Seminar “Mycoremediation 2003”, Prague, Czech Republic, October 9th–10th, 2003

The seminar was organised by joint Commission for Experimental Mycology of the Czechoslovak Microbiological Society and the Czech Scientific Society for Mycology together with a group of experts collaborating under NATO project No. 978297 “Evaluation of composting and fungal treatment technology for remediation of PAH-contaminated soil”. The purpose of the seminar was to provide insight into the complexity of application of fungi in remediation of polluted soils. Only 17 participants took part in the seminar representing 6 countries (Czech Republic, Estonia, Germany, Italy, Norway, Slovak Republic).

Application of fungi in soil remediation (mycoremediation) is a very complex issue that comprises many different aspects, both theoretical and practical. During the first day of the seminar, two plenary lectures and seven specialised communications were presented. In the opening plenary lecture V. Šašek (Czech Republic) tried to sum up current state and perspectives of application of fungi in remediation of contaminated soils pointing out both positive results and several drawbacks. All these aspects were supported by the contribution by A. Majcherczyk (Germany), who explained why the sophisticated technology developed at the University of Göttingen, Germany as a pilot-scale treatment of contaminated soil by cultures of ligninolytic fungi, has not been brought real practice. On the other hand, T. Eggen (Norway) showed a successful (although lab-scale) application of spent oyster mushroom (Pleurotus ostreatus) substrate in remediation of industrial soil highly polluted with polycyclic aromatic hydrocarbons. Vanessa Leonardi (Italy) draw the attention to one important point in soil bioremediation, i.e. improvement of mycoremediation efficacy by pre-treatment of long-term contaminated soil with surfactants. A comparison of mycoremediation with the method of composting in the clean-up of soils contaminated with polycyclic aromatic hydrocarbons (PAHs) was presented by T. Cajthaml (Czech Republic); both the processes were proved to be successful, composting being more efficient.

The second part of the first day was opened by a plenary lecture presented by Č. Novotný (Czech Republic). The lecture dealt with one of the crucial topics in the research of the biodegrading potential of ligninolytic basidiomycetes, i.e., the correlation between activities of individual ligninolytic enzymes and the potential of the fungus to degrade organopollutants. This lecture was followed by a contribution presented by T. Cajthaml, who documented integrated research of both ligninolytic enzyme activities and degrading potential in the model white rot fungus Irpex lacteus (Fr.: Fr.) Fr. The last two contributions concerned another important factor of the bioremediation business. The goal of the treatments is a decrease in toxicity of the contaminated matrix. Anne Kahru (Estonia)
compared changes in toxicity and mutagenicity of soil polluted with polycyclic aromatic hydrocarbons after treatment by mycoremediation and composting. Tomáš Hubálek (Czech Republic) pointed out the problems of ecotoxicological evaluation, when different methods of bioremediation are applied. Abstracts of all the presentations are part of this report.

During the second day of the meeting the participants visited two localities close to the town of Soběslav (South Bohemia). The first one was a site where remediation of aged-contaminated soil took place (the soil was polluted during long-term timber preservation), the other place was an oyster mushroom farm. The reason of the visit was that the large-scale production of lignocellulosic material colonised by fungal mycelium (that can be applied in field bioremediation trials) is basically the same as the preparation of compost for growing oyster mushrooms. The trip was concluded with a short mushroom foray in south Bohemian forests.

ACKNOWLEDGEMENT

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Václav Šašek and Jiří Kunert
Applications of mycoremediation in practice
Použití mykoremediace v praxi

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Ligninolytic fungi belonging to the class of Basidiomycetes have developed unique mechanisms for the degradation of recalcitrant compounds such as lignin. Due to the non-specific character of the radical-mediated reaction of ligninolytic enzymes, the biodegradation of a wide variety of xenobiotic compounds, having an aromatic structure like lignin, have become subjects of extensive research. Most important environmental pollutants, such as chlorophenols, polycyclic aromatic hydrocarbons, polychlorinated biphenyls and dioxins, trinitrotoluene and other nitroaromatic explosives, different synthetic dyes and pesticides, have proven to be degradable by ligninolytic fungi. Most of the experiments were performed using liquid culture media. In soil conditions the fungal degrading potential is only one prerequisite, and other factors influence the degradation process. Many of the parameters (chemical, physiological and biological soil properties, chemical structure and bioavailability of the pollutant) are similar to those generally influencing any soil bioremediation process. Other conditions (ability to colonise the soil matrix and compete with the indigenous soil microflora, as well as the resistance to toxic compounds present in the polluted soil) are more or less specific for the application of ligninolytic fungi.

Field applications of fungi in soil remediation (mycoremediation) were not always successful. This indicates that more research is needed to establish mycoremediation as an effective and reliable soil-remediation technology. Both positive and negative field-scale experiences of fungal treatment of polluted soils using mycelia of *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm., *Phanerochaete chrysosporium* Burds. and other *Phanerochaete* species that have been performed in the Czech Republic and the USA were described in the lecture. Individual requirements (de-colourising potential, growth parameters, ability to degrade respective pollutants, ability to colonise non-sterile toxic soil) that the fungus has to meet on the way from the first screening to application in field remediation trials were evaluated in the lecture.
Lignin degrading white rot fungi have been demonstrated to be able to degrade numerous recalcitrant environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dioxins, DDT, chlorophenols, nitrotoluene, and different pesticides, in sterile, liquid culture as well as in complex soil systems. Application of these wood-inhabiting fungi for soil bioremediation requires methods that establish a growth of fungus in soil by adding lignocellulosic substrates or mixing soil with already fungus-colonised materials. However, contaminated soils originating from former industrial sites are usually not polluted in a uniform way and display areas of high concentration of chemicals prohibiting any microbial activity. Mixing of soil samples and disrupting of contaminated aggregates is in many cases unavoidable but also difficult to realise, e.g. in case of wet soil or a high clay content.

We developed a large-scale soil preparation system based on: 1. preparation of soil slurry by addition of water, 2. supplementation of the slurry with additives (e.g. potato pulp, tensides), 3. solidification of the slurry by adding wood chips, and 4. inoculation of the solid mixture with a millet culture of white rot fungi. The method overcame any problems of soil/contaminant inhomogeneity and composition, delivered a uniform size of soil particles as wood chips covered with soil, and resulted in a homogenous inoculation with the fungus. This treatment was successfully applied for bioremediation of soil contaminated with PAHs and PCBs in scales ranging from 1 dm$^3$ to 1 m$^3$. The growth of fungus was extremely uniform within the soil particles and the degradation rates obtained at a large scale were corresponding to the laboratory experiments showing up to 80 % degradation of PAHs after 3 months. However, in several cases the degradation was not successful due to a low availability of the contaminants. This problem could not be overcome by addition of surfactants, emulsifiers, solvents or modification of the treatment.
Use of spent mushroom substrate for bioremediation of polluted soil

Využití vyplozeného houbového substrátu k bioremediaci znečištěných půd

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In the middle of the 1980s it was demonstrated that some ligninolytic basidio­
mycetes were able to degrade recalcitrant environmental pollutants (polycyclic
aromatic hydrocarbons (PAHs), polychlorinated biphenyls, synthetic dyes and
dioxins). This was the start of an effort to apply these fungi for remediation of soil
contaminated with hazardous organic compounds. In the beginning most studied
fungus was Phanerochaete chrysosporium, but soon other fungi were evaluated for
their potential to degrade organopollutants, among others also edible fungi such
as Pleurotus ostreatus (oyster mushroom) and Lentinula edodes (shiitake).

Edible mushroom production is a large industry in several countries, and use of
spent fungal substrate, which still contains active fungal mycelium, represents an
agroindustrial byproduct and is available nearly for free. Experiments with spent
fungal substrate from oyster mushroom production were performed to investigate
its capability as an inoculum in bioremediation processes.

Experimental design and initial PAH concentration have shown to be influen­
cing factors. In soil with a high initial PAH concentration (e.g. 16,000 ppm of 16
PAHs) a low reduction of PAHs with 4 or more rings was observed. On the contrary,
fungal treatment of soil with a lower initial PAH concentration (e.g. 2,000 ppm
of 16 PAH), the 2-ring PAHs were reduced to less than 1 % of the original total
concentration; simultaneously a significant reduction of 4-ring compounds and also
of some 5-ring compounds was documented. Additional reinoculation of fungal
substrate into already mycoremediated soil stimulated further PAH degradation.

The effects of surfactants on mycoremediation
of aged PAH-contaminated soil

Vliv surfaktantů na mykoremediaci půd ze starých zátěží kontaminovaných
polycyklickými aromatickými uhlovodíky

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The use of white rot fungi for decontamination of PAH-contaminated soils
has been studied for many years. Ligninolytic fungi posses a great potential for
PAH removal. However, bioremediation of aged contaminated soils is often limited by low bioavailability of pollutants. The addition of surfactants to increase the diffusive mass-transfer rate of soil pollutants has received considerable attention in the last years.

Pretreatment of aged PAH-contaminated soil with four types of surfactants: soya oil, Tween 80, Tween 20 and olive-mill wastewater, was studied at laboratory scale in Erlenmeyer flasks thus simulating an on-site mycoremediation treatment. Two white rot fungi, Irpex lacteus strain 617/93 and Pleurotus ostreatus strain 3004 from the Culture Collection of Basidiomycetes, Institute of Microbiology, Prague were selected for their efficiency in degrading PAHs. Contaminated soil originated from a site of a former gasholder in Prague-Měcholupy, Czech Republic and contained PAH sum 2526 ppm. Before use in experiments the soil was pretreated with 5 % water dispersion of individual surfactants and left for 6 days at 4 °C.

Experimental setup. The fungi were grown in Erlenmeyer flasks on moistened and sterilised wheat straw for 21 days and after that the pretreated contaminated soil was put on top of the culture. The flasks were incubated at 24 °C for 6 weeks during which the humidity of the flask contents was maintained by regular addition of distilled water, and fungal development documented by photography. At the end of the experiment the material (straw grown mycelium + contaminated soil) of each flask was harvested and the contents of PAHs was estimated by a standard HPLC analysis (see the following abstract).

The results showed that: (i) in flasks with soil without any pretreatment (non-treated controls) a good growth of fungal mycelium appeared only on straw, not on contaminated soil; (ii) soil pretreated with surfactants was colonised by mycelium, the most pronounced colonisation was observed in soils pretreated with soya oil or olive-mill waste water; (iii) in flasks with pretreated soil a more abundant mycelial growth was observed also on straw; (iv) air mycelium over the pretreated soil turned to a greyish colour indicating that some material was translocated from soil into fungal hyphae; (v) both fungi under study behaved in the same, above described way.

The performance of fungi in soil bioremediation depends not only on their capacity to degrade respective pollutants but also on the capability of the fungal mycelium to colonise the soil matrix.
Mycoremediation versus composting of soil polluted with PAHs
Srovnání mykoremediace a kompostování při remediaci půd obsahujících polycyklické aromatické uhlovodíky

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Composting has been used to treat solid waste such as agricultural wastes, sewage sludge and food wastes. The technique was also used for bioremediation of contaminated soils originating from different industrial sites. Composting matrices are rich sources of microorganisms including bacteria, actinomycetes and fungi that can degrade pollutants.

Another perspective method is the application of fungal technology (mycoremediation) for the cleanup of contaminated soils. Several strains of white rot fungi have proven to attack many organopollutants including PAHs. However, most of the studies have been carried out using Phanerochaete chrysosporium and out of many hundreds of species possessing ligninolytic activity, only few have been studied in detail. That is why fungal remediation techniques have only slowly been brought into practice.

In our investigation we tried to compare the efficiency of degradation of polycyclic aromatic hydrocarbons (PAHs) in four different industrial, contaminated soils by composting and mycoremediation. The soils originated from a former gas-works in Prague-Michle (total PAH content 1466 mg/kg dry soil), a former tar-producing plant in Ostrava (total PAH content 2832 mg/kg dry soil), a former gasholder in Prague-Měcholupy (total PAH content 2526 mg/kg dry soil), and a wood-treatment plant in Soběslav (total PAH content 1987 mg/kg dry soil). All the sites are situated in the Czech Republic.

The results showed that both techniques are promising and both were able to reduce contamination during the several-month treatment. However, we found that after long-term post-composting maturation the level of contamination dropped significantly more than mycoremediation could reach. Average degradation (sum of PAHs) was 50 % by fungi but in the case of composting it was 75 % and in soil from the wood-treatment plant even 95 %.
Pollution with recalcitrant xenobiotic chemicals has become one of the major environmental problems. Some of these chemicals are highly resistant to biodegradation by native microflora. Ligninolytic fungi responsible for the white rot of wood have proven to decompose and mineralise a broad range of persistent chemicals as a result of the non-specificity of their extracellular enzyme system. Many of those chemicals are major pollutants: ammunition waste, pesticides, organochlorines, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), synthetic dyes, wood preservatives and synthetic polymers.

