ČESKÁ ZEMĚDĚLSKÁ UNIVERZITA V PRAZE
Fakulta agrobiologie, potravinových a přírodních zdrojů
Katedra agroenvironmentální chemie a výživy rostlin

Bioremediace polycyklických aromatických uhlovodíků přítomných v popelu po spalování biomasy pomocí rostlin a kompostováním

doktorská disertační práce

Autor: Ing. Zdeněk Košnář

Školitel: prof. Ing. Pavel Tlustoš, CSc.

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Prohlášení

Prohlašuji, že jsem disertační práci na téma: **„Bioremediace polycyklických aromatických uhlovodíků přítomných v popelu po spalování biomasy pomocí rostlin a kompostováním“** vypracoval samostatně a použil jen pramenů, které cituji a uvádím v přiloženém seznamu literatury.

V Praze dne ……..

Podpis

Poděkování

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Obsah

1 Úvod

Životní prostředí představuje podle Listiny základních práv a svobod České republiky jednu z nejvyšších hodnot lidské společnosti, proto je nezbytné dbát na jeho ochranu a udržitelnost, jelikož každý má právo na příznivé životní prostředí (Česko, 1993). Z dlouhodobého hlediska je zřejmé, že využívání obnovitelných zdrojů energie je nezbytnou podmínkou trvale udržitelného rozvoje. Zvyšování podílu obnovitelných zdrojů energie na výrobě tepla a elektrické energie spalováním biomasy je výhodné zejména z hlediska snižování podílu celkových emisí skleníkových plynů (Hustad et al., 1995; McKendry, 2002).

Avšak Morf et al. (2002) uvedli, že nedokonalé spalování biomasy představuje riziko tvorby a emisí perzistentních organických polutantů, mezi které řadíme i polycyklické aromatické uhlovodíky (dále jen PAU). Tyto organické sloučeniny se mohou významně akumulovat i ve vznikajícím popelu po spalování biomasy (Janvijitsakul et Kuprianov, 2007; Enell et al., 2008; Straka et Havelcová, 2012; Freire et al., 2015; Masto et al., 2015; Orecchio et al., 2016; Rey-Salgueiro et al., 2016; García-Alonso et al., 2017; Li et al., 2018).

Jednotlivé PAU mohou být karcinogenní, mutagenní nebo teratogenní (Lewtas, 1993). Naftalen jako zástupce skupiny PAU s nejnižší molekulovou hmotností obsahující dvě benzenová jádra (cykly) v molekule je nejvíce akutně toxický. Dále například vysokomolekulární benzo[a]pyren s pěti cykly ve sloučenině je prokázaný karcinogen člověka. Dalších sedm sloučenin z 16 prioritních PAU je považováno za možné karcinogeny člověka (King et al., 2004; Bojes et Pope, 2007). Avšak, kromě organických polutantů se v popelu mohou akumulovat i rizikové prvky (As, Cd, Cr, Cu, Ni, Pb), které také mohou představovat vážné riziko pro životní prostředí (Száková et al., 2013).

Vzniklý popel po spalování biomasy se využívá v mnoha odvětvích průmyslu (Ahmaruzzaman, 2010). Z důvodu poměrně vysokého obsahu minerálních živin, především Ca, K, Mg a P, lze popel z biomasy za určitých podmínek využívat i v zemědělství jako půdní aditivum za účelem navrácení živin z popelu zpět do půdy (Park et al., 2012; Ochecová et al., 2014; Ochecová et al., 2016; Mercl et al., 2016; Perná et al., 2016; Ochecová et al., 2017; Mercl et al., 2018). V současné době je aplikace popelu na zemědělskou půdu legislativně upravována (Johansson et van Bavel, 2003a). V České republice (dále jen ČR) je, mimo jiné, limitní obsah PAU v popelu z biomasy stanoven ve výši 20 mg/kg suché hmotnosti (dále jen jednotky) popelu (MZe ČR, 2014).

Popel s vysokým obsahem PAU je možné ukládat na skládky nebezpečného odpadu, vitrifikovat nebo využít jako přísadu při výrobě cementu (Demirbas et al., 2009; Sarenbo, 2009). Vysoký obsah PAU v popelu brání jeho využití v zemědělství (Reijnders, 2005). S tím souvisí i potřeba hledat možnosti, jak tyto nebezpečné látky z uvedené matrice odstranit nebo snížit a přitom využít živiny obsažené v popelu (Pitman, 2006; James et al., 2012). V současné době popel s vysokým obsahem PAU je remediován pouze fyzikálně – chemickými metodami, které jsou obecně považovány za nákladné a nešetrné k životnímu prostředí (Hustad, 1995; Sarenbo, 2009).

Na remediaci PAU v popelu by mohly být aplikovatelné některé bioremediační techniky, které jsou běžně využívané pro šetrné odstraňování PAU z životního prostředí nebo které vedou ke zmírnění jejich vlivu na jednotlivé složky životního prostředí (Gan et al., 2009; Ram et Masto, 2014). Během bioremediace se využívá působení biologických procesů, které jsou schopny degradovat PAU až na konečné produkty oxid uhličitý a vodu (mineralizace PAU). Nicméně, účinnost biodegradace se odvíjí od vlastností kontaminované matrice, faktorů prostředí a dané struktury PAU (Gerhardt et al., 2009). Mezi konkrétní bioremediační opatření, které by mohly být použitelné v případě popelu kontaminovaného PAU, můžeme zařadit zejména: 1) fytoremediace PAU po aplikaci popelu do půdy, 2) kombinované využití fytoremedace půdy kontaminované PAU s přídavky kompostu, vermikompostu nebo substrátů obsahujících ligninolytické houby, 3) bioremediace PAU v popelu během kompostování a vermikompostování (Newman et Reynolds, 2004; Antizar-Ladislao et al., 2007; Di Gennaro, 2009; Winquist et al., 2014).

Pro úspěšné zvládnutí řešené problematiky bylo potřebné získat poznatky o možném obsahu PAU v popelu z různých druhů biomasy vzniklých za reálných podmínek spalování a následně zjistit vliv vybraných bioremediačních metod na změnu obsahu PAU přítomných v popelu po spalování biomasy. Předkládaná disertační práce je souborem komentovaných publikovaných článků, které vznikly na základě řešení dané problematiky.

2 Literární přehled

2.1 Spalování biomasy

Nároky společnosti na životní úroveň se neustále zvyšují. S tím souvisí i rostoucí produkce a spotřeba energie. Mezi hlavní zdroje energie stále patří spalování fosilních paliv. Výroba energie spalováním fosilních paliv je spojena s významným negativním vlivem na životní prostředí včetně bioty. Spotřebu fosilních paliv lze snížit využíváním obnovitelných zdrojů energie (OZE), mezi které řadíme i biomasu. Využívání OZE je považováno za jeden ze základních předpokladů pro zachování trvale udržitelného hospodářství (Jenkins et al., 1998; Forbes et al., 2016). K hlavním výhodám využívání biomasy k energetickým účelům patří zmírnění negativních dopadů na životní prostředí z hlediska neutrální bilance tvorby skleníkových plynů a šetrného hospodaření v krajině (Gil et al., 2010).

Kvantifikace současného celosvětového využití OZE je značně odlišná (van den Broek et al., 1996; Dare et al., 2001; Michel et al., 2017). V ČR je dle Národního akčního plánu z roku 2012 cílová hodnota podílu energie z OZE na celkové konečné spotřebě energie v roce 2020 stanovena na 15,3 % (MPO ČR, 2016). Podíl obnovitelných zdrojů energie na konečné spotřebě činil v roce 2016 podle mezinárodní metodiky výpočtu 15,1 %. Na spotřebě elektřiny se OZE podílely 14 %, na spotřebě v dopravě 6 % a na konečné spotřebě při vytápění 20 % (MPO ČR, 2017b). Spalováním biomasy pro energetické účely se dle Saidur et al. (2015) rozumí zejména přímé spalování biomasy a spoluspalování biomasy s fosilními palivy za účelem výroby elektrické energie nebo užitkového tepla, ale i za účelem kombinované výroby elektřiny a tepla. V roce 2016 podíl přímého včetně kombinovaného využití biomasy na výrobě elektřiny z OZE tvořil 22,0 % a na výrobě tepla 86,4 % (MPO ČR, 2017a).

Mezi paliva z biomasy využívaná v elektrárnách a teplárnách pro energetické účely se řadí zejména: palivové dříví, dřevní štěpka, kůra, piliny, dřevní odpad a posklizňové zbytky, brikety a pelety z rychle rostoucích dřevin nebo bylin, ostatní biomasa a jiné biologické materiály (Skoblia et al., 2006). Nevýhodou spalování biomasy mohou být procesy spékání, koroze a aglomerace popelovin tvořící nespalitelnou anorganickou část paliva ve spalovacím zařízení. Dále emise nespálených látek uvolňované při nedokonalém spalování biomasy, které mohou vést k negativnímu vlivu na životní prostředí a ke snížení efektivity výroby energie z OZE (Di Blasi, 2008).

Účinnost spalovací technologie dosahuje přibližně 90 % z celkového možného zisku energie ze spalování biomasy a je významně ovlivňována typem používaného spalovacího

zařízení, vlhkostí biomasy, výhřevností paliva, podílem těkavých látek a prchavé hořlaviny vznikajících při spalování, obsahem alkalických kovů a popelovin v biomase nebo jejich reziduí ve spalovacím zařízení (Demirbas, 2005). Nehořlavá část paliva je tvořena zejména vodou a popelovinami, které obecně snižují výhřevnost paliva. Během spalování může docházet k aglomeraci popelovin ve spalovací komoře. Spékání popelovin během spalování způsobuje nerovnoměrný přístup vzduchu k palivu s následkem nedostatečného spálení organické hmoty (Werther et al., 2000; García et al., 2015).

Existují dva základní druhy průmyslových kotlů na spalování biomasy – zařízení pro spalování biomasy na pevném loži (roštové spalování) a zařízení pro spalování biomasy s fluidním ložem (fluidní spalování). Roštové spalování v rozmezí 500 – 900 °C je vhodné pro spalování biomasy s vyšší vlhkostí, různou velikostí a s vysokým obsahem popelovin (Koornneef et al., 2007; Yin et al., 2008). Během fluidního spalování při teplotách vyšších než 650 °C se biomasa spaluje společně s inertním materiálem tvořícím fluidní lože, které je často složeno z křemičitého písku nebo dolomitu. Spalovací vzduch je do fluidního spalovacího zařízení vháněn zespod a uvádí tak do pohybu směs biomasy s fluidním ložem (Latva-Somppi et al., 1998; Steenari et al., 1999; Chirone et al., 2008).

2.1.1 Popel ze spalování biomasy

V elektrárnách a teplárnách, vybavenými roštovými nebo fluidními kotly, které spalují biomasu, se jako odpadní produkt spalovacího procesu tvoří popel. V zařízeních na spalování biomasy vybavenými jak roštovými, tak i fluidními kotly, můžeme obvykle rozlišit dva základní druhy popelu: roštový a úletový. Roštový popel je často tvořen spečenou směsí anorganických látek a hrubou frakcí minerálních nečistot spalovaných společně s biomasou, jako je například písek nebo zbytky půdy. Úletový popel je tvořen jemnou frakcí anorganických látek smíchanou s nespálenými organickými částmi paliva. Nejvyšší produkce roštového popela je v zařízeních s roštovými kotly, kde se popel hromadí ve spodní části kotle pod pevnými rošty (Vassilev et al., 2010). V případě roštových kotlů s občasným nebo trvalým pohybem spalované biomasy je popel odváděn do strusky. Úletové popele v roštových kotlích se zachytávají zejména na multicyklonu (filtru spalin) a následně jsou odváděny do kontejneru. V zařízeních s fluidními kotly je produkce roštových popelů minimální, avšak možná. Tyto popele se ukládají jako spečené ve spodní pórovité části fluidního kotle na tzv. fluidním loži (Vassilev et al., 2014). Z více než 90 % ve fluidním kotli vzniká úletový popel, který může být deponován uvnitř spalovacího zařízení na vláknitém, eletrostatickém nebo

cyklónovém precipitátoru (filtru spalin). Úletové popele se také mohou zachytávat na ekonomizéru a chladiči spalin (Lanzerstorfer, 2015).

Vznikající popel v závislosti na parametrech spalování, efektivitě spalování, typu kotle, druhu biomasy, může tvořit 1 – 10 % původní hmotnosti biomasy. Roční celosvětová produkce popela z biomasy byla v roce 2010 odhadnuta na 476 milion tun a v Evropské unii (dále jen EU) byla odhadnuta v roce 2009 na 5,6 milionu tun s možným nárůstem produkce až na 15,5 miliónů tun v roce 2020 (Obernberger et Supanic 2009; Vassilev et al., 2010). Odhad roční produkce popela z biomasy se v ČR v roce 2012 pohyboval kolem 70 tisíc tun a v roce 2016 přibližně 112 tisíc tun (Tlustoš et al., 2012; MPO ČR, 2017a).

Se zvyšující se spotřebou biomasy pro energetické účely se zvyšuje i produkce popela. S tím souvisí také otázka nakládání se vzniklým popelem a jeho možného dalšího využití. Popel obsahuje poměrně vysoké množství Ca, Si, ale také může obsahovat P, K, Mg a jiné prvky, proto mnozí autoři popisují popel z biomasy jako cenný zdroj minerálních živin. Mnohé studie se zabývaly možnou aplikací popela na lesní a zemědělské půdy. Popel se může používat i jako příměs rozložitelných organických materiálů určených ke kompostování (Basu et al., 2009; Vamvuka et Kakaras, 2011).

Hlavním důvodem recyklace popela v zemědělství je kompenzace ztráty živin, úspora využívání minerálních hnojiv, úprava hodnot pH půdy, meliorační schopnosti a zajištění dlouhodobě udržitelného hospodářství (Ram et Masto, 2014). Rozdíly v kvalitě popela se hodnotí dle fyzikálních a chemických vlastností – zejména hodnoty pH a vyluhovatelnosti makro a mikro živin (Demeyer et al. 2001). Byla popsána řada alternativních možností využití popela v průmyslu, ve stavebnictví nebo při stavbě silnic a dálnic (Rajamma et al., 2009). Využití popelů je výhodné také z ekonomického hlediska, protože v případě jejich využití v zemědělství by provozovatelé zařízeních na spalování biomasy k energetickým účelům nemuseli vynakládat vysoké finanční prostředky spojené s jejich ukládáním na skládky odpadů (Jala et Goyal, 2006; Tlustoš et al., 2012).

Mezi limitující faktory ovlivňující kvalitu popela a jeho využití v zemědělství patří vysoké pH, možný výskyt vysokého obsahu rizikových prvků (zejména As, Cd, Cr, Ni, Pb) a možný výskyt vysokého obsahu organických polutantů – především polycyklických aromatických uhlovodíků (Ribbing, 2007; Száková et al., 2013; Nabeela et al., 2015; Masto et al., 2016; Magdziarz et al., 2016; Li et al., 2018).

2.3 Polycyklické aromatické uhlovodíky (PAU)

2.3.1 Základní charakteristika a vlastnosti PAU

Polycyklické aromatické uhlovodíky jsou organické polutanty, které vznikají a jsou následně emitovány do prostředí především při nedokonalém spalování organických materiálů. Do prostředí se PAU mohou dostávat jak z přírodních, tak i antropogenních zdrojů (Abdel-Shafy et Mansour, 2016).

Mezi významné přírodní zdroje emisí PAU se dle Ravindra et al. (2008) řadí:

1) vulkanická činnost, lesní a prérijní požáry
2) sedimentované horniny v místech těžby fosilních paliv
3) chemická syntéza PAU mikroorganismy
4) působení kosmického záření v atmosféře a jiné

Mezi výzmamné antropogenní zdroje emisí PAU se dle Ravindra et al. (2008) řadí:

1) zdroje nedokonalého spalování nebo pyrolýza organických materiálů:
   (a) stacionární zdroje: elektrárny, teplárny, spalovny odpadů, domácí topeniště a jiné
   (b) mobilní zdroje: automobilová, letecká doprava a jiné
2) průmyslové zpracovávání fosilních paliv, metalurgický a dřevozpracovatelský průmysl:
   (a) zpracování ropy, zkapalňování a zplyňování uhlí, koksárenství
   (b) výroba hliníku, barviv, plastů a jiné
3) využívání materiálů obsahujících PAU:
   (a) ropa, zemní plyn, dehty, asfalty a jiné ropné deriváty
   (b) čistírenské kaly, rybniční a říční sedimenty, půdy a jiné

Sloučeniny typu PAU se v životním prostředí nejčastěji vyskytují jako směsi jednotlivých skupin PAU rozlišitelných dle počtu cyklických struktur, které tvoří danou sloučeninu. PAU se mohou skládat ze dvou až sedmi kondenzovaných sloučenin (cyklů) obsahující benzenová jádra v lineárním, klastrovém nebo angulárním uspořádání. Benzenová jádra jsou spojena do konjugovaných systémů isomerního charakteru s možností substituce různými funkčními skupinami. Aromatické označení se vztahuje pouze ke struktuře a chemické stabilitě molekuly benzenu (Gan et al., 2009).

Struktura PAU je nejčastěji tvořena pouze uhlíkem a vodíkem, ale benzenová jádra mohou být také substituována dusíkem, sírou nebo kyslíkem. Existují stovky sloučenin typu

PAU a jejich derivátů, proto agentura United States Environmental Protection Agency – Agentura ochrany životního prostředí spadající pod federální vládu Spojených států amerických (US EPA) stanovila seznam 16 prioritních polutantů PAU (16 US EPA PAU) životního prostředí (Liu et al., 2001).

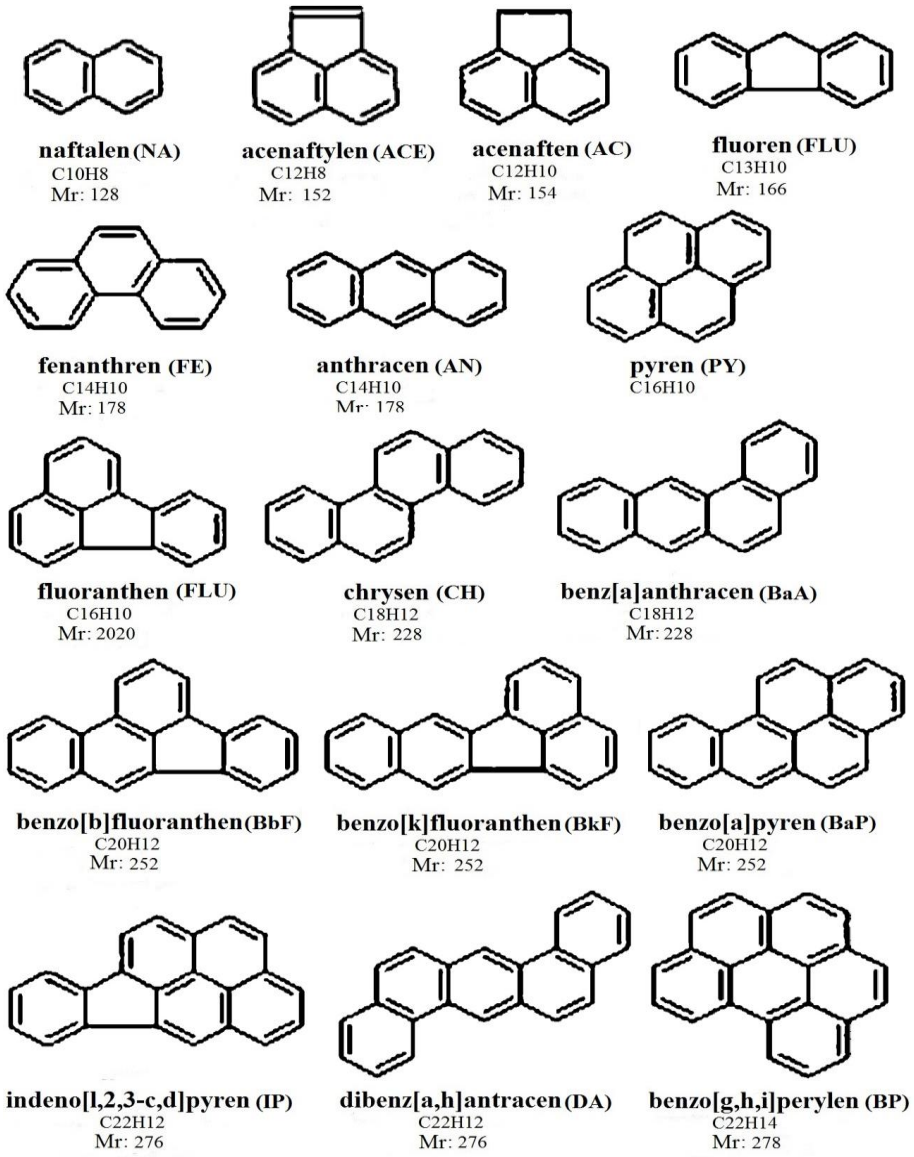
Názvy jednotlivých sloučenin typu PAU byly dle US EPA (1999) vytvořeny organizací Mezinárodní unií pro čistou a užitou chemii (International Union for Pure and Aplied Chemistry). Přehled 16 prioritních PAU, jejich názvy a zkratky, strukturní vzorce, číslování řetězců a relativní hmotnosti jsou uvedeny na Obrázku 1 (strana 8).

Na základě molekulové hmotnosti lze jednotlivé PAU dle Eisler (2000) rozdělit do skupin:

1) Nízkomolekulární PAU (NM PAU) – naftalen, acenaftylen, acenaften, fluoren, fenantren a antracen s 2 až 3 cyklickými řetězci ve sloučenině.
2) Středněmolekulární PAU (SM PAU) – fluoranten, pyren, benz[a]antracen a chrysen se 4 cyklickými řetězci ve sloučenině.
3) Vysokomolekulární PAU (VM PAU) – benzo[b]fluoranten, benzo[k]fluoranten, benz[a]pyren, indeno[1,2,3-c,d]pyren, dibenz[a,h]antracen a benzo[g,h,i]perylen s 5 až 6 cyklickými řetězci ve sloučenině.

Fyzikálně – chemické vlastnosti PAU se odvíjí od jejich molekulové hmotnosti a chemické struktury dané látky. Za standardních podmínek se jedná o bezbarvé, bílé až jemně žluté pevné krystalické látky (Liu et al., 2001). Rozpustnost PAU ve vodě je velmi nízká, jejich rozpustnost se zvyšuje v organických rozpouštědlech, kdy nejvyšší rozpustnosti dosahují v nepolárních rozpouštědlech – hexanu, toulenu, dichlormethanu nebo jiných (Wilson et Jones, 1992). V polárních rozpouštědlech je nejvíce rozpustný naftalen, zatímco se zvyšujícím se počtem cyklů ve sloučenině a rostoucí molekulovou hmotností PAU se jejich rozpustnost ve vodě snižuje. Díky omezené rozpustnosti ve vodě jsou PAU považovány za lipofilní (hydrofobní) látky, které mají vysokou afinitu k organické hmotě. S tím souvisí i významná schopnost sorpce PAU na půdní a prachové částice (Bakker et al., 2000).

Obrázek 1. Přehled 16 prioritních US EPA PAU, jejich názvy, strukturní vzorce, číslování řetězců a relativní hmotnosti (Anyakora a kol., 2005; Atkins et al., 2010).



V životním prostředí jsou PAU vysoce perzistentní a neustále podléhají redistribuci mezi jednotlivými složkami životního prostředí (Wei et al., 2015). Ze spalovacích procesů jsou PAU nejprve emitovány do atmosféry, primárně jako sorbované na úletové nespálené částice, a následně se mohou dostávat i do všech složek životního prostředí (Salam et al., 2011). V atmosféře PAU podléhají mnoha atmosférickým procesům a mohou se dostávat i na velmi vzdálená místa od zdroje znečištění (Williams et al., 2001; Bignal et al., 2008; Jumpponen et al., 2013). Vlivem suché a mokré atmosférické depozice se PAU dostávají z atmosféry do půdy, vody a bioty. Do vodního prostředí s následnou akumulací v sedimentech,

se PAU dostávají přímo z atmosféry nebo zpětnou resorpcí PAU ze sedimentů a půd (Manoli et Samara, 1999; Dong et al., 2012).

Ke vstupu PAU do půdy dochází zejména suchou a mokrou depozicí z atmosféry (Srogi, 2007; Cachada et al., 2016). Dále ke kontaminaci zemědělské půdy PAU může docházet při záplavách a vyplavováním říčních sedimentů v průmyslových oblastech nebo aplikacemi materiálů obsahující PAU, především čistírenských kalů a rybničních sedimentů (Podlešáková et al., 1998; Vácha et al., 2003; Vácha et al., 2005; Vácha et al., 2011). Na základě monitorování obsahu PAU zemědělských půd zatížených významnou antropogenní činností bylo zjištěno, že průměrný obsah PAU je v Ústeckém kraji 0,9 mg/kg a v Severomoravském kraji 8,0 mg/kg (Vácha et al, 2014; Vácha et al., 2015). Preventivní obsah PAU pro zemědělské půdy ČR je 1 mg/kg půdy (MŽp ČR, 2016).

Schopnost vazby PAU na organickou hmotu v půdě a možnost následné akumulace PAU v živých organismech představuje velmi významnou vlastnost související s dostupností kontaminantu pro organismy, která je důležitým předpokladem pro možné bioremediace PAU v životním prostředí (Jiao et al., 2007). Sorpce PAU na půdní organickou hmotu vyjadřuje rozdělovací koeficient organický uhlík/voda (K_{OC}), který popisuje rozdělení látky mezi organickou hmotu a vodu. PAU s vysokou schopností se sorbovat na půdní organickou hmotu dosahují hodnot log K_{OC} vyšších než 3 (Lamichhane et al., 2016).

Významnou možností kontaminace bioty PAU, zejména rostlin, se uvádí atmosférická depozice (Meharg et al., 1998). Lin et al. (2007) zmínili možný způsob vstupu PAU do rostlin prostřednictvím epidermálního povrchu nadzemních částí rostlin. Příjem PAU kořenovým systémem rostlin z půdy je možný, avšak velmi omezený (Vácha et al., 2008; Vácha et al., 2010). Stomatální příjem je méně obvyklou možností vstupu PAU do rostlin ve formě plynného skupenství (Liste et Alexander, 2000; Dias et al., 2016). PAU se mohou dostávat i do potravního řetězce živočichů včetně člověka (Khillare et al., 2012). Rozdělovací koeficient mezi *n*-oktanolem a vodou (K_{OW}) vyjadřuje míru tendence látky se akumulovat v živých organismech. Hodnota log K_{OW} prioritních 16 PAU se pohybuje od 3,37 do 7,66. PAU s vysokou tendencí se akumulovat dosahují hodnoty log K_{OW} vyšší než 4, zatímco PAU s hodnotou log K_{OW} nižší než 4 jsou více hydrofilní, a tedy více biodostupné (Tao et al., 2006).

Mnohé sloučeniny patřící mezi PAU jsou toxické pro organismy. Při posuzování toxicity PAU pro organismy jsou důležité jejich vlastnosti a vzájemné interakce (Halek et al., 2008). Velké množství živočichů včetně člověka je schopno v živočišných tkáních akumulovat PAU, které do organismů mohou vstupovat z atmosféry inhalačně, dermálně,

sliznicemi nebo zažívacím traktem společně s potravou obsahující PAU (Samantha et al., 2002). Pro mnohé organismy mohou PAU být například karcinogenní, mutagenní, teratogenní nebo embryotoxické (Chen et Liao, 2006). Benzo[a]pyren je prokázaný karcinogen člověka a dalších šest PAU (benz[a]antracen, chrysen, benzo[b]fluoranten, benzo[k]fluoranten, indeno[1,2,3-c,d]pyren a dibenz[a,h]antracen) je dle US EPA řazeno mezi potenciální karcinogeny člověka (Johansson et van Bavel, 2003b). Z tohoto důvodu je nutné sledovat PAU v popelu. V případě akumulace PAU v popelu je potřeba hledat možnosti jejich šetrného odstranění nebo zmírnění jejich vlivu na biotu včetně člověka (Haritash et Kaushik, 2009).

2.3.2 Vznik PAU během spalování biomasy a jejich akumulace v popelu

Vznik PAU začíná pyrolýzou organického materiálu a pokračuje následnou pyrosyntézou uhlíkatých fragmentů vzniklých během nedokonalého spalování biomasy. Při pyrolýze probíhající za vysokých teplot nad 700 °C se organické molekuly (celuóza, hemiceluóza a lignin) tvořící spalovanou biomasu štěpí na jednotlivé fragmenty – nestabilní produkty spalovacího procesu. Jedná se zejména o volatilní acetylenové a propylové radikály, které spolu následně při vysokých teplotách od 500 do 800 °C reagují za vzniku stabilních látek benzenu. Tato fáze se označuje jako pyrosyntéza (Chagger et al., 1998; McGrath et al., 2001; Ross et al., 2002). Jeden z možných způsobů syntézy naftalenu je postupná adice dvou uhlíkatých reaktantů acetylenu za vzniku molekuly benzenu. Syntéza více cyklických PAU probíhá jak postupnou syntézou dvou uhlíkatých fragmentů, tak i polykondenzací dvou nebo více již vzniklých sloučenin například benzenu (Richter et Howard, 2000; Williams et al., 2012). Vznikající PAU během nedokonalého spalování organického materiálu se mohou akumulovat i ve vznikajících popelech. Množství vznikajících PAU se odvíjí od fyzikálně – chemických vlastností paliva, podmínek vlastního spalovacího procesu a druhu spalovacího zařízení (Khan et al., 2009).

Mezi významné faktory ovlivňující vznik PAU patří teplota spalování a obsah kyslíku ve spalovacím zařízení (Chagger et al., 2000; McGrath et al., 2007). V mnoha studiích byly publikovány rozdílné obsahy PAU v popelu vzniklé během různých teplot spalování. Jelikož se teplota během reálných podmínek spalování neustále mění, uvádí se pro danou sumu vzniklých PAU často pouze teplotní interval (Enell et al., 2008; Sarenbo, 2009). Masto et al. (2015) stanovili nejvyšší obsah PAU v popelech při teplotě kolem 850 °C. Rey-Salgueiro et al. (2016) uvedli, že během teplot spalování nižších než 400 °C byl obsah PAU v popelu na hranici detekce (nižší než 0,003 mg/kg). Levendis et al. (1998) zjistili, že během spalování v

rozmezí 1000 až 1300 °C nebyly v popelu z biomasy stanoveny žádné PAU nebo byly pod mezí detekce. Vlhkost biomasy vyšší než 60 % je také považována za významný faktor ovlivňující vznik a akumulaci PAU v popelu. Vysoká vlhkost paliva způsobuje vyšší spotřebu tepla na odpaření vody. V důsledku toho dochází ke snížení teplot spalování až do možného intervalu 500 – 800 °C vedoucích ke vzniku PAU spojenou s jejich akumulací v popelu (Simoneit, 2002; Hays et al., 2005).

2.3.3 Obsah PAU v popelu ve vztahu k obsahu nespáleného organického uhlíku

Vzniklé PAU se mohou akumulovat v produkovaném popelu po spalování biomasy (Morf et al., 2002). Obsah PAU v úletovém a roštovém popelu po spalování biomasy dosahuje značné variability. Výsledky sledování obsahu PAU v popelu vzniklého za reálných podmínek spalování biomasy ukazují, že úletový popel dosahuje významně vyššího obsahu PAU než roštový popel. Úletový popel obvykle dosahuje vyššího specifického povrchu než roštový, z tohoto důvodu je sorpce PAU v úletovém popelu významnější (Masto et al., 2015). Zatímco Straka et Havelcová (2012) stanovili celkový obsah PAU v úletovém popelu přibližně 6,1 mg/kg, tak Johansson et van Bavel (2003a) uvedli sumu obsahu PAU v úletovém popelu až ve výši 77 mg/kg. Rey-Salgueiro et al. (2016) zjistili, že v popelu z biomasy převládají obsahy NM PAU nad SM PAU a VM PAU, zatímco Singh et al. (2013) prokázali, že v plynné fázi uvolňované během spalování převládají SM PAU. García-Falcón et al. (2006) uvedli, že obsahy PAU souvisí s množstvím nespáleného organického uhlíku v popelech. Organický nedopal v popelu je místem, kde se vzniklé PAU mohou snadno sorbovat (Janvijitsakul et Kuprianov, 2007). Bylo zjištěno, že popel z různých druhů biomasy dosahuje širokého rozmezí hodnot nespáleného uhlíku v závislosti na typu spalovacího zařízení a druhu spalované biomasy. Dále bylo zjištěno, že obsah nespáleného uhlíku v popelech z biomasy byl značně variabilní (0,6 – 20,3 %) (Gómez-Barea et al., 2009; Duan et al., 2012). Enell et al. (2008) ve své práci naznačili, že mezi množstvím nespáleného organické uhlíku a obsahem PAU v popelu může být přímá závislost. Haglund (2008) považoval popel s obsahem nespáleného uhlíku vyšším než 5 % za rizikový a navrhl, že takovýto popel musí být před jeho následným využitím v zemědělství podroben analýze obsahu PAU.

2.3.4 Využití popelu z biomasy s ohledem na jeho obsah PAU

Dle Baumard et al. (1998) se popele po spalování fytomasy (obilné slámy) mohou definovat jako silně náchylné k vysoké akumulaci PAU, zatímco popele po spalování dřevní

štěpky jsou obecně považovány za méně rizikové. Limity obsahu PAU v popelu jsou dány v jednotlivých státech EU zcela odlišně. Švédská EPA vydala pravidla pro aplikaci popelů na zemědělskou půdu. Pro nejšetrnější aplikaci je stanoven limit obsahu sumy možných karcinogenních PAU v popelu maximálně 0,3 mg/kg a sumy nekarcinogenních PAU v hodnotě 20 mg/kg (Johansson et van Bavel, 2003b). V ČR je podle MZe ČR (2014) limit obsahu PAU v popelu pro využití na zemědělskou půdu 20 mg/kg a v Norsku dle Haglund et al. (2008) je limit obsahu PAU 3,0 mg/kg. V případě splnění všech požadavků na kvalitu popela je možná jeho aplikace přímo na zemědělskou půdu jako podpůrné minerální hnojivo nebo jako půdní aditivum (MZe ČR, 2014). Někteří autoři navrhli, že v případě popelu s vysokým obsahem PAU, jehož aplikace na zemědělskou půdu je zakázána, může být popel vitrifikován, uložen na skládky nebezpečného odpadu nebo může být spoluspalován v cementárnách s vápencem a jílem při výrobě cementu (Xiao et al., 2008; Demirbas et al., 2009; Gómez-Barea et al., 2009; Berra et al., 2015).

2.4 Možnosti remediace životního prostředí kontaminovaného PAU

Ve státech, kde není využívání popela legislativně upraveno, je popel aplikován na zemědělskou půdu bez ohledu na jeho obsah PAU. Tato praxe může znamenat vážně ohrožení životního prostředí včetně bioty (James et al., 2012). Z tohoto důvodu je potřeba hledat možné způsoby remediace půd po aplikaci popela kontaminovaného PAU nebo remediace samotné matrice popelu obsahující PAU z důvodu navrácení živin zpět do půdy (Reijnders, 2005; Mahmoudkhani et al., 2007). Remediace jsou využívány v sanačních technologiích pro kompletní odstranění polutantů nebo v postupech, které vedou ke zmírnění vlivu a toxicity polutantů na životní prostředí (Vidali, 2001).

Remediace se dle Semple et al. (2001) a Megharaj et al. (2011) rozdělují na:

a) Fyzikálně – chemické metody remediace, mezi které jsou řazeny například izolační metody, imobilizační metody, chemické stabilizace, fyzikální separace a extrakce (promývání, elektrokinetická úprava). Tyto druhy remediací se obecně využívají při velmi vysokém znečištění a tehdy, když hrozní neodkladné řešení situace, které představuje velmi vážné ohrožení lidského zdraví. Tyto remediace jsou považovány za velmi finančně nákladné a nešetrné k životnímu prostředí.
b) Biologické metody remediace jsou postupy s cílem odstranit kontaminant nebo snížit jeho obsah s ohledem na zachování základních funkčních vlastností kontaminované matrice.

Tyto remediace jsou označovány jako „bioremediace“, protože se při nich využívá činnosti mikroorganismů, hub, rostlin nebo živočichů.

Iwamoto et Nasu (2001) a Kumar et al. (2011) uvedli, že remediace se dle místa aplikace rozlišují:

a) Remediace „*in situ*“ – jsou aplikovány přímo v místě kontaminace. Mají nižší náklady na realizaci, avšak jejich účinnost je nižší a průběh je obtížně kontrolovatelný.
b) Remediace „*ex situ*“ – jsou aplikovány mimo kontaminované místo, kdy je kontaminovaná matrice (nejčastěji půda) vytěžena a remediace probíhá, buď v místě těžby („*on site*“) nebo je vytěžená kontaminovaná matrice převezena a remediace probíhá mimo místo těžby („*off site*“). Nevýhodou zmíněných metod je zvyšování finančních nákladů spojených s těžbou kontaminované plochy a s náklady potřebnými na její převoz, avšak průběh remediace je snadno kontrolovatelný.

2.5 Možnosti bioremediace životního prostředí kontaminovaného PAU

Bioremediace PAU jsou všeobecně vnímány jako finančně dostupné a environmentálně šetrné *in situ,* ale i *ex situ* sanační postupy. Nicméně, i bioremediace mají své limitující faktory – zejména rychlost a omezenou biodostupnost PAU. Předpokladem účinné bioremediace je zajištění optimálních podmínek pro organismy, které se podílejí na bioremediační procesech. Mezi specifické podmínky pro organismy se řadí optimální vlhkost, pH, teplota prostředí, přítomnost živin a zajištění aerobních podmínek, i když jsou známy také případy bioremediace PAU probíhající za anaerobních podmínek (Boopathy, 2000). Mezi vybrané metody bioremediace PAU v půdě patří například:

1) Přirozená atenuace – tato metoda je řazena mezi bioremediační metody, i když bioremediace probíhá bez cíleného zásahu, usnadnění či podpory, protože je založena na biologické schopnosti autochtonních organismů degradovat PAU v místě znečištění (Mulligan et Yong, 2004).
2) Biostimulace – je metoda, kdy se do půdy kontaminované PAU přidávají minerální živiny s cílem zvýšit účinnost a rychlost mikrobiální degradace PAU (Liebeg et Cutright, 1999).
3) Bioventing – je to metoda remediace PAU v půdě, při které se vhání vzduch nebo slabý proud čistého kyslíku přímo do kontaminované půdy, a tím dochází k obohacování prostředí s mikroorganismy o kyslík potřebný k aerobní mikrobiální degradaci PAU.

Podmínkou úspěšného bioventingu je přítomnost mikroorganismů v půdě schopných degradace PAU (García Frutos et al., 2010).

4) Landfarming – metoda je založena na principu aerobního mikrobiologického rozkladu PAU v tenké vrstvě kontaminované půdy. Tato metoda je často aplikována jako *ex situ*, kdy je vytěžená kontaminovaná zemina bioremediována v místě těžby. Landfarming spočívá v navezení vrstvy kontaminované zeminy (20 – 30 cm) na nekontaminovanou půdu s její následnou pravidelnou orbou a kypřením společně s dodávanými živinami. Tato metoda je nevýhodná zejména z důvodu nekontrolovatelnosti podmínek a možné volatilizace PAU do atmosféry (Hamdi et al., 2007).

Další vybrané bioremediační metody PAU jsou uvedeny pro přehlednost v rámci samostatných podkapitol.

2.6 Bioremediace PAU pomocí rostlin (fytoremediace)

Fytoremediace představuje jednu z mnoha bioremediačních metod, ve které se používají vyšší druhy rostlin, zejména bylin a dřevin, pro odstranění, stabilizaci nebo snížení vlivu PAU na životní prostředí včetně bioty. Fytoremediace PAU se nejčastěji využívá na *in situ*, ale i *ex situ* remediaci povrchově kontaminovaných půd. Fytoremediace by mohla být perspektivní a účinná metoda bioremediace půdy kontaminované PAU z popelů (Glick, 2003; Fazel Todd et al., 2016).

Metoda využití rostlin pro odstranění PAU zatím široké uplatnění v praxi nenašla, avšak je stále velice atraktivní a žádanou oblastí výzkumu, protože je nutné stále nalézat nové druhy rostlin, které by byly schopny účinně a rychle degradovat či dekontaminovat PAU v půdě nebo v jiných materiálech kontaminovanými PAU. Potřeba přesného poznání metabolismu kontaminantů v rostlinách schopných akumulace kontaminantů z půd je stále velice aktuální, protože poznání metabolického odbourávání kontaminantů v rostlinách umožní vhodný výběr rostlinných druhů využitelných v praxi (Chromá et al., 2002). Limitující může být kontaminovaná biomasa rostlin po ukončení fytoremediačního opatření, pokud by se jednotlivé PAU nedegradovaly, ale pouze akumulovaly v rostlinných pletivech. V tomto případě je nutné takovou kontaminovanou biomasu spalovat, odkládat na skládky nebezpečného odpadu nebo dále bioremediovat například kompostováním (Susarla, 2002). I přes mnohé limity jsou fytoremediační technologie přijímány veřejností s velikou oblibou, protože jsou považovány za šetrné a ohleduplné k životnímu prostředí. Nespornou výhodou

je, že ve srovnání s konvenčními fyzikálně – chemickými remediačními metodami jsou výrazně finančně levnější (Cunnigham et al., 1995; Cunningham et Ow, 1996).

Fytoremediační procesy jsou založeny na podobných principech, které probíhají v přírodě zcela volně a přirozeně. Ve vývoji fytoremediačních metod je nutné se zabývat mechanismem interakce PAU s rostlinami (Olson et al., 2001; Liu et al., 2014). Nezbytné z hlediska širokého a cíleného využití fytoremediací v běžné praxi je poznání samotného enzymatického aparátu podílejícího se na metabolické degradaci a následné transformaci PAU prostřednictvím biochemických procesů (Huang et al., 2004).

Cílem fytoremediačního opatření je degradace PAU v rostlině nebo jejich stabilizace s důrazem na zamezení dalšího transportu kontaminantu do míst, kde by mohlo dojít k bezprostřednímu ohrožení životního prostředí včetně bioty. Nicméně, je třeba brát ohled na možnost, že ne všechny metabolity PAU, které mohou být netoxické pro rostlinu, nemusejí vykazovat nezávadnost pro živočichy včetně člověka (Macek et al., 2000).

Fytoremediace zahrnuje soubor jednotlivých fytoremediačních procesů, které mohou být realizovány v návaznosti na sebe nebo mohou být zcela na sobě nezávislé. Z hlediska mechanismu vedoucího k odstranění PAU z půdy pomocí rostlin lze fytoremediační metodu dle Abhilash et al. (2009) rozdělit na jednotlivé děje nebo procesy. Jednotlivé procesy fytoremediace jsou uvedeny samostatně v následujících podkapitolách.

2.6.1 Fytostabilizace

Fytostabilizace (fytosekvestrace) představuje sorpci PAU na rhizodermálním povrchu kořenového systému rostlin. Tímto způsobem dochází k imobilizaci PAU v půdě a zamezuje se tak dalšímu pohybu PAU v životním prostředí (Schwitzguébel, 2017).

2.6.2 Fytovolatilizace

Fytovolatilizace označuje možný příjem volatilních PAU kořenovým systémem rostlin s jejich následným vyloučením stomaty v modifikované nebo nemodifikované formě. Tento proces je v případě PAU velmi omezený a v zásadě možný pouze pro NM PAU (Alkorta et Garbisu, 2001).

2.6.3 Fytodekontaminace

Fytodekontaminace označuje proces částečného odstraňování PAU z půdy, při kterém nedochází ke kompletní mineralizaci PAU. Dochází pouze ke snížení obsahu PAU, a tím i ke

zmírnění jejich vlivu na životní prostředí. Fytodetoxifikací se označují možné substituce PAU, které vedou ke snížení jejich toxicity, avšak v rámci metabolických substitucí mohou vzniknout ještě toxičtější substituenty PAU než původní sloučeniny (Meagher, 2000).

2.6.4 Fytoextrakce a fytoakumulace

Fytoextrakce představuje vlastní příjem PAU z půdy kořenovým systémem rostlin a fytoakumulace označuje děj, při kterém dochází k akumulaci PAU v jednotlivých buněčných kompartmentech po jejich fytoextrakci. PAU jsou nejčastěji akumulovány v rostlinných vakuolách nebo jsou stabilizovány v buněčných stěnách. Tímto procesem dochází ke stabilizaci PAU na buněčné úrovni (Cristaldi et al., 2017).

Důležitým předpokladem fytoremediace PAU v půdě je jejich biodostupnost. Rostlina přijímá PAU z půdy zejména kořenovým vlášením ze zdrojového místa kontaminace společně s živinami v půdním roztoku, kde jsou PAU rozpuštěné na základě jejich rozpustnosti ve vodě. Naftalen a ostatní NM PAU se 2 až 3 cykly ve sloučenině jsou relativně mobilní v půdním prostředí. PAU rozpuštěné v půdním roztoku mají schopnost přestupu z půdy do kořenového systému rostlin apoplastickou cestou nebo omezeně symplastickou cestou. Do nadzemních částí rostlin se PAU mohou dostávat xylémem, avšak musí nejprve překonat bariéru v podobě pericyklu, který se nachází ve středním válci kořenové soustavy. Schopnost translokace PAU z kořenového systému do nadzemní biomasy je považována za velmi omezenou. Rostlina může přijímat PAU v plynném skupenství stomaty, avšak tento způsob přijmu je také velmi omezený. Častou možností přijmu PAU rostlinou z atmosféry je atmosférická depozice. V takovém případě dochází zejména k adsorbci PAU a jejich akumulaci v kutikule na povrchu listů. Transport PAU floémem je také velmi omezený (Jordahl et al., 1997).

Příjem PAU rostlinami z půdy se odvíjí od biologické dostupnosti PAU, která je ovlivňována: a) fyzikálními a chemickými vlastnostmi a charakteristikami kontaminované půdy (půdní druh, pH, teplota, vlhkost, obsah organického uhlíku a živin), b) fyzikálně – chemickými vlastnostmi PAU (počet benzenových cyklů ve sloučenině, relativní molekulová hmotnost, rozpustnost ve vodě a těkavost). Biodostupnost je také závislá na hloubce zdroje kontaminace, velikosti a hustotě kořenového systému. Pro povrchovou kontaminaci jsou využívány byliny a pro hloubkovou kontaminaci přibližně od dvou metrů se využívají přednostně dřeviny (Mueller et Shan, 2006). Biologická dostupnost pro příjem PAU z půdy se vyjadřuje jako podíl koncentrace rozpuštěných PAU v půdním roztoku a obsahu PAU v

půdě. Množství akumulovaných PAU v rostlině z půdy se vyjadřuje pomocí biokoncentračního faktoru, který je dán podílem obsahu PAU v rostlině a v půdě (Mackay et Fraser, 2000).

2.6.5 Fytodegradace

Fytodegradace zahrnuje dva hlavní procesy – transformaci a mineralizaci PAU. V první řadě musí dojít ke změně nepolárního charakteru PAU, tak aby v rámci metabolismu rostlin došlo k postupné transformaci PAU převedením na méně toxickou formu. Úplná mineralizace PAU v rostlinách až na oxid uhličitý a vodu je možná, ale velmi omezená, a proto častěji dochází k transformacím PAU (Saier et Trevors, 2010).

Degradace PAU spočívá v postupném enzymatickém štěpení jednotlivých cyklů ve sloučenině PAU. V rostlinné buňce existuje mnoho enzymů, které jsou schopny metabolizovat PAU. Jedná se například o cytochrom P450 monooxygenázy, glutathiontransferázy, peroxidázy, hydroxylázy nebo také glukoronyltransferázy. Působením zmíněných enzymů vznikají hydroxylované a karboxylované PAU, ale i epoxidy PAU (Coleman et al., 1997). Jednotlivé deriváty PAU po metabolické přeměně jsou následně vyloučeny ve formě rostlinných exudátů nebo mohou být ukládány v rostlinných pletivech, protože rostliny postrádají efektivní exkreční mechanismus, než je tomu u živočichů. PAU s větším počtem benzenů a vyšší molekulovou hmotností jsou poměrně rezistentní k odbourávání, a proto dochází přednostně k jejich transformaci (Soudek et al., 2008).

Transformace PAU je vnímána jako složitý metabolický proces, kterým se za pomocí enzymů přetváří původní sloučeniny PAU na molekuly odpovídajícím primárním (substituované PAU) a sekundárním metabolitům (konjugované PAU). Proces transformace PAU může být obecně několika fázový a může probíhat současně s jejich degradacemi. V první fázi dochází ke zvýšení hydrofility PAU substitucí hydroxylových, methylových a jiných skupin. V konjugační fázi mohou vzniklé substituenty PAU reagovat například s různými deriváty kyseliny glukuronové nebo glutathionem za vzniku methylsulfonylových derivátů, které jsou katalyzovány glutathion-S-transferásou (Stiborová et al., 2004). V případě metabolické přeměny PAU u rostlin existuje určitá analogie s metabolismem organických polutantů u živočichů. V případě degradací PAU bakteriemi a houbami v půdě jsou metabolické přeměny PAU dobře prozkoumány, zatímco informace o degradaci PAU v rostlinných buňkách nejsou tak komplexní, jelikož dochází jejich vícenásobným transformacím (Kučerová et al., 1999).

2.6.6 Rhizodegradace

Hlavní procesy enzymatické degradace a mikrobiální transformace PAU probíhají v půdě v bezprostřední oblasti kořenové zóny rostlin, nazývané rhizosféra. Takovýto proces se označuje jako rhizosférní biodegradace neboli rhizodegradace (Nagendran et al., 2006). V rhizosféře se nachází přibližně $10^6 – 10^8$ bakterií, 10^5 aktinomycet a 10^3 hub na gram půdy, které jsou schopny odbourávat organické polutanty (Macek et al., 2000). Rostliny napomáhají mikrobiální degradaci PAU v rhizosféře tím, že uvolňují do půdy látky (exudáty), které mohou sloužit mnohým mikroorganismům jako primární zdroj uhlíku a energie na štěpění vazeb organického polutantu. Tento děj se často označuje jako kometabolismus rostliny s půdními mikroorganismy. Rostlinné exudáty obsahují především enzymy, terpeny, fenoly, flavonoidy, aminokyseliny, sacharidy a jiné látky, které mohou působit jako induktory mikrobiálního metabolismu PAU (Nichols et al., 1997).

To je ve shodě s Johnsen et al. (2005), kteří zjistili pozitivní vztah mezi rostlinými exudáty a degradací PAU v půdě. Účinnost rhizodegradace PAU je ovlivňována především jejich strukturou, molekulovou hmotností a rozpustností ve vodě (Nam et al., 1998). To povrdili Stroud et al. (2007), když se jednotlivé PAU ze skupiny NM PAU degradovaly snáze než jednotlivé PAU ze skupiny VM PAU z důvodu větší preference nízkomolekulárních PAU mikroorganismy jako primárního zdroje uhlíku. Andreoni et al. (2004) popsali možnost synergického štěpení nízkomolekuárních PAU například pomocí bakterií *Rhizobium galegae* a *Rhodococcus aetherovorans*, které byly izolované ze substrátu kontaminovaného PAU.

2.6.7 Příklady využití bylin a dřevin ve fytoremediacích

Ve fytoremediačních technologiích se upřednostňují ty druhy rostlin, které jsou schopny vysoké exkrece rostlinných exudátů. Vylučované exudáty mají charakter jednoduchých organických alifatických rozpouštědel, které zvyšují biodostupnost kontaminantů tím, že zvyšují jejich rozpustnost ve vodě. Mnohé studie fytoremediací PAU byly zaměřeny na použití jednoděložných trav z čeledi lipnicovitých (*Graminacea*) z důvodu schopnosti vysoké exudace. Tyto druhy rostlin mají zároveň svazčitý až vláknitý kořenový systém, který může prorůstat do hloubek až několika metrů (Larsson et al., 2013). Schopnost rostlin biodegradovat PAU v půdě je silně ovlivňována složením rostlinných exudátů v půdě. Bylo zjištěno, že vybrané rostlinné exudáty (alanin a vzniklé acetáty) v půdě korelovaly s enzymy katechol-2,3-dioxygenázou a naftalen dioxygenázou zodpovědnými za degradaci vybraných nízkomolekulárních PAU v půdě (Phillips et al., 2012).

V kombinovaném využití fytoremediace a bakteriální augmentace se při pěstování jílku vytrvalého (*Lolium perenne* L.) s přidanými gramnegativními hlízkovými bakteriemi rodu *Rhizobium*, schopných fixace vzdušného dusíku, docílilo snížení celkového obsahu PAU v půdě o 17,6 % po 180 dnech experimentu (Johnson et al., 2005). V práci Rezek et al. (2008) byly sledovány změny obsahu PAU v kontaminované půdě během 18 měsíčního nádobového pokusu s jílkem vytrvalým (*Lolium perenne* L.). Po ukončení pokusu došlo k významnému snížení PAU téměř o 50 % s výjimkou jednotlivých vysokomolekulárních PAU, které jsou obecně považovány za vysoce odolné vůči fytoremediacím. Cofield et al. (2008) prokázali schopnost kostřavy rákosovité (*Festuca arundinacea* Schreb.) a prosa prutnatého (*Panicum virgatum*, L.) fytoremediovat PAU v průměru o 40 % kromě indeno[1,2,3-c,d]pyrenu, jehož obsah v půdě se snížil pouze o 1,5 %. Lee et al. (2008) publikovali, že ježatka kuří noha (*Echinogalus crus-galli* L.) je schopna snížit v půdě obsah fenantrenu z 99 % a obsah pyrenu až o 94 % za 80 dnů především z důvodu vysoké produkce extracelulárních enzymů, které jsou schopny poměrně efektivně štěpit jednotlivé cykly PAU. Během pěstování kořenové zeleniny bylo zjištěno, že příjem PAU z půdy rostlinou je velmi omezený a pohyboval se v rozmezí od 0,05 do 0,3 mg/kg (Kipopoulou et al., 1999).

Mnohé studie naznačily, že kukuřice setá (*Zea mays* L.) je schopna růst na půdě kontaminované PAU (Liao et al., 2015; Liao et al., 2016). Xu et al. (2006) ve své studii nezjistili žádné významné rozdíly ve výnosu biomasy kukuřice pěstované na půdě kontaminované PAU ve srovnání s kontrolní variantou. K podobným závěrům došli i Wu et al. (2011), kteří pěstovali kukuřici na půdě kontaminované fenantrenem (12 mg/kg) a pyrenem (17 mg/kg). To je ve shodě s Panda et al. (2015), kteří zkoumali morfologické změny kukuřice pěstované na půdách s různými dávkami kontaminovaného popelu. Až přídavek popela v půdě vyšší než 50 % způsobil významné snížení růstu rostlin stejně jako redukci fotosyntetické aktivity vlivem sníženého obsahu chlorofylu v listech. Významné snížení výnosu biomasy zaznamenali také Dupuy et al. (2015), kteří pěstovali kukuřici na půdě s velmi vysokým obsahem fenantrenu v rozmezí od 50 do 750 mg/kg. Teprve obsah fenantrenu v půdě vyšší než 250 mg/kg prokazatelně snížil fotosyntetickou aktivitu. Nicméně, Xu et al. (2006) ve své studii navrhli kukuřici jako vhodnou rostlinu na fytoremediace půd s vysokým obsahem PAU. Kacálková et Tlustoš (2011) zaznamenali během *in situ* fytoremediace schopnost kukuřice snížit obsah PAU v dlouhodobě kontaminované půdě odebrané z průmyslové oblasti. Feng et al. (2014) pěstováním kukuřice na půdě s kalem z odpadních vod kontaminovaným PAU snížili jejich obsah v půdě o více než 40 % za 126 dnů experimentu.

Liao et al. (2015) prokázali schopnost kukuřice snížit jednotlivé PAU v půdě o více než 90 %. Navíc, Zhang et al. (2017) uvedli, že kukuřice je také schopna extrahovat PAU z půdy do nadzemní biomasy.

Přestože k nejvíce využívaným rostlinám ve fytoremediačních technologií patří byliny, rychle rostoucí dřeviny mohou být ještě účinnější, zejména z důvodu vysoké produkce biomasy, rozsáhlého kořenového systému a možnosti jejich využití na kontaminované lokalitě po více let (Burken et Schnoor, 1998; Vervaeke et al., 2003). Mezi druhy dřevin, které úspěšně snížily obsah fenantrenu a antracenu v půdě se dle Mueller et Shan (2006) řadí: morušovník červený (*Morus rubra* L.), topol černý (*Populus nigra* L.) a vrba jíva (*Salix caprea* L.). Použitím různých druhů borovic (*Pinus banksiana* Lamb., *Pinus resinosa* Lawson, *Pinus strobus* L.) se dosáhlo během 8 týdenního nádobového pokusu snížení obsahu pyrenu v půdě až o 74 % oproti variantě bez rostlin (Liste et Alexander, 2000).

Mnozí autoři popsali možné uplatnění rychle rostoucích druhů vrb a topolů zejména na extrakce Cd a Zn (ostatní rizikové prvky velmi omezeně) z půdy do nadzemní biomasy rychle rostoucích dřevin (Tlustoš et al., 2007; Vondráčková et al., 2017; Zárubová et al., 2015). Nicméně, Oleszczuk et Baran (2005) uvedli, že vrba košíkářská (*Salix viminalis* L.) byla schopna extrakce PAU z půdy až do nadzemních částí (stonků a listů), kdy v rámci polního pokusu byl sledován obsah 16 individuálních PAU v půdě. Po pěstování vrby došlo za prvních 6 měsíců k poklesu obsahu PAU v půdě o více než 50 %. Během následujících tří let se obsah PAU v půdě již významně nelišil. Fytoextrakce PAU z půdy byla velmi omezená. Obsah PAU ve stoncích a listech byl významně nižší než 0,04 mg/kg. Avšak, Kacálková et Tlustoš (2011) ve své práci uvedli, že rychle rostoucích dřeviny hybridních vrb (*Salix* × *smithiana* Willd.) a topolů (*Populus maximowiczii* Henry) byly schopny v rámci *in situ* fytoremediace akumulovat PAU z půdy až v hodnotě 3,4 mg/kg během jednoho vegetačního období.

2.7 Bioremediace PAU pomocí kompostování

Proces kompostování představuje soubor biochemických reakcí, při kterých dochází k aerobnímu rozkladu a transformaci organických látek působením činnosti mnohých mikroorganismů, kdy vznikají stabilní organické látky (Insam et de Bertoldi, 2007). Kompostováním se biologicky rozložitelné materiály postupně modifikují na organické materiály – substráty s vysokou hnojivou hodnotou označované jako komposty. Během kompostování probíhají analogické procesy jako v přirozeném půdním prostředí. Vytvářením

vhodných podmínek se celý děj urychluje a intenzifikuje (Tang et al., 2006; Habart et al., 2010).

Průběh kompostování představuje složitý kontinuální proces, který se rozděluje do tří základních fází. V první fázi, která trvá přibližně 3 až 4 týdny, dochází k rozkladu snadno rozložitelných látek (fáze rozkladu). Vlivem působení mikroorganismů, schopných rozkládat celuózu a lignocelulózu, dochází k vysokému růstu teploty na 50 – 70 °C. Během vysokých teplot dochází k hygienizaci kompostu a likvidaci patogenních organismů. V této fázi se organický materiál rozkládá až na aminokyseliny, polysacharidy, uhlíkaté fragmenty a minerální živiny. Tento proces se obecně nazývá mineralizací. Při pozvolném klesání teploty začíná druhá fáze, která probíhá přibližně od 4. do 10. týdne kompostování. Během této doby jsou uvolněné látky transformovány do stabilních struktur humusových látek s typickým hnědým zabarvením vznikajícího kompostu (fáze přeměny). Ve třetí fázi (dozrávací fáze) teplota klesá až na okolní teplotu a kompost získává zemitou strukturu (Hanč et al., 2016; Vaněk, 2012).

Z hlediska remediačních technologiích je kompostování řazeno mezi *ex situ* bioremediační metody, které je možné využívat například na bioremediaci půdy kontaminované PAU, kdy je půda vytěžena a následně kompostována nejčastěji ve směsi s biologicky rozložitelnými odpady. Bioremediace PAU kompostováním může být také využitelná na kontaminované sedimenty, kaly, inertní materiály včetně popela (Sayara et al., 2010). Pro dosažení vysoké účinnosti bioremediace kontaminantů během kompostování musí být dosaženo vhodného poměru C:N (většinou 25 až 35:1), jak uvedl Crecchio (2004).

Biologická degradace PAU během kompostování probíhá obdobně jako v případě degradace PAU v půdě vlivem účinku aerobních mikroorganismů – bakterií, hub, plísní a dalších organismů. Kompostování může probíhat na *on site* nebo *off site* statických hromadách. Důležitými faktory ovlivňujícími průběh bioremediace PAU pomocí kompostování je intenzivní aerace kompostovaného materiálu, které může být dosaženo manuální nebo automatickou překopávkou materiálu a nuceným provzdušňováním (Cajthaml et al., 2002; Bernal et al., 2009).

Dostatečná aerace materiálu zvyšuje biologickou aktivitu mikroorganismů schopných degradace PAU a podporuje stabilizaci PAU v kompostech vazbou na vznikající humínové látky. Důležité je také během kompostování udržování optimální teploty a vlhkosti kompostovaného materiálu (Steger et al., 2005; Cai et al., 2007).

Bioremediace PAU v popelu pomocí kompostování nebyla dosud zkoumána. Většina publikací byla zaměřena na degradace PAU v kontaminovaných půdách, kalech z odpadních vod a dřevních materiálech během jejich společného kompostování s organickým materiálem (Atagana, 2004; Löser et al., 2004; Amir et al., 2005; Oleszczuk, 2007; Hafidi et al., 2008). Antizar-Ladislao et al. (2004) ve své studii, která se zabývala degradacemi PAU během kompostování kontaminované půdy odebrané z okolí uhelné elektrárny ve Velké Británii, došli k závěru, že optimální podmínky na biodegradace PAU jsou mezofilní během stabilizační fáze kompostování při vlhkosti materiálu 60 až 80 %.

Antizar-Ladislao et al. (2008) se zabývali změnami složení jednotlivých skupin mikroorganismů v průběhu kompostování zeminy kontaminované PAU. Rozdělením fosfolipidických mastných kyselin zjistili, že nejvíce aktivní mikrobiální společenstva podílejících se na degradaci PAU jsou houby za mesofilních podmínek. Bioremediace PAU pomocí kompostování byla zkoumána jak v laboratorních podmínkách, tak i v poloprovozních podmínkách, tedy ve speciálně vytvořených bioreaktorech, boxech nebo tunelech s monitorovanými podmínkami. Tyto způsoby kompostování jsou v případě degradací polutantů nejefektivnější, avšak mnohem finančně náročnější (Potter et al., 1999).

Byla provedena studie na kompostování zeminy dlouhodobě kontaminované PAU. Kompostování bylo provedeno ve speciálních nádobách (reaktorech) umístěných v laboratoři s kontrolovanými podmínkami. Během kompostování půdy kontaminované PAU s organickým materiálem v různém poměru se sledovala změna obsahu 16 PAU po dobu 8 týdnů. Po ukončení experimentu došlo k prokazatelnému poklesu obsahu PAU o 75 % v případě kompostování zeminy a biologického materiálu v poměru 0,8:1 (Antizar-Ladislao et al., 2005).

Podobných výsledků dosáhli také Kapanen et al. (2013), kdy během kompostování směsi organické hmoty a kontaminovaného kalu došlo ke snížení obsahu PAU o 84 % z původního obsahu PAU ve výši 0,5 mg/kg. Bylo také zjištěno, že půdu kontaminovanou PAU lze bioremediovat pomocí kombinovaného využití fytoremediace s přídavky kompostu (Cai et al, 2008). Feng et al. (2014) zjistili, že 10 % obohacením půd kontaminovaných PAU o kompost dochází ke zvýšení přístupnosti jednotlivých nízkