with one beaker containing contaminated soil and uninoculated autoclaved straw (straw treatment) and another with only soil (soil alone), to obtain additional information. The degradation conditions were 80-90% relative air humidity, 20-23°C, and darkness. No irrigation was provided.

Analytical procedure

Duplicates were randomly selected for extraction at 0, 50, 106 and 141 days. The soil samples (which weighed between 20 and 23 g) were extracted without further treatment (drying, etc.). All the soil in the beaker was transferred to a cellulose extraction thimble and extracted for 4 h by Soxhlet with 250 ml analytical grade dichloromethane (Riedel de Häen). The extracts were kept in the dark at -20°C in volumetric flasks until the end of the experiment in order to analyze them simultaneously. A solution of phenylhexane (Sigma) was added to the extracts as an internal standard before adjusting the final volume.

The GC-MS used was a Varian Ion Trap Saturn II C system, with a 30 m x 0.32 mm i.d., DB5 (non-polar) column (J&W Scientific Co.). The manual injection (0.4 μL) was splitless and, after 1 min, the split was opened (1:20). The temperature programs were: injector 260°C, ion trap 220°C, column 35°C to 160°C at 20°/min, then to 300°C at 10°/min. The scanning frequency was 1 scan/sec.

Qualitative and quantitative determinations

The compounds were identified from the reference mass spectra in the MS library (NIST) and from GC retention data of reference compounds (except for the dimethylnaphthalenes and the intermediate fungal degradation products).

The total ion mass of each compound was used for quantitative determination. The concentration (in arbitrary units) of a compound was obtained from dividing the total ion current of the compound by that of the internal standard. The figure for relative abundance in Table 3.3.1 was calculated from the proportion between the total ion current of a compound and the sum of the ion currents of all selected ions.

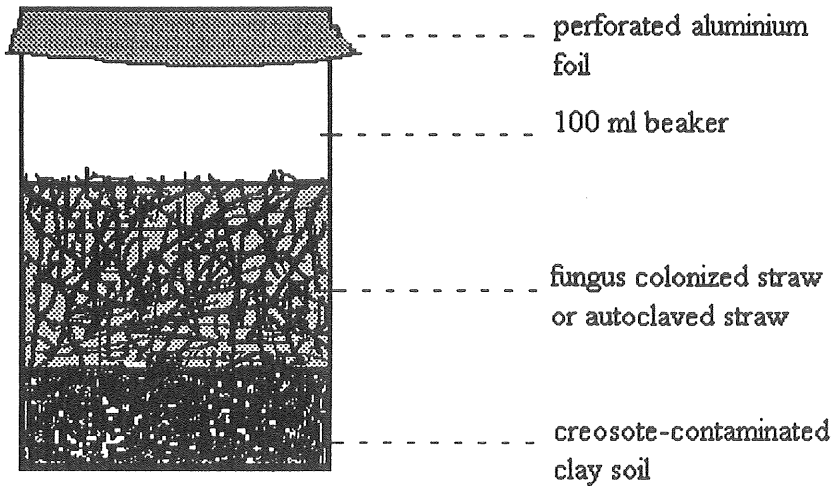

Figure 3.3.1. Setup of PAH biodegradation experiment 2

Figure 3.3.2 Humidity chamber used for degradation experiments 2 and 3

3.3.3 Results and discussion

Quantitative and qualitative determinations

A total of 33 compounds were detected in this particular type of creosote with the GC-MS method used. 16 of these compounds (accounting for 93% of the total concentration) were identified. 2 compounds, which were not present at the beginning (probably 9H-fluoren-9-one, which is a suspected tumorigenic compound, and an unidentified one), increased in concentration after day 50 of fungal treatment. The identified compounds, their relative abundances (as percent of total concentration of compounds) and the percentage degradation of individual compounds at the end of fungal treatment are presented in Table 3.3.1. Data for the beakers with uninoculated straw and soil alone are also included in this table since the results were considered of interest.

PAH degradation

If all the 33 compounds initially present were considered, 68% degradation occurred with the fungal treatment and 85% with the straw treatment. Considering only the 16 identified compounds, 68% and 86% degradation occurred with each respective treatment (Table 3.3.1). With respect to the total unidentified and minor compounds (not shown), 65% and 73% reductions was obtained with these treatments.

With respect to the soil alone, it can be seen that the concentration of total identified compounds increased by 10% after 133 days, and that the concentration of 2-ring PAHs (naphthalene, methylnapthalene and biphenyl) decreased by about 50% (Table 3.3.1). This was probably due to volatilization or biodegradation. It has been reported that PAHs with more than 2 rings show negligible volatilization from soils (Davis *et al.*, 1993) and that biodegradation affects PAH persistence in soil more than volatilization (Sims and Overcash, 1983). The disappearance of large PAHs can thus be attributed to biodegradation. The increase in concentration of other compounds in the "soil alone" sample (*e.g.*, anthracene increased by 28% and fluoranthene by 65%) could be explained by variations in the creosote content and homogeneity in the soil.

The ranges of percentage degradation of individual identified and unidentified (minor) compounds were:

- Identified compounds: 49-100% with fungal treatment and 60-100% with straw treatment, and

- Minor compounds: 17-90% with fungal treatment and 7-100% with straw treatment.

In Figure 3.3.3 the degradation dynamics of the total identified compounds are illustrated. A tendency towards reduction of concentration may be seen, with the exception of an increase of concentration between days 50 and 106, possibly owing to sample inhomogeneity. Further sampling time would be needed to observe if whether or not the degradation continues. There was no intention of finding the optimum

Table 3.3.1. Identified PAHs (in order of elution) and their relative abundance at day 0 and percentage degradation after 141 days of fungal treatment and 133 days of straw treatment and no treatment (experiment 2)

Ccompound	Relative abundance (%)	Percentage degradation after treatment with:		
		fungus	straw	soil alone[a]
naphthalene	1.0	74	87	54
methylnaphthalene	3.0	69	88	49
1,1-biphenyl	0.6	73	86	41
dimethylnaphthalene	0.7	100	100	28
dimethylnaphthalene	0.8	65	85	7
dibenzofuran	3.3	64	86	7
fluorene	11.3	71	87	0.5
dibenzothiophene	1.3	62	88	0
phenanthrene	13.1	60	86	+7
anthracene	39.6	70	86	+28
carbazole	11.6	69	87	2
methylphenanthrene	1.9	73	88	70
fluoranthene	1.8	57	79	+65
pyrene	1.5	59	83	+13
benz[a]anthracene	0.4	70	60	+33
chrysene[b]	1.0	49	72	+28
total, selected PAHs	92.9	68	86	+10

[a] + sign denotes an increase in concentration
[b] heaviest PAH detected

degradation conditions, but it is possible that additional supplementation of fungus, nutrients and oxygen could accelerate the process.

Two compounds increased in concentration after day 50. They accounted for 24% of the total concentration at the end of the experiment. They are probably fungal

intermediate degradation products since they were not found in the straw treatment or in the soil alone. Their formation dynamics are illustrated in Figure 3.3.4. Further sampling and analyses would be necessary to determine if these degradation products are intermediate or final.

Two pairs of PAHs detected have the same molecular weight, similar structure, mass spectra and retention times (phenanthrene/anthracene and benz[a]anthracene/chrysene). In some cases, both compounds were taken as one by the computerized integrator so manual integrations were done in these cases. This could be avoided if better peak resolution could be obtained.

Straw treatment

The straw treatment was 20% more efficient than the fungal treatment and no intermediate degradation products were detected. The addition of straw to the soil might have provided nutrients directly or indirectly. The latter could result from microbial straw degradation products, or from disintegrating microorganisms growing in the straw, stimulating resident microorganisms in the contaminated soil to degrade PAHs cometabolically.

Evidence has been presented by some researchers indicating that agricultural or contaminated soil resident microorganisms may have PAH degradation abilities comparable with WRF (Martens and Zadrazil, 1992; Sack and Günther, 1993). The full scale bioremediation of creosote-contaminated sediments with composting technique reported by Seman and Rydergren (1991) also suggests that these relatively simple methods of bioremediation (and PAH degrading soil fungi) merit further research.

3.3.4 Conclusions and future work

The fungus *Pleurotus ostreatus* degrades PAHs in the creosote-contaminated soil. This treatment, under the conditions used here, generates large amounts of intermediate degradation products. These compounds must be characterized chemically and toxicologically, and tested for further degradability. It also seems that nutrient stimulation of resident microorganisms (straw treatment) degrades PAHs as efficiently or better than the fungal treatment, and generates no detectable intermediate degradation products. Straw treatment must be carefully repeated to confirm its behavior. Finally, it seems that no major reduction in total PAH concentration occurs in soil without fungal or nutrient supplementation treatment.

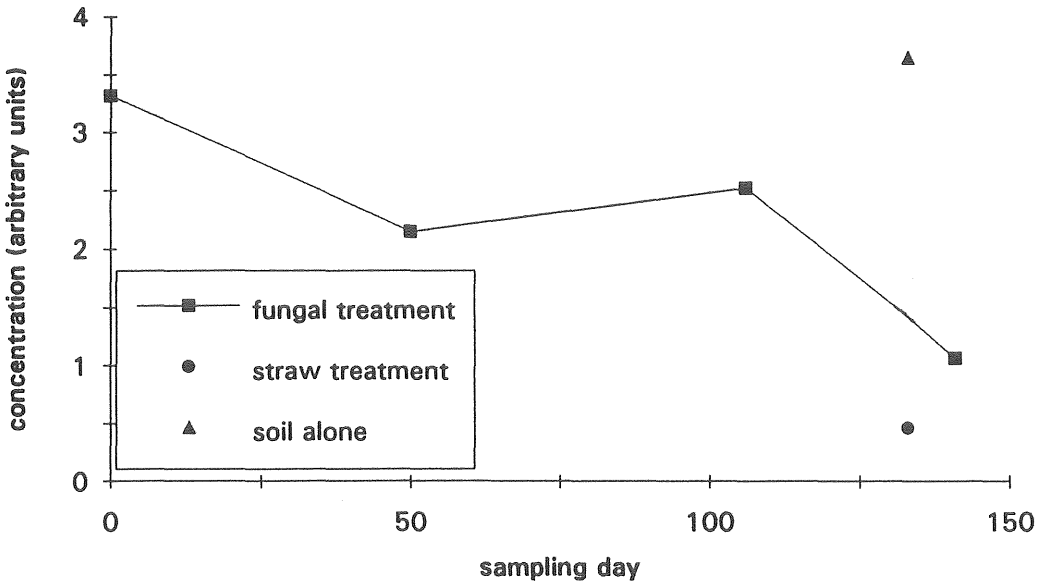

Figure 3.3.3. Degradation dynamics of total identified PAHs after 141 days of fungal treatment and their concentrations (in arbitrary units) after 131 days of straw treatment and no treatment (experiment 2)

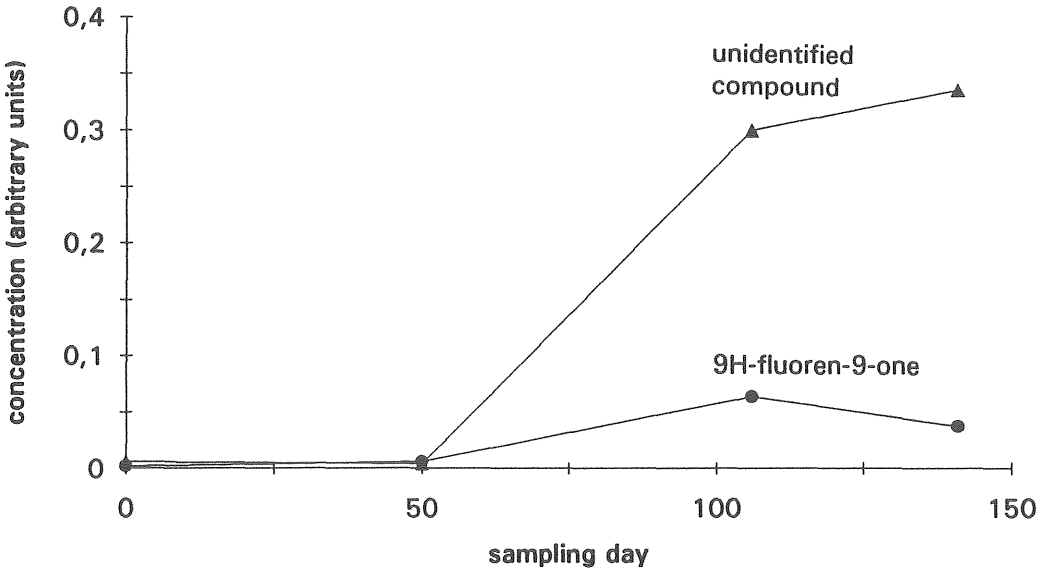

Figure 3.3.4. Formation dynamics of the fungal intermediate degradation products (experiment 2)

4 Recent work (experiment 3)

4.1 Introduction

A publishable approach, with interesting results, came out of the preliminary studies. The main purpose of the present experiment was to confirm that straw treatment more efficiently degrades PAHs than the WRF method (under the specific laboratory conditions used). This was confirmed, and so was the PAH degrading ability of *Pleurotus* spp. The large amounts of intermediate degradation products generated by the fungus (30% of the total concentration detected at the end of this experiment) was also confirmed. However, no practical consequences for larger scale use of the straw treatment or similar treatments can be inferred from these laboratory scale experiments.

4.2 Methodology

Fungal strain and inocula preparation

Pleurotus pulmonarius (INRA 3300) was obtained from a commercial mushroom grower in Frillesås, Sweden. It was propagated on 2% malt extract agar plates (DIFCO). Next, 2 cm^2 young mycelium + underlying agar were subdivided into 8 pieces and transferred to 100 ml of 2% malt extract liquid medium in 250 ml Erlenmeyer flasks with gauze and cotton plugs. After 4 weeks of static incubation, in the dark and at room temperature (20-23°C), the developed mycelium was homogenized with a tissue homogenizer (Ultra Turrax TP 18/10, IKA Werk, Germany) at 10,000 rpm for 30 seconds in an ice bath. The contents of the Erlenmeyer flasks were mixed after homogenization and kept under continuous stirring. 1 ml homogenate or 1 ml sterile liquid medium was transferred under aseptic conditions with a pipette (from the same depth) to 9 ml of 2% malt extract liquid medium in test tubes. After 2 weeks, the contents of the test tubes were emptied onto 10 g (dry weight) autoclaved (120°C for 20 minutes) chopped wheat straw (1-2 cm long), which had been placed in clean plastic bags after autoclaving. The fungal biomass stayed on the straw while some liquid medium reached the bottom of the bags. Two weeks later, the contents of the bags were applied to the soil. No microbial growth was observed in the straw to which sterile medium had been added. Both straw preparations contained about 80% (by weight) water. After inoculation, the soil had 25% (by weight) water content.

Degradation test

The soil used was what remained after experiment 2.

20 g of the mixed soil was placed in each 100 ml beaker, which had previously been autoclaved with an aluminium foil cover. After inoculation, the aluminium foil was perforated. The degradation experiment took place in a humidity chamber with 80-90% relative humidity at 20-23°C and in the dark. No irrigation was provided.

Controls

The straw treatment resembles the controls used by Stroo *et al.* (1989) and Hansson (1991). However, no major controls were used since the effect of WRF has been proved, as found in the literature. Additionally, other previous experiments, although slightly different from this one, suggested that controls were not necessary.

In a preliminary control, no major changes were observed after 12 weeks in beakers containing the sand-soil mixture alone. The sand and soil were not sterilized and the sand did not appear to enhance PAH degradation. In another preliminary experiment, batches of 15 g of sand + soil mixture, non-sterile or gamma-radiation sterilized for 36 hours (Nordisk Sterilisering AB, Skärhamn, Sweden), were supplemented with 100 ml autoclaved (for 3 consecutive days at 120°C for 25 min) 2% malt extract liquid medium (with succinate buffer) + 0.5 g wheat straw dust. The sterilization only inhibited microbial activity, but no PAH degradation was detected in either sterile or non-sterile soil.

Fungus-colonized straw was extracted after being in contact with creosote-contaminated soil for 12 weeks and no PAHs were detected (experiment 1). Davis *et al.* (1993) reported a negligible (*ca.* 1%) sorption of PAHs by wood chips mixed with a creosote-contaminated soil. In the present experiment, the sand and extraction thimbles used were also free of PAHs.

Analytical procedure

After adding the straw preparations to all the beakers, samples (quadruplicates) were randomly selected for extraction at 0, 4, 8, 12 and 16 weeks. The soil was air dried in the beakers in a draft hood in the dark for 10 to 14 hours, until it reached its original weight (20 g). All the soil in the beaker was transferred to a cellulose extraction thimble and extracted for 4 h by Soxhlet with 250 ml analytical grade dichloromethane (Riedel de Häen). The extracts were kept in the dark at -20°C in volumetric flasks until the end of the experiment, to make it possible to analyze them simultaneously. A solution of phenylhexane (Sigma) was added to the extracts as an internal standard before adjusting the final volume.

The extracts were analyzed using GC-MS (Varian Saturn 3 ion trap GC-MS system) at the end of the experiment without further treatment. A non-polar GC column was used (DB-5ms, 30 m x 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific Co.). The injection (0.5 μL) was splitless, using a Varian 8200 Cx autosampler. The temperature programs were: a) Injector: 50°C to 280°C at 200°/min; and b) Column: 50°C for 1 min, then to 100°C at 20°/min, and to 280°C at 8°/min. The temperature of the transfer line to the ion trap was 280°C and that of the ion trap 250°C. The scan frequency was 1 scan/sec and the scan range was mass number 40-300. The carrier gas (He) pressure was 77 kPa [11 psi].

Qualitative determination

The compounds were identified from the reference mass spectra in the MS library

(NIST) and from GC retention data of reference compounds (except for the dimethylnaphthalenes and the degradation products).

Quantitative determination

For each compound, including the internal standard, one abundant ion at a high mass number (in most cases the molecular ion) was selected for quantitative determination. The concentration (in arbitrary units) of a compound was obtained from dividing the selected ion current of the compound by that of the internal standard. The figure for relative abundance in Table 4.3.1 was calculated from the proportion between the ion current of a selected ion and the sum of the ion currents of all selected ions.

Statistical analysis

The hypothesis to be proved was: the straw treatment is better than the fungal treatment to degrade PAHs in the creosote-contaminated soil. Therefore, a one-sided unpaired Student's t-test was used for the statistical analysis, assuming that the data were normally distributed. The data of the fungal treatment were analyzed including and excluding the degradation products when considering the total PAH concentration. Statistical analyses of 5 selected (carcinogenic/mutagenic) PAHs were also conducted. For each test, a significance level of 5% was used and the analyses were performed using a statistics program (Stat View).

To compare within treatments, the values from weeks 4, 8, 12 and 16 of each treatment were considered as one set of data. To compare between treatments, only the data from week 16 were used. Although the risk of making an erroneous decision owing to random effects (*i.e.*, the significance) is very small in each separate statistical analysis, mass errors may still occur when a large number of analyses are carried out. Therefore, the results from the statistical analyses were considered separately rather than simultaneously.

4.3 Main results and discussion (résumé of the appended manuscript)

Qualitative and quantitative determinations

The initial total PAH concentration of the contaminated soil was considerably lower in experiment 3 than in experiment 2, although it was the same sand-soil mixture, kept in the dark at 4°C, for several months. Perhaps the PAHs sorbed more strongly to the soil particles over time. It was not considered important since only a change in concentration (in arbitrary units) was of interest, and not the real concentration of PAHs.

41 compounds were detected in the extracts on day 0, and only 16 compounds were identified (90% of the total concentration). All identified compounds were degraded to a greater extent by the straw treatment, except acenaphthene (which was degraded more by the fungal treatment) and pyrene (which was degraded to the same extent by both

treatments). Considering the concentration of all identified (and identified + unidentified) compounds, the straw treatment was more efficient (Table 4.3.1). This difference was statistically significant. The ranges of percentage degradation of the compounds were:

- Identified compounds: 32-82% with fungal treatment and 55-91% with straw treatment,

- Unidentified compounds: 36-86% with fungal treatment and 20-93% with straw treatment.

Unidentified compounds

Out of the 25 unidentified compounds (many of them probably methyl-derivatives of PAHs), the 3 most abundant were present in amounts comparable to acenaphthene (1-1.6%). Seven of these compounds were degraded to a greater extent by the fungal treatment, 15 were degraded to a greater extent by the straw treatment, 2 increased after fungal treatment and one increased after both treatments (Figure 4.3.1). These increasing compounds were already present at the beginning of the experiment (in very small amounts) so it is not possible to say that they are degradation products.

It is probable that some of the unidentified compounds are PAHs with 5 or more rings. One of them (1.1% of all compounds) was tentatively identified as benzo[k]fluoranthene by the MS library (5 before the last in Figure 4.3.1). Its mass (252) corresponds to that of benzo[k]fluoranthene. This compound was degraded to a greater extent by the straw treatment but other presumably large PAHs were degraded to a greater extent by the fungal treatment. The only problem is that the concentration of these compounds was so small that quantification errors could have been made. Interestingly, Grimmer *et al.* (1992) showed that some soil fungi degraded benzo[ghi]perylene (6 rings) by 60% and coronene (7 rings) by 40% after 30 days in submerged culture (cometabolically).

PAH degradation

The degradation rates obtained in the present experiment, similar to those obtained in experiment 2, were obtained despite half the amount of fungus colonized straw was used (1:2 inoculum to soil ratio). It could be of interest to study the effect of the amount of initial fungus inoculum before adding it to the substrate and the effect on the extent of degradation of the amount of fungus colonized substrate used. However, the best results reported by Stroo *et al.* (1989) were using 25 and 50% fungus inoculum addition and 25% by Morgan *et al.* (1993).

Variation in analyte concentration in contaminated soils bioremediation experiments is a common problem (*e.g.*, Davis *et al.*, 1993). A very high variation in concentration of PAHs was obtained between sample replicates, especially at day 0 (see Figure 1 in appended manuscript). In repeated GC-MS runs of one same sample, a 1% variation was found, so this variation may not be attributed to the chromatography. Instead, it may be explained by uneven homogenization of the soil. Despite the variation, the concentration of PAHs tended to decrease. Contrary to the PAH concentration values,

Table 4.3.1. Identified compounds (in order of elution), their relative abundance at day 0 and reduction of concentration after 16 weeks of fungal and straw treatment (experiment 3)

Compound	Relative abundance (%) at day 0	Reduction of concentration (%) after 16 weeks of	
		Fungal treatment	Straw treatment
2-ring			
naphthalene	1.0	63	79
2-methylnaphthalene	1.9	71	85
1,1-biphenyl	0.6	52	60
dimethylnaphthalene	0.6	72	86
dimethylnaphthalene	0.8	65	86
3-ring			
acenaphthene	0.1	82	71
dibenzofuran	2.7	69	83
fluorene	8.9	74	84
dibenzothiophene	1.1	63	82
phenanthrene	12.5	59	91
anthracene[a]	35.9	66	79
carbazole	12.2	66	83
4-ring			
fluoranthene[b]	3.1	48	60
pyrene	2.0	55	55
benz[a]anthracene[a,b]	1.0	65	66
chrysene[a,b]	2.1	32	57
total, 16 selected PAHs	86.5	64	80

[a] carcinogenic in laboratory animals
[b] mutagenic (Ames test)

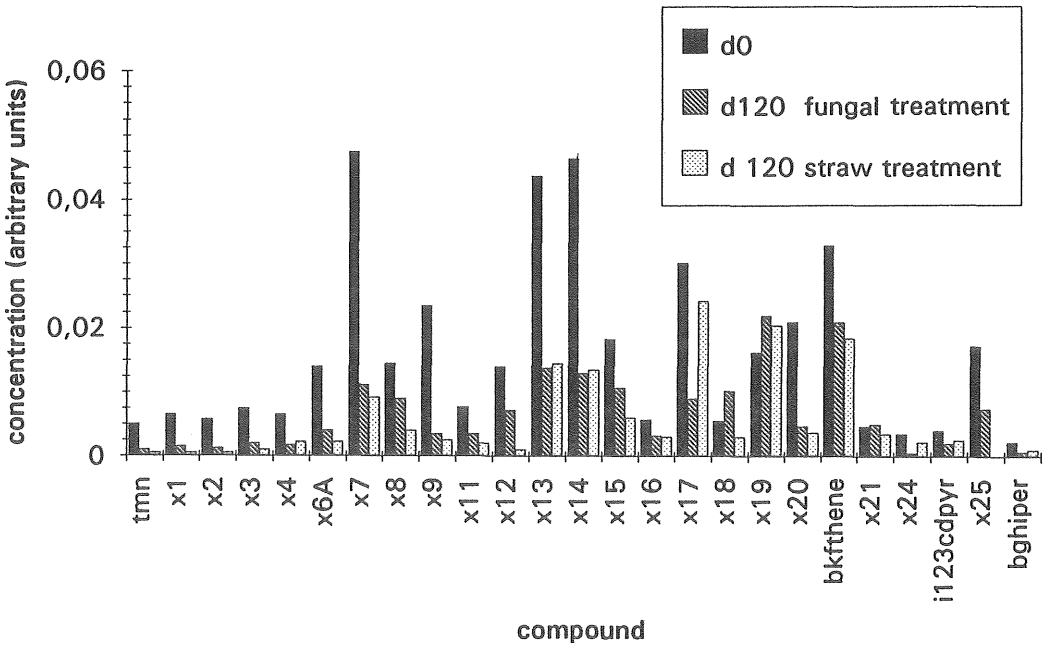

Figure 4.3.1. Concentration (arbitrary units) of individual minor unidentified creosote compounds after fungal and straw treatment (experiment 3) (abbreviations correspond to tentative identities)

very small variation in the concentration of degradation products was obtained.

Degradation products

The degradation products in this experiment could be intermediate metabolic products of fluorene and anthracene. However, further sampling is necessary to determine their degradation rate. It is very probable that the minor degradation product (tentatively identified as 9H-fluoren-9-one) is derived from fluorene, since the mass spectrum of the compound and of the NIST library matched quite well. But the second one comes most probably from anthracene since it was the most abundant compound (about 40%) and, after simple mass balance calculations, no other PAHs could possible provide enough substrate. The net reduction of concentration (probably owing to mineralization to carbon dioxide) of fluorene and anthracene after fungal treatment could then be 67% and 23% respectively, compared to 74% and 66% of their total reduction of concentration.

Straw treatment

The straw treatment was better (statistically significant) in PAH degradation than the WRF. The statistical significance of the results of the treatments compared are summarized in Table 4.3.2.

Table 4.3.2. Statistical significance of: A) reduction of total PAH concentration (including and excluding intermediate fungal degradation products) and B) reduction of concentration of 4 mutagenic/carcinogenic PAH, after 16 weeks of fungal and straw treatment (experiment 3)

	Within treatments		Between treatments
	fungus	straw	(straw better)
A) total PAHs			
including degradation products	s[a]	s	s
excluding degradation products	s	s	ns
B) carcinogenic/mutagenic PAHs			
anthracene	s	s	ns
fluoranthene	s	s	ns
benz[a]anthracene	s	s	ns
chrysene	s	s	s

[a] $p < 0.05$
s = significant
ns = not significant

The main advantage of the straw treatment over the fungal treatment is that no degradation products were formed by the straw treatment. Some limitations of the straw treatment used in this experiment are that the degradation rates obtained might be attributable to the presence of a special microbial population in this specific site, and/or to the specific conditions in which the experiment were carried out. Some researchers (*e.g.*, Davis *et al.*, 1993) consider the performance of contaminated-soil resident microorganisms unpredictable.

Four species of unidentified mold-like fungi were isolated from the original soil. They probably play a role in the degradation by the straw treatment. Bacteria might also play an important role in this process. It would be very useful to identify these fungi and test their ability to degrade PAHs in pure culture.

4.4 Conclusions

Under the experimental conditions used, the fungus degrades PAHs in the CS, generating large amounts of unidentified intermediate degradation products. The straw treatment is also more efficient than the fungal treatment in degrading PAHs in this CS and has the additional advantage that no detectable intermediate degradation products are formed by it.

5 Final remarks

5.1 Work in the immediate future

My work in the immediate future will include the following:

1) The extracts from CS treated with 3 additional WRF will be analyzed shortly and reported on.

2) **Methodological improvements**: It is necessary to find a better soil homogenization and sampling technique to reduce the sample variation obtained. One suggestion is to homogenize slurries of 300-400 ml water/kg soil-straw mixture (Hansson, 1994). This or any other homogenization approach, however, does not seem applicable to large scale bioremediation. It was also found to be necessary to test and develop creosote extract clean up methods because, after all the GC-MS analyses were finished, the column performed very poorly, probably owing to accumulation of dirty material from the extracts. The problem was solved by cutting off a few decimeters from the distal end of the column.

3) **Test more organisms**: Additional WRF and controls (including the straw treatment) will be tested. The soil fungi isolated from the contaminated soil will be identified and tested for PAH degradation together with other resident microorganisms.

4) **Intermediate degradation products**: Chemical characterization of the intermediate fungal degradation products will be made. Their toxicological properties will also be studied. Toxicological studies of the soil before and after bioremediation will be carried out with Microtox and Mutatox.

5) A medium-sized commercial *Pleurotus* farm produces 134 tons/year (wet weight) of waste (spent substrate) (Pind, 1994). This waste is of no use and it contains living fungus mycelium which is still viable for several months. Bioremediation trials using this waste will be made.

5.2 Recommendations and long-term future work

For future experiments, the following controls should be taken into account and analyzed at least once (*e.g.*, at the end of the experiment) just to be sure that they are not necessary in every experiment:

- Contaminated soil + sterilized fungus-colonized straw (to control the effect of the fungus on the overall degradation),

- Contaminated soil + nitrogen and/or carbohydrate source (to confirm if this kind of nutrients stimulate the resident microorganisms), and

- Gamma radiation sterilized soil + uninoculated straw or fungus-colonized straw (to control the effects of soil resident microorganisms).

Other factors which need special attention are:

- Quantification of the initial fungus inoculum dose (mycelium dry weight) before applying it to the straw, since inoculation with different amounts of fungus could cause variations in the degradation rates in replicate samples,

- Checking again as to whether or not PAHs are taken up by the fungus or its growth substrate, although this was done in experiment 1, and it has been reported in the literature that wood chips added to creosote-contaminated soil take up less than 1% of total PAHs (Davis *et al.*, 1993).

- The mobility of the creosote and its biodegradation products should also be determined in soil column leaching experiments.

Half lives of individual and total PAHs could be determined when the PAH-content variability problem has been solved.. A statistical correlation between PAH-structure and degradation rate could also be made.

Additional future work could include:

- Soil screening with toxicity, analytical and laboratory scale degradation tests, to evaluate the remediation method of choice (follow protocols).

- Test WRF for degradation of other important organic pollutants in different matrices, especially in liquids.

- Other technologies that should be tested are: biological methods with resident microorganisms making a succession of natural populations of microorganisms, simulating wood degradation (even in combination with WRF), immobilized fungi and enzymes in liquid or slurry reactors, and low temperature-tolerating organisms.

- Although it is far from the objectives of this work, bench scale degradation experiments should be attempted to check if the results of both treatments are reproducible (with respect to the degradation abilities of each treatment and with respect to the formation of fungal degradation products).

- Extraction methods should be revised, especially with respect to the optimum water content for extraction. It would be ideal to test SFE and microwave extraction. This would avoid using large amounts of hazardous solvents like dichloromethane. New solvents and mixtures of solvents should also be tested.

- The impregnation plant wastes are normally incinerated. Therefore, it could be interesting to make an environmental impact assessment of different waste treatment methods in the wood preserving industry.

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Appendix I Manuscript to be submitted to an environmental journal. Polycyclic aromatic hydrocarbon degradation in contaminated soil by resident microorganisms and the white-rot fungus *Pleurotus pulmonarius*

POLYCYCLIC AROMATIC HYDROCARBON DEGRADATION IN CONTAMINATED SOIL BY RESIDENT MICROORGANISMS AND THE WHITE-ROT FUNGUS *PLEUROTUS PULMONARIUS*

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ABSTRACT

Polycyclic aromatic hydrocarbon (PAH) degradation in creosote-contaminated soil, either through treatment with the white-rot fungus *Pleurotus pulmonarius* or by nutrient supplementation with autoclaved wheat straw to stimulate the resident microorganisms, was carried out at laboratory scale. After incubation for 0, 4, 8, 12 and 16 weeks, soil samples were extracted with dichloromethane and the extracts were analyzed using gas chromatography-mass spectrometry.

The concentration of PAHs decreased notably after 16 weeks of fungal and straw treatment. However, the concentrations of two unidentified compounds (intermediate degradation products) increased significantly after 4 weeks of fungal treatment. These were not detected during the straw treatment. They comprised 30% of the total concentration of compounds detected after fungal treatment. At the end of the experiment, the straw treatment caused a 78% reduction of total PAH concentration initially present. The fungal treatment caused a 48% including the intermediate degradation products and 64% reduction excluding them. The findings suggest that, at least in the laboratory conditions of this experiment, the microorganisms present in this contaminated soil have PAH degrading capacities comparable to or better than *Pleurotus pulmonarius*.

Key words: polycyclic aromatic hydrocarbons, creosote-contaminated soil, white-rot fungi, bioremediation

INTRODUCTION

Many polycyclic aromatic hydrocarbons (PAHs) are important environmental pollutants owing to their persistence, toxicity, mutagenicity and/or cancer related effects. They are formed in natural or anthropogenic processes such as the incomplete combustion of organic material, and are widely distributed in the environment (1, Blumer, 1976; 2, Lee *et al.*, 1981). Their fate and effects in the soil and aquatic environments have been studied (3, Sims and Overcash, 1983; 4, Varanasi, 1989).

PAH contamination of soils and sediments, associated with wood impregnation with creosote, has been reported in many industrialized countries (5, Sundström *et*

al., 1986). Methods have been developed to confine contaminated soils within an area with suitable geologic material, as well as a range of physical, chemical and biological methods to transform organic contaminants into innocuous products. The use of white-rot fungi (WRF) and nutrient addition to stimulate the resident microorganisms (land farming) are among the biological methods tested to clean creosote-contaminated soils (6, Stroo *et al.*, 1989; 7, Borazjani *et al.*, 1990).

Since the 1940's, it has been known that certain bacteria, yeasts and molds have the capacity of degrading PAHs (8, Sisler and ZoBell, 1947). Recently, more groups of microorganisms with this capacity have been recognized. Among them are species of blue-green algae, bacteria, diatoms and eukaryotic microalgae, and additional species of yeasts and filamentous fungi (9, Cerniglia, 1984). Microorganisms acclimated to PAH-contaminated sediments have the capacity of using up to 3-ring PAHs as the sole source of carbon and energy (10, Herbes and Schwall, 1978; 11, Herbes, 1981). However, *some bacteria may utilize pyrene (4-ring PAH) as sole source of carbon and energy (Heitkamp *et al.*, 1988). Other organisms, such as certain fungi, may degrade these PAHs when grown on an alternative substrate (9, Cerniglia, 1984) (*i.e.*, cometabolically).

Early works have reported the capacity of WRF to degrade recalcitrant organic pollutants (12, Bumpus *et al.*, 1985; 13, Eaton, 1985). Compounds that may be degraded by WRF include chlorinated and non-chlorinated aromatic and polyaromatic compounds, non-aromatic chlorinated compounds, biopolymers and lignin models (14, Bumpus and Aust, 1987).

In the laboratory-scale study reported here, the ability of the white-rot fungus *Pleurotus pulmonarius* (fungal treatment) to degrade PAHs in a creosote-contaminated clay-soil was compared with nutrient supplementation with autoclaved wheat straw (straw treatment) to stimulate the resident microorganisms. The genus *Pleurotus* was selected, owing to its general availability and its reported capacity of degrading PAHs in soil (15, Loske *et al.*, 1990; 16, Hansson, 1991). Preliminary experiments showed that the straw treatment gave better degradation of creosote in contaminated soil than the fungal treatment. Therefore, these early experiments were carefully repeated to confirm the hypothesis: The soil microorganisms from this particular contaminated soil degrade PAHs in soil with comparable or more efficiency than the WRF *Pleurotus pulmonarius*. In addition, the experiments were designed to allow a consideration of the biological degradation dynamics of PAHs in creosote-contaminated soil.

METHODOLOGY

Contaminated soil

The soil was obtained from a hazardous waste landfill (owned by Reci Industri AB, Göteborg, Sweden). It originated from a mixed waste contaminated site where creosote cistern train cars had been washed, over a period of several years. Some areas of this site contained creosote in concentrations greater than 200 mg kg^{-1} dry weight, which exceeds the Swedish maximum allowable PAH concentration in soil. The soil was defined as a sandy-silty clay, through dry sieving and particle size distribution analysis. The Ph of the original soil was 6.7 and its organic matter content 11%. It also contained Cu and Pb at 100 and 1500 mg kg^{-1} ashed soil, respectively.

Ten kg of soil, from which coarse material (rocks, nails, wood) was previously removed, was thoroughly mixed with sand (10% coarse, 57% medium and 37% fine particles) (1:1 by weight, dry sand to wet soil ratio) in a construction material mixing machine (Tecnotest, S.N.C. di Coughi & C., Italy). The mixture was kept in a plastic bag in the dark at 4°C until used. The soil was mixed with sand to make it more porous because in preliminary tests the fungal mycelium did not penetrate it, and creosote PAHs were not easily extracted from unmixed clay soil. In field scale tests this mixing could be very expensive since thousands of cubic meters of soil are involved.

Fungal strain and inocula preparation

Pleurotus pulmonarius (INRA 3300) was obtained from a commercial mushroom grower in Frillesås, Sweden. It was propagated on 2% malt extract agar plates (DIFCO). Next, 2 cm^2 young mycelium + underlying agar were subdivided into 8 pieces and transferred to 100 ml of 2% malt extract liquid medium in 250 ml Erlenmeyer flasks with gauze and cotton plugs. After 4 weeks of static incubation, in the dark and at room temperature (20-23°C), the developed mycelium was homogenized with a tissue homogenizer (Ultra Turrax TP 18/10, IKA werk, Germany) at 10,000 rev. min^{-1} for 30 seconds in an ice bath. The contents of the Erlenmeyer flasks were mixed after homogenization and kept under continuous stirring. 1 ml homogenate or 1 ml sterile liquid medium was transferred under aseptic conditions with a pipette (from the same depth) to 9 ml of 2% malt extract liquid medium in test tubes. After 2 weeks, the contents of the test tubes were emptied onto 10 g (dry weight) autoclaved (120°C for 20 minutes) chopped wheat straw (1-2 cm long), which had been placed in clean plastic bags after autoclaving. The fungal biomass stayed on the straw while some liquid medium reached the bottom of the bags. Two weeks later, the contents of the bags were

applied to the soil. No microbial growth was observed in the straw to which sterile medium had been added. Both straw preparations contained about 80% (by weight) water. After inoculation, the soil had 25% (by weight) water content.

Degradation test

20 g of the mixed soil was placed in each 100 ml beaker, which had previously been autoclaved with an aluminium foil cover. After inoculation, the aluminium foil was perforated. The degradation experiment took place in a humidity chamber with 80-90% relative humidity at 20-23°C and in the dark. No irrigation was provided.

Controls

Adding uninoculated straw + liquid medium to the soil yielded better PAH degradation than by addition of a WRF (*Pleurotus* sp.) in preliminary experiments and was carefully repeated to confirm this behavior.

In a preliminary control, no major changes were observed after 12 weeks in beakers containing the sand-soil mixture alone. The sand and soil were not sterilized and it seems that the sand did not enhance PAH degradation.

In another preliminary experiment, batches of 15 g of sand + soil mixture, non-sterile or gamma-radiation sterilized for 36 hours (Nordisk Sterilisering AB, Skärhamn, Sweden), were supplemented with 100 ml autoclaved (for 3 consecutive days at 120°C for 25 min) 2% malt extract liquid medium (with succinate buffer) + 0.5 g wheat straw dust. The sterilization only inhibited microbial activity, but no PAH degradation was detected in either sterile or non-sterile soil.

Fungus-colonized straw was extracted after being in contact with creosote-contaminated soil for 12 weeks and no PAHs were detected. Davis *et al.* (17, Davis *et al.*, 1993) report a negligible (*ca.* 1%) sorption of PAHs by wood chips mixed with a creosote-contaminated soil. In the present experiment, the sand and extraction thimbles used were also free of PAHs.

Analytical procedure

After adding the straw preparations to all the beakers, samples (quadruplicates) were randomly selected for extraction at 0, 4, 8, 12 and 16 weeks. The soil was air dried in the beakers in a draft hood in the dark for 10 to 14 hours, until it reached its original weight (20 g). All the soil in the beaker was transferred to a cellulose extraction thimble and extracted for 4 h by Soxhlet with 250 ml analytical grade dichloromethane (Riedel de Häen). The extracts were kept in the dark at -20°C in volumetric flasks until the end of the experiment, to make it possible to analyze them simultaneously. A solution of phenylhexane (Sigma) was added to the extracts as an internal standard before adjusting the final volume.

The extracts were analyzed using GC-MS (Varian Saturn 3 ion trap GC-MS system)

at the end of the experiment without further treatment. A non-polar GC column was used (DB-5ms, 30 m x 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific Co.). The injection (0.5 μL) was splitless, using a Varian 8200 Cx autosampler. The temperature programs were: a) Injector: 50°C to 280°C at 200° min^{-1}; and b) Column: 50°C for 1 min, then to 100°C at 20° min^{-1}, and to 280°C at 8° min^{-1}. The temperature of the transfer line to the ion trap was 280°C and that of the ion trap 250°C. The scan frequency was 1 scan sec^{-1} and the scan range was mass number 40-300. The carrier gas (He) pressure was 77 kPa [11 psi].

Qualitative determination

The compounds were identified from the reference mass spectra in the MS library (NIST) and from GC retention data of reference compounds (except for the dimethylnaphthalenes and the intermediate degradation products).

Quantitative determination

For each compound, including the internal standard, one abundant ion at a high mass number (in most cases the molecular ion) was selected for quantitative determination. The concentration (in arbitrary units) of a compound was obtained from dividing the selected ion current of the compound by that of the internal standard. The figure for relative abundance in Table 1 was calculated from the proportion between the ion current of a selected ion and the sum of the ion currents of all selected ions.

Statistical analysis

The hypothesis to be proved was: the straw treatment is better than the fungal treatment to degrade PAHs in the creosote-contaminated soil. Therefore, a one-sided unpaired Student's t-test was used for the statistical analysis, and it was assumed that the data were normally distributed. The data of the fungal treatment were analyzed including and excluding the intermediate degradation products when considering the total PAH concentration. Statistical analyses of 5 selected (carcinogenic/mutagenic) PAHs were also conducted. For each test, a significance level of 5% was used and the analyses were performed using a statistics program (Stat View).

To compare within treatments, the values from weeks 4, 8, 12 and 16 of each treatment were considered as one set of data. To compare between treatments, only the data from week 16 were used. Although the risk of making an erroneous decision owing to random effects (*i.e.*, the significance) is very small in each separate statistical analysis, mass errors may still occur when a large number of analyses are carried out. Therefore, the results from the statistical analyses were considered separately rather than simultaneously.

RESULTS AND DISCUSSION

Fungal growth in soil

Pleurotus pulmonarius colonized the soil after 1 week. After 12 weeks it began to develop fruit body primordia on the straw and also on the soil. No growth of contaminant molds was seen in unused fungus or in unused straw inoculum bags at the end of the experiment. In the straw treatment, conidia-producing filamentous fungi and some red and black stains were observed. These were present as diffuse spots on the straw and soil in most beakers. 4 species of unidentified fungi were isolated from the soil. Some bacterial colonies were also formed.

The upper quarter of the straw in both treatments dried out but the rest of the straw and the soils had sufficient humidity, even at the end of the experiment. The soil from the straw treatment contained more water than that from the fungal treatment. For this reason, plus the fact that no drainage was provided, the beakers were not irrigated.

Qualitative and quantitative determinations

On day 0, in this particular type of creosote extracted from the soil, 41 compounds were separated and distinguished above the baseline noise level by the GC-MS method used. Of these, 16 compounds (87% of the total PAH concentration) were identified. These compounds, their initial relative abundance and decreases in concentration at the end of fungal and straw treatment, are presented in Table 1.

If the 41 initial compounds are considered, the total PAH concentration had decreased, on the last sampling day, by 64% with the fungal treatment and by 78% with the straw treatment. Corresponding, 64% and 80% decreases in concentration were obtained taking into account only the 16 identified PAHs. With respect to the unidentified compounds, 58% and 65% decreases in concentration were observed in each treatment. The degradation dynamics, considering the total PAH concentration throughout the experiment, are shown in Figure 1.

Intermediate degradation products

No intermediate degradation products were detected after straw treatment. However, during the fungal treatment, 2 compounds (one could be identified as 9H-fluoren-9-one from its mass spectrum) which were not present at the beginning, increased in concentration between day 0 and week 4. 9H-fluoren-9-one reached its maximum concentration in week 4 and the other compound in week 12, after which both compounds began to decrease. These 2 compounds corresponded to 1.2 and 29% of the total concentration, respectively, in week 16. Their formation dynamics are illustrated in Figure 2. If these compounds are included in the total concentration after fungal treatment, a 48% decrease of the total initial PAH concentration is

Table 1. Identified compounds, their relative abundance (%) on day 0 and reduction of concentration (%) after 16 weeks of fungal and straw treatment

Compound	Relative abundance (%)	Reduction of concentration (%) after	
		Fungal treatment	Straw treatment
2-ring			
naphthalene	1.0	63	79
2-methylnaphthalene	1.9	71	85
1,1-biphenyl	0.6	52	60
dimethylnaphthalene	0.6	72	86
dimethylnaphthalene	0.8	65	86
3-ring			
acenaphthene	0.1	82	71
dibenzofuran	2.7	69	83
fluorene	8.9	74	84
dibenzothiophene	1.1	63	82
phenanthrene	12.5	59	91
anthracene	35.9	66	79
carbazole	12.2	66	83
4-ring			
fluoranthene	3.1	48	60
pyrene	2.0	55	55
benz[a]anthracene	1.0	65	66
chrysene	2.1	32	57
Total, 16 identified PAH	86.5	64	80

obtained.

It is still necessary to identify these intermediate degradation products, investigate their toxic properties and degradation dynamics, and observe whether or not they are formed in larger scale experiments. No intermediate degradation products were reported in small and field-scale experiments using *Pleurotus* for creosote PAH degradation in soil (15, Loske *et al.*, 1990; 16, Hansson, 1991). This suggests that the conditions of the present experiment could have favored the formation of these compounds.

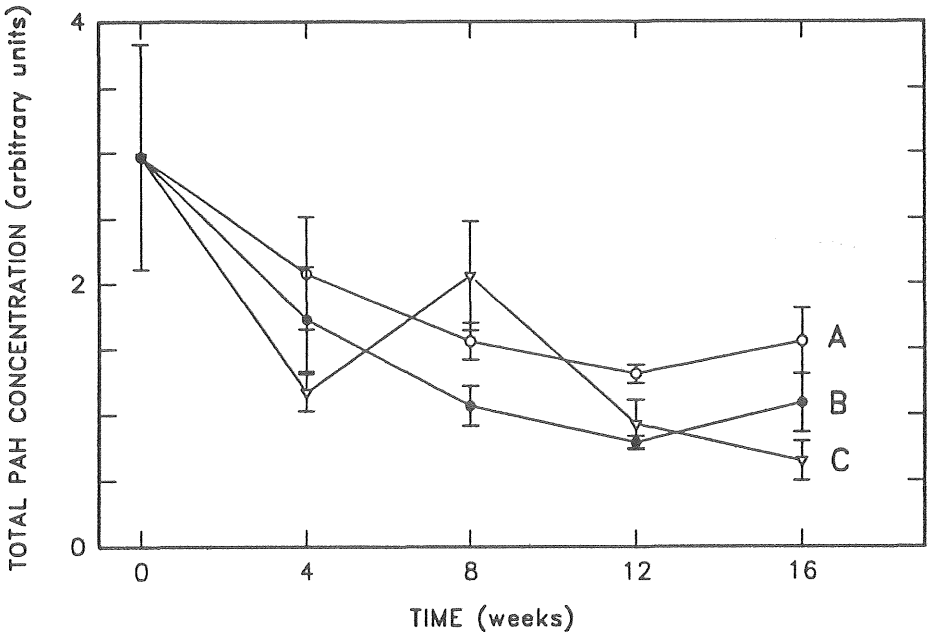

Figure 1. Degradation dynamics during: A) Fungal treatment (including intermediate degradation products); B) Fungal treatment (excluding intermediate degradation products); and C) Straw treatment (no intermediate degradation products detected). Each point is the arithmetic mean of 4 observations and the bars are the standard error of the mean.

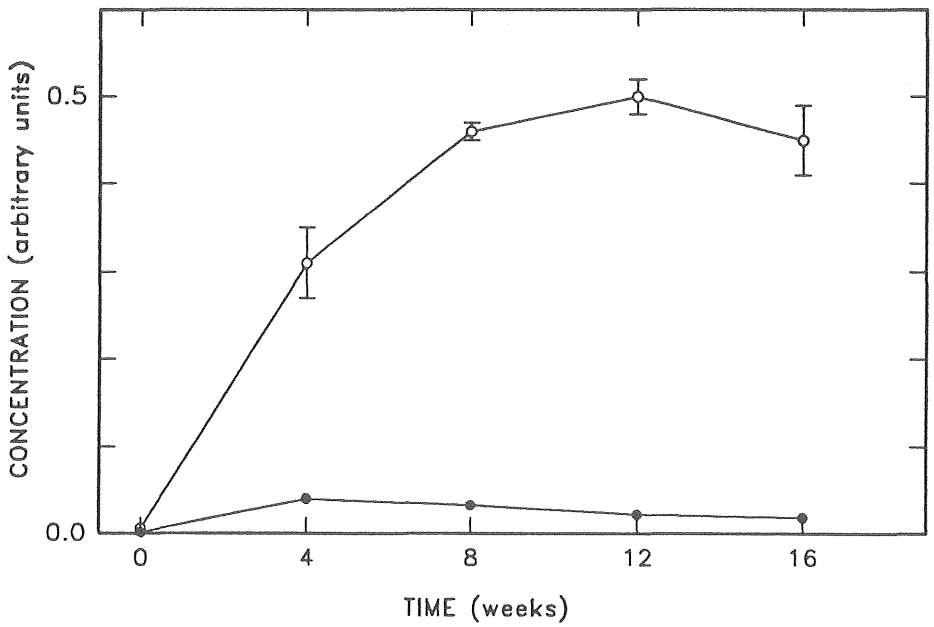

Figure 2. Formation dynamics of two unidentified fungal intermediate degradation products. Each point is the arithmetic mean of 4 observations and the bars are the standard error of the mean. The minor compound has the same mass spectrum as 9H-fluoren-9-one.

Sutherland (18, Sutherland, 1992) has reviewed the formation and toxicity of fungal metabolic products of PAHs. Most of the knowledge in this field comes from fungi such as *Cunninghamella elegans*, and to a lesser extent from others such as *Aspergillus* spp., *Rhizotocnia solani* and several species of yeasts and macrofungi. The most studied WRF is *Phanerochaete chrysosporium*, and quinones, glucose conjugates and unidentified metabolites are among its major PAH degradation products (18, Sutherland, 1992). PAH methoxy derivatives formed by *Pleurotus* have also been reported (19, Grimmer *et al.*, 1992). Most fungal PAH metabolites are usually less mutagenic (18, Sutherland, 1992) and more polar than the parent compound (20, Bumpus, 1989).

Statistical analysis

Despite the fact that the total and individual PAH concentrations tend to decrease in both treatments, some of the data do not follow this trend. The source of error might be the soil homogenization process, yielding a variation in mean concentration values. Extreme variability of concentration of PAHs in soil samples, affecting the statistical significance of the results of biodegradation studies, has been reported by other researchers (17, Davis *et al.*, 1993).

The straw treatment yielded comparable or better degradation than the fungal treatment, for most individual selected PAHs and for total PAHs, respectively (Table 1, Figure 1). Statistically, both the straw and fungal treatment (including and excluding intermediate degradation products) caused a significant decrease of total PAH concentration with respect to day 0. All 5 selected PAHs (anthracene, fluoranthene, pyrene, benz[a]anthracene and chrysene) were also significantly degraded by both treatments. Excluding the intermediate degradation products, there was no significant difference between the treatments, but including them, the straw treatment was significantly better (*i.e.*, higher percentage of total PAH degradation). The straw treatment was significantly better than the fungal treatment in degrading chrysene, but no significant differences were found for the other 4 selected compounds.

Fate of PAH

The biotic or abiotic fate of the PAHs in this experiment is unknown. PAH degradation experiments by WRF using radio-labelled compounds have revealed useful information (20, Bumpus, 1989; 21, Qiu and McFarland, 1991). In a liquid culture experiment, radio-labelled phenanthrene was degraded by *Phanerochaete chrysosporium* (20, Bumpus, 1989) and an important part of its metabolic products (92% of the organic phase) consisted of polar metabolites with only 8% transformed to CO_2. In a solid phase experiment with radio-labelled benz[a]pyrene (21, Qiu and McFarland) the

major fate of the contaminant carbon was humification (*i.e.*, polymerization to soil humic components) rather than conversion to CO_2. This humification could be compared with the repolymerization process observed in isolated lignins reacted with WRF ligninolytic enzymes (peroxidases and laccases) (Haemmerli *et al.*, 1986, cited in 22, Muheim *et al.*, 1991). Bartha (1981) (cited in 15, Loske *et al.*, 1990) reported that aromatic and chlorinated compounds added to a soil with normal microbiota end up in the high molecular weight humic acid fraction. After binding irreversibly to humus, the pollutants may continue the natural course of humus metabolism without endangering or damaging the biota (21, Qiu and McFarland, 1991).

Straw treatment

Some researchers have suggested or shown that microorganisms from agricultural (23, Martens and Zadrazil, 1992) or PAH-contaminated soil (24, Sack and Günther, 1993) have good cometabolic PAH degrading ability, comparable to or better than that of certain WRF. Other researchers report that their fungal treatment was better than stimulation of resident microorganisms by addition of sterile uninoculated substrate (6, Stroo *et al.*, 1989; 16, Hanson, 1991), and the latter also caused degradation of 5 and 6-ring PAHs (6, Stroo *et al.*, 1989).

One reason for using WRF for PAH-contaminated soil clean up is that WRF are capable of degrading PAHs with 5 or more rings. This has been achieved in aqueous conditions (12, Bumpus *et al.*, 1985) and in some solid phase (contaminated soil) experiments (6, Stroo *et al.*, 1989; 16, Hansson, 1991). However, Davis *et al.* (17, Davis *et al.*, 1993) found that the fungus *Phanerochaete sordida* failed to degrade these PAHs in soil. They suggest that this was due to the preferential degradation of the most abundant PAHs (which had less than 5 rings) by ligninolytic enzymes and due to too low a fungus to soil (1:10) ratio. In contrast, a recent study (19, Grimmer *et al.*, 1992) showed that the zygomycetous fungi *Cunninghamella elegans* and *Mucor circinelloides* (the latter isolated from PAH-contaminated soil) degraded PAHs such as benzo[ghi]perylene (6 rings) and coronene (7 rings) by 60% and 40%, respectively, after 30 days in submerged culture. In the present experiment, the initial fungal dose (dry mycelial weight) added to the beakers with contaminated soil was not quantified possibly resulting in variations in the overall initial fungal dose and in the degradation rate.

The experimental data of the present study confirms the finding of previous experiments in which the straw treatment was more efficient in degrading PAHs in creosote-contaminated soil than the *Pleurotus* species used, under the conditions of this experiment. This result, added to the experimental data of other researchers, suggests that the straw treatment could be as efficient as or better than treatment

with *Pleurotus* species. This conclusion may be subject to the existence of a particular microbial community at a specific site, and the fungal methodology utilized. Some researchers (17, Davis *et al.*, 1993) consider the performance of contaminated-soil resident microorganisms unpredictable. These two factors could be limitations of the resident microorganisms method. In the current study, however, there is the additional advantage that no metabolic products were detected after the straw treatment.

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