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Comparing the removal of polycyclic aromatic hydrocarbons in soil after different bioremediation approaches in relation to the extracellular enzyme activities

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ABSTRACT

A 120-day experiment was conducted to compare the removal of polycyclic aromatic hydrocarbons (PAHs) from agricultural soil after natural attenuation (NA), phytoremediation (P), mycoremediation (M), and plant-assisted mycoremediation (PAM) approaches in relation to the extracellular enzyme activities in soil. The NA treatment removed the total soil PAH content negligibly. The P treatment using maize (*Zea mays*) enhanced only the removal of low and medium molecular PAHs. The *Pleurotus ostreatus* cultivated on 30–50 mm wood chip substrate used in M treatment was the most successful in the removal of majority PAHs. Therefore, significantly ($p < 0.05$) highest total PAH removal by 541.4 μg/kg dw (dry weight) (36%) from all tested M treatments was observed. When using the same fungal substrate together with maize in PAM treatment, the total PAH removal was not statistically different from the previous M treatment. However, the maize-assisted mycoremediation treatment significantly boosted fungal biomass, microbial and manganese peroxidase activity in soil which strongly correlated with the removal of total PAHs. The higher PAH removal in that PAM treatment could be reflected in the following post-harvest time. Our suggested M and PAM approaches could be promising *in situ* bioremediation strategies for PAH-contaminated soils.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are associated with the volatile combustion phase that occurs during the incomplete combustion of organic materials (Bragato et al., 2012). The PAHs are organic pollutants with condensed aromatic rings of special interest due to their toxicity and ubiquitous presence in the environment. In addition is their carcinogenicity, as some of priority 16 PAHs are suspected carcinogens with acute and chronic health effects (Larsson et al., 2013).

The PAHs are lipophilic compounds with a chemical arrangement which predicts their stability and persistence in the environment (Bojes and Pope, 2007). The PAHs tend to accumulate in soils (García-Sánchez et al., 2018), sediments (Dvořák et al., 2017), and sewage sludge (Vácha et al., 2005). The

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content of PAHs in Central European arable soils ranged from 30 to 4108 μg/kg dw (Maliszewska-Kordybach et al., 2009). Thus, agricultural areas should receive satisfactory attention in regard to contamination with PAHs because the preventive limit of total PAHs is 1000 μg/kg dw for arable soils in the Czech Republic (Public Notice No. 153/2016).

The accumulation of PAHs in soil can be attributed to the high persistence of PAHs in the environment due to their strong adsorption onto soil organic matter and recalcitrance of PAHs to degradation (Wei et al., 2014). Most of the biological approaches considered for the restoration of PAH-contaminated sites depend on their availability for plants and microorganisms in soil. The water solubility of PAHs is low, but some studies suggested that the PAHs are transported in association with dissolved organic matter which increased their bioavailability as the basic requirement of PAH bioremediation (Gerhardt et al., 2017). The ability of maize (*Zea mays* L.) to grow on PAH contaminated sites was reported by Lin et al. (2008), and Dupuy et al. (2015) indicated that maize exudates enhanced the PAH removal from soil during the phytoremediation approach. This process is based on the synergisms of the plant and their associated microorganisms in the rhizosphere helping to extract, immobilize, accumulate, and/or remove PAHs within the *in* or *ex planta* degradation in soil (Kuppusamy et al., 2017).

A promising option for the biodegradation of PAHs are ligninolytic fungi, *e.g.*, *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, and *Bjerkandera adusta*, due to their production of ligninolytic enzymes, such as manganese peroxidase, lignin peroxidase, and laccase (Kadri et al., 2017). Nevertheless, Marco-Urrea et al. (2015) reviewed the potential of extracellular oxidation of PAHs also due to some of non-ligninolytic ascomycetes in soil.

The predominant products of PAH degradation derived from the action of lignin-modifying enzymes are several PAH derivatives such as quinones, dicarboxylated, and their ring fission derivatives. Whereas, *e.g.*, hydroxylated derivatives of anthrone and phenanthrene 9,10-dihydrodiol suggested the possible involvement of a cytochrome P-450-epoxide hydrolase system as showed by the *in vitro* experiments of Covino et al. (2010). A study by Sack et al. (1997) indicated that using white-rot fungi, such as *P. chrysosporium* and *Trametes versicolor* to breakdown the PAHs within the complete PAH mineralization may occur. Moreover, Eggen and Majcherczyk (1998) described the removal of [^{14}C]benzo[a]pyrene by 49% after 3 months of incubation in aged creosote-contaminated soil applied with *P. ostreatus*. Unfortunately, only 1% completely mineralized to $^{14}CO_2$, but it was still significantly higher in comparison to the unsterile control soil (0.1%) without the white-rot fungus.

Besides the root exudates (carboxylic and amino acids, carbohydrates, secondary metabolites, polysaccharides, and proteins) and the stimulation of the degradative potential of the rhizosphere microflora, another important rhizodegradation mechanism is the root exudation of enzymes (peroxidases, proteases, laccases, hydrolases, lipases, *etc.*) involved in the biodegradation of PAHs (Dubrovskaya et al., 2017). Moreover, Shi et al. (2017) suggested that the combination of phytoremediation using suitable plants with fungi assisted bioremediation can have a positive role in the rhizosphere degradation of PAHs.

The PAH-polluted soil is still one of the most intractable environmental problems today and studies focused on cost-effective and environmentally friendly bioremediation strategies of PAHs in contaminated soils are still needed and welcome (Feng et al., 2017). Therefore, the main aims of this work were: (1) To compare the decrease of PAH content after natural attenuation, phytoremediation, mycoremediation, and plant-assisted mycoremediation of agricultural PAH-spiked soil; (2) to investigate the influence of maize (*Zea mays* L.) and *P. ostreatus* (Jacq.) P. Kumm., strain HK35 cultivated on waste lignocellulosic substrates on microbial activity, fungal biomass, and selected extracellular enzymes activities in soil in comparison to bare soil; and (3) to evaluate the relationship between the removal of PAHs from soil and enzyme activities in order to develop a feasible treatment for the bioremediation of PAH-contaminated agricultural soil.

