Degradation of Polycyclic Aromatic Hydrocarbons by Actinomycetes

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Abstract

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The potential of some actinomycetes to degrade polycyclic aromatic hydrocarbons (PAH) and the effect of co-substrates, plants and other additives on their degradation and bioavailability was studied.

A glass bead system for growth of PAH-degrading actinomycetes in liquid culture was developed and used for the screening of strains for biosurfactant activity and phenanthrene degradation in the presence of different co-substrates. Indication of biosurfactant production by all tested strains was obtained with hexadecane and rapeseed oil as co-substrates but not with glucose.

Rhodococcus sp. DSM 44126 was identified as *R. wratislaviensis* and found to be able to degrade phenanthrene and anthracene. An actinomycete with a high capacity to degrade phenanthrene and pyrene was isolated from an agricultural soil and identified as *Mycobacterium* LP1. The catabolic activity of both strains was studied in liquid cultures and in soil.

Several additives were also tested for their effect on PAH degradation in soils. The surfactant Triton X-100, but not wheat straw, promoted PAH degradation in a soil with aged creosote by increasing the bioavailability of the compounds. The presence of plants increased the proportion of active microorganisms and enhanced PAH degradation, likely due to root exudates provided by the plants. In a PAH-spiked soil, the addition of rapeseed oil (1% w/w) stimulated the degradation of anthracene and benzo(*a*)pyrene mainly as a result of abiotic processes, but negatively affected the degradation of phenanthrene and pyrene, probably due to limitations in nutrient and oxygen supply.

Based on these results, a new sequential treatment in two steps for cleaning PAH-contaminated soil was designed and tested using four different PAH as model substances. The first step consisted of the inoculation with *Mycobacterium* LP1, favouring biological degradation of low-molecular-weight PAH, and the second step consisted of the addition of rapeseed oil, which promoted the abiotic transformation, and probably also the solubilisation, of the high-molecular-weight PAH.

Keywords: bioavailability, bioremediation, co-substrate, *Mycobacterium*, PAH, phytoremediation, polycyclic aromatic hydrocarbons, rapeseed oil, *Rhodococcus*

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A los "mejores"

What is essential is invisible to the eye.

Antoine de Saint-Exupéry

Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Pizzul, L., Castillo, M.d.P. & Stenström, J. 2006. Characterization of selected actinomycetes degrading polyaromatic hydrocarbons in liquid culture and spiked soil. *World Journal of Microbiology & Biotechnology,* in press DOI 10.1007/s11274-005-9100-6.

II. Pizzul, L., Castillo, M.d.P. & Stenström, J. Effect of rapeseed oil on the degradation of PAH in soil by *Rhodococcus wratislaviensis*. (In revision with International Biodeterioration & Biodegradation).

III. Pizzul, L., Sjögren, Å, Castillo, M.d.P. & Stenström, J. Degradation of polyaromatic hydrocarbons in soil by a two-step sequential treatment. (Manuscript).

IV. Hultgren, J., Granhall, U., Pizzul, L & Castillo, M.d.P. Phytoremediation of polycyclic aromatic hydrocarbons by means of *Salix viminalis* - a greenhouse experiment with creosote contaminated soils. (Manuscript).

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My contribution to the papers in this thesis has been as follows:

I. Took part in planning the study together with the other authors. Performed the laboratory work, analysis of the results and writing of the manuscript.

II. Took part in planning the study together with the other authors. Performed the laboratory work, analysis of the results and writing of the manuscript.

III. Took part in planning the study together with the other authors. Performed most of the laboratory work, analysis of the results and writing of the manuscript.

IV. Took part in the laboratory work concerning determination of surfactant activity. Took part in the analysis of the results and in the writing of the manuscript concerning the degradation of PAH.

Introduction

Human activities have an important impact on the environment, especially during the last two centuries with the increase of industrial activities. Nowadays there is a general awareness about the detrimental effects of certain products and sub-products from industrial processes and that their release and disposal to the environment should be controlled or even prevented. However, there are large extensions of soils and sediments with high levels of pollution, as a heritage from activities in the past or as a result of current releases. In any case, pollutants pose a risk to all living forms and therefore must be removed.

Several techniques are used to clean contaminated soils, such as incineration and chemical treatments. During the last decades bioremediation has gained acceptance as an alternative for pollutant removal. It is defined as the use of microorganisms or plants to detoxify an environmental compartment by degradation of the pollutants (Bollag & Bollag, 1995).

Before the application of bioremediation in the field and in order to achieve a successful result, some aspects have to be considered. The presence of a microorganism with the catabolic potential to degrade the target pollutant is indispensable. Many efforts are made to isolate novel microorganisms that can degrade different compounds. Moreover, it is important to understand the complex interrelations between the degrading microorganism, the pollutant and the soil and the factors limiting and/or promoting the biodegradation process, *e.g.* bioavailability, toxicity, temperature and nutrients. Once such basic information is obtained, the methodology applied in the bioremediation programme can be optimised.

The present thesis explores the potential of some actinomycetes to degrade polycyclic aromatic hydrocarbons, a group of hazardous organic pollutants. The information obtained will contribute to a better understanding of the processes involved and constitutes a contribution to the potential application of bioremediation of contaminated soils.

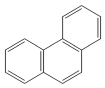
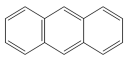
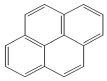
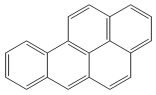
Polycyclic aromatic hydrocarbons

General properties

Polycyclic aromatic hydrocarbons (PAH) are non-polar organic compounds made up of two or more fused benzene rings, arranged in linear, angular or clustered structures. They are hydrophobic with very low water solubility and high octanol-water partition coefficient (K_{ow}). Generally, their hydrophobicity increases and their volatility decreases with increasing molecular weight (Mackay & Callcott, 1998). PAH are classified according to the number of rings, the type of ring and the atom composition. The low molecular weight (LMW) PAH contain two or three aromatic rings and the high molecular weight (HMW) ones more than three. Alternant PAH are composed of aromatic rings with six carbon atoms and nonalternant ones of rings with less or more than six carbons (*e.g*. fluorene and fluoranthene). By definition, PAH contain only carbon and hydrogen atoms in their structure, but some carbons can be substituted by nitrogen, sulphur and oxygen, resulting in polycyclic heteroaromatic hydrocarbons, *e.g.* carbazole and dibenzofuran. The structure and some physicochemical properties of selected LMW and HMW alternant PAH, which also were used as model compounds in this work, are shown in Table 1.

The toxicity of PAH was first recognized in the second half of the 18th century. In 1761 the physician John Hill documented a high incidence of nasal cancer in tobacco snuff consumers and in 1775 Percival Pott reported a high rate of scrotal skin cancer in chimney sweeps (Cerniglia, 1984). It is known today that LMW PAH are acutely toxic and HMW PAH are considered genotoxic (Table 1). Epidemiological studies show direct evidence of the carcinogenic effects of PAH in occupationally exposed persons and demonstrate that the risk of lung and bladder cancer is dose related (Mastrangelo *et al*., 1996). Due to their mutagenic, carcinogenic and genotoxic activities, PAH are classified as priority environmental pollutants by the United States Environmental Protection Agency (US EPA) and the European Community (Wattiau, 2002).

Table 1. The structure and some physicochemical properties of selected LMW and HMW alternant PAH.

	Phenanthrene	Anthracene	Pyrene	Benzo(*a*)pyrene
Structure				
Chemical formula	$C_{14}H_{10}$	$C_{14}H_{10}$	$C_{16}H_{10}$	$C_{20}H_{12}$
Molecular weight (g mol^{-1})	178.2	178.2	202.2	252.3
Solubility in water at 25˚C (mg l^{-1}) [a]	1.10	0.045	0.132	0.0038
Octanol-water partition coefficient (log K_{ow})	4.57	4.54	5.18	6.04
Ionization potential (eV)	8.03	7.43	7.53	7.21
Genotoxicity[b]	Toxic[b]	Toxic[b]	± Ames + UDS + Carcinogen	+ Ames + CA + UDS + DA + SCE + Carcinogen

[a] From Mackay & Callcott (1998)
[b] Data for phenanthrene and anthracene were obtained from Mueller *et al.* (1996) and for pyrene and benzo(*a*)pyrene, from Cerniglia (1992). (Ames) *Salmonella typhimurium* reversion assay, (CA) chromosomal aberrations, (UDS) unscheduled DNA synthesis, (DA) DNA adducts, (SCE) sister chromatid exchange

PAH in the environment - sources and fate

PAH are formed whenever organic substances are exposed to high temperatures (pyrolysis) and the composition of the products depends on the nature of the starting material and on the transformation temperatures (Blumer, 1976). They are naturally produced during the burning of vegetation in forest and bush fires, by volcano activity and by plant and bacterial reactions (Blumer, 1976; Wilson & Jones, 1993). However, the main sources of PAH in the environment are anthropogenic. An ice core study from a site in Greenland, with records over the past 400 years, shows an increase in PAH levels from 1900 to 1980 (Kawamura *et al.*, 1994). Moreover, the analysis of soils collected from an uncontaminated site at different intervals from the mid-1800s to 1989, revealed that the atmospheric deposition of PAH increased four-fold since the 1880s (Jones *et al.*, 1989).

PAH are released to the atmosphere as the result of inefficient combustion activities and uncontrolled emissions (Wilson & Jones, 1993). Local

contaminations usually originate from petroleum refining and transport activities, through discharge of industrial effluents or accidental release (Kanaly & Harayama, 2000), from industries related to the transformation of fossil fuels and derived products (*e.g.* coal tar and carbon black) and from wood-treatment processes and wood-preservative production (Wilson & Jones, 1993).

Possible fates of PAH in the environment are volatilisation, photo-oxidation, chemical oxidation, bioaccumulation, interaction with the soil matrix and biodegradation (Cerniglia, 1992). The importance of these processes varies depending on the environment, *i.e.* atmosphere, soil or water. In soils, PAH can undergo abiotic reactions (photo-oxidation and chemical oxidation) and some, *i.e.* naphthalene and alkyl naphthalene, are partly lost by volatilisation (Park *et al.*, 1990). However, the main transformation is the result of microbial degradation (Cerniglia, 1992) and a relevant fate is the absorption to the soil matrix.

Microbial degradation of PAH in soils

Many different bacteria and fungi are able to partially or completely metabolise PAH. Fungal degradation of PAH occurs through two different pathways. White rot fungi produce unspecific extracellular ligninolytic enzymes, peroxidases and laccases that initiate a free radical attack by a single electron transfer, leading to formation of quinones (Cerniglia, 1993). Degradation of PAH by non-ligninolytic fungi involves the activity of the cytochrome P-450 monooxygenases. These enzymes catalyze a ring epoxidation to form an unstable arene oxide, which is further transformed to *trans*-dihydrodiol by enzymatic hydration or rearranged to phenols by non-enzymatic reactions (Sutherland, 1992).

In general, the first step in the aerobic bacterial degradation is the hydroxylation of an aromatic ring via a dioxygenase, with the formation of a *cis*-dihydrodiol. The *cis*-dihydrodiol is then dehydrogenated to give a cathecol, which undergoes further ring cleavage and is transformed into intermediates that enter the central pathways of metabolism and are used for energy production and biosynthesis (Mueller *et al.*, 1996). Bacteria can also degrade PAH via the cytochrome P-450 pathway, with production of *trans*-dihydrodiols (Sutherland *et al.*, 1990; Moody *et al.*, 2004). PAH have also been shown to be biodegradable anaerobically, *e.g.* under nitrate-reducing conditions (Eriksson *et al.*, 2003).

PAH may be degraded in the rhizosphere of some plants and direct plant uptake of pyrene and phenanthrene has been observed (Gao and Zhu, 2004). However, the most common reason for the degradation of PAH in the presence of plants is the enhancement of the activity of PAH-degrading microorganism in the vicinity of the roots, where they find an environment rich in root exudates and nutrients (Chaudhry *et al*., 2005)

Bioavailability of PAH in soil

The PAH, as other hydrophobic organic pollutants, are persistent in the environment. In spite of the presence in the soil of microorganisms with the capacity to degrade the compounds, they are generally slowly transformed. This slow biodegradation is the result of the generally low bioavailability of these compounds, *i.e.*, as defined by Volkering *et al.* (1998), "its uptake rate by organisms is limited by a physicochemical barrier between the pollutant and the organisms" and the microorganisms may "live a famine existence" even at a highly polluted site (Bosma *et al.*, 1997).

Most PAH are highly hydrophobic and have low water solubility (Table 1). When they reach the soil, they leave the aqueous phase, are sorbed into the organic matter and sequestered (Semple *et al*., 2003). This sequestration is a time-dependent process by which the PAH first contaminate the macropores and the particle surfaces, where the potential degrading microorganisms are found, then diffuse into micropores, and finally reach the nanopores, which are inaccessible to the microorganisms (Bosma *et al*., 1997) and where the compounds remain entrapped. This decrease in the pollutant bioavailability and extractability that is obtained when the pollutant-soil contact time increases is called "ageing". In addition, the PAH can also be strongly directly adsorbed to soil particles (Hatzinger & Alexander, 1995).

Microorganisms have developed different strategies to overcome the problem of the low bioavailability of hydrophobic substrates, basically by three mechanisms: a) high-affinity uptake systems (Sokolovská *et al*., 2003; Miyata *et al.*, 2004); b) adhesion to the solid substrate, sometimes with formation of a biofilm (Eriksson *et al*., 2002; Wick *et al.*, 2002); c) production of biosurfactants or emulsifiers (Neu, 1996; Lang, 2002)

Surfactants are amphiphilic molecules consisting of a hydrophilic and a hydrophobic domain. They partition between two phases in a heterogeneous system and increase the apparent solubility of a hydrophobic compound in water (Georgiou *et al.*, 1992). The three general characteristics of the surfactants are the enrichment at interfaces, the lowering of interfacial tension and the formation of micelles (Neu, 1996). They can be synthetic or of microbial origin (biosurfactants). It has been established that the carbon source plays an important role in the production of surface-active compounds. Usually the presence of water-immiscible substances, *e.g.* hydrocarbons, is required (Rapp *et al*., 1979, Hommel, 1990; Bredholt *et al.*, 1998) but some bacteria even produce surfactants when grown on ordinary carbohydrates (Guerra-Santos *et al*., 1984, Person & Molin, 1987).

Moreover, the biodegradation of PAH can be enhanced by the addition of a co-substrate. The term co-substrate often refers to a compound that supports the growth of microorganisms and enables the degradation of a target compound, a process denominated cometabolism (Alexander, 1999). In the present work, the term co-substrate is used in a wider sense, and includes organic or inorganic

supplements that affect the microbes or the pollutant or both. The co-substrate may affect the microbial processes in different ways: increasing of the microbial biomass when it can be used as an additional carbon and energy source (Carmichael & Pfaender, 1997), activating a particular enzyme activity (Kanaly *et al*., 2000) or stimulating the production of biosurfactants and emulsifiers (Ramsay *et al.*, 1988; Ferraz *et al*., 2002). In the case of some hydrophobic co-substrates, they act by solubilising the pollutant and thereby facilitate its transfer to the bacterial cells. The co-substrate can also be a synthetic surfactant that enhances the solubility of the PAH and sometimes be used as a carbon source.

Vegetable oils are good examples of such co-substrates. They have been used as additional carbon sources (Pannu *et al*., 2003) and for promoting the production of biosurfactants (Sim *et al.*, 1997; Vance-Harrop *et al.*, 2003), besides acting as surfactants themselves (Desai & Banat, 1997). The addition of oils or lipids stimulates the activity of enzymes by microorganisms, such as manganese peroxidases (Watanabe *et al*., 2000) and phenoloxidases in biobeds (Castillo, unpublished data). Because of their efficiency for the solubilisation and extraction of PAH, together with the fact that they are biodegradable, non-volatile and non-toxic, they also have been used to replace organic solvents (Berg Schuur & Mattiasson, 2003, Pannu *et al.*, 2004; Gong *et al.*, 2005).

Actinomycetes

Far, far in the forest, or sauntering later in summer, before I think where I go,
Solitary, smelling the earthy smell, stopping now and then in the silence,
Walt Whitman, 1900

Over the years, many poets have been inspired by the earthy smell. Little did they know that they were being touched by microscopic muses. In 1965, two inspired scientists, Gerbe & Lechevalier, isolated an earthy smelling substance, geosmin, produced by a distinct group of microorganisms: the actinomycetes.

General characteristics

Actinomycetes are a group of diverse bacteria. They have in common that they all are Gram-positive and have a high content of guanine plus cytosine in their DNA (>55 mol %). Most are aerobic and neutrophilic. The name actinomycete derives from the Greek “actys” (ray) and “mykes” (fungus) and the actinomycetes were initially regarded as minute fungi because of their mycelium-like growth. The attention paid to this group rose notably after the discovery of streptomycin by Waksman and Schatz in 1943 (Erikson, 1949) and as the number of studies and the scientific data accumulated, they were finally recognized as bacteria. Their morphology, however, varies among the different genera, from cocci and pleomorphic rods to branched filaments that break down into spherical cells or

aerial mycelium with long chains of spores. The spores are formed as a result of nutrient depletion and can survive prolonged desiccation until nutrients are again available (McCarthy & Williams, 1992). This ability to sporulate is important for their survival in the environment.

Actinomycetes are ubiquitous in soils, where they usually are present in numbers of 10^5-10^6 colony-forming units per gram of soil. They play an important role in the recycling of organic carbon and are able to degrade complex polymers (Goodfellow & Williams, 1983). Many strains have the ability to solubilise lignin and degrade lignin-related compounds by producing cellulose- and hemicellulose-degrading enzymes and extracellular peroxidases (Ball *et al*., 1989; Pasti *et al.*, 1990, Mason *et al*., 2001). They also occur in other environments rich in organic matter such as composts, in both the mesophilic and thermophilic phases (Steger, 2006), and sewage sludge, where, notably the mycolic acid-containing actinomycetes are associated with the extensive and undesirable formation of stable foams and scum (Seong *et al*., 1999, Goodfellow *et al*., 1996).

In general the optimal conditions for their growth are temperatures of 25-30°C (50°C for the thermoactinomycetes) and neutral pH, but many species have been isolated from extreme environments. For example, the psychrophilic *Arthrobacter ardleyensis* was isolated from sediment from an Antarctic lake and is able to grow at temperatures as low as 0°C (Chen *et al*., 2005) and *Nocardiopsis alkaliphila* was isolated from desert soil in Egypt and grows at pH 9.5-10 (Hozzein *et al*., 2004). A number of mycolate actinomycetes were isolated from the deep-sea bed, an environment with high pressure, low temperatures, lack of light and variable concentration of oxygen and salinity (Colquhoun *et al.*, 1998).

Some actinomycetes are important as human pathogens. *Mycobacterium tuberculosis* is the etiologic agent of tuberculosis, a disease that, according to data from the World Health Organization in 2004 caused 1.7 million deaths. Another *Mycobacterium, M. leprae,* causes leprosy and *Corynebacterium diphteriae,* diphtheria. Although they are not frequent in clinical practice, other genera have the potential to cause serious human and animal infections, *e.g. Nocardia, Rhodococcus, Gordonia* and *Actinomadura* (McNeil & Brown, 1994). Some actinomycetes are also phytopathogenic and belong mainly to the genera *Corynebacterium* and *Streptomyces* (Young *et al.,* 1992, Locci, 1994,).

Probably most of the interest in this group of microorganisms lies in their ability to produce secondary metabolites. Two thirds of the microbial antibiotics known today are produced by actinomycetes (Bèrdy, 1995), mainly by *Streptomyces* species, although the number of rare actinomycetes (non-*Streptomyces*) is increasing due to the application of new selective isolation methods (Lazzarini *et al.,* 2000). Actinomycetes also produce secondary metabolites that show bioactivities other than antibiotics, such as enzyme inhibitors, immunosuppressors, phytotoxins and pesticides (Bèrdy, 1995; Park *et al.*, 2002; Imada, 2005).

Actinomycetes in bioremediation

Actinomycetes possess many properties that make them good candidates for application in bioremediation of soils contaminated with organic pollutants. They produce extracellular enzymes that degrade a wide range of complex organic compounds and spores that are resistant to desiccation. In addition, the frequently occurring filamentous growth favours the colonization of soil particles (Ensign, 1992).

Another interesting feature within this group of microorganisms, especially regarding the degradation of hydrophobic compounds, is their surfactant-producing activity. In the case of the actinomycetes, the surfactant activity is due to a) the production of extracellular biosurfactants, specially glycolipids, like the trehalose lipids produced by *Rhodococcus* species (Rapp *et al.*, 1979; Kretschmer *et al.*, 1982; Singer & Finnerty, 1990) or the lipopeptide produced by a *Arthrobacter* sp. strain MIS38 (Morikawa *et al.*, 1993) and, b) cellular biosurfactants such as mycolic acids that give adherence of the microbial cells to hydrophobic phases in two-phase systems (Neu, 1996).

Many actinomycetes can degrade different pollutants, including several pesticides. For example, members of the genus *Arthrobacter* degrade 4-chlorophenol (Westerberg *et al.*, 2000), atrazine (Strong *et al.*, 2002) and monocrotophos (Bhadbhade *et al.*, 2002) and *Streptomyces* sp. degrades alachlor (Durães Sette *et al.*, 2004). The nocardioform actinomycetes (*e.g. Rhodococcus*, *Gordonia*, *Mycobacterium*) are known hydrocarbon degraders (Kästner *et al.*, 1994; Iwahori *et al.*, 1995; Whyte *et al.*, 1999) and degrade PAH in soil. Also, in some contaminated sites they represent the dominant group among the degraders (Kästner *et al.*, 1994, Johnsen *et al.*, 2002).

The present study

Objectives of the thesis

The main purpose of this thesis was to design a system for the degradation of PAH in soil by actinomycetes and by the addition of co-substrates to enhance their bioavailability. The particular objectives were to:

- Study the effect of hydrophilic and hydrophobic co-substrates on the degradation of PAH by actinomycetes in liquid cultures (**Paper I**).
- Evaluate the effect of the addition of different co-substrates on the degradation of PAH in spiked and aged contaminated soils (**Paper I, II, III and IV**).
- Design a system for the treatment of a PAH-contaminated soil combining the inoculation with a PAH-degrading actinomycete with the addition of a co-substrate **(Paper III)**.
- Evaluate the effect of plants on the degradation of PAH (**Paper IV**).

Actinomycetes used

The actinomycete group comprises a large number of genera. This section briefly describes the strains that were involved in the different projects of this thesis. In addition, the development of a growth methodology for the cultivation of microorganisms with hydrophobic cell surfaces in liquid medium is presented.

Rhodococcus wratislaviensis

Rhodococcus wratislaviensis was originally obtained from the German Collection of Microorganisms as *Rhodococcus* sp. DSM 44126 and had been previously reported as able to degrade naphthalene (Grund *et al.*, 1992). Our results revealed that it was also an efficient phenanthrene degrader and that it could transform anthracene when these two compounds were present as the sole carbon sources in liquid cultures. In addition, *R. wratislaviensis* showed some biosurfactant activity when growing on hydrophobic carbon sources (**Paper I**). Because of its potential for degradation of PAH, we chose this strain for further studies in soil, where it also expressed catabolic activity (**Paper I and II**). The strain was identified to the species level by complete sequencing 16S rRNA (**Paper I**).

Other *Rhodococcus* and *Gordonia* strains were included in the initial screening for biosurfactant production and phenanthrene degradation. The *Gordonia* strains formed emulsions, an ability that could be useful for enhancing PAH bioavailability. In addition *Gordonia* sp. APB degrades phenols (Pérez-Bercoff, 2003), compounds that are often found as co-contaminants together with PAH in creosote-contaminated sites.

Mycobacterium LP1

The high mineralisation and degradation values obtained in the control soil (**Paper I and II**) revealed the presence of an active PAH-degrading microflora. A pyrene

degrader was isolated from that soil by enrichment culture. According to the results of the 16S rRNA sequencing, our isolate was closely related to *Mycobacterium gilvum* and it was designated *Mycobacterium* LP1. In liquid cultures, *Mycobacterium* LP1 was able to degrade phenanthrene and pyrene as sole carbon sources. Anthracene and, to a smaller extent, benzo(*a*)pyrene was also degraded when phenanthrene and pyrene also were present. Further mineralisation studies in soil demonstrated the ability of the strain to metabolise phenanthrene and pyrene to a large extent. *Mycobacterium* LP1 was used as inoculum in the sequential treatment of a contaminated soil, and proved to be a good degrader (**Paper III**).

Other actinomycetes

Several actinomycetes probably were involved in the degradation of PAH in the presence of willows in the phytoremediation experiment, since they were present at high numbers in almost all the treatments with the highest degradation values (**Paper IV**). The classification was preliminary, based on few characteristics, and the isolates were all assigned to the group *Clavibacter-Arthrobacter-Corynebacterium*, according to a previous classification scheme (Cambours *et al.*, 2005). The results, however, indicate that there could be interesting PAH-degraders among these isolates.

A method for the cultivation of microorganisms with hydrophobic cell surfaces in liquid medium containing hydrophobic substrates

The genera *Rhodococcus* and *Gordonia* belong to the taxonomical group coryneform bacteria. Mycolic acids are a major constituent of the cell wall of these bacteria and their presence and chain length give varying hydrophobic and adhesive properties to the cell surface (Bendinger *et al.*, 1993). We encountered a practical problem when we tried to grow these hydrophobic cells in media containing hydrophobic carbon sources. Rapeseed oil and hexadecane deposited on the walls of the Erlenmeyer flask and so did the microorganisms. As a result most of the growth occurred on the glass surfaces, especially at the air-liquid interface (Fig. 1).

Based on this tendency of the cells and the hydrophobic carbon sources to adhere to the glass surfaces, we developed a growth system using glass beads immersed in liquid medium. Approximately 200 g of 5-mm-diameter glass beads and 50 ml of liquid medium were placed in a 250-ml Erlenmeyer flask, so that the space between the glass beads was filled with liquid medium (Fig. 1). The flask was then placed on a shaking table at 40 rev min^{-1} to allow oxygen exchange. The system was adapted for the phenanthrene degradation experiments by replacing the flasks with 50-ml test tubes, 20 g of glass beads and 10 ml of liquid medium. The tubes were placed on a shaking table at 40 rev. min^{-1} with an inclination of about 10° to the horizontal, to maximize the surface in contact with the liquid.

The method was simple and allowed a good microbial growth. The hydrophobic compounds and the cells adhered to the surfaces uniformly and the growth was confined to the liquid medium and glass beads matrix. Moreover, it was suitable

for the quantification of PAH since the solvents could directly be added to the tubes and the presence of the glass beads did not affect the extraction procedure. One disadvantage of the system is that bacterial growth over time cannot be quantified by the traditional methods, *i.e.* by optical density or plate counting. Therefore, a destructive method has to be used instead, since the attached cells have to be released first and then analysed.

Methods involving the growth on glass beads have been used for other purposes. A cultivation system consisting of smaller glass beads (265 to 325 µm) immersed in liquid medium placed in syringes was applied for the growth and the study of *Streptomyces* differentiation (Nguyen *et al.*, 2005). On a larger scale, glass beads in bioreactors for the growth of biofilms (Park *et al.*, 2005) and immobilization of cells (Hou, 1984, Ehlers & Rose, 2005) have been used. Our method is recommended for the growth of microorganisms in liquid media supplemented with hydrophobic substances.

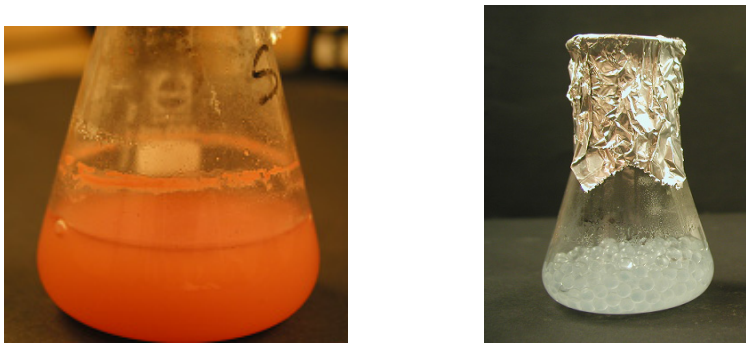


Figure 1. Growth on walls in an ordinary flask and the glass-bead system.

PAH studied

The degradation of PAH was studied in three different systems, *viz.* liquid cultures (**Paper I**), spiked soil (**Paper I, II and III**) and aged creosote-contaminated soil (**Paper IV**).

Phenanthrene, anthracene, pyrene and benzo(a)pyrene were used as model compounds and studied in liquid cultures, individually and in a mixture. In the spiked soil they were studied as a mixture. They were chosen as representatives for PAH with different number of rings, water solubilities and recalcitrance (Table I). Labelled [9-^{14}C] phenanthrene, [4, 5, 9, 10-^{14}C] pyrene and [7,10-^{14}C] benzo(a)pyrene were used in the studies of mineralisation in spiked soil. The mixture of PAH was added to a non-polluted agricultural soil. It is worth mentioning that this soil turned out to harbour an active microflora with the ability

to degrade PAH, a potential that essentially remains unexplored (**Paper II and III**).

A creosote-contaminated soil was used in the phytoremediation study (**Paper IV**). Creosote is a common wood-preservative chemical, which can contain 85% of PAH, 10% phenolic compounds and 5% N-, S- and O-heterocyclic compounds (Carriere & Mesania, 1995). The soil used in this experiment was collected from a site in Vansbro, Dalarna, Sweden. This location was used from 1953 to 1967 by the Swedish State Railway for impregnation of sleepers with creosote oil. The total PAH content was ~10 mg kg^{-1} and consisted probably of less bioavailable PAH.

Effect of different co-substrates on biosurfactant activity and degradation of PAH

This section discusses the effect of different substrates on a) the production of biosurfactants (**Paper I**) and b) the degradation of PAH by selected actinomycetes (**Paper I**) and by microorganisms present in an aged creosote contaminated soil (**Paper IV**).

The effect of hydrophilic (glucose) and hydrophobic substrates (rapeseed oil and hexadecane) on the production of surface-active substances by three *Rhodococcus* and two *Gordonia* strains was studied (**Paper I**). All five strains showed some biosurfactant activity, but only in the presence of a hydrophobic carbon source (Table 2 in **Paper I**). The activity was measured as the percentage of decrease in surface tension in the whole medium or in the supernatant from time 24 to 168 h. Only the *Gordonia* species showed emulsifying properties. Emulsions were formed by the whole culture but not in the cell-free supernatant. This indicates that the effect was due to cell-related compounds or to the high hydrophobicity of the cell surface. The decrease in surface tension, both in the whole culture and the supernatant, indicates that these strains also produce biosurfactants. It has been shown that a combination of extracellular biosurfactants and cell properties induced the formation of emulsions by *G. amarae* (Iwahori *et al.*, 2001). The *Rhodococcus* species exhibited surfactant activity but did not form emulsions (**Paper I**). Surfactants produced by Rhodococci are predominantly glycolipids (Lang & Philp, 1998). These molecules are involved in the lowering of surface and interfacial tensions in liquids but stable emulsions are not a usual trait for these surfactants (Christofi & Ivshina, 2002).

The surface tension of the media without microorganisms was 71 mN m^{-1} in the presence of glucose or hexadecane, similar to pure water. However, in the medium with only rapeseed oil the values before and after centrifugation were 49.2 mN m^{-1} and 59.7 mN m^{-1}, which indicates that the rapeseed oil acted as a surfactant itself. The phospholipids, fatty acids and neutral lipids present in the vegetable oil probably contributed to this surfactant effect (Desai & Banat, 1997; Mulligan, 2005). The ability of rapeseed oil to lower surface tension was also observed in soils (**Paper II**).

The strains tested for surfactant activity were known for their ability to degrade several aromatic compounds (Table 1 in **Paper I**) but there was almost no information available about their ability to transform PAH. Our results revealed that, among the five strains, only *R. wratislaviensis* was able to utilize phenanthrene as the sole carbon source, degrading ~80% in 14 days. When glucose, hexadecane or rapeseed oil was used as co-substrate all strains showed some degradation of phenanthrene, although to different extents and with values lower than 20% in all cases except for *R. wratislaviensis* (Fig. 2 in **Paper I**). Most likely this enhancement was due to an increase in microbial biomass and probably, in the case of the hydrophobic compounds, by cometabolism or increased solubilisation.

Two different co-substrates, wheat straw and an octylphenol polyethoxylate surfactant (Triton X-100), were evaluated within the framework of the phytoremediation study (**Paper IV**). The addition of straw as an additional carbon source stimulated both the growth and the activity of the soil microflora but did not enhance the degradation of aged PAH present in soil (Figure 3 in **Paper IV**). The addition of straw usually favours the production of unspecific extracellular enzymes by ligninolytic fungi (Castillo *et al*., 1997), which can transform the hydrocarbons (Andersson & Henrysson, 1996) but do not stimulate availability, which probably was the limiting factor for degradation in this study, as discussed below.

The addition of Triton X-100 enhanced the degradation of phenanthrene and pyrene. Surfactants act as solubilisation agents but can also sometimes function as carbon source (Chen *et al.*, 2004). However, in our study no increased respiration was obtained by the addition of Triton X-100 but a tendency of decreased amounts of active microorganisms were obtained with increasing Triton concentrations indicating that the surfactant could be toxic to the soil microflora. The increase in degradation was probably the result of an increased bioavailability.

According to the results of the phytoremediation study (**Paper IV**), higher degradation of PAH was obtained in the presence of the willows. In the treatments with plants, the number of viable counts increased and there was a clear shift from dormant to active microorganisms. Likely, the root exudates, *e.g.* organic acids, acted as a "natural" amendment, providing carbon to the microorganisms, including the PAH-degrading microflora.

Effect of rapeseed oil on the degradation of PAH by *R. wratislaviensis* in soil

The next step after the studies in liquid medium was to evaluate the performance of *R. wratislaviensis* in a soil contaminated with phenanthrene, anthracene, pyrene and benzo(*a*)pyrene in the presence of rapeseed oil as co-substrate, added at 0.1% and 1% w/w.

In the soils without the addition of rapeseed oil, treatments with *R. wratislaviensis* degraded phenanthrene and anthracene at a rate higher than those in the control soil without inoculum (**Paper I and II**). This gave indirect evidence of the ability of the strain to survive in the soil environment and to compete with the autochthonous microflora. At the end of the incubation time, phenanthrene and anthracene were almost completely degraded in both inoculated and non-inoculated soils. *R. wratislaviensis* did not degrade pyrene or benzo(*a*)pyrene. However, the indigenous microflora degraded 90% of pyrene and 30% of benzo(*a*)pyrene after 49 days (**Paper II**). Except for an initial delay in the degradation of phenanthrene and anthracene by the indigenous microflora, the results obtained with the addition of 0.1% rapeseed oil did not differ much from those in the soils without rapeseed oil. Therefore, hereafter the discussion will be based on the results obtained in the soils without or with 1% of rapeseed oil.

The addition of 1% rapeseed oil produced a degradation profile different from that in the soil without co-substrate. The transformation of phenanthrene and pyrene was negatively affected, reflected in both the mineralisation and the degradation values, irrespective of the presence of *R. wratislaviensis*. Figure 2 shows the mineralisation levels of ^{14}C-phenanthrene and ^{14}C-pyrene during incubation at 30°C in the inoculated soil, with or without oil. In the presence of the oil there was a decrease in mineralisation of both compounds, more marked for pyrene than for phenanthrene. The degradation values were also lower (Fig. 3), falling from 94 to 30% for pyrene and from 99 to 66% for phenanthrene with the addition of 1% rapeseed oil. Conversely, the degradation of benzo(*a*)pyrene was enhanced from 35 to 74% with the addition of the oil (Fig. 3).

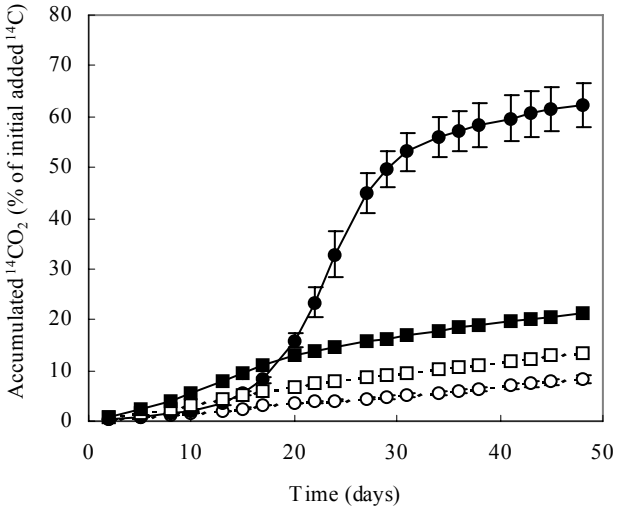


Figure 2. Phenanthrene (squares) and pyrene (circles) mineralisation (as % of added ^{14}C) in soil incubated at 30˚C and inoculated with *Rhodococcus wratislaviensis* and with addition of 1% (open symbol) or without (solid symbol) rapeseed oil. The values are means ± SD (*n*=3).

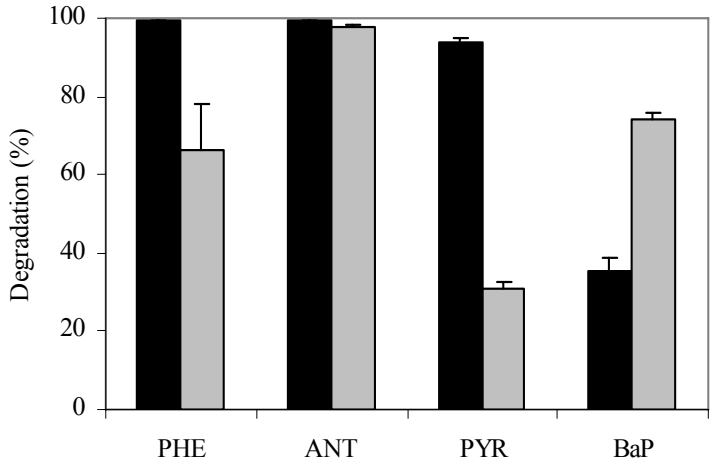


Figure 3. Degradation of phenanthrene (PHE), anthracene (ANT), pyrene (PYR) and benzo(*a*)pyrene (BaP) in inoculated soils without (black bar) and with 1% rapeseed oil (grey bar) after 49 days of incubation at 30°C. The values are means ± SD (*n*=3).

The degradation levels for anthracene were similar with or without the addition of the oil, but different pathways might be involved in the degradation of this compound. In the presence of 1% oil, anthraquinone was formed at high amounts and accumulated (**Paper II**) but was produced in small amounts and degraded completely in the soils with 0.1% or without rapeseed oil. Anthraquinone is a metabolite of anthracene oxidation and both biotic and abiotic *(e.g.* by photooxidation) degradation of anthracene with formation of anthraquinone are well known (Mallakin *et al.*, 2000).

The activity of specific soil organisms might be involved in the formation of anthraquinone as a metabolite of anthracene degradation. Indeed, 9,10-anthraquinone has been found as a dead-end product in the degradation of anthracene by *Mycobacterium* sp. PYR-1 (Moody *et al.,* 2001) and a mycobacterium able to degrade anthracene was isolated from the control soil used in this experiment (**Paper III**). However, the contribution of this pathway to the production of anthraquinone was not high, since only low concentrations of this metabolite were found in the non-inoculated soil without or with 0.1% of rapeseed oil, regardless of the extensive degradation of anthracene. Moreover, anthraquinone is an important metabolite from the degradation of anthracene by white-rot fungi. One of the enzymes of the lignin-degrading system of these fungi, manganese peroxidase, promotes lipid peroxidation of unsaturated fatty acids (Bao *et al.*, 1994; Moen and Hammel, 1994). It has been shown that this process can give decomposition of recalcitrant PAH and non-phenolic lignin model

compounds (Bogan *et al*., 1996; Watanabe *et al*., 2000). The presence of rapeseed oil may have stimulated this kind of reaction in the soil.

The same mechanisms may be involved in the oil-dependent transformation of benzo(*a)*pyrene. Even though it was not possible to identify any metabolites, *e.g.* quinones, a change in colour in the toluene layer was observed after extraction in the treatments containing 1% rapeseed oil, where a reduction in the concentration of benzo(*a*)pyrene was observed (Fig. 4). The participation of an abiotic process is supported by the results obtained in liquid culture, using sterile controls, where a similar transformation of anthracene and benzo(*a*)pyrene took place in the presence of 1% rapeseed oil, which in addition, was lower in dark conditions (**Paper I**).

The reason why phenanthrene and pyrene were not transformed in the presence of the oil may lie on their higher ionization potentials compared to those of anthracene and benzo(*a*)pyrene (Table 1). Bogan and Lamar (1995) found a strong correlation between the disappearance of PAH and ionization potential in a manganese peroxidase-lipid peroxidation system, where PAH with low ionization potential were the most susceptible to degradation.

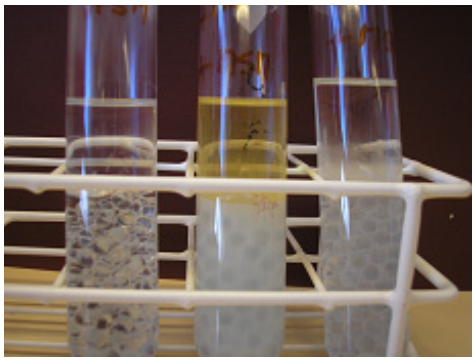


Figure 4. Colour development in the toluene layer after extraction of benzo(*a*)pyrene in sterile liquid medium containing 1% rapeseed oil (centre), compared to the treatments in absence of the oil and with benzo(*a*)pyrene (left) and with 1% oil and in absence of benzo(*a*)pyrene (right).

Regarding the detrimental effect of the highest concentration of rapeseed oil on the degradation of phenanthrene and pyrene, the supply of such large amounts of a carbon source probably resulted in the depletion of nutrients, and possibly also of oxygen. This limitation was evident one to two weeks after the addition of the oil, when the degradation of phenanthrene by *R. wratislaviensis* ceased. There was also almost a total inhibition of the autochthonous microorganisms able to degrade phenanthrene and pyrene. As seen in Figure 5, in the soils without addition of the co-substrate, there was a 2- and 3-week lag phase for phenanthrene and pyrene,

respectively, before degradation became substantial. At that time, in the soil with rapeseed oil, the nutrients might already be exhausted. This also explains the results obtained in the mineralisation study shown in Figure 2. The negative effect of the oil was stronger on the mineralisation of pyrene in the inoculated soil, which mainly was due to the activity of the autochthonous microflora, than on phenanthrene, mainly due to *R. wratislaviensis*.

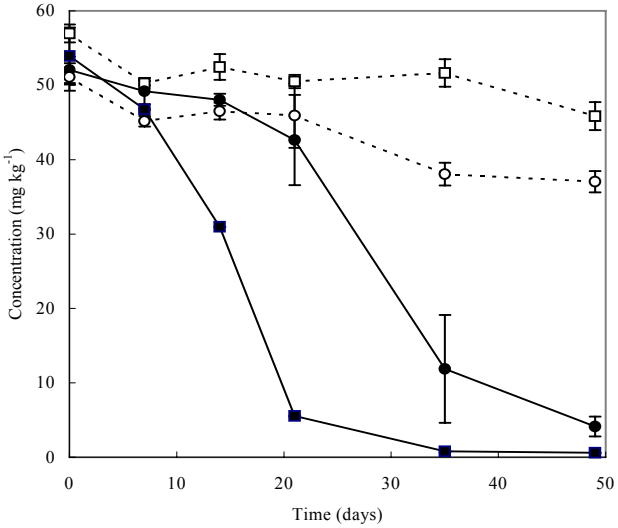


Figure 5. Concentration of phenanthrene (squares) and pyrene (circles) in non-inoculated soil incubated at 30˚C, with addition of 1% rapeseed oil (open symbols-dashed lines) or without addition (solid symbols-solid lines). The values are means ± standard deviations (*n*=3).

One important aspect in the degradation of PAH is the formation of metabolites that may be more toxic than the parent compounds (Mallakin *et al.*, 1999) and that can accumulate in the soils (Wischmann & Steinhart, 1997). Furthermore, it has been proposed that partially oxidized PAH show an increased bioavailability and biodegradability (Meulenberg *et al.*, 1997) and that most transformations performed by fungi, *e.g.* to quinones and phenols, reduce the mutagenicity and toxicity of PAH (Sutherland, 1992). We found that anthraquinone, which accumulated at high concentrations, could be eventually degraded by the indigenous microflora (**Paper II**). However, further information is needed about the production and fate of other metabolites after the addition of rapeseed oil.

In summary, two main processes took place in the soil depending on the amount of oil added and the compound in question. In absence of rapeseed oil, phenanthrene, anthracene and pyrene were biologically degraded by *R. wratislaviensis* and/or the indigenous microflora and the degradation of benzo(*a*)pyrene was low. In presence of 1% rapeseed oil, the biological processes were probably retarded by

nutrient and oxygen limitations. However, a high transformation of benzo(*a*)pyrene and anthracene occurred, mainly due to oxidation processes, initiated by chemical or biological processes, and partially due to biological degradation. When initiated, these oxidation processes apparently can continue independently of the nature of the initial step (*e.g.* photooxidation or fungal enzymes). Even though biological activity may be involved at the beginning of the process, we refer to it as "abiotic" because of its predominance in the transformation of the compounds during the whole process.

A two-step sequential treatment using *Mycobacterium* LP1 and 1% rapeseed oil

Based on the results from **Paper II**, a two-step sequential treatment was proposed as an alternative for cleaning up PAH-contaminated soil, combining the biological and the "abiotic" processes. The first step was based on the biological transformation of the compounds, enhanced by the inoculation with an efficient PAH degrader. The second step consisted of the addition of rapeseed oil (1% w/w) aiming for the "abiotic" transformation of the PAH remaining in the soil after the biological degradation.

The course of degradation for each of the spiked compounds during the whole treatment is shown in Figure 6. During the first 12 days (after inoculation but before the addition of rapeseed oil), 96.3% of phenanthrene, 78.4% of anthracene and 92.2% of pyrene was degraded, compared to 72.2, 34.2 and 17.6% respectively in the non-inoculated soil (**Paper III**). The degradation of benzo(*a*)pyrene after the first step was about 23% in both soils. After the second step, almost all phenanthrene, anthracene and pyrene and 85% of the benzo(*a*)pyrene were degraded.

The transformation of benzo(*a*)pyrene, one of the most beneficial effects of the oil treatment, is time dependent and higher degradation values can be obtained with longer incubation times. Figure 7 compares the degradation values obtained with the application of the sequential treatment to those obtained in soils after the simultaneous addition of inoculum and rapeseed oil at the beginning of the incubation time or those obtained with only inoculation. After 34 days of incubation in the presence of *Mycobacterium* LP1 and of rapeseed oil, 96.6% of the benzo(*a*)pyrene was transformed. The sequential treatment degraded 85% of the benzo(*a*)pyrene but the "abiotic" period was of only 22 days. Probably, higher degradation can be obtained with longer "abiotic" treatment times. The advantage of the two-step treatment is evident in Figure 6. The simultaneous addition of *Mycobacterium* LP1 and rapeseed oil gave a high degradation of benzo(*a*)pyrene and anthracene but affected negatively the degradation of phenanthrene and pyrene, as seen previously with *R. wratislaviensis* (**Paper II**). The inoculation without addition of rapeseed oil resulted in high degradation of phenanthrene, anthracene and pyrene, but low transformation of benzo(*a*)pyrene (Fig. 7).

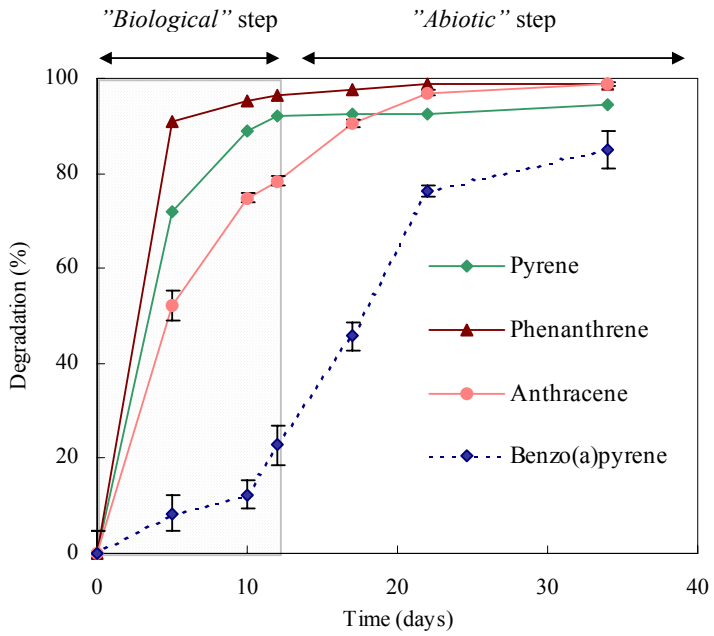


Figure 6. Degradation of the four PAH during the two-step sequential treatment. The shadowed area represents degradation before the addition of rapeseed oil at day 12. The values are means ± SD (*n*=3).

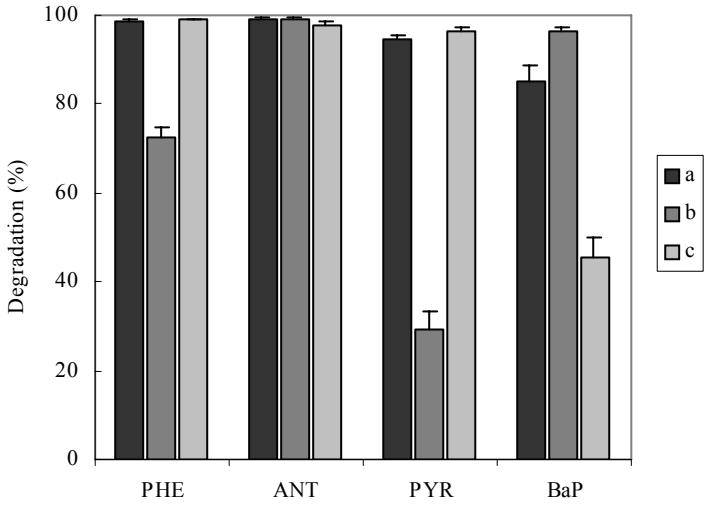


Figure 7. Degradation of phenanthrene (PHE), anthracene (ANT), pyrene (PYR) and benzo(*a*)pyrene (BaP) in soils after 34 days of incubation at 30°C and after sequential treatment (a), simultaneous inoculation with *Mycobacterium* LP1 and addition of rapeseed oil at day 0 (b) or only inoculation (c). The values are means ± standard deviations (*n*=3).

Vegetable oils have been applied in remediation of soils contaminated with PAH in diverse systems, and with different purposes, *e.g.* as solvents or substrates for the enhancement of biodegradation of the pollutants by the soil microflora (Table 2). The interactions between the oil, the pollutant and the microorganisms are highly affected by the concentration of oil added to the system. Low amounts of vegetable oil (0.1-0.2%) enhance the microbial degradation (Pannu *et al.*, 2003; **Paper II**) probably as a result of one or more of the following mechanisms: a) increase of the solubility of the pollutants by their dissolution into the oil; b) stimulation of the production of biosurfactants (Sim & Ward, 1997); c) increase of microbial growth by the presence of an additional carbon source; d) attachment of the microbial cells to a hydrophobic surface; or e) function as surfactants themselves. In our system the oil is used to stimulate the "abiotic" oxidative transformation of the PAH. After this stage is completed, the oil might as well play the same roles as mentioned above, however, addition of nutrients and oxygen may be necessary to re-establish the balance with the carbon source.

Table 2. Methods for treating PAH contaminated soils involving vegetable oils.

Oil type	Description of the system	Oil:soil	PAH Removal (%)
Sunflower [a]	a) oil percolation through a column of soil	150 ml in 75-150 g	>90
Rapeseed[b]	a) bioslurry b) abiotic release in a slurry system	1.5-7.5 ml in 28 g	86
Peanut[c]	a) double extraction in a bioreactor b) cleaning of contaminated oil with activated carbon c) bioregeneration or thermal treatment of activated carbon	5 + 5% (w/w)	91.4
Peanut [d]	50% w/v slurry in water (proposed as a complement of the system described above as an alternative to step b)	0.2% (v/v)	42 to 94
Corn and palm kernel [e]	a) oil addition to soil slurry b) treatment with Fenton's reagent	1-5% w dry soil	
Rapeseed [f]	a) biological degradation b) addition of oil for abiotic treatment	1% (w/w)	86 to 99

[a] Gong *et al*., 2005; [b] Berg Schuur & Mattiasson, 2003; [c] Pannu *et al.*, 2004; [d] Pannu *et al.*, 2003; [e] Bogan *et al*., 2003; [f] this study

Some of the systems in Table 2, even if efficient in the removal, have some disadvantages. Some are complex, involving several steps and equipment *e.g.* bioreactors or special columns, and the amounts of oil applied often are high.

In summary, it is possible to obtain high degradation values by applying the sequential treatment to a PAH-contaminated soil, including those compounds that have low availability and are recalcitrant, *e.g.* benzo(*a*)pyrene. The method has potential for scaling up: it is simple, fast and recycling of the oil is not needed since the amounts applied are low.

Concluding remarks and future perspectives

The major findings of this work can be summarized as follows:

- A glass-bead system for culture in liquid medium was developed for the growth of microorganisms with hydrophobic cell walls growing on hydrophobic carbon sources.
- *Rhodococcus* sp. DSM 44126 was characterized as *Rhodococcus wratislaviensis* and new PAH-degrading abilities of this strain were reported, *i.e.* phenanthrene and anthracene degradation and surfactant-producing activity.
- A *Mycobacterium* strain, denominated *Mycobacterium* LP1, able to degrade pyrene, phenanthrene and anthracene was isolated from an agricultural soil.
- Rapeseed oil showed surfactant properties and stimulated the formation of emulsions and the biosurfactant activity of the actinomycete strains in liquid cultures. In soils, when present at high concentrations it decreased the degradation of phenanthrene and pyrene, but had a stimulatory effect on abiotic and biological processes leading to a high transformation of anthracene and benzo(*a*)pyrene.
- A two-step sequential treatment, consisting of the inoculation with a PAH degrader, followed by the addition of 1% rapeseed oil, was designed and proved to be effective for the degradation of PAH in a spiked soil.
- A large effect of willows on PAH degradation and on the microbial activity was obtained. The presence of plants stimulated the PAH-degrading activity and changed the physiological state of the microorganisms from dormant to active, a change that also increased their culturability.

Many questions remain to be answered and new questions arise from this work. For instance, the ability of *Mycobacterium* LP1 to degrade four different PAH was tested. Members of this genus often are able to degrade a wide range of PAH metabolically or co-metabolically and probably the catabolic capacity of our isolate is much broader than reported here.

The mechanisms behind the transformation of the PAH in the presence of the rapeseed oil are not yet elucidated. Such knowledge is crucial for optimising conditions that favour the transformations. The effect on other pollutants has to be tested and also information on products formed during the process and their properties, *e.g.* toxicity and recalcitrance, is needed.

Promising results have been obtained with the application of a sequential treatment to a spiked soil. The next and decisive step is to implement this method on aged PAH-contaminated soil, with low bioavailability. One probable improvement of the method is to invert the order of the steps by first making the compounds available and some of them oxidized with the oil and then transform them by the addition of nutrients included in the following clean-up step.

The presence of plants seemed to favour the growth of specific PAH-degrading actinomycetes. The identification of those microorganisms and the evaluation of

their catabolic ability and the dramatically changed physiological state of the microorganisms from dormant to active as a response to the presence of plants inspire to more studies.

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