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A comparative study to evaluate natural attenuation, mycoaugmentation, phytoremediation, and microbial-assisted phytoremediation strategies for the bioremediation of an aged PAH-polluted soil

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ABSTRACT

Biological treatments are considered an environmentally option to clean-up polluted soil with polycyclic aromatic hydrocarbons (PAHs). A pot experiment was conducted to comparatively evaluate four different strategies, including natural attenuation (NA), mycoaugmentation (M) by using *Crucibulum leave*, phytoremediation (P) using maize plants, and microbial-assisted phytoremediation (MAP) for the bioremediation of an aged PAH-polluted soil at 180 days. The P treatment had higher affinity degrading 2–3 and 4 ring compounds than NA and M treatments, respectively. However, M and P treatments were more efficient in regards to naphthalene, indeno [*l*,2,3-*c*,*d*]pyrene and benzo[*g*,*h*,*i*]perylene degradation respect to NA. However, 4, 5–6 rings undergo a strong decline during the microbe-assisted phytoremediation, being the treatment which determined the highest rates of PAHs degradation. Sixteen PAH compounds, except fluorene and dibenzo[*a*,*h*]anthracene, were found in maize roots, whereas the naphthalene, phenanthrene, anthracene, fluoranthene, and pyrene were accumulated in the shoots, in both P and MAP treatments. However, higher PAH content in maize biomass was achieved during the MAP treatment respect to P treatment. The bioconversion and translocation factors were less than 1, indicating that phystabilization/phytodegradation processes occurred rather than phytoextraction. The microbial biomass, activity and ergosterol content were significantly boosted in the MAP treatment respect to the other treatments at 180 days. Ours results demonstrated that maize-*C. laeve* association was the most profitable technique for the treatment of an aged PAH-polluted soil when compared to other bioremediation approaches.

1. Introduction

The industrial pollution around the Czech city of Ostrava has dramatically increased during the last two decades as consequence of mining, metallurgical activities, and atmospheric deposition from fossil fuel power plants, resulting in serious and harmful accumulation of polycyclic aromatic hydrocarbons (PAH) in agricultural areas (Podlešáková et al., 1998; Vácha et al., 2015). In this regard, PAHs have generated a great deal of interest in recent decades due to their mutagenic, and carcinogenic properties and their recalcitrance into the environment (Luch, 2009; EPA, 2015; IARC, 2010). Some physicochemical properties of PAHs, such as high hydrophobic character, and/or stable polycondensed aromatic structures determine their sequestration to the soil particle in which the prolonged contact time promote the phenomenon of "soil aging", thereby leading to their recalcitrance (Reid et al., 2000; Boopathy, 2002; Yap et al., 2010).

The selection of an appropriate remediation approach for PAH-polluted soils is not an easy choice. Bioremediation strategies such as natural attenuation, mycoaugmentation, and phytoremediation offer a particularly attractive option for the cleanup of contaminated sites, especially for the remediation of PAH-polluted soils (Mirsal, 2008). Natural attenuation of soil allows the biodegradation of recalcitrant compounds by autochthonous microbial communities, which is commonly considered to be the primary mechanism for the natural removal

Abbreviations: ACE, Acenaphthylene; ACEN, Acenaphthene; ANT, Anthracene; BaA, Benzo[*a*]anthracene; BbF, Benzo[*b*]fluoranthene; BkF, Benzo[*k*]fluoranthene; BghiP, Benzo[*g*,*h*,*i*] perylene; BaP, Benzo[*a*]pyrene; CHR, Chrysene; DH, Dehydrogenase; DBA, Dibenz[*a*,*h*]anthracene; FLU, Fluorene; FLUO, Fluoranthene; FDA, Fluorescein diacetate; IYP, Indeno[*l*,2,3-*c*,*d*] pyrene; LME, Lignin-modifying enzymes; MAP, Microbe-assisted phytoremediation; M, Mycoaugmentation; NAP, Naphthalene; NA, Natural attenuation; PHE, Phenanthrene; P, Phytoremediation; PAH, Polycyclic aromatic hydrocarbons; PYR, Pyrene