1. Materials and methods

1.1. Chemicals, reagents, and materials

Acetone, dichloromethane, and *n*-hexane, each GC/MS grade, were purchased from Chromservis, Czech Republic. Standards of 16 individual US EPA priority PAHs containing acenapthylene (ACY), acenaphtene (ACE), anthracene (ANT), benzo[a]anthracene (BaA), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[g,h,i]perylene (BghiP), benzo[a]pyrene (BaP), chrysene (CHR), dibenz[a,h]anthracene (DBA), fluorene (FLU), fluoranthene (FLUO), indeno[l,2,3-c,d]pyrene (IPY), naphthalene (NAP), phenanthrene (PHE), and pyrene (PYR) in a 2000 mg/L mixture solution of each PAH species (SV Calibration Mix 5, Restek, USA) were purchased from Chromservis, Czech Republic. Deuterated *p*-TER-d_{14} (IS) and 2-FBP (SS) solutions at 2000 mg/L (Restek, USA) were purchased from Chromservis, Czech Republic. All working PAH, IS, and SS solutions were diluted with hexane (V/V). The Strata SI-1 Silica SPE cartridges (Phenomenex, USA) were purchased from Chromservis, Czech Republic. The used glassware was prewashed with distilled water, then acetone, and followed by hexane and dried in an oven at 150°C for 2 hr before use.

1.2. Characterization of experimental soil

The soil was collected from a long-term trial site (49°33′16″N, 15°21′2″E) close to the city of Humpolec in the Czech Republic. The site characteristics were described elsewhere in Černý et al. (2010). The total soil sample was obtained by mixing different sub-samples collected from different zones of the field area at a depth of 0–20 cm. The non-sterilized experimental soil was homogenized, air-dried at room temperature, and passed through a 5 mm stainless steel sieve. The soil texture of our experimental soil (Cambisol) was sandy loam (clay, 5.8%; silt, 43.6%; sand, 50.6%, W/W) and the main physicochemical properties of the soil were: pH ($CaCl_2$), 5.2; CEC, 90.3 $mmol_{(+)}$/kg; C_{tot}, 18 g/kg; N_{tot}, 1.7 g/kg; $P_{pseudotot}$, 0.87 g/kg; $K_{pseudotot}$, 9.55 g/kg; $Ca_{pseudotot}$, 2.71 g/kg; $Mg_{pseudotot}$, 8.18 g/kg dry weight (dw) basis; individual PAHs were below the quantifiable limit in range between 1.8 and 5.6 μg/kg dw for individual PAH compounds.

1.3. Characterization of plant seeds, lignocellulosic substrates, and fungal inoculum

The experimental plant in our study was tested maize (*Zea mays* L. var. Colisee) purchased from KWS (Germany). Before sowing, maize seeds were surface-disinfected according to Smith et al. (2006). The wood chips (10–30 mm) of waste apple tree branches (S1), wood chips (30–50 mm) of waste apple tree trunks (S2), and wood chips (10–50 mm) of an S1 and S2 mixture in 1:1 ratio (W/W) (S3) were tested in this study as a lignocellulosic substrate carrier for ligninolytic fungi–*P. ostreatus* (Jacq.) P. Kumm., strain HK35 (*P. ostreatus*) obtained from the Crop Research Institute in Prague, Czech Republic.

The preparation of the inoculum followed the procedure described by García-Delgado et al. (2015). Briefly, *P. ostreatus* culture was maintained at 4°C and pre-cultured at 24°C on four 2% (W/V) malt extract-glucose agar plates for 2 weeks in order to obtain fresh inoculum containing mycelium of *P. ostreatus*. This culture was used for the production of *P. ostreatus*-spawn on wheat grain. The grain was half-cooked, drained of excess water, supplemented with 5% (W/W) of gypsum, filled in 1 L bottles, and sterilized in an autoclave at 121°C for 2 hr. After sterilization, the grain was inoculated with four agar pieces of *P. ostreatus* mycelium and cultivated at 24°C for 14 days. Before inoculation of the wood chips, each cultivation substrate with 60% (W/W) of moisture content, adjusted by the addition of distilled water, was placed in a 6 L glass container covered with Al-foil and sterilized in an autoclave (121°C, 2 hr). After inoculation with grain containing *P. ostreatus* spawn, the culture was grown for 4 weeks at 24°C until the whole substrate was fully colonized by mycelium of *P. ostreatus*. The main properties of "ready to use" fungal substrates with grown *P. ostreatus* were: dry matter, 39.3% (W/W); pH (H_2O), 7.8; C_{tot}, 449 g/kg dw; N_{tot}, 12.0 g/kg dw; C/N, 44.1.

1.4. Experimental design setup

The 120-day experiment was conducted in a roofed, outdoor, atmospheric precipitation-controlled, vegetation hall with natural temperature and light using a series of 6 L polypropylene pots (h = 20.5 cm, d_{top} = 21.0 cm, d_{bottom} = 18.0 cm). Each pot contained 5 kg dw of experimental soil. The pot experiment was set up in 8 treatments each in four replications to simulate different bioremediation approaches of PAH contaminated soil as follows: (1) Natural attenuation of PAHs in contaminated bare soil (NA); (2) Mycoremediation of PAHs in soil using *P. ostreatus* cultivated on i) 10–30 mm substrate (M + S1), ii) 30–50 mm substrate (M + S2), iii) 10–50 mm substrate (M+S3); (3) phytoremediation of PAHs using maize in non-amended contaminated soil (P); (4) Plant-assisted mycoremediation of PAHs in soil using maize together with *P. ostreatus* cultivated on i) 10–30 mm substrate (PAM+S1), ii) 30–50 mm substrate (PAM+S2), iii) 10–50 mm substrate (PAM+S3). The dose of respective substrate with colonized *P. ostreatus* applied to PAH-spiked soil was set to 5% (W/W) in accordance with Li et al. (2012). Before the experiment was established, the soil was spiked with a synthetic mixture of 16 individual US EPA priority PAHs (SV Mix 5, Restesk, USA) diluted with hexane as a carrier solvent to provide the 100 μg/kg dw content of each PAH species following the procedure described by Smith et al. (2006). The resulted average initial contents of low molecular weight (LMW), medium molecular weight (MMW), high molecular weight (HMW) PAHs, and total PAHs in soil (μg/kg dw) of each treatment are shown in Table 1. An individual pot was also fertilized (per 1 kg soil dw) as follows: 100 mg N (NH_4NO_3 water solution); 32 mg P and 80 mg K (K_2HPO_4 water solution).

In the case of 16 planted pots, maize seeds were sown directly in soil at a 2–3 cm depth at a rate of 8 seeds per pot. After 15 days of germination, maize plants were thinned to three of uniform size. The location of pots was randomly changed once a week. The moisture of soil was kept at 60%–70% (V/W) of the maximum water holding capacity (MWHC) by weighing the pots regularly and adding demineralized water as necessary. The MWHC was calculated according to Mercl et al. (2016). During the vegetation period, weeds were removed to avoid interplant competition.