Ligninolytic fungi (LF) can be used for the biodegradation of pollutants in both contaminated water and soil. These fungi secrete one or more of the three major ligninolytic enzymes, lignin peroxidase (LiP, EC 1.11.1.14), Mn-dependent peroxidase (MnP, EC 1.11.1.13) and phenol oxidase (laccase) (LAC, EC 1.10.3.2). Each fungus has its typical enzyme pattern. Extracellular peroxidases and laccases have repeatedly shown to oxidise recalcitrant compounds in vitro but the importance of in vivo enzyme levels for biodegradation efficiency remains unclear. The question of correlability of enzyme activities, responsible for degradation of recalcitrant pollutants, with the degradation rate is thus relevant as other factors can become rate limiting in the biodegradation process due to its complexity.

Our study documented levels of MnP, LiP and LAC in various LF species cultivated in liquid media or colonising soil with explorative mycelium. Their effect on degradation of PAHs, PCBs and synthetic dyes was studied. Submerged and stationary cultures of *Irpex lacteus* were compared with respect to extracellular enzyme synthesis and the corresponding capability of decolorisation of RO16 azo dye and RBBR anthraquinone dye. In the former cultures, productions of MnP, LiP and LAC were significantly reduced. The difference in enzyme activities correlated with a lower rate of decolorisation of RO16, but not of RBBR.

A comparison of cultures of *I. lacteus* immobilised on polyurethane or wood showed differences between the production of extracellular ligninolytic activities, comparable to those observed in stationary and submerged cultures. The decolorisation of RBBR was similar in both immobilised cultures, which was in accordance with the observation in liquid cultures where a significant reduction in the synthesis of MnP did not result in a decrease of the RBBR decolorisation rate. A similar decolorisation efficiency of the two immobilised cultures was also
observed in the case of textile colouring bath liquids containing the dye mixtures Drimaren Blue and Drimaren Red. The respective decolorisation rates measured in the polyurethane culture after 7 days were 83 ± 6 and 94 ± 4 % of the initial absorbance value, compared to 99 ± 1 and 82 ± 9 % in the wood growing culture. In contrast, the ability to decolorise the Acid Black dye-containing colouring bath liquid strongly correlated with a higher synthesis of MnP in the polyurethane culture, where 95 ± 3 % of the initial dye absorbance was decolorised within 7 days, compared to only 18 ± 9 % in the wood culture. Decolorisation of the textile dye mixture Remazol Green was rather low in both immobilised cultures, irrespective of the MnP level produced.

Soil cultures of Pleurotus ostreatus, Phanerochaete chrysosporium and Trametes versicolor, where pre-sterilised soil was spiked with PAHs and colonised by explorative mycelium of a fungal organism growing from wheat straw, were used and degradation of PAH was investigated. The fungal explorative mycelium was able to secrete ligninolytic enzymes into soil. Correlability between the enzyme levels in soil and PAH degradation was very poor, probably due to other factors such as a low bioavailability of PAH molecules due to sorption to soil particles, hydrophobicity of the pollutant molecule, etc.

The study showed that the importance of high enzyme levels for efficient degradation of recalcitrant chemicals was better demonstrable in liquid medium cultures compared to cultures growing in soil.

**Biodegradation of selected PAHs by the ligninolytic fungus Irpex lacteus**

Biodegradace vybraných polycyklických aromatických uhlovodíků lyginolýtickou houbou Irpex lacteus

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The study of metabolites of polycyclic aromatic hydrocarbons (PAHs) degraded by ligninolytic fungi is a prerequisite for the application of fungal technology in practice because some of their metabolites can also represent serious environmental pollutants which may have mutagenic and carcinogenic potential.

In our work we demonstrated that representatives of PAHs (benzo[a]anthracene, benzo[a]pyrene, benzo[g,h,i]perylene) were degraded by the ligninolytic fungus *Irpex lacteus* in liquid nutrient medium. The products were analysed by GC-Ion
trap mass spectrometry. The combination of full scan mass spectra, product ion scans (MS-MS) and derivatisation of the degradation products provided further insight in the degradation mechanism initiated by I. lacteus. Particularly the daughter ion scans enabled the interpretation of unknown degradation products, even though they were only produced at trace level. Most of the structures suggested were later confirmed with authentic standards.

The results indicated that besides a strong potential of the fungus to degrade PAHs no dead-end metabolites were accumulated. We proposed a pathway for the degradation of benzo[a]anthracene, corresponding with the decomposition of anthracene where, except for anthraquinone, we detected 7,12-benzo[a]anthracenedione. Another parallel pathway appeared via 6-hydroxy-1,2-naphthalenedione.

**Toxicity and mutagenicity of PAH-polluted soils during composting and fungal treatment**

Sledovaní toxicity a mutagenicity půd kontaminovaných polycyklickými aromatickými uhlovodíky v průběhu kompostování a mykoremediace

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Polycyclic aromatic hydrocarbons (PAHs) are an important class of environmental contaminants because some of them are toxic, (pro)mutagenic and resist biodegradation. It has also been shown that some of the decomposition products of PAHs are more toxic or mutagenic than the parental compounds. In this work the toxicity and mutagenicity of PAHs-polluted soils during composting and fungal bioremediation was studied. The soils (1987 mg PAHs per kg of soil) originated from the territory of a wood-treatment plant in Soběslav, Czech Republic. The comparative remediation study was carried out in Norway (small-scale composting and fungal treatment) and also in Prague (pilot scale composting). The change of (geno)toxicity during bioremediation was studied analysing the samples in the beginning, middle and at the end of the treatment process. Two different extractants were applied for the extraction of toxicants from the soil: extraction of the soil with water (to mimic the hazard via the soil-water path) and extraction with methanol (to predict the potential hazard of less soluble and soil-bound pollutants). The ratio of the soil and extractant was 1+10 in both cases. For toxicity testing the photobacterial (**Vibrio fischeri**) bioassays (Microtox and Solid-Phase Flash-Assay) were used. Mutagenicity was studied using Ames assay (**Salmonella typhimurium TA98**) with and without metabolic activation (S9).
In all samples analysed (initial soil and all treatment samples, altogether 10 samples) the water-extracted toxicity was absent or low and no mutagenicity was observed. The methanol-extracted toxicity exceeded water-extractable toxicity about 70-fold. Mutagenicity was observed only in the case of methanol extracted samples of pilot-scale composting (Prague): mutagenicity was developed in the middle of composting and was not removed by the end of the treatment (after maturation). It was shown that the change of mutagenicity and toxicity during the treatments was dependent on the technique applied and was not directly correlated to the removal of PAHs.

Problems in ecotoxicological estimation of soils after bioremediation

Otázky ekotoxikologického měření půd po bioremediaci

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Toxicity tests characterising ecotoxicity of bioremediated soil are sometimes not in accordance with each other. We faced such a situation in the case of pilot-scale composting when we used soil containing 1987 mg PAHs per kg, originating from the territory of a wood-treatment plant in Soběslav, Czech Republic. We observed a decrease in ecotoxicity according to the bioluminescence test with *Vibrio fischeri* (EC$_{20}$ = 49 % at start versus EC$_{20}$ = 80 % after composting). The small decline in ecotoxicity (around 10 %) was seen also with the seed germination test using *Sinapis alba*. However, the results from the test with earthworms of the species *Eisenia fetida* showed a lasting ecotoxicity effect. There are other drawbacks in application of ecotoxicity tests for evaluation of bioremediation of soil. Using the seed germination test for estimating actual soil ecotoxicity before and after composting was found to be unsuitable. Already the original fresh substrate used for a compost-soil mixture showed toxicity to plant seeds, however, this was not related to toxicity of the polluted soil. This phenomenon was even more pronounced in tests with earthworms when no earthworms survived in freshly mixed substrate. If the substrate was left to dry for 2 weeks prior to performance of the earthworm test, ecotoxicity decreased dramatically (the earthworm survival was around 90 %). Also the part of the compost pile from which the samples were taken for testing played a significant role. The necessity to perform different ecotoxicity tests at the beginning of remediation and creating a suitable battery of ecotests can result in a better evaluation of the ecotoxicity during bioremediation.