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of contaminants (Declercq et al., 2012). Mycoaugmentation, through the use of white-rot fungi, has been suggested to be a profitable approach for cleaning up polluted soils, as previously reported by Covino et al. (2010a, 2010b, 2010c). White-rot fungi are efficient degraders of a wide range of organic contaminants through non-specific lignin-modifying enzymes (LME) which are released into the extracellular environment. Moreover, the hyphal growth of white-rot fungi makes them able to extensively penetrate into soil and to serve, at the same time, as dispersion vectors of autochthonous pollutant-degrading bacteria (Kohlmeier et al., 2005). The use of white-rot fungi requires the concomitant addition of lignocellulosic substrates to improve their ability to compete with the autochthonous microbiota. It has been observed that the use of lignocellulosic inoculum carriers such as wheat straw, corn cobs, and straw pellets significantly increased the growth capacity and PAH degradation performance of *Dichomitus squalens*, *Pleurotus ostreatus*, *Coprinus comatus*, *Lentinus tigrinus*, and *Irpex lacteus* (Covino et al., 2010a, 2010b). Phytoremediation comprises a group of technologies that use plants and their associated microorganism to remove pollutants from the environment or to make them less harmless (Salt et al., 1998). This technology is particularly suited to the treatment of large areas of surface contamination, when other methods may not be as feasible (Boer and Wagelmans, 2016). Among the different approaches for phytoremediation of pollutants, phytoextraction (uptake of organic pollutants), phytodegradation (biotransformation/biodegradation of organic molecules) and phytovolatilization (release of the volatile organics or their metabolites into the atmosphere), seem to be less appropriate for the bioremediation of PAH-polluted soils, because until now, there has been no evidence that plants may act as PAH-hyperaccumulators (Alagić et al., 2015). Nevertheless, the phytostabilization and/or rhizodegradation has been shown as one of the most powerful tool in PAHs removal which represents the process of synergistic nature occurring between plants and rhizospheric microorganisms (Haritash and Kaushik, 2009; Alagić et al., 2015). A multitude of changes take place in soil in the presence of roots which increase the aeration, provide a habitat for microbial population through their exudates, thereby allowing ideal conditions for stimulating the growth of specific autochthonous microbial populations involved in the PAH transformation into more bio-accessible compounds (Larsson et al., 2013). Thus, the aerobic degradation occurs also in deeper layers, as previously reviewed by Alagić et al. (2015). Natural attenuation, mycoaugmentation and phytoremediation approaches can be used not only as remediation technologies in themselves but also in combination. Thus, microbe-assisted phytoremediation optimizes the synergic effect of plants and microorganisms and has been used for the removal of organic contaminants (Glick, 2010). So far, some studies have addressed the combined use of plants and biodegradative bacteria with the aim to remove PAHs (Lin et al., 2008; Agnello et al., 2016), but none of these have been conducted through the combination between plants and white-rot fungi. In this view, the aim of this study was to comparatively investigate the feasibility, in regards to biodegradation outcome and evolution of the autochthonous microbial functionality, of four different bioremediation approaches: a) natural attenuation (NA); b) mycoaugmentation (M); c) phytoremediation (P); and d) microbe-assisted phytoremediation (MAP) in an aged PAH-polluted soil. To do the M approach, the white-rot fungus, namely *Crucibulum laeve* was selected according to its previously reported ability to colonize degraded PAH-environments (Tornberg et al., 2003). Meanwhile, maize plants were used for performing the P strategy due to its suitability for stabilizing/degrading PAH (Kacálková and Tlustoš, 2011; Chirakkara et al., 2016). The results derived from this study will allow us to gain new insight in the applicability of biological strategies to deal with the removal of PAH-polluted soil.

2. Material and methods

2.1. Chemicals

Acetone and *n*–hexane were purchased from Chromservis (Czech Republic) with the highest purity available. Standards of priority 16 US EPA PAHs and internal standard solution (IS) containing napthalene − *d8*, acenaphthene − *d10*, phenanthrene − *d10*, chrysene − *d12*, and perylene − *d12* were purchased from Restek (USA) in a solution of methylene chloride.

2.2. Contaminated soil description

The aged PAH-polluted soil was collected from an agricultural field close to the Olza River (49°50'08"N; 18°17'33"E, Ostrava, Czech Republic). The total soil sample was obtained by mixing different subsamples collected from different zones of the field area at a depth of 0–20 cm. Subsequently, the soil was homogenized, air-dried at room temperature and finally passed through a 5 mm mesh-sieve. The soil was stored in polythene bags at 4 °C until its use. According to the US textural classification, the soil was sand loamy soil (clay, 11%; silt, 6%; sand, 83%) and the main properties of soil were: pH, 7.5; CEC, 7.6 mmol g^{-1}; C_{tot} 36.7 g kg^{-1}; N_{tot} 2.9 g kg^{-1}. Sixteen priority US EPA PAHs were detected in the polluted soil and concentrations are given in a detailed table in Supplementary material (Table S1).

2.3. Plant and fungal inocula preparation

Maize seeds (*Zea mays* L., var. Colisee) were surface disinfected by immersion in 2% (v/v) hydrogen peroxide for 8 min (García-Sánchez et al., 2012). Then, seeds were thoroughly rinsed three times with sterile water and used for the pot experiment.

Crucibulum laeve was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ 8451). The strain was maintained at 4 °C and pre-cultured at 24 °C on 2% malt extract agar for 2 weeks in order to obtain fresh inoculum.

Barley seeds were chosen as lignocellulosic substrate carrier for fungal inocula following the methodology previously described by Reina et al. (2013). Before the inoculation, 18 g of barley seeds and 30 mL of distilled water were placed in Erlenmeyer flasks (250 mL) and covered with cotton-wool stoppers and subsequently sterilized by autoclaving (121 °C, 45 min). The barley seeds were inoculated with 10 mL of the content of 4 fungal agar plates homogenized in 80 mL sterile water (55% v/w) and were grown and incubated for 4 weeks at 24 °C under stationary conditions. In order to test the production of LME by *C. laeve*, the laccase activity was measured after 4 weeks of incubation by monitoring the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) ($\varepsilon_{420\ nm}$: 36 $mM^{-1}cm^{-1}$) using a combination assay in 50 mM sodium malonate buffer at pH 4.5 (Reina et al., 2013). The dose of application to polluted soil was used in a ratio of 0.6:10 [*C. laeve* inoculum:soil (w/w)] as previously described by Lladó et al. (2012).

2.4. Experimental design setup

PAH degradation experiment was carried out in a series of identical polypropylene pots with a total volume of 5 L (20.5 cm length, 21 cm width, and 18 cm height). Approximately 5 kg of contaminated soil was individually stacked in each pot. Four treatments were conducted as follows: a) natural attenuation (intrinsic cleanup ability of soil) (NA); b) mycoremediation (soil inoculated with *C. laeve*) (M); c) phytoremediation (soil vegetated with maize) (P); d) microbial-assisted phytoremediation (soil vegetated with maize and inoculated with *C. laeve*) (MPA). The moisture of the soil was kept to 60% of the field water-holding capacity by weighing the posts regularly and adding sterile distilled water as necessary. Each treatment was run in four replicates,