Each soil sample was collected by thoroughly mixing three sub-samples randomly collected from the whole soil profile of each pot referred as replication of each respective treatment using a stainless steel tool at the end of the 120-day experiment. Before the analysis of PAHs, the total of 32 soil samples were divided into equal halves and used as technical replications for PAH measurement, then freeze–dried, ground with a mortar, and subsequently sieved through 2 mm stainless steel mesh and stored at −20°C in Petri dishes covered with Al-foil before the analysis of PAHs. Moist soil samples for analyses of microbial and extracellular enzyme activities, and fungal biomass analyses were stored in a refrigerator at 4°C. The plant and *P. ostreatus* biomass was harvested at the end of the experiment and plants were divided into roots and shoots. Roots were gently washed with distilled water to remove attached soil particles. Roots and shoots were composites of three maize plants of each planted pot and were cut into small pieces together, respectively. Thereafter, roots, shoots, and *P. ostreatus* samples, 16 of each, were halved into equal parts for technical replications, homogenized, and oven dried at 35°C for 72 hr, and milled to a fine powder.

1.5. Analytical methods

1.5.1. Analysis of microbial activity and soil fungal biomass

The microbial activity (MA) of living microbial biomass in soil was assayed by the quantification of dehydrogenase activity (DHA, EC 1.1) measured with triphenyl formazan (TPF) formed by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) in same frame as Fu et al. (2012). Briefly, 1 g soil was incubated with 1 mL of 1.5% (W/V) TTC solution (0.1 mol/L Tris-HCl buffer, pH 7.6) with shaking at 150 r/min in the dark for 6 hr at 30°C. Blanks without the soil and samples without TTC addition were run simultaneously for control purposes. The reaction mixture was blended with 4 mL of acetone, filtered and measured at 485 nm using a spectrophotometer (HachLange 3900, USA). Results were expressed as μg TPF/(g soil dw·6 hr).

The fungal biomass (FB) in soil was quantified by non-alkaline extraction of free ergosterol in living fungal cells according to Djajakirana et al. (1996). Briefly, 1 g soil was suspended in 25 mL ethanol in amber bottles and shaken for 30 min at 250 r/min on an orbital shaker in the darkness. The soil suspension was filtered and evaporated in a vacuum rotary evaporator at 40°C.

Table 1 – Initial content and residual content of the sum of LMW, MMW, and HMW PAHs, and total PAHs in soil at 0 days (initial) and at 120 days (residual) of an experiment.

PAH content (μg/kg dw)	Days	NA	M + S1	M + S2	M + S3	P	PAM+S1	PAM+S2	PAM+S3
LMW PAHs	0	562.2 ± 31aA	561.2 ± 9.4aA	546.0 ± 23aA	572.5 ± 7.3aA	574.1 ± 15aA	549.2 ± 4.0aA	565.7 ± 13aA	567.8 ± 22aA
	120	527.9 ± 19eA	430.2 ± 12cdB	339.4 ± 23aB	397.7 ± 7.1bcB	452.4 ± 5.8 dB	424.2 ± 8.2bcdB	329.6 ± 27aB	390.8 ± 9.7bB
MMW PAHs	0	376.7 ± 4.0aA	377.3 ± 13aA	372.7 ± 17aA	377.5 ± 5.4aA	379.6 ± 9.5aA	376.4 ± 12aA	379.6 ± 12aA	366.4 ± 20aA
	120	354.5 ± 8.1cB	294.4 ± 5.8bB	215.0 ± 24aB	296.4 ± 5.4bB	361.6 ± 10cB	275.7 ± 29bB	220.2 ± 23aB	269.9 ± 20bB
HMW PAHs	0	566.1 ± 7.7aA	565.3 ± 14aA	572.2 ± 34aA	572.1 ± 36aA	564.0 ± 23aA	573.3 ± 15aA	569.6 ± 15aA	564.8 ± 27aA
	120	549.7 ± 10cA	484.6 ± 18bB	408.0 ± 16aB	490.0 ± 25bB	541.1 ± 28cB	492.1 ± 19bB	406.0 ± 14aB	488.5 ± 23bB
Total PAHs	0	1505 ± 32aA	1504 ± 1.0aA	1491 ± 50aA	1522 ± 39aA	1518 ± 37aA	1499 ± 21aA	1515 ± 24aA	1499 ± 59aA
	120	1432 ± 29d	1209 ± 25bB	962.4 ± 20aB	1184 ± 21bB	1355 ± 31cB	1192 ± 40bB	955.7 ± 34aB	1149 ± 38bB

Means ± standard deviations estimated from four replications within the same column followed by different lowercase letters indicate significant differences ($p < 0.05$) among the respective PAH removal of each treatment as determined by Tukey's test. NA: natural attenuation; M+S1, S2, and S3: mycoremediation using fungal substrates S1, S2, and S3; P: phytoremediation; PAM+S1, S2, and S3: plant-assisted mycoremediation using fungal substrates S1, S2, and S3. PAH: polycyclic aromatic hydrocarbon; LMW: low molecular weight; MMW: medium molecular weight; HMW: high molecular weight.

The dry extract was dissolved in 1 mL ethanol and percolated through a syringe filter (cellulose-acetate, 0.45 μm pore size) into a vial. Quantitative determination of ergosterol was performed by an HPLC analysis on a 1260 Infinity HPLC system (Agilent Technologies, USA) equipped with a diode array detector and Phenomenex C18 column (250 mm × 4.60 mm; particle size 5 μm; pore size 100 Å), mobile phase 97% methanol/water (V/V), flow rate of 1 mL/min, and detection at 282 nm. Ergosterol was quantified using a calibration curve (10–1000 μg/L) of pure standard solution (Sigma-Aldrich, USA). Results were expressed as μg ergosterol/g soil dw.

1.5.2. Analysis of selected extracellular enzyme activities

Assays of selected ligninolytic (laccase, manganese peroxidase) and hydrolytic (β-D-glukosidase, acid phosphatase, arylsulfatase, and lipase) enzyme activities in soil extracts were done according to Štursová and Baldrian (2011). Briefly, 0.2 g soil in a 50 mL Erlenmayer flask was extracted with 20 mL of 50 mmol/L sodium acetate buffer (pH 5.0) at room temperature. The reaction mixture was homogenized using an Ultra-Turrax (IKA Labortechnik, Germany) for 30 sec at 8000 r/min. The extracts for ligninolytic activities were filtrated and desalted using PD-10 columns (Pharmacia, Sweden) to remove inhibitory compounds. Individual enzyme activities were measured in four replicates in 96-well microplates spectrophotometrically using the plate reader Infinite M200 (Tecan, Switzerland). One unit of enzyme activity per 1 g soil dw (U/g soil dw) was defined as the amount of enzyme catalyzing the formation of 1 μmol of reaction product per min under the assay conditions.

Laccase (LAC, EC 1.10.3.2) activity was measured in 50 μL soil extract by monitoring the oxidation of ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) immediately after the addition of 50 μL of 0.08% (W/V) ABTS solution with 150 μL citrate–phosphate (100 mmol/L citrate, 200 mmol/L phosphate) buffer (pH 5.0) at 420 nm.

Manganese (–dependent) peroxidase (MnP, EC 1.11.1.13) activity was measured in 50 μL soil extract in the presence of 200 μL substrate for a MnP measurement solution using the succinate–lactate buffer with MBTH (3-methyl-2-benzothiazolinonehydrazone) and DMAB (3,3-dimethylaminobenzoic acid). The substrate for MnP measurement was prepared through the mixing of 42 mL succinate–lactate buffer (100 mmol/L, pH 4.5), 5.6 mL DMAB solution (25 mmol/L), 2.8 mL MBTH solution (1 mmol/L), 2.8 mL $MnSO_4$ solution (2 mmol/L), and 2.8 mL peroxide solution (0.08 mmol/L). MBTH and DMAB are oxidatively coupled by the enzyme, and the resulting purple indamine dye is detected at 595 nm. The results were corrected by the activities of the samples without $MnSO_4$ being substituted by an equimolar amount of EDTA.

The activities of β-D-glukosidase (β-D-G, EC 3.2.1.21), acid phosphatase (AP, EC 3.1.3.2), arylsulfatase (AS, EC 3.1.6.1), and lipase (LPS, EC 3.1.1.3) in soil extracts were measured according to Baldrian (2009). Briefly, hydrolytic enzymes activities were measured in 200 μL of soil extract in the presence of the respective substrate (40 μL) in dimethylsulfoxid (DMSO) at a final concentration as follows: (1) 2.75 mmol/L MUFG (4-methylumbellyferyl-β-D-glucopyranoside) for β-D-G, (2) 2.75 mmol/L MUFP (4-methylumbellyferyl-phosphate) in DMSO for AP, (3) 2.50 mmol/L MUFS (4-methylumbellyferyl sulphate potassium salt) in DMSO for AS, and (4) 2.50 mmol/L MUFY (4-methylumbellyferyl-caprylate) in DMSO for LPS. The soil extracts in 96-well microplates were incubated at 40°C and measurements of fluorescence were recorded with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The background fluorescence measurement, standard for corrections of fluorescence quenching, and quantification of enzymatic activities based on standard curves has been described elsewhere in Štursová and Baldrian (2011).

1.5.3. Analysis of PAHs

The extraction of PAHs in soil samples was carried out according to US EPA (2007) using the ultrasonic bath extraction procedure with a continuous three re-extraction cycle. For the determination of PAHs in soil samples, an aliquot sample of 5 g (accuracy ±0.001 g) was weighed into the glass-capped flask (100 mL). Ultrasonic extraction of the sample in the flask was performed with 30 mL hexane–acetone mixture (1:1, V/V) in the ultrasonic bath system (Bandelin Sonorox Digitec DT510/H, Germany) with addition of SS solution at 500 μg/L and sonicated for 30 min at a bath temperature of 35°C. The reaction mixture was then filtered through the filtrate paper and rinsed with 5 mL of hexane. The sample was

re-extracted twice with 30 mL of hexane–acetone (1:1, V/V) following the same procedure. The extracts were collected together and evaporated on a rotatory evaporator (Büchi rotavapor R-300, Switzerland) at 40°C to near dryness (>1 mL), dissolved in 5 mL of hexane and concentrated to 1–2 mL for further purification according to US EPA (1996). The silica of SPE cartridges was conditioned using 10 mL 15% (V/V) dichloromethane–hexane, washed with 10 mL of hexane, and followed by the elution of 10 mL 15% (V/V) dichloromethane–hexane. The eluate was reconcentrated to 1 mL, and after the addition of the IS solution at 500 μg/L, it was analyzed for PAHs. For the analysis of PAHs in roots, shoots, and *P. ostreatus* the samples weighing 5, 5, and 2 g, respectively, were extracted and treated using the same procedure as was described above for soil samples.

Analysis of individual PAHs was performed by gas chromatography coupled with a mass spectrometric detector (GC/MS) according to US EPA (2014). The PAHs were analyzed in an Agilent HP 6890 N gas chromatograph (Agilent Technologies, USA) connected to an Agilent HP 5975 inert mass selective detector (Agilent Technologies, USA) equipped with an Agilent 7683B autosampler and DBEUPAH (20 m × 0.18 mm inner diameter, 0.14 μm film thickness) capillary column (Agilent J&W Scietific, USA). Pure helium (HiQ, 6.0, Linde, Czech Republic) was used as the carrier gas at a constant ramped flow rate of 1.0 mL/min. The samples were injected under the pulsed splitless condition mode (1 μL, purge flow 70 mL/min at 0.75 min). The mass spectrometer was operated using electron ionization (70 eV). The temperatures of inlet, transfer line, ion source, detector, and column have been described elsewhere in Košnář et al. (2016). The PAHs in soil extracts were identified based on the retention times of PAH standards and quantified using the response factors related to the respective internal standards based on an external five-point calibration curve (10–1000 μg/L) for each individual PAH compound. The calibration curves showed acceptable linearity ($R > 0.9985$) for each of the PAHs. The quantification limits were calculated in the range of 1.8 (ACE) and 5.6 (BghiP) μg/kg dw. The SS recoveries of 2-FBP and *p*-TER-d_{14} ranged from 89.7%–98.0% and 90.5%–117.8% in the analyzed samples.

1.6. Data processing and statistical analysis

In this study, the sum of 16 individual US EPA priority PAHs represented the content of total PAHs which were divided into three groups according to their molecular weight and number of rings as follows: (1) LMW PAHs — the sum of NAP, ACY, ACE, FLU, PHE, and ANT; (2) MMW PAHs — the sum of FLUO, PYR, BaA, and CHR; and (3) HMW PAHs — the sum of BbF, BkF, BaP, IPY, DBA, and BghiP. The removal of PAHs in soil (r_{PAH}, μg/kg dw) was calculated as follows:

$$r_{PAH} = C_{initial} - C_{residual}$$

where, $C_{initial}$ (μg/kg dw) refers to the content of PAHs at 0 days and $C_{residual}$ (μg/kg dw) refers to the content of PAHs at 120 days. The bioaccumulation factor (BF) of PAHs were calculated as a ratio of respective PAH content in a biomass to its PAH content in soil, whereas the translocation factor (TF) of PAHs were estimated as the ratio of respective PAH content in maize shoots and roots. A pair-wise comparison (one-way ANOVA at $p < 0.05$) followed by Tukey's *post-hoc* test ($\alpha = 0.05$) was performed to evaluate the statistical differences. The conditions of one-way ANOVA were confirmed using tests of normality and homogeneity of variance (Shapiro–Wilk and Levene tests). All statistical analyses and figures were conducted in Microsoft Excel 2010 (Microsoft Corporation, USA) and Statistica 12.0 CZ software (StatSoft, USA). A principal component analysis (PCA), in Canoco 4.5 software (Microcomputer Power, USA) was applied to make visible similarity of different treatments and correlations among the removal of total PAHs, microbial activity, fungal biomass, and extracellular enzyme activities. Results of PCA were visualized in the form of a bi-plot ordination diagram using CanoDraw software (Microcomputer Power, USA).

2. Results and discussion

2.1. Natural attenuation and phytoremediation of PAHs

In natural attenuated PAH-contaminated soil (NA) the changes of tested individual PAHs were negligible after the period of 120 days (Fig. 1a–c) which is contradictory to the results of Larsson et al. (2013) who reported, that most of the low and medium molecular weight PAHs are readily biodegradable by the autochthonous soil microbiota. Furthermore, using NA treatment, the lowest removal (significantly, $p < 0.05$) of total PAHs by 72.9 μg/kg dw (4.8%) of all investigated bioremediation strategies was observed. Simultaneously, the soil microbial activity (MA) in Table 2 decreased significantly at the end of NA treatment and soil fungal biomass (FB) was the lowest in comparison to other biological treatments. Moreover, investigated ligninolytic enzymes (LAC and MnP) and other extracellular enzymes activities in soil (Table 3) were not significantly different ($p < 0.05$) in comparison with their respective values at initial time. The results of the principal component analysis (PCA) in Fig. 2 showed that the data of PAH removal in NA treatment were clearly separated from other treatments within the opposite vectors of recorded data. It indicated that the bare soil did not provide sufficient conditions for the PAH biodegradation.

Contradictorily, the phytoremediation treatment (P) using maize plants (*Z. mays*) cultivated on the spiked soil increased the removal of NAP, ACY, ACE, and FLU in a range from 18.8 to 31.4 μg/kg dw significantly ($p < 0.05$) in comparison to NA treatment (Fig. 1a). The sum of low and medium molecular weight PAHs decreased almost four times more than in NA treatment and this resulted in the removal of total PAHs (Fig. 3) by 162.6 μg/kg dw (10.7%). The increased removal of total PAHs in P treatment could be related to the increased fungal biomass (FB) and lipase activity (LPS) in soil (Tables 2 and 3) because the PCA showed (Fig. 2) a positive correlation with the total PAH soil removal. It seems that soil LPS enzymes could also be involved in the PAH degradation pathway, as well the soil ligninolytic enzymes, which is in concordance with the study by Balajil et al. (2014). The higher fungal biomass than in NA treatment (Table 2) could be caused by the autochthonous fungi growth stimulated by plant exudates released in the maize rhizosphere which were not investigated in this study. As the results of PCA showed strong correlation among fungal biomass, ligninolytic enzyme activities (MnP and LAC), and the total PAH soil removal

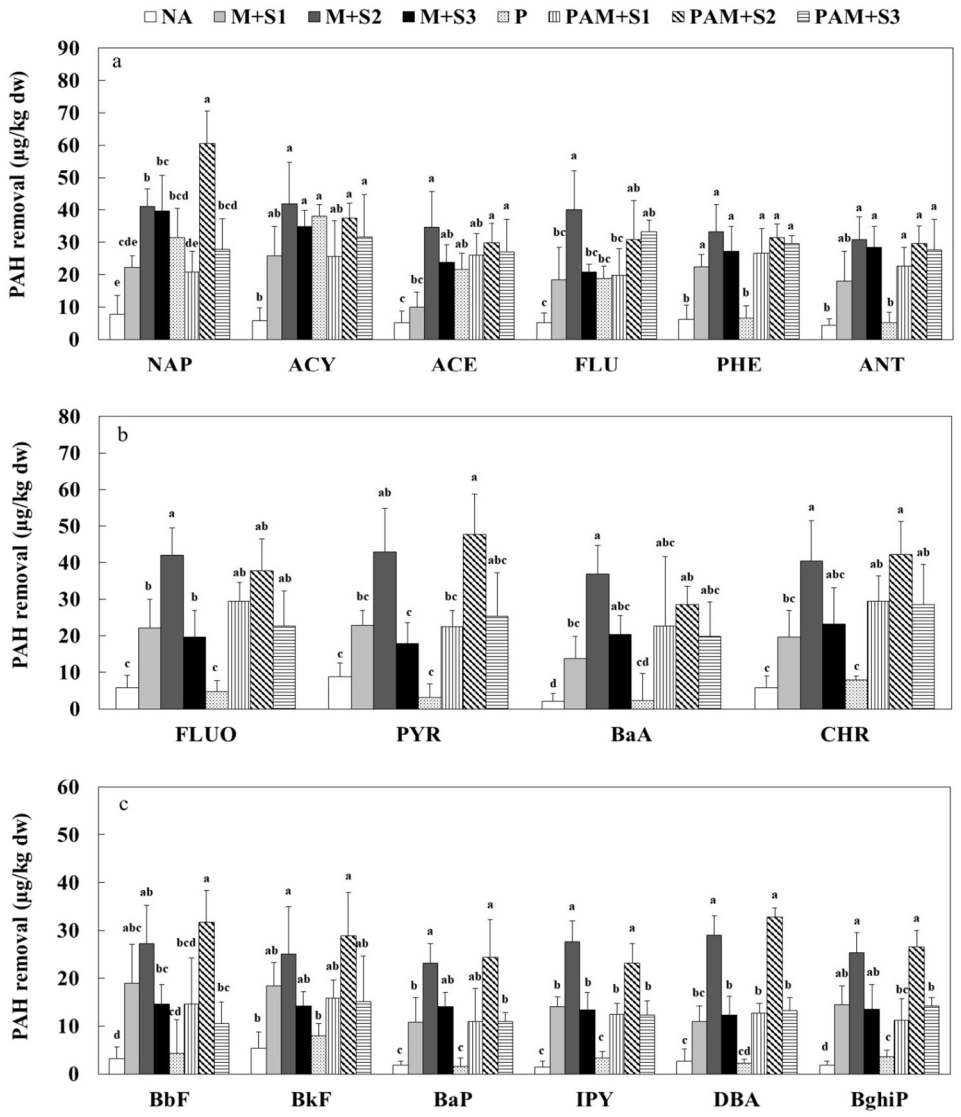

Fig. 1 – The PAH removal of (a) individual LMW PAHs, (b) individual MMW PAHs, and (c) individual HMW PAHs from soil, at the end of the 120-day experiment. The columns of each respective PAH removal (means; $n = 4$) sharing different lowercase letters indicate significant differences ($p < 0.05$) between the treatments as determined by Tukey's test. Error bars indicate SD values of $n = 4$. experiment.

it seems that boosted fungi biomass in our P treatment provoked the production of soil ligninolytic enzyme activities which are known to be involved in PAH biodegradation. Unfortunately, there was no statistical difference between the P and NA treatment in the production of MnP and LAC, as shown in Table 3. This lead to the suggestion that these ligninolytic

Table 2 – Microbial activity (MA) and fungal biomass (FB) in PAH-contaminated soil at 0 and 120 days of an experiment (means ± SD; $n = 4$).

	Days	NA	M+S1	M+S2	M+S3	P	PAM+S1	PAM+S2	PAM+S3
MA	0	40.7 ± 2.7bA	59.3 ± 1.0aA	59.6 ± 1.6aB	58.2 ± 1.7aA	40.1 ± 3.0bA	58.3 ± 2.1aA	60.1 ± 2.2aB	58.5 ± 1.5aA
(μg TPF/	120	24.9 ± 2.1 dB	61.2 ± 1.8bA	66.4 ± 1.8aA	59.1 ± 2.4bA	33.3 ± 1.9cB	60.7 ± 2.4bA	67.6 ± 2.4aA	59.6 ± 1.4bA
g soil dw·6 hr)									
FB	0	0.2 ± 0.1aA	0.1 ± 0.0aA	0.2 ± 0.1aA	0.2 ± 0.1aA	0.2 ± 0.1aA	0.1 ± 0.0aA	0.2 ± 0.0aA	0.1 ± 0.0aA
(μg ergosterol/	120	7.8 ± 3.3cB	48.0 ± 3.3abB	45.1 ± 8.2bB	41.9 ± 3.9bB	37.3 ± 5.1bB	66.5 ± 7.4aB	65.8 ± 15aB	54.4 ± 12abB
g soil dw)									

Means ± standard deviations estimated from four replications within the same row followed by different lowercase letters indicate significant differences ($p < 0.05$) among each treatment as determined by Tukey's test. The different uppercase letters indicate significant differences ($p < 0.05$) between 0 and 120th day of the experiment. Recorded data abbreviations: FB: fungal biomass; MA: microbial activity.

Table 3 – The activities of selected extracellular enzymes in PAH-contaminated soil at 0 and 120 days of an experiment (means ± SD; n = 4).

Enzyme (U/g soil dw)	Days	NA	M + S1	M + S2	M + S3	P	PAM+S1	PAM+S2	PAM+S3
LAC	0	0.3 ± 0.2aA	0.4 ± 0.4aA	0.2 ± 0.1aB	0.2 ± 0.1aA	0.1 ± 0.1aB	0.4 ± 0.3aA	0.4 ± 0.3aA	0.2 ± 0.1aA
	120	0.2 ± 0.1bA	0.2 ± 0.1bA	0.8 ± 0.3abA	0.2 ± 0.1bA	0.4 ± 0.1abA	0.2 ± 0.1bA	0.3 ± 0.0bA	0.2 ± 0.2bA
MnP	0	0.4 ± 0.2aA	0.3 ± 0.1aA	0.3 ± 0.1aA	0.4 ± 0.1aA	0.3 ± 0.1aA	0.4 ± 0.1aB	0.3 ± 0.1aB	0.4 ± 0.0aB
	120	0.4 ± 0.1cA	0.5 ± 0.1bcA	0.9 ± 0.4bA	0.5 ± 0.0bcA	0.6 ± 0.2bcA	0.5 ± 0.0bcA	1.5 ± 0.4aA	0.5 ± 0.0bcA
β-D-G	0	1.2 ± 0.5aA	1.2 ± 0.3aA	0.8 ± 0.6aB	1.0 ± 0.3aB	1.4 ± 0.5aA	1.1 ± 0.3aA	1.1 ± 0.1aA	1.0 ± 0.3aA
	120	1.1 ± 0.2aA	1.4 ± 0.9aA	1.9 ± 0.1aA	2.2 ± 0.7aA	2.1 ± 0.9aA	1.8 ± 0.7aA	2.2 ± 0.9aB	2.6 ± 1.1aB
AP	0	2.7 ± 0.4aA	2.6 ± 0.3aA	2.3 ± 0.3aB	2.4 ± 0.3aB	2.3 ± 0.3aB	2.7 ± 0.4aB	2.3 ± 0.4aB	2.7 ± 0.3aB
	120	2.5 ± 0.3cA	3.2 ± 0.9abcA	4.1 ± 0.4bcA	3.2 ± 0.4bcA	4.5 ± 0.1abcA	4.1 ± 0.1abcA	5.3 ± 1.0aA	4.8 ± 1.4abA
AS	0	0.1 ± 0.1aB	0.0 ± 0.0aB	0.1 ± 0.1aA	0.1 ± 0.1aB	0.1 ± 0.0aB	0.1 ± 0.1aB	0.1 ± 0.0aB	0.1 ± 0.1aB
	120	0.7 ± 0.1abA	0.2 ± 0.0cdA	0.2 ± 0.1dA	0.4 ± 0.1cdA	0.6 ± 0.1abA	0.7 ± 0.3abA	0.8 ± 0.1aA	0.9 ± 0.1aA
LPS	0	6.0 ± 1.2aA	6.1 ± 0.9aB	6.3 ± 0.3aB	5.7 ± 2.0aB	6.2 ± 1.0aB	6.5 ± 1.4aB	5.9 ± 1.3aB	6.6 ± 1.7aB
	120	8.7 ± 2.4dA	14.8 ± 4.4bcdA	10.8 ± 2.1dA	13.1 ± 2.5cdA	19.3 ± 4.2abcA	26.2 ± 11abcA	29.1 ± 8.0aA	26.3 ± 5.0abA

Means ± standard deviations estimated from four replications within the same row followed by different lowercase letters indicate significant differences ($p < 0.05$) among each treatment as determined by Tukey's test. The different uppercase letters indicate significant differences ($p < 0.05$) between 0 and 120th day of the experiment. Recorded data abbreviations: β-D-G: β-D-glukosidase; MnP: manganese peroxidase; LAC: Laccase; AP: acid phosphatase; AS: arylsulfatase; LPS: lipase.

enzymes were increased only in the initial stages of tested P treatment, which can be supported by Wang et al. (2009).

2.2. *Mycoremediation and plant-assisted mycoremediation of PAHs*

Using the fungal substrates in mycoremediation (M) and plant-assisted mycoremediation (PAM) treatments significantly ($p < 0.05$) removed PHE, ANT, and FLUO in a range from 18.0 to 42.0 μg/kg dw in comparison to the non-amended soils of NA and P treatment (Fig. 1a). The most appropriate fungal substrate for the removal of individual PAHs in M and PAM treatments was the S2 substrate (30–50 mm wood chips with *P. ostreatus*). When using this fungal substrate the CHR and BkF, BaP, and DBA from HMW PAHs group (Fig. 1c) were significantly ($p < 0.05$) decreased from 10.8 to 42.2 μg/kg dw in comparison to the respective treatments using S1 and S3 fungal substrates. Furthermore, using the S2 substrate also significantly decreased the content of BbF, IPY, and BghiP from HMW PAH group (Fig. 1c) in comparison to NA and P

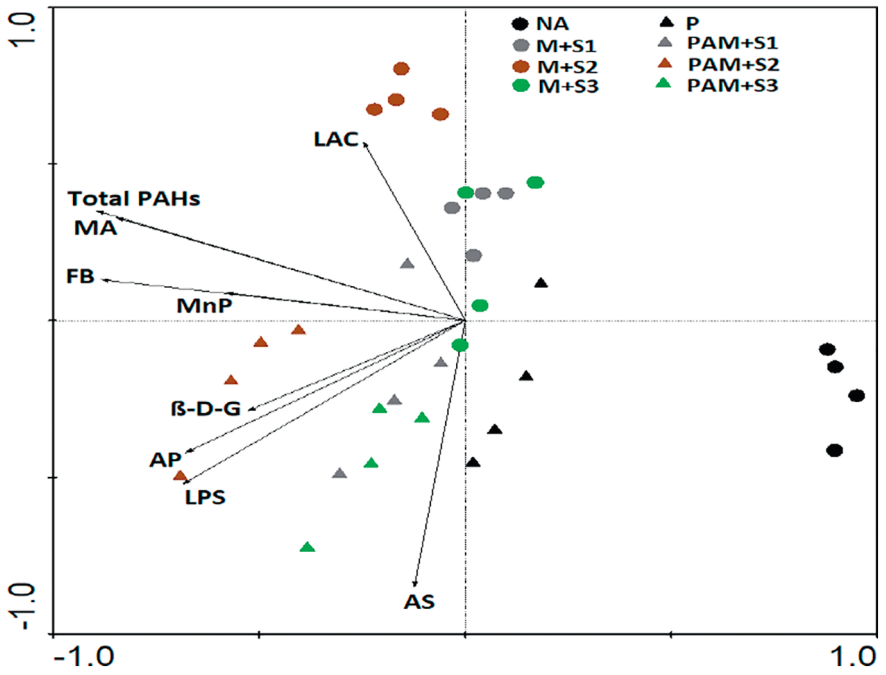

Fig. 2 – Ordination diagram showing the results of a principal component analysis (PCA) to evaluate the multivariate data of total PAH removal, microbial activity, fungal biomass, and extracellular enzyme activities in PAH–contaminated soil at the end of 120–days experiment. The first axis of the PCA of total PAHs removal and presence of individual enzymes explained 43.5% of the data variability, while the first two axes combined explained 64%. total PAHs: removal of total PAHs.

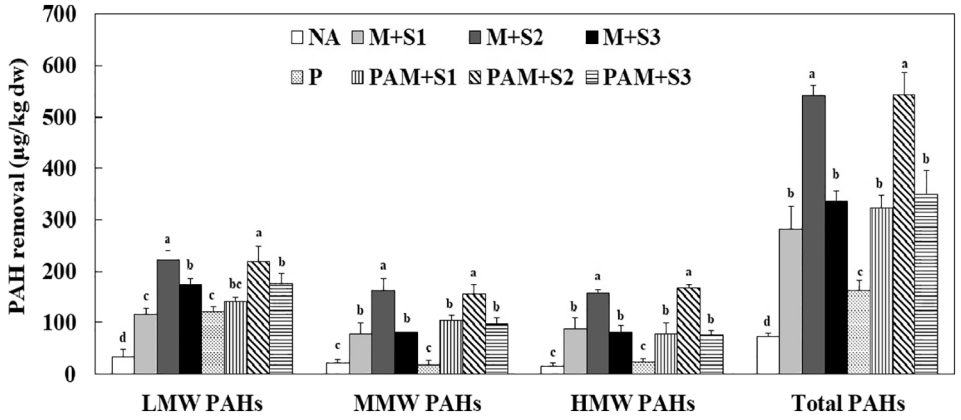

Fig. 3 – The PAH removal of the sum of LMW, MMW, and HMW PAHs and total PAHs from soil, at the end of the 120–day experiment. The columns of each respective PAH group removal (means; $n = 4$) sharing different lowercase letters indicate significant differences ($p < 0.05$) between the treatments as determined by Tukey's test. Error bars indicate SD values of $n = 4$.

treatments. The fungal substrates applied into the soil could stimulate the removal of PAHs due to the rich colonization of *P. ostreatus* in soil as was indicated by the highest growth of fungal biomass (FB) and microbial activities in soil (Tables 2 and 3) which correlated strongly with the removal of total PAHs according to the results of PCA (Fig. 2). Therefore in M and PAM treatments, the total PAH content dissipated in soil 4–7 times more than in NA treatment, and 2–3 times more than in P treatment (Fig. 3).

The significantly ($p < 0.05$) higher removal of LMW, MMW, HMW, and total PAH content from soil (Fig. 3) was observed after mycoremediation and plant-assisted mycoremediation using the S2 substrate containing the mycelium of *P. ostreatus* (M+S2 and PAM+S2) than in other investigated treatments. Therefore these suggested treatments could be the most efficient bioremediation strategies of agricultural soil contaminated by PAHs. Unfortunately, when using the same fungal substrate in M and PAM treatments there were no statistical differences ($p < 0.05$) in the removal of individual PAHs, except NAP which was removed by 60.6 μg/kg dw in PAM treatment using S2 substrate (Fig. 1a). Therefore, the removal of total PAHs in M + S2 (541.4 μg/kg dw; 36%) and PAM+S2 (543.4 μg/kg dw; 36.2%) in Fig. 3 was not statistically different ($p < 0.05$) after the period of 120 days. This was not expected because in PAM+S2 treatment the microbial activities, fungal biomass, and soil enzyme activities such as manganese peroxidase (MnP), arylsulfatase (AS), acid phosphatase (AP), and lipase (LPS) were significantly ($p < 0.05$) increased than in M+S2 treatment (Tables 2 and 3). Especially when the PCA in Fig. 2 revealed the association of AP and LPS with the boosted fungal biomass and MnP activity which correlated strongly with the removal of total PAHs. This lead to the suggestion, that the PAH removal in PAM treatment was enhanced only by the fungal substrate. It is also possible that the positive effect of maize to increase the removal of PAHs from soil of PAM+S2 treatment will be reflected in post-harvest time as was indicated in Table 3 by the significantly higher ($p < 0.05$) activities of soil MnP (1.5 U/g soil dw), AP (5.3 U/g soil dw), and LPS (29.1 U/g soil dw).

Considering our M and PAM treatments using S2 fungal substrate, the residual content of total PAHs at the end of the experiment in Table 1 was lower than the limit of total PAHs (1000 μg/kg dw) according to the Public Notice No. 153/2016 for agricultural soils in the Czech Republic. This could provide support for the development of fungal based bioremediation of PAH contaminated agricultural soils.

2.3. *Influence of soil PAHs on maize and* P. ostreatus *in the development of bioremediation approaches*

The PAH contaminated soil had no adverse effects on maize growth (139.8–155.5 cm) as well on the biomass of roots (14.6–17.4 g/(dw·pot)) and shoots (101.7–118.8 g/(dw·pot)) as there was no significant differences ($p < 0.05$) between each of the respective parameters investigated after the harvest at day 120 of the experiment. This is in concordance with Dupuy et al. (2015) who reported that the yields of maize biomass planted on PAH contaminated soil were reduced only when the PAH content in soil was higher than 250 mg/kg dw.

The fungal substrates in cooperation with maize exudates could increase the bioavailability of PAHs in soil because the total PAH content in the roots of PAM treatments from 51.4 to 56.2 μg/kg dw were significantly higher than the 15.3 μg/kg dw achieved in P treatment. The content of total PAHs was found only in maize roots. However, the calculated BF values of total PAHs in roots were very low (0.01–0.06) for each planted treatment. The PAHs in shoots were not detected therefore the ability of PAHs to transport from maize roots to shoots was not confirmed. Due to this the TF values were not defined in our study. This could indicate that the PAHs during the phytoremediation, as well during the plant-assisted mycoremediation using fungal substrates containing the mycelium of *P. ostreatus*, tended to be biodegraded in the maize rhizosphere and blocked by root surfaces, which was also indicated by Binet et al. (2000).

It was expected that the fungal amendments in PAH-spiked soil would only increase the overall fungal biomass in soil, but surprisingly *P. ostreatus* was able to create the oyster-shaped cap biomass on the soil surface of M and P+M treatments. The biomass of *P. ostreatus* of M and PAM treatments ranged between 2.1 and 2.3 g/(dw·pot) without any significant

differences ($p < 0.05$) between them. The individual PAHs in *P. ostreatus* biomass were not detected, and the BFs of total PAHs were not defined, therefore the uptake of PAHs from soil by *P. ostreatus* biomass was not confirmed. Concerning the efficiency of PAH extraction the ultrasonic extraction with three continuous re-extraction cycles is mostly recommended for low PAH contaminated solid samples according to US EPA (2007). The ultrasonic bath system is commonly used for the PAH extraction from the environmental samples such as sewage sludge (Oleszczuk and Baran, 2003), sediments (Dvořák et al., 2017), soils (García-Sánchez et al., 2018), and organic soil amendments (Košnář and Tlustoš, 2018). Moreover the ultrasonic extraction method was optimized by Song et al. (2002) with no statistical differences in PAH extraction efficiency between the ultrasonic, shaking and Soxhlet extraction for less polluted solid samples. Therefore, the ultrasonic extraction is also frequently used for the PAH extraction from plant and animal samples with acceptable PAH recovery (Li et al., 2017; Wang et al., 2018).

In our experiment PAH content in maize aboveground biomass extracted using the ultrasonication was below the limit of detection and PAH content in maize roots was lower than 56.2 μg/kg dw. The low PAH content in maize biomass is comparable with PAH content extracted from maize biomass using a Soxhlet extraction presented by Feng et al. (2014). Košnář et al. (2018) observed that the ability of maize roots to take up individual PAHs from soil is significantly lower than 0.1%. The not detected PAH content in biomass of *P. ostreatus* in our study is in concordance with the suggestion of Andersson and Henrysson (1996) that *P. ostreatus* can stimulate the removal of PAHs from soil only due to the production of ligninolytic enzymes by the mycelium of *P. ostreatus* in soil. Therefore, the harvested aboveground plant biomass can be used as fodder and *P. ostreatus* could safely be edible, because the total PAHs content was lower than the limit of PAHs (10 μg/kg dw) according to the Public notice No. 53/2012 in the Czech Republic.

3. Conclusions

This study compared different approaches for the bioremediation of a soil contaminated by PAHs. The natural attenuation decreased the content of total soil PAHs by 4.8% while the phytoremediation using maize (*Zea mays* L., var. Colisee) by 10.7%. The tested *Pleurotus ostreatus* (Jacq.) P. Kumm., strain HK35 (*P. ostreatus*) cultivated on the apple wood chips substrate (30–50 mm) significantly ($p < 0.05$) removed the total PAH content by 36% in mycoremediation treatment. This treated soil reached the lower total PAH content than the limit required for agricultural soils in the Czech Republic. When using the same fungal substrate together with maize in plant-assisted mycoremediation treatment, the removal of total PAHs was not different from the previous treatment. Nevertheless, this plant-assisted mycoremediation provided the highest soil microbial activity, fungal biomass, and manganese peroxidase activity in the end of the 120-experiment, which correlated strongly with the removal of total PAHs in soil. This could be promising in further improving development, directions, and management of bioremediation methods based on plant–fungal soil systems because the higher PAH removal could be reflected in the post-harvest time, but this needs further research. Moreover, there was no adverse effect of PAHs in soil on maize cultivation and quality of harvested aboveground biomass and *P. ostreatus* biomass does not present any environmental risk.

Acknowledgments

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