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Ph.D. Thesis

Bioremediation of Creosote-contaminated Soil

Marius Byss

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University of South Bohemia
Faculty of Science
Department of Ecosystem Biology
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Annotation

Bioremediation of creosote-contaminated soil was studied employing the methods of soil microbial biology and using new gas chromatography-mass spectrometry-mass spectrometry analytical approach. The changes of the soil microbial community under the polycyclic aromatic hydrocarbons (PAH) pollution impact were analyzed and described, as well as the changes during the bioremediation experiments. Laboratory-scale bioremediation experiments using the soil microbial community (consisted of bacterial inoculum capable of degrading PAH and of original soil microbial community) and employing ligninolytic fungi *Pleurotus ostreatus* and *Irpex lacteus* were conducted.

I hereby declare that this Ph.D. thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement has been made in the text.

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List of papers

The thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Byss, M.**, Elhottová, D., Tříska, J., Baldrian, P. (2008): Fungal Bioremediation of the Creosote-contaminated Soil: Influence of *Pleurotus ostreatus* and *Irpex lacteus* on Polycyclic Aromatic Hydrocarbons Removal and Soil Microbial Community Composition in the Laboratory-scale Study. **CHEMOSPHERE**. DOI:10.1016/j.chemosphere.2008.07.030.

II **Byss, M.**, Tříska, J., Elhottová, D. (2007): GC-MS-MS Analysis of Bacterial Fatty Acids in Heavily Creosote-contaminated Soil Samples. **ANALYTICAL AND BIOANALYTICAL CHEMISTRY** 387, 1573-1577.

III **Byss, M.**, Elhottová, D., Tříska, J.: Soil Microbial Community Changes During Bioremediation of Extensively Creosote-contaminated Soil. *Manuscript.*

Co-author agreement

We hereby declare that Marius Byss had a major contribution to each of these three papers.

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Jan Tříska	Dana Elhottová	Petr Baldrian

Contents

Prologue

This Ph.D. thesis was focused on bioremediation of soil extensively contaminated by creosote. Creosote, as a mixture of polycyclic aromatic hydrocarbons (PAH), is a wide-spread preservative used for wood treatment and represents a serious problem due to its carcinogenicity and persistence in soil. Various *in situ* and *ex situ* techniques have been employed to treat creosote-contaminated sites and decontamination procedures based on biological treatment have become quite successful option, especially for favourable cost/effective ratio. Nevertheless, there are still certain severe constraints of these biological approaches, e.g. recalcitrance of PAH with more than four aromatic rings within the molecule or strong PAH sorption to the soil particles/soil organic matter, etc. The general aim of this thesis was to contribute to the present knowledge of creosote bioremediation and improve *status quo* by highlighting new promising opportunities.

There are various organisms capable of PAH degradation, however PAH with more than four aromatic rings within the molecule remain severe problem in the environment due to their molecular structure resistant to simple degradation. These PAH pose high-level health risk, because of their carcinogenic, mutagenic and teratogenic effects on humans and animals. Ligninolytic fungi were found to be capable of removing these problematic compounds, because of their non-specific enzymes capable of lignin (molecular structure similar to the structure of PAH) degrading. This ability constitutes opportunity how to deal with high molecular weight PAH recalcitrance.

A part of the thesis was dedicated to the development of new method allowing distinguishing the soil microbial community signature molecules (phospholipid fatty acids) in interfering hydrophobic background signal caused by PAH presence in the sample during the gas chromatography analyses. Developed technique was derived by adding new substantial improvement to the practice in present use, was successful in distinguishing fatty acids in extensively creosote-contaminated soil samples and is applicable for the lipophilic signature molecules present in the soil in general.

Introduction

Polycyclic aromatic hydrocarbons (PAH) - short overview

Polycyclic aromatic hydrocarbons are toxic pollutants that have accumulated in the environment due to a variety of anthropogenic activities. PAH are also major constituents of creosote: about 85% is comprised by PAH, while heterocyclic aromatic hydrocarbons and phenolic compounds are also present (ATAGANA, 2004a).

PAH are the group of various substances based on two or more aromatic (fused benzene) rings with the possibility to form derivatives with various radicals (alkyl-, hydroxy-, nitro- derivates etc.). Sixteen PAH selected as priority pollutants by the United States Environmental Protection Agency (U.S.EPA) are made up of two or more aromatic rings and/or pentacyclic heterocycles, arranged in linear, angular, and cluster arrangements (DABESTANI ET IVANOV, 1999).

Molecular weight ranges from 128 to 178 $g.mol^{-1}$. PAH are thermodynamically stable because of their large resonance energy. These neutral compounds have generally very low aqueous solubility. The most soluble PAH are naphthalene (31 $mg.l^{-1}$) and acenaphthylene (16 $mg.l^{-1}$). PAH of higher molecular weight are much less soluble (benzo[*a*]anthracene 0,009 $mg.l^{-1}$, benzo[*g,h,i*]perylene 0,00026 $mg.l^{-1}$). They are highly hydrophobic (log P = 3,3 at naphthalene to 6,75 at dibenzo[*a,h*]anthracene). They tend to be associated strongly with particle surfaces in the environment (K_{oc} = 2.10^3 at naphthalene to $3{,}8.10^6$ at dibenzo[*a,h*]anthracene). In addition, most of them present low sensitivity to volatilization and photolysis. As a result, they are generally persistent under many natural conditions, with half-lives in soils amounting to 26 days for phenanthrene (a volatile and degradable compound) and 6250 days for benzo[*a*]anthracene, representing a recalcitrant compound (SIMS ET OVERCASH, 1983; ALEXANDER, 1994).

Environmental sources of PAH are multiple. Formed during almost all combustion reactions, as well as being natural components of fossil fuels, PAH have been produced throughout geological time and widely distributed in the biosphere (HOLOUBEK, 1996). Some PAH may have natural origin, as biogenic compounds, they are components of surface waxes of leaves, plant oils and cuticles of insects. They are also formed from biosynthetic pathways in plants, including carotenoids, lignin, alkaloids, terpenes and flavonoids, which are continuously cycled through the biosphere (HARVEY ET AL., 2002). PAH of geochemical origin also occur in the environment. They are formed when organic substances are exposed to high temperature (pyrolysis), and by the aromatization of biological compounds during

humification. Petroleum, coal (all fossil fuels) and ancient sediments provide the largest source of petrogenic polyaromatic compounds. Finally, PAH of pyrogenic origin are released into the environment during vegetation fires and volcano eruptions (MUELLER ET AL., 1996).

PAH also have anthropogenic origin in many industrially developed countries where point source contaminations are common results of spills or leakages due to mismanaged industrial operations. Contaminated areas are generally small, but PAH concentrations are high and often associated with many other types of xenobiotics and heavy metals. Industrial pollution includes steelworks, manufactured gas plants and wood-preservation treatment plants (KANALY ET HARAYAMA, 1996).

Concern over PAH is due mainly to the fact that some PAH and their transformation products are toxic to living cells, with respect to ring number and condensation degree. Generally, PAH are classified into three categories: probably carcinogenic, possibly carcinogenic, and not classifiable. For example, benzo[*a*]pyrene, chrysene and benzo[*a*]anthracene are probably carcinogenic for mammals, mutagenic and/or teratogenic in bacterial and animal cells (DABESTANI ET IVANOV, 1999).

Interactions between PAH and the soil

PAH-soil interactions determine the fate, transport and bioavailability of the hydrocarbons for degrading organisms in aquatic and terrestrial ecosystems. Bioremediation techniques have been developed and improved due to the ability of degrading organisms to grow and multiply in polluted soils. Nevertheless, direct contact between PAH and organisms, regulated by chemical bioavailability, is essential and crucial for effective treatment (EGGEN ET SVEUM, 1999). Several complex physico-chemical and biological factors are responsible for PAH limited availability to microbial populations in the environment and for increasing PAH recalcitrance with time (TÖRNEMAN ET AL., 2008).

It is unlikely that the microorganisms are exposed to single aromatic hydrocarbon in the environment, because mixtures are much more likely to occur. It is well established that certain mixtures are more rapidly biodegraded than the others. For example, naphthalene stimulates the biodegradation of phenanthrene, but not that of anthracene (MOLLEA ET AL., 2005). However, most of the laboratory studies are mainly based on work with non-growth-supporting (secondary) substrates, with biomass being created by one or more easily degradable primary substrate, present in high concentration (CARMICHAEL ET PFAENDER, 1993; CAI ET AL., 2007).

Several mechanisms condition the partitioning of the pollutants between water, dissolved humic matter and soil or sediment humic matter. Humic acids constitute major fraction of soil natural organic matter, which is dominant sorbent of hydrophobic compounds in soil. Sorption of pollutants to humic acids associated with soils usually inhibits transport and bioavailability, whereas binding to dissolved humic material facilitates desorption from the solid phase. Binding to soils of nonionic compounds such as PAH depends on the organic carbon concentration of the soil. The affinity for association of a contaminant with the organic material of soil (expressed as the organic carbon partition coefficient K_{oc}) is a function of the hydrophobicity of the compound (expressed as the octanol-water partition coefficient K_{ow} or P), thus naturally occurring organic matter is an excellent PAH sorbent. Moreover, the molecular size appears to be an important factor determining the binding of solids to the organic matter. PAH exist in soil not only sorbed to solids but also in liquids immiscible with water, called non-aqueous phase liquids (NAPL). As such, the availability of the contaminants to biodegradation may be drastically reduced (KRAATZ ET AL., 1993; MAHRO ET KÄSTNER, 1993). Two kinetically distinct processes were found to be associated with PAH binding onto soil material, a "fast" and a "slow" process. The initial fast adsorption process is considered to reflect rapid adsorption of PAH onto hydrophobic areas of soil surfaces, whereas the following slow adsorption process is considered to be based on the migration of PAH to less accessible sites within the soil matrix. Thus, longer pollution periods result in migration of an increasingly large fraction of the pollutant into the organic material (CERNIGLIA, 1984).

Two views exist on how hydrophobic molecules are retained by the organic matter. The first view maintains that the process is physical sorption by the organic matter, in which physical binding of the solute to the organic solids occurs. The second view holds that hydrophobic molecules exist in the organic matter because they diffuse and partition into the solid organic matter, within the physical matrix of the solid organic phase functioning as a sorbent. This is similar to process of partition of the hydrophobic compound from aqueous solution into an organic solvent in which it is highly soluble (CHIOU ET AL., 1986; CHIOU 1989). Sequestration of PAH is still not fully understood process of PAH becoming physically unavailable to microorganisms (WICKE ET AL., 2008). This process is a time dependent, aged PAH are more entrapped than the pollutants which got to the soil recently. Pollutant concentration is one of the variables, but the presence of numerous pollutants in polluted soil may affect the sequestration of a specific chemical. In short-term contaminated soil, PAH biodegradation competes with migration of PAH into non-accessible soil compartments (WEISSENFELS ET AL., 1992). PAH may also be converted partially to bound residues in the

soil. They thus may represent new molecular species formed from the parent compound of transformation products, often as complexes with organic constituents of soils or living organisms. The residues are stable, often considered as releasing the xenobiotics in concentrations too low to represent a hazard. Nevertheless, it is not completely clear whether bound residues present a problem of toxicological significance (WILSON ET JONES, 1993).

PAH polluted soils may be classified into three main types, two including PAH of pyrolytic origin (fluoranthene : pyrene ratio > 1) and one with petroleum hydrocarbons origin (fluoranthene : pyrene ratio < 1). All these soils are subjected to slow PAH release (BAUMARD ET AL., 1998; LEHTO ET AL., 2000; AHTIAINEN ET AL., 2002). First type of soil is found around wood-preserving treatment plants, where wood is impregnated by creosote. It comprises mainly 3-ring PAH (phenanthrene and anthracene) and 4-ring compounds (fluoranthene, pyrene). Soil organic carbon content is generally low (less than 2%) and pollutants are moderately aged. Their potential for bioremediation is high (ATAGANA ET AL., 2006). Soils from manufactured gas plants (MGP) are the second main type of PAH-polluted soils. High-residue content (up to 25 000 $mg.kg^{-1}$) is commonly found on the sites after plant demolition. PAH are mainly 4-ring (fluoranthene, pyrene, benzo[*a*]anthracene) and 5-ring compounds (benzo[*b*]fluoranthene, benzo[*a*]pyrene). Residues are subjected to long-term aging, sometimes more than one century. They can be very recalcitrant to bioremediation (GRAMMS ET AL., 1999). The third soil is contaminated by petroleum pollutants including PAH, and can be found near oil field batteries. Amounts of unsubstituted PAH are generally moderate, and the pollutants are associated with low-molecular-weight hydrocarbons (benzene, toluene, xylenes) and methylated forms of PAH. Main encountered PAH comprise 3-ring (phenanthrene) and all 4-ring (pyrene, chrysene) compounds. Petroleum-polluted soils in the vicinity of refineries are closely related by their characteristics to soils from MGP (SABATÉ ET AL., 2006).

Soil microorganisms capable of PAH degradation

There are three main groups of soil microorganisms actually involved in marketed soil bioremediation processes, bacteria, fungi and actinobacteria (CHANG ET AL., 2007).

Bacteria can be rod-shaped, coccoidal, helical or pleomorphic, with a size ranging from 0.1 to 2 µm. Characterized by a complex cell envelope which contains cytoplasm and a few cell organelles, they can be aerobic or anaerobic (BLAKELY ET AL., 2002). Their deoxyribonucleic acid is circular in cytoplasm, but plasmidic DNA is frequent. Bacteria are

capable of rapid growth and reproduction which occurs by binary fission. Genetic exchange is predominant by conjugation (cell to cell contact), transduction (exchange via virus) or transformation (transfer of naked DNA). While most bacteria are able to oxidize PAH using dioxygenase enzymes, a few bacteria are also capable of oxidizing PAH by the action of the cytochrome P450 monoxygenase enzyme and there is also a report of an ability of marine methanotrophs in degrading PAH via the action of the methane monoxygenase gene (LOZADA ET AL., 2008). It is thought however that these are minor mechanisms compared with the activity of the dioxygenase enzymes. The most commonly used genera are *Arthrobacter*, *Pseudomonas* and *Bacillus* (HO ET AL., 2000).

Fungi are second major group of soil organisms. These eukaryotic organisms comprise molds, mildews, rusts and mushrooms (all aerobic), as well as yeast (fermenting organisms). Filamentous fungi are characterized by extensive branching and mycelial growth, as well as by the production of sexual and asexual spores. Deuteromycetes lack sexual reproduction, but a sexual stage is quite often discovered in which case these organisms are reclassified into other classes. Fungi are more tolerant to acidic soils and low moisture than bacteria. In particular, white-rot fungal species have significant potential for the treatment of contaminated wood for their ability to grow on ligninocellulosic materials and to degrade phenols and PAH. Their degradative activity is linked to the production of ligninolytic, extracellular enzymes (laccase, lignin peroxidase and manganese peroxidase), released by fungal hyphae into the lignin matrix (BALDRIAN ET GABRIEL, 2002). The most common genera are *Penicillium*, *Aspergillus*, *Fusarium*, *Rhizoctonia*, *Pleurotus, Alternaria* and *Rhizopus* (REID ET AL., 2002; ŠNAJDR ET BALDRIAN, 2006).

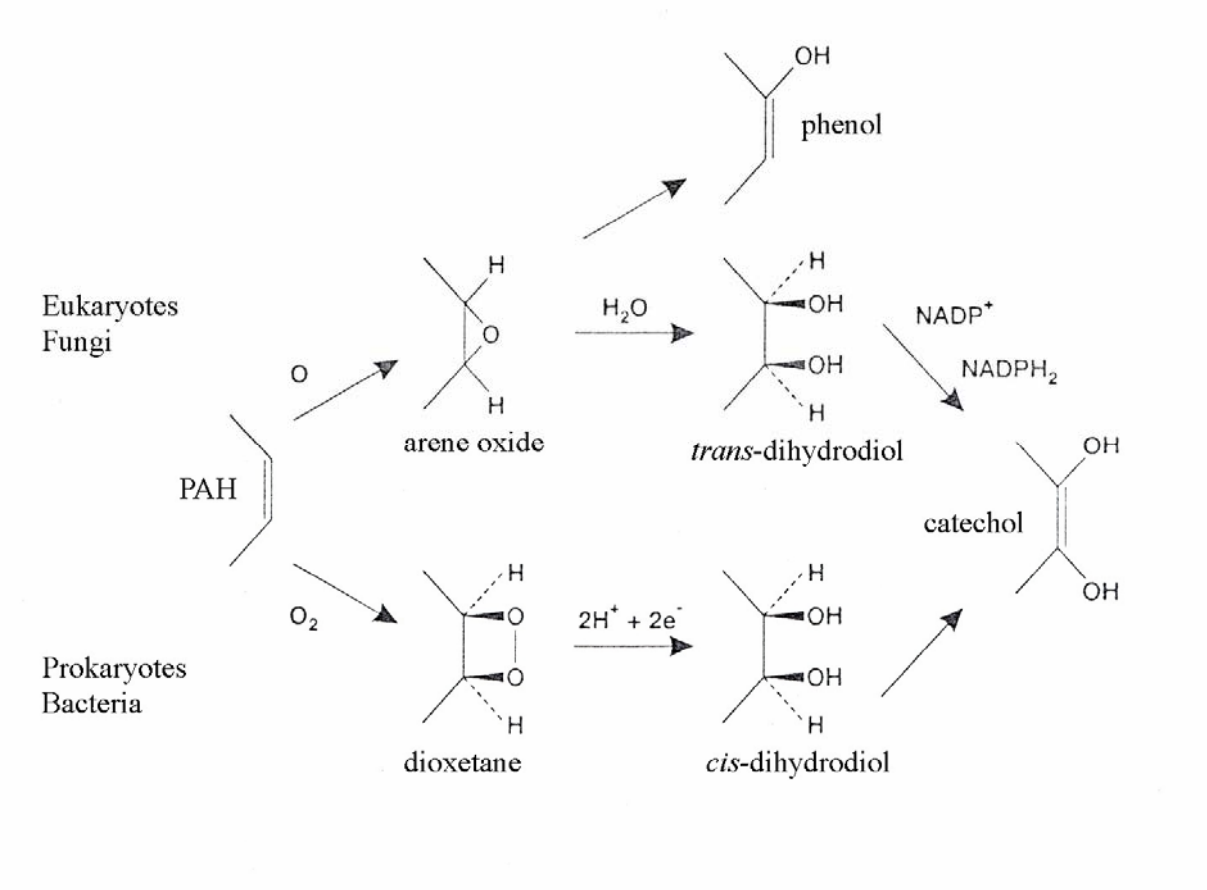
Actinobacteria are classified as bacteria. Nevertheless, despite some characteristics in common with bacteria, they are also similar to fungi. Mostly aerobic they grow as elongated single cells, branched into filaments or hyphae, which are similar to those of fungi although with a smaller diameter (0.5 - 2 μm). Their cellular organization and DNA material are similar to those of other bacteria. They are more tolerant to alkaline soil pH and low moisture content than bacteria, thus actinobacteria population tend to be high in desert soils. Actinobacteria are a group of biodegradative bacteria which make an important contribution to the degradation of the polymeric components of lignocellulosic basis. *Streptomyces* genera are the most dominant species in the populations (BIDAUD ET TRAN-MINH, 1998; JOHNSEN ET KARLSON, 2005).

Principles of PAH biotransformation

The biotransformation of PAH through bacterial and fungal pathways includes three processes: biodegradation, cometabolism and synthesis (McLaren et Skujinš, 1971, Sylvia et al., 1999). During the biodegradation, one or several interacting organisms metabolize PAH into carbon dioxide and other inorganic components. In this way, the organisms obtain their requirements for growth and energy by mineralizing the molecule. The prevalent form of PAH metabolism in the environment is cometabolism, in which organisms grow at the expense of a co-substrate to transform PAH without deriving any nutrient or energy for growth from the process (Atagana, 2004b). Cometabolism is a partial and fortuitous metabolism and enzymes involved in the initial reaction lack substrate specificity. Generally cometabolism results only in minor modifications of the structure of the xenobiotic, but different organisms can transform a molecule by sequential co-metabolic attack or one organism can use co-metabolic products of another as a growth substrate. Minor modifications resulting from cometabolism could greatly influence pollutant bioavailability and mobility in the soil. In addition, intermediate products with their own bio- and physico-chemical properties can accumulate, thus causing some adverse effects on the environment (Potin et al., 2004). The last process, synthesis, includes conjugation and oligomerization. PAH are transformed into compounds with chemical structures more complex than those of the parent compounds. During conjugation a PAH (or one of its transformation product) is linked to hydrophilic endogenous substrates, resulting in the formation of methylated, acetylated, or alkylated compounds (Bamforth et Singleton, 2005).

The biodegradation pathways of PAH can be performed via bacterial or fungal metabolism, under aerobic or anaerobic conditions (Fig. 1).

FIG. 1: Initial reactions of aromatic ring cleavage by oxygen incorporation (HOLOUBEK, 1996).



Wide variety of bacteria metabolizes PAH and uses them as their sole source of carbon and energy (LI ET AL., 2008). The complete bacterial degradation pathways of several PAH have been already elucidated (CERNIGLIA, 1981). However, until recently, only a few genera of bacteria have been isolated with the ability to utilize 4-ring PAH as sole carbon and energy sources while only cometabolism has been reported for 5-ring containing compounds (ZELLES, 1999). The bacterial metabolism of PAH, under aerobic conditions, starts with the attack of a single terminal ring by a dioxygenase, although G+ and G- bacteria degrade PAH in the different ways. Bacteria initiate anthracene degradation, for example, by hydroxylation of the aromatic ring to yield *cis*-1,2-dihydroanthracene-1,2-diol. This intermediate is converted to anthracene-1,2-diol, which is cleaved at the *meta*- position to yield 4-(2-hydroxynaph-3-yl)-2-oxobut-3-enoate. This compound may spontaneously rearrange to form 6,7-benzocoumarin or be converted to 3-hydroxy-2-naphthoate, from which degradation proceeds through 2,3-dihydroxynaphthalene to salicylate (EVANS ET AL., 1965). This pathway has been identified in G- bacteria and the upper stage of the pathway, through the *meta*-cleavage of anthracene-1,2-diol, has been observed in G+ bacteria (MOODY ET AL., 2001).

Numerous fungi among zygomycetes, deuteromycetes, ascomycetes and basidiomycetes are able to metabolize PAH (EGGEN, 1999). Fungal metabolism of PAH is being extensively studied at present because of the increasing development of remediation processes using filamentous fungi (EICHLEROVÁ ET AL., 2000). In contrast to bacteria, non-ligninolytic fungi produce metabolites that include *trans*-dihydrodiols, phenols, quinones, tetralones and dihydrodiol epoxides, whereas ligninolytic basidiomycetes are able to cleave the aromatic rings and mineralize them in addition (SUTHERLAND, 1992). Most of the metabolites produced from PAH by fungi are less toxic than the parent compounds and detoxification results. Nevertheless, small amounts of mutagenic and carcinogenic compounds can be formed during fungal metabolism of unsubstituted and methyl-substituted PAH. Fungal metabolites can be retained within the cells, or released in their environment. In general, fungal metabolites have higher water solubility and enhanced chemical reactivity (CERNIGLIA, 1997; BALASHOVA ET AL., 1999).

Unsubstituted polyaromatic compounds can be also degraded in anoxic environment under nitrate-reducing, iron-reducing, sulfate-reducing and methanogenic conditions (CHANG ET AL., 2006). Anaerobic pathways are frequent when aerobic transformation of PAH exhausts dissolved oxygen and decreases redox potential, which becomes the crucial for prevalent processes. Although some halogenated aromatic compounds are degraded more easily via anaerobic pathways, anaerobic transformation of aromatics remains limited to a small range of PAH (ROCKNE ET STRAND, 1998; AMELLAL ET AL., 2001).

Bioremediation of PAH by bacteria and fungi - current knowledge

Bioremediation using various microorganisms is one of the approaches tested for the removal of PAH from the environment. Over the past 35 years, research on the microbial degradation of PAH has resulted in the isolation of numerous strains of bacteria, fungi and algae capable of degrading PAH containing two or three aromatic rings (MACGILLIVRAY ET SHIARIS, 1993; ANDERSSON ET HENRYSSON, 1996). PAH containing four or more aromatic rings are generally recalcitrant to microbial attack, although some fungi and algae were found to be capable of transforming these compounds (CERNIGLIA ET HEITKAMP, 1989). Bioremediation technologies have commonly relied on bacterial activity to decontaminate the environment. While knowledge of bacterial bioremediation has continued to improve, relatively less attention has been paid to fungal bioremediation of contaminated environment (ATAGANA ET AL., 2006).

Bioremediation using ligninolytic fungi has been intensively studied since 1980s (BALDRIAN, 2008). Due to heterogeneous structure of lignin, ligninolytic fungi produce extracellular enzymes with very low substrate specificity (GABRIEL ET AL., 1994). Moreover, in contrast to bacteria, fungi are able to extend extracellular enzymes production through hyphal growth, thus they are able to grow under stressful conditions in their environment (DAVIES ET WESTLAKE, 1979). The capability of mycelial fungi to penetrate insoluble substances such as oil tar balls was found as a profitable ability allowing the succession of other microorganisms and thus enhancing degradation of the creosote (KIRK, 1969). Low substrate specificity of oxidative enzymes produced by the white rot fungi like *Pleurotus ostreatus* (EICHLEROVÁ ET AL., 2000)*, Irpex lacteus* or others is supposed to be advantageous for the degradation of recalcitrant xenobiotic compounds, e.g. PAH with four or more aromatic rings (BOGAN ET LAMAR, 1995; BOONCHAN ET AL., 2000; BABOROVÁ ET AL., 2006).

Microbial community within aromatic hydrocarbon contaminated ecosystems tend to be dominated by those organisms capable of utilizing and/or surviving toxic compounds (VIÑAS ET AL., 2005). Generally, on a site contaminated by PAH, microbial community are typically less diverse than those in non-stressed systems and - as a result of contamination - their composition tends to shift from primarily eukaryotic biomass rather to G- bacterial biomass, belonging generally to a very limited number of taxonomic groups (KÄSTNER ET AL., 1994). Microbial community composition is selected towards organisms not only surviving under the impact of toxic compounds, but even utilizing those (HAMDI ET AL., 2007).

Studies of the creosote contaminated soil from wood preserving plants show that the microbial community is usually limited (GALLI ET AL., 2006). Most common limitations are the lack of nutrients (nitrogen, phosphorus), moisture content, pH and bioavailability of PAH. Nitrogen content seems to be ideal in ratio C:N about 10:1 (ATAGANA ET AL., 2003). Phosphorus additions (as well as nitrogen addition) are conducted in the best way by mixing the soil with the slow release fertilizers (GUERIN, 1999). However, further studies are needed for the explanation of the role of both nutrients, nitrogen and phosphorus, in the degradation of high molecular weight hydrocarbons for the successful application of bioremediation at PAH contaminated sites (BREEDVELD ET SPAREVIK, 2000). Maintaining the stable moisture content and pH (between 6 and 8) was shown to be important too (ATAGANA, 2003). Stirring or mechanical mixing of the soil (GUERIN, 1999) as well as use of surfactants (CARRIERE ET MESANIA, 1995) significantly increase the biodegradation rates.

PAH bioremediation outlook

Though a variety of microorganisms that degrade different PAH have been isolated and characterized and though new pathways for PAH-degradation have been elucidated, further research is needed to explore the microbial interactions within the PAH-degrading consortia and the regulatory mechanisms of PAH with various molecular structures biodegradation as well as the co-metabolic biodegradation of PAH. At the same time the factors affecting the environment and the mathematical model of PAH-biodegradation are worthy of more attention in future studies (ZHANG ET AL., 2006).

New advances in molecular biology can aid not only detection of PAH-degrading organisms from environmental samples, but also clarification of principles of microbial PAH-degradation (WATANABE, 2001). DNA-DNA hybridization, quantitative competitive polymerase chain reaction (QC-PCR), denaturing gradient gel electrophoresis (DGGE), and the 16S rRNA sequence technique have been directly applied to detect and monitor the microbial populations recovered from soil (KANALY ET AL., 2000; WIDADA ET AL., 2002; HAO ET AL., 2003). At the same time, by means of a molecular biology approach, many new biodegradation pathways of PAH have been discovered (JOHNSEN ET AL., 2005). Therefore, it is evident that molecular biology, as an advanced approach, will play a more prominent role in the future field of microbial degradation of PAH.

Usually, in comparison with laboratory conditions, adverse environmental conditions in natural soils cause less efficient biodegradation of PAH. It is possible that enhancing the enzymatic activity of biochemical pathways using genetic engineering (resulting in higher expression of key enzymes) could improve degradation of many PAH in soil (TORRES ET AL., 1995; SAMANTA ET AL., 2002). On one hand, genetic engineering has provided an adequate strategy to develop genetically engineered microorganisms that are able to sense an environmental contaminant and respond to it through easily detectable signals such as bioluminescence (PARK ET AL., 2002). On the other hand, several genetically engineered tools, such as gene conversions, gene duplications, transpositions, and bio-vehicles (plasmids and transposons), could also have vital roles in the rearrangement of DNA fragments, in the activation or inactivation of cryptic genes, and in facilitating the crossover of significant genetic information from host cells to recipients (CHENG ET AL., 2002; LIU ET AL., 2006). In conclusion, modern biological techniques will play important role in the future of bioremediation, and should be employed more frequently to achieve higher efficiency of microbial PAH-degradation as well as various new degradation pathways.

Experimental

Soil samples contaminated with creosote were collected under the former factory hall used for creosote treatment in the area of wood-preserving plant in Soběslav in the Czech Republic. Control samples were collected in the agricultural grass field under conventional management close to the plant.

The excavated soil was moved to the remediation field nearby the wood-preserving plant. The PAH concentrations in the excavated soil ranged widely, typically from 10-10 000 $mg.kg^{-1}$ soil dry mass. During the remediation, soil underwent repetitive inoculation (mixed culture of *Pseudomonas* sp. cultivated on the medium enriched by naphthalene), pH and moisture adjustment and regular mixing, aerating and irrigating.

Basal respiration and substrate-induced (glucose) respiration were employed for basic soil activity measurement. The phospholipid fatty acids analysis (PLFA) was used to qualitatively and quantitatively assess the total active soil microbial community. Counts of culturable bacteria (CFU), the species identification of the bacterial isolates (MIS Sherlock System) and bacterial growth strategy analyses were performed in selected soil samples.

The wood-rotting basidiomycetes *Pleurotus ostreatus* and *Irpex lacteus* were used for fungal bioremediation experiments. Experimental setup consisted of wide glass tubes filled with the layer of milled wheat straw plus layer of contaminated soil, incubated under stable water content in the dark.

Main objectives

This thesis addresses following concerns:

Aim 1

Description of the changes of the soil microbial community influenced by an extensive creosote contamination. Laboratory-scale bioremediation experiments - evaluation and comparison of bacterial and fungal inoculum performance.

How extensive soil pollution of creosote origin affects the present soil microbial community? How selected bacterial inoculum capable of PAH degradation perform biodegradation of extensively creosote-contaminated soil? Are selected ligninolytic fungi capable of degrading complex aromatic compounds from extensively creosote-contaminated soil and how they affect the present soil microbial community? Are selected ligninolytic fungi capable of degrading the polycyclic aromatic hydrocarbons with 4-6 aromatic rings in more efficient pathways than bacteria?

Phospholipid fatty acids analysis (PLFA) and selected methods of soil microbial biology (respiration rate and substrate-induced respiration rate measuring) were used for examination of the complex soil microbial community. Selected PLFA markers were used for bacterial, actinobacterial and fungal populations. Bacterial characterization was completed by CFU counts, growth strategy and species identification. PAH contents in the soil samples were evaluated using gas chromatography-mass spectrometry (GC-MS) apparatus (see **Paper I** and **Paper III**).

Aim 2

Analytical procedures of extensively creosote-contaminated soil samples - constraint evaluation and new analytical approach concept.

How to analyze soil microbial community characteristic molecules (phospholipid fatty acids) in the samples containing extensive hydrophobic compounds of creosote origin inducing severe complications during the sample preparation and gas chromatography evaluation?

Extensive PAH pollution of creosote origin in the soil samples represents severe complications in distinguishing the particular fatty acids in the chromatogram in full-scan mode due to the lipophilic character of PAH compounds which are co-extracted with the lipids. Scanning of selected ions in the samples in MS-MS scan mode was developed in order to suppress interfering aromatic hydrocarbons signal and to obtain clear chromatogram record of fatty acids (see **Paper II**).

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Results

The results of this thesis are presented in two published research papers and in one manuscript ready for publication.

Paper I summarizes the influence of two ligninolytic fungi (*Pleurotus ostreatus* and *Irpex lacteus*) on bioremediation of creosote-contaminated soil. It describes the polycyclic aromatic hydrocarbon concentration decrease during the laboratory-scale experiments and reveals the changes in the present soil microbial community under the influence of either fungus. The article compares different impact on PAH concentrations and soil microbial community depending on the fungus applied. Both fungi were found to be capable of degrading PAH more efficiently than the soil microbial community (consisted of bacterial inoculum capable of degrading PAH and original soil microbial community) themselves alone, although the PAH with 5-6 aromatic rings remained recalcitrant. It was proposed that even better results in PAH removal could be achievable, if the ligninolytic fungi and mixed cultures of bacteria and actinobacteria are maintained together in favourable conditions.

Paper II characterizes new approach allowing analyzing the soil microbial community characterized by phospholipid fatty acids in the soil samples extensively contaminated with polycyclic aromatic hydrocarbons of creosote origin. It includes the description of the preparation of the soil samples for gas chromatography-mass spectrometry-mass spectrometry detection of bacterial fatty acids. The article contains ion specifications important for mass spectrometry-mass spectrometry scanning mode and other details needed for successful measuring of characteristic fatty acids in the extensive polycyclic aromatic hydrocarbon background signal. Used technique was successful in distinguishing eleven fatty acids in

extensively creosote-contaminated soil samples, nevertheless is applicable for the fatty acids present in the soil microbial community in general.

Paper III (*Manuscript*) contains the description of the fundamental influence of extensive polycyclic aromatic hydrocarbons (creosote) pollution on the soil microbial community. It also describes the changes occurred during the remediation of such soil. The microbial community was found much stressed and poorly developed; furthermore disfavourable growth conditions of affected microbial community were confirmed. Nevertheless, glucose addition experiments proved the potential of such community to activate rapidly when easily degradable substrate is available. The polycyclic aromatic hydrocarbons concentration decreased substantially, although the most hazardous PAH compounds with 5-6 aromatic rings remained recalcitrant too. Gradual return of present microbial community to the nature-close composition was revealed during the remediation.

PAPER I

BYSS, M., ELHOTTOVÁ, D., TŘÍSKA, J., BALDRIAN, P.

Fungal Bioremediation of the Creosote-contaminated Soil: Influence of *Pleurotus ostreatus* and *Irpex lacteus* on Polycyclic Aromatic Hydrocarbons Removal and Soil Microbial Community Composition in the Laboratory-scale Study.

CHEMOSPHERE (2008). DOI:10.1016/j.chemosphere.2008.07.030.

Abstract

The aim of this study was to determine the efficacy of selected basidiomycetes in the removing of polycyclic aromatic hydrocarbons (PAH) from the creosote-contaminated soil. Fungi *Pleurotus ostreatus* and *Irpex lacteus* were supplemented with creosote-contaminated (50-200 $mg.kg^{-1}$ PAH) soil originating from a wood-preserving plant and incubated at 15 °C for 120 d. Either fungus degraded PAH with 4-6 aromatic rings more efficiently than the microbial community present initially in the soil. PAH removal was higher in *P. ostreatus* treatments (55-67%) than in *I. lacteus* treatments (27-36%) in general. *P. ostreatus* (respectively *I. lacteus*) removed 86-96% (47-59%) of 2-rings PAH, 63-72% (33-45%) of 3-rings PAH, 32-49% (9-14%) of 4-rings PAH and 31-38% (11-13%) of 5-6-rings PAH. MIS (Microbial Identification System) Sherlock analysis of the bacterial community determined the presence of dominant Gram-negative bacteria (G-) *Pseudomonas* in the inoculated soil before the application of fungi. Complex soil microbial community was characterized by phospholipid fatty acids analysis followed by GC-MS-MS. Either fungus induced the decrease of bacterial biomass (G- bacteria in particular), but the soil microbial community was influenced by *P. ostreatus* in a different way than by *I. lacteus*. The bacterial community was stressed more by the presence of *I. lacteus* than *P. ostreatus* (as proved by the ratio of the fungal/bacterial markers and by the ratio of *trans*/*cis* mono-unsaturated fatty acids). Moreover, *P. ostreatus* stimulated the growth of Gram-positive bacteria (G+), especially actinobacteria and these results indicate the potential of the positive synergistic interaction of this fungus and actinobacteria in creosote biodegradation.

Abstrakt (překlad do českého jazyka)

Cílem této práce bylo zjistit efektivitu degradace polycyklických aromatických uhlovodíků (PAU) pocházejících z půdy znečištěné kreozotovým olejem u vybraných basidiomycet. Houby *Pleurotus ostreatus* a *Irpex lacteus* rostly na půdě znečištěné kreozotovým olejem o koncentraci 50-200 $mg.kg^{-1}$ PAU, pocházející z dřevozpracujícího

závodu, po dobu 120 dní při 15 °C. Obě houby degradovaly PAU s 4-6 aromatickými kruhy efektivněji než půdní mikrobiální společenstvo přítomné v půdě. Celkově, *P. ostreatus* degradoval více PAU (55-67%) než *I. lacteus* (27-36%). *P. ostreatus* (respektive *I. lacteus*) degradoval 86-96% (47-59%) PAU s dvěma aromatickými kruhy, 63-72% (33-45%) PAU s třemi aromatickými kruhy, 32-49% (9-14%) PAU se čtyřmi aromatickými kruhy and 31-38% (11-13%) PAU s 5-6 aromatickými kruhy. Analýza MIS (Microbial Identification System) Sherlock půdního mikrobiálního společenstva prokázala přítomnost dominantního G- kmene *Pseudomonas* v půdě před aplikací hub. Půdní mikrobiální společenstvo bylo charakterizováno analýzou fosfolipidických mastných kyselin následovanou GC-MS-MS. Obě houby způsobily pokles bakteriální biomasy (zejména G- bakterií), ale celkově bylo půdní mikrobiální společenstvo ovlivněno každou houbou jinak. Bakteriální společenstvo bylo více stresováno přítomností *I. lacteus* než *P. ostreatus* (což bylo prokázáno pomocí poměru houbových/bakteriálních markerů a poměrem mezi *trans*/*cis* formami nenasycených mastných kyselin). *P. ostreatus* navíc stimuloval růst G+ bakterií, zvláště aktinobakterií a tyto výsledky naznačují potenciál pozitivních synergických interakcí mezi touto houbou a aktibakteriemi při degradaci kreozotového oleje.

Marius Byss had a major (approx. over 70%) contribution to this paper.

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PAPER II

BYSS, M., TŘÍSKA, J., ELHOTTOVÁ, D.
GC-MS-MS Analysis of Bacterial Fatty Acids in Heavily Creosote-contaminated Soil Samples.
ANALYTICAL AND BIOANALYTICAL CHEMISTRY (2007) 387: 1573-1577.

Abstract

PLFA (phospholipid fatty acid) profiles of soil samples offer rapid and reproducible measurements and characterization of the dominant portion of soil microbial communities. When an extensive pollution caused by polycyclic aromatic hydrocarbons (PAHs) is present in the soil, it is very difficult or even impossible to distinguish the particular fatty acids in the chromatogram in full-scan mode due to the lipophilic character of PAHs which are co-extracted with the lipids. Scanning of selected ions in the samples in MS-MS scan mode was performed in order to suppress the aromatic hydrocarbons signal and obtain the clear chromatogram record of fatty acids. By using the described technique it was possible to clearly distinguish at least 11 fatty acids in extensively creosote contaminated soil samples (PAHs concentration about 15 $g.kg^{-1}$ of dry weight soil).

Abstrakt (překlad do českého jazyka)

Profily fosfolipidických mastných kyselin (PLFA) v půdních vzorcích nabízejí rychlé a reprodukovatelné měření a charakterizaci dominantních částí půdního mikrobiálního společenstva. V případě přítomnosti silného znečištění půdy polycyklickými aromatickými uhlovodíky (PAU) je velmi těžké nebo přímo nemožné rozlišit jednotlivé mastné kyseliny v chromatogramu ve full-scan módu kvůli lipofilnímu charakteru PAU, které jsou extrahovány spolu s lipidy. Bylo provedeno skenování vybraných iontů ve vzorcích v MS-MS módu, aby byl potlačen signál polycyklických aromatických uhlovodíků a aby bylo možné obdržet čistý chromatografický záznam mastných kyselin. Použitím popsané techniky bylo možné rozlišit nejméně 11 mastných kyselin v půdách velmi znečištěných kreozotovým olejem (koncentrace PAU okolo 15 $g.kg^{-1}$ suché půdy).

Marius Byss had a major (approx. over 80%) contribution to this paper.

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PAPER III

SOIL MICROBIAL COMMUNITY CHANGES DURING BIOREMEDIATION OF EXTENSIVELY CREOSOTE-CONTAMINATED SOIL

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Abstract

The aim of this study was to describe the substantial influence of extensive polycyclic aromatic hydrocarbons (of creosote origin) pollution on the soil microbial community and to describe the changes occurred during the remediation of such soil. Polycyclic aromatic hydrocarbons contamination level (15-15 662 mg.kg^{-1}soil) has shifted present microbial community to low biomass levels capable of surviving such conditions. The microbial community was also very stressed and poorly developed, as was confirmed by the detection of limited number (8 of 28) of analyzed signature molecules - fatty acids and by the absence of microbial fungal markers. Furthermore, disfavourable growth conditions of affected microbial community were confirmed by the stress indicators (*trans/cis*-fatty acid ratio, production of cyclopropyl fatty acids). Nevertheless, glucose addition experiments prove the potential of such community to activate rapidly when easily degradable substrate is available.

The polycyclic aromatic hydrocarbons (PAH) concentration decreased to 17.3% of the original concentration during the remediation, although the most hazardous PAH compounds with 5-6 aromatic rings remained recalcitrant. Gradual return of present microbial community (stimulated by repetitive inoculation by mixed culture of *Pseudomonas* sp.) to the nature-close composition was revealed during the remediation by the principal component analysis. The microbial community respiration rate was also positively correlated with the increasing duration of remediation process.

Keywords

Soil microbial community; PLFA; creosote; PAH

Introduction

There are numerous methods developed for characterization of soil microbial community, e.g. respiration and microbial (fluorescein diacetate, dehydrogenase) activity measuring, chemical methods - lipid liquid chromatography (LLC), denaturing gradient gel electrophoresis (DGGE), or genetics-based methods - reverse sample genome probing (RSGP), etc. However, only a few methods, within some modifications (e.g. phospholipid fatty acid analysis – PLFA) are suitable for assessing and characterization of the microbial community in the presence of extensive polycyclic aromatic hydrocarbons (PAH) pollution of creosote origin, because of its hydrophobic background seriously disturbing the analytical procedure. Creosote consists of mixture of hydrocarbons and their derivatives; about 85% is comprised by polycyclic aromatic hydrocarbons, while heterocyclic aromatic hydrocarbons and phenolic compounds are the other substances (ATAGANA, 2004).

Microbial communities in the ecosystems contaminated by PAH develop themselves in such way that they are not only surviving under the impact of toxic compounds, but even utilize them as a substrate (JOHNSEN ET AL., 2002). As a result, these communities are typically less diverse than those in non-stressed systems and they tend to shift from primarily eukaryotic biomass rather to Gram-negative (G-) bacterial biomass, belonging generally to a very limited number of taxonomic groups (KÄSTNER ET AL., 1994). Most common limitations are the lack of nutrients (nitrogen, phosphorus), moisture content, pH and bioavailability of PAH (LI ET AL., 2008). Nitrogen content calculated as C:N ratio should be about 10:1 as confirmed by ATAGANA ET AL. (2003). The best way how to assure phosphorus addition (as well as nitrogen addition) is by mixing the soil with the slow release fertilizers (GUERIN, 1999). However, further studies are needed for the explanation of the role of both nutrients in the degradation of higher molecular weight polycyclic aromatic hydrocarbons for the successful bioremediation application on the sites contaminated by PAH (BREEDVELD ET SPARREVIK, 2000). Maintaining the stable moisture content and pH (between 6 and 8) was shown to be important for successful remediation (ATAGANA, 2003). Stirring or mechanical mixing of the soil as well as use of surfactants (CARRIERE ET MESANIA, 1995) significantly increase the biodegradation rates.

PLFA analysis can supplement traditional microbiological methods focused more on soil microbial activity by different types of information (WIDMER ET AL., 2001). Total amount of PLFAs (or ester-linked PLFAs), as well as their characteristics (for example the length, position of substitution, or unsaturation) have been used as indicators of different microbial

strains in the environmental samples. The straight-chain fatty acids are generally widely distributed in organisms, however some of them, especially the long straight-chain fatty acids are mainly characteristic for eukaryotes and higher plants. Branched-chain fatty acids (*i*, *a*, and branched chain) are largely found in Gram-positive (G+) and sulphate-reducing G- bacteria (BALKWILL ET AL., 1988; ZELLES ET AL., 1994). Cyclopropyl fatty acids are common in some G- strains (HAACK ET AL., 1994), as well as in some anaerobic strains of G+ bacteria (RATLEDGE ET WILKINSON, 1988). Methyl branching on the tenth carbon atom in the molecule is specific for actinobacteria (KROPPENSTEDT, 1992).

Shifts in the whole soil PLFA profile indicate changes in microbial community composition. Changes in certain PLFAs or in their ratios can provide information about the nutritional and/or physiological status of microbial communities and indicate environmental stress. A high *trans/cis*-PLFA ratio is the indicator of metabolic stress due to a toxicity or starvation (KIEFT ET AL., 1994). Similarly, G- bacteria convert certain monoenoic PLFAs to cyclopropyl fatty acids, as growth rate slows from an exponential to a stationary phase. Also, total amount of PLFA was proposed as a good measure of viable microbial biomass (SMIT ET AL., 2001).

The aims of this study were following: a) to collect the soil samples from the polluted wood-preserving plant and the control sample, together with the samples from the remediation field, of a different age during the remediation process b) to find out the types and concentration of pollutants in the samples c) to characterize a microbial community found in the polluted, non-polluted (control) and remediated soil samples d) to assess the degradation process with respect to the microbial communities of analyzed soil samples.

Material and Methods

Soil samples

Soil samples contaminated with PAH were collected under the former factory hall used for creosote treatment in the area of wood-preserving plant in Soběslav in the Czech Republic. Soil samples had pH 2.8, 4.78% C and 0.1% N. The loamy soil was very dark and oily because of extensive PAH pollution. The soil samples from the maximum pollution point are marked as “A”, the samples from the other points of the plant are marked as “B” and “C”.

Control samples (marked as “X”) were collected in the agricultural grass field under conventional management 200 m from the plant. PAH concentrations of Czech republic background level (HOLOUBEK ET AL., 2007) were expected in these samples and that was confirmed by the analyses of the PAH content. The soil had loamy character, normal appearance (of grassland soil) and had pH 6.9, 3.3% C and 0.3% N. Viable microbial biomass

measured as total amount of PLFAs reached the concentration 75.1 nmol/g dry weight soil, which is comparable to the natural level (VIÑAS ET AL., 2005).

The remediation process was taking place nearby the wood-preserving plant. Firstly, the freshly excavated soil from the polluted plant was mixed with inert material in order to facilitate the machine manipulation. During the remediation, soil underwent repetitive inoculation (mixed culture of *Pseudomonas* sp. cultivated on the medium enriched by naphthalene), pH and moisture adjustment and regular mixing, aerating, irrigating. The soil pH was set between 6.9 and 7.2 by lime adding. The carbon content reached the values between 2.2% (fourth month of the remediation) and 4.6% (twelfth month – the end of the remediation process), the nitrogen content was in range between 0.08% and 0.2%, respectively. The remediation soil samples were taken after 4, 8, 10 and 12 months of the remediation process, they are marked as "4M", "8M", "10M" and "12M", respectively.

Soil samples were homogenized and passed through a 2-mm sieve. The soils were stored in glass bottles in the dark at 8 °C to preserve their biological activity. Soil moisture was determined gravimetrically after drying to the constant weight at 105 °C.

Reagents, solvents, standards

All solvents (hexane, methanol, acetone for trace organic analysis, SupraSolv Grade, Merck, Germany) and reagent-grade chemicals (Merck, Fluka - Switzerland) were used without further purification. FAMEs (fatty acid methyl esters) standards (total number of 27 FAMEs including internal standard) were obtained from Supelco (USA) or Larodan Fine Chemicals (Malmö, Sweden). PAH standards were obtained from Dr.Ehrenstorfer (Augsburg, Germany). SPE columns LiChrolut Si 60, 200 mg, were obtained from Merck.

Methods

The soil samples for PAH analyses were sonicated in the mixture of hexane and acetone 3:2 and the solvent was evaporated to dryness by flow of nitrogen, then redissolved in toluene and used for the GC-MS analysis on Finnigan GCQ (Generalized Cascaded Quadruplet, Finnigan MAT, Austin, USA) apparatus equipped with ion trap with an external ionization. The oven temperature, initially at 60 °C, was gradually increased (20 °C.min^{-1}) to 200 °C and then gradually increased by 4 °C.min^{-1} to 275 °C and held at this temperature for 15 min, total time of analysis was 40 min (HOLOUBEK ET AL., 2000). The individual PAH molecules identification was based on the comparison with the standard containing 16 priority PAH as identified by U.S.EPA as carcinogenic for animals and humans.

Basal respiration and substrate-induced respiration were employed for basic soil activity measurement as described in WEST ET AL. (1987). The samples were amended with 0.6% (w/w) of glucose.

The phospholipid fatty acids analysis (PLFA) was used to qualitatively and quantitatively assess the total active soil microbial community. The analysis was performed according to WHITE ET AL. (1979) with some modification. FAMEs, resulting from mild alkaline methanolysis of phospholipids in the methanolic fraction, were extracted with hexane and were analyzed by GC-MS-MS as described previously by BYSS ET AL. (2007). The separation parameters were following: a 30 m fused silica capillary column Zebron ZB-5MS (Phenomenex, USA) I.D. 0.25 mm, film thickness 0.25 μm. The volume of 1 μl of each sample was injected in the splitless mode. The injection temperature was 250 °C, temperature of ion source 200 °C and transfer line 275 °C. Helium velocity was 40 cm.s^{-1}. The oven temperature, initially at 60 °C, was gradually increased (20 °C.min^{-1}) to 160 °C and then gradually increased by 3 °C.min^{-1} to 275 °C and held at this temperature for 10.5 min, total

time of analysis was 56 min. The measurements were performed using GCQ Xcalibur software.

Signature lipid biomarkers were used for defined microbial group proportion description, details in VAN ELSAS ET AL. (2007). Terminally branched fatty acids (*a*, *i*) were used as markers for G+ bacteria; the methyl branching in the middle of the fatty acid molecule (10Me18:0) for actinobacteria; the cyclopropyl branched fatty acid for G- bacteria; the unsaturated fatty acid 18:2ω6,9 for fungi. The total bacteria were evaluated as a sum of *i*-15:0, *a*-15:0, 15:0, 16:1ω7c, *i*-17:0, cy17:0, cy19:0. The ratio of *trans*/*cis* isomers of monoenoic PLFA (18:1ω9) was used as indicator of stressful growth conditions. The typical stress growth response of bacteria is an increased *trans*/*cis* PLFA ratio with the critical value higher than 1.0 (KIEFT ET AL., 1997). Microbial biomass was estimated from the sum of phospholipid fatty acids as described previously (FROSTEGÅRD ET AL., 1993).

Results and Discussion

The soil pollution found in the area of the wood-preserving plant is extensive, however typical is the point character of pollution (data not presented). PAH concentration in the plant sample A reaches very high value, but this level is comparable to the contamination levels described in the literature in the wood-preservation plants (HANSEN ET AL., 2004). The plant neighborhood is not polluted, the PAH concentration of control sample X reaches the level of background concentration of the Czech agricultural soil (HOLOUBEK ET AL., 2007). Also, there's a significant loss of pollutants during the remediation. The PAH content decreased significantly during the twelve months of the remediation process (TABLE 1).

TABLE 1: Polycyclic aromatic hydrocarbons concentration in the soil samples, sum of PAH, in $mg.kg^{-1}$ dry weight soil, mean±SD.

Sample Description	***Sum of PAH [$mg.kg^{-1}$]***
Plant Sample A	*15662.2±3429.3*
Plant Sample B	*15±11.4*
Plant Sample C	*672.7±152.6*
Control Sample X	*0.9±0.3*
Remediation Sample 4M	*1057.5±462.8*
Remediation Sample 8M	*431.4±53.6*
Remediation Sample 10M	*471.5±253.1*
Remediation Sample 12M	*182.3±16.6*

The clear negative correlation between the number of aromatic rings and PAH concentration decrease during the remediation is observable: more rings the compound has, slower the biodegradation is. PAH with the 5-6 aromatic rings (including the carcinogenic ones) remain mostly recalcitrant (SABATÉ ET AL., 2006). However, the overall PAH soil concentration during the remediation experiment decreased to 17.3% of the original concentration (TABLE 2).

TABLE 2: The concentration decrease of the particular PAH during twelve months of the remediation process, depending on the number of the aromatic rings, in % (100% responds to the original contamination at the beginning of the remediation process).

PAH	Number of Aromatic Rings	Decrease [%]
Naphthalene	2	90.2
Acenaphthylene	2	73.7
Acenaphthene	2	87.8
Fluorene	2	95.5
Phenanthrene	3	94.3
Anthracene	3	96.5
Fluoranthene	3	77.3
Pyrene	4	58.9
Benzo[*a*]anthracene	4	56.5
Chrysene	4	79.3
Benzo[*b*]fluoranthene	4	43.5
Benzo[*k*]fluoranthene	4	49.9
Benzo[*a*]pyrene	5	30.0
Indeno[1,2,3-*c*,*d*]pyrene	5	26.7
Dibenzo[*a*,*h*]anthracene	5	42.6
Benzo[*g*,*h*,*i*]perylene	6	14.0
Sum of PAH	**2-6**	**82.7**

Basal respiration (TABLE 3) is limited in the most polluted sample (A) from the plant and is cca ten times lower than the control sample X. The extensive pollution has deep impact on respiration level due to extensive concentration of high molecular weight PAH, that are not easily degradable (ZHANG ET AL., 2006). Basal respiration in the control sample is comparable to the respiration level observable in similar soils (low in available carbon content) in the

Czech republic (ŠANTRŮČKOVÁ, 1993). However, the respiration in the plant sample C is higher than the respiration in the control sample, which is probably due to overall wet environment supporting the microbial existence (SALMINEN ET AL., 2002), where samples C were taken from.

There is gradual increase in basal respiration during the whole remediation process. This effect is explainable by the repetitive mixed culture of *Pseudomonas* sp. inoculation occurring during the remediation process, when the total amount of microbial biomass was slowly arising.

The highest respiration potential (TABLE 3) after the glucose addition was recorded in the control sample X, which confirms natural healthy microbial community capable of increasing the respiration immediately after the adding of easily degradable substrate (glucose), limited by the carbon content until that (CURIEL YUSTE ET AL., 2007).

The lowest active microbial biomass was measured in the samples from the plant, thus indicating limited microbial community poorly developed in stressful conditions and therefore not capable of utilizing the glucose immediately after their additions as was also demonstrated by LI ET AL. (2007). The substrate-induced respiration in the sample C is also higher than in the samples taken from the other parts of the plant due to more developed microbial community because of the wet environment. Active microbial biomass remains comparable during the whole remediation process.

TABLE 3: Basic soil microbial community parameters: The basal respiration of the soil, in μg $C\text{-}CO_2$.g $soil^{-1}.h^{-1}$ (mean±SD), and the active microbial biomass calculated as substrate induced respiration of the soil, in μg C.g $soil^{-1}$ (mean±SD).

Sample Description	***Basal Respiration*** *[μg $C\text{-}CO_2$. g $soil^{-1}.h^{-1}$]*	***Active Microbial Biomass*** *[μg C.g $soil^{-1}$]*
Plant Sample A	*0.08±0.002*	*167.1±32*
Plant Sample B	*0.1±0.005*	*90.8±20.7*
Plant Sample C	*1.4±0.2*	*293.3±28.2*
Control Sample X	*0.8±0.2*	*636.1±37.2*
Remediation Sample 4M	*0.9±0.3*	*435.9±18.7*
Remediation Sample 8M	*1.1±0.08*	*371.4±71.4*
Remediation Sample 10M	*1.4±0.06*	*411±31.4*
Remediation Sample 12M	*1.5±0.05*	*337±24.6*

A high *trans/cis*-PLFA ratio is the indicator of metabolic stress due to the PAH toxicity, as shown in TABLE 4. This stress indicator shows stressful environment in all samples, however the value in the control sample X is very low in comparison to the extreme values in the most polluted plant samples where maximum stress occurs, as was also observed by TÖRNEMAN ET AL. (2008). There is comparable stress in all the samples from the remediation field.

Similarly, G- bacteria convert certain PLFAs to cyclopropyl fatty acids, as a result of toxic environment. This stress indicator confirms the stressful environment in the most contaminated samples, however all other values do not confirm stress. Also, plant sample C differs from all the other samples due to wet conditions of the sampling spots.

TABLE 4: PLFA stress indicator ratios (GUCKERT ET AL., 1986)

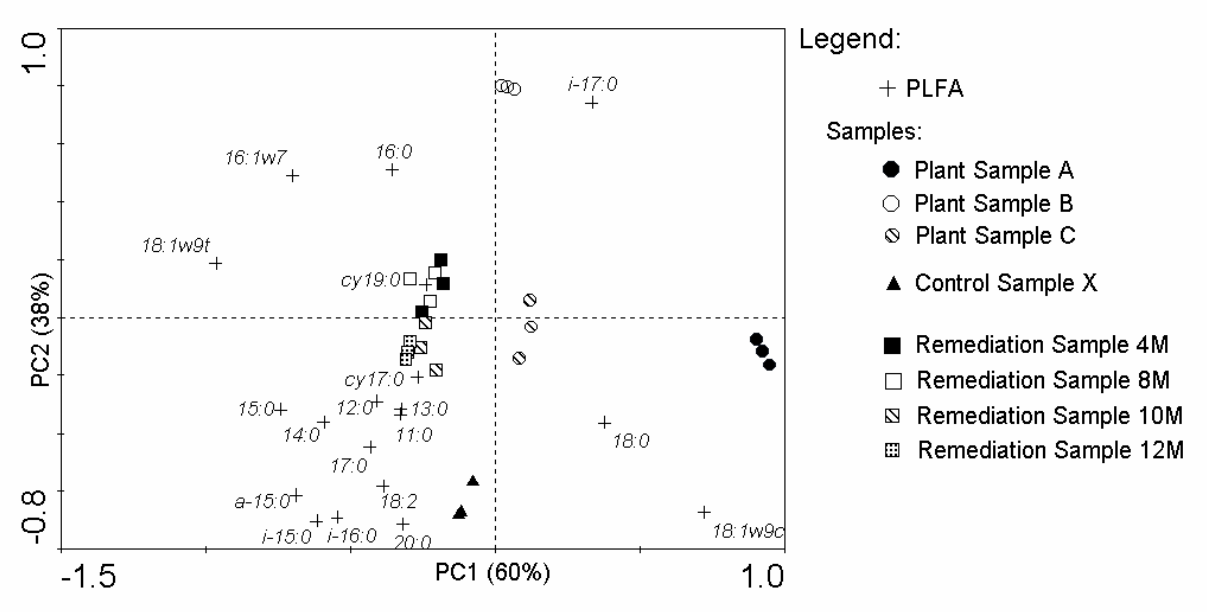
Sample Description	**18:1ω9t/18:1ω9c**	**cy19:0/18:1ω9c**
Plant Sample A	x	x
Plant Sample B	x	x
Plant Sample C	2.6	0.01
Control Sample X	1.3	1.5
Remediation Sample 4M	14.3	1.9
Remediation Sample 8M	15.3	1.7
Remediation Sample 10M	11.2	1.6
Remediation Sample 12M	11.5	1.8

x = *cis*-form not detected, *trans*-form found in high concentration

The PCA (principal component analysis) illustrates the previous results. It reveals that the most polluted samples (plant sample A and plant sample B) are unique and extreme in comparison to all the other samples according to the PLFA profile. The control sample C and X are unique as well and they differ from each other and they also differ from the previous two samples. Furthermore, the control sample X is correlated closely to the large number of PLFA (including fungal markers), which confirms healthy natural composition of present microbial community (TÖRNEMAN ET AL., 2008).

All the samples from the remediation field are closely correlated to each other and also differ from all the other samples in the PLFA profile. The correlation of these samples to the large number of PLFA increases with the increasing time of remediation and thus confirms slow return of microbial community to the natural structure, similar process as POTIN ET AL. (2004) observed in the research focused on filamentous fungi isolated from an aged PAH-contaminated soil.

FIGURE 1: Score plot of principal component analysis (PCA) showing the separation of all the samples using PLFA data.



Conclusions

The microbial community was influenced by the presence of extensive creosote (i.e. PAH) pollution substantially. The samples from the plant demonstrated significantly reduced microbial biomass as well as poor PLFA profile without the presence of fungal markers, in comparison to the control samples. Stress indicators also confirmed disfavourable growth conditions.

The return of microbial community to the nature-close composition was observed during the remediation process, where significant loss of polycyclic aromatic hydrocarbon pollution occurred. However, some of higher molecular weight PAH compounds remained recalcitrant. They may be degraded by the wood-decaying white-rot fungi, e.g. *Pleurotus* or *Irpex*, as was demonstrated in our recent study (BYSS ET AL., 2008).

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Conclusions

The following summary can be concluded from the results presented in this thesis:

Extensive polycyclic aromatic hydrocarbon pollution of creosote origin in the soil influenced the present soil microbial community fundamentally, both in their quantity and quality. On a site contaminated by PAH (concentration ranges from tens to ten thousands $mg.kg^{-1}$soil), soil microbial community were typically less diverse than those in non-stressed systems and – as a result of contamination – their composition shifted from primarily eukaryotic biomass rather to G- bacterial biomass, belonging generally to a very limited number of taxonomic groups. Typical soil microbial community profile as represented by the phospholipid fatty acids analysis demonstrated significantly reduced microbial biomass without the presence of fungal markers, in comparison to the control samples. Stress indicators also confirmed disfavourable growth conditions. Nevertheless, glucose addition experiments prove the potential of such community to activate rapidly when easily degradable substrate is available.

The laboratory-scale bioremediation experiments demonstrated the ability of selected bacterial inoculum (*Pseudomonas* sp.) to degrade PAH efficiently. Maintaining the suitable conditions (stable moisture content and pH) was shown to be important for successful remediation. Mixture of bacterial inoculum and original soil microbial community were capable of decreasing PAH concentrations to 17.3% of the original concentration during the four month remediation experiment. The clear negative correlation between the number of aromatic rings of the PAH compound and PAH concentration decrease during the remediation was observed. Most hazardous PAH compounds (identified by U.S.EPA as carcinogenic for humans and animals) with 5-6 aromatic rings remained recalcitrant. Gradual return of

microbial community to the nature-close composition was observed during the remediation process, thus the shifts in the soil microbial community affected by extensive creosote pollution were found reversible.

The laboratory-scale bioremediation experiments employing ligninolytic fungi *Pleurotus ostreatus* and *Irpex lacteus* followed the previous experiments. Either fungus was found capable of degrading PAH compounds even more efficiently than the soil microbial community (consisted of bacterial inoculum capable of degrading PAH and original soil microbial community) during the four months remediation experiment. Either fungus was more efficient than the soil microbial community themselves alone in degrading 4-6 aromatic rings PAH compounds particularly. *Pleurotus ostreatus* was also accented as better PAH-degrader than *Irpex lacteus* and was capable of degrading PAH compounds with two aromatic rings completely. On the other hand, more complex PAH compounds with 5-6 aromatic rings remained recalcitrant, as well as in the previous experiments, but the degradation rate was significantly better. Complete biodegradation, nevertheless, was not achievable, because of the physico-chemical properties of the soil. The important role of actinobacteria was also elucidated.

Overall results indicate promising biodegradative potential of the synergistic interaction among the ligninolytic fungi and mixed cultures of bacteria and actinobacteria maintained together in favourable conditions, thus even better creosote bioremediation results could be achievable in the future. For this, successful implementation of pieces of knowledge recently acquired in genetics, microbiology, biochemistry, physiology and ecology is desirable as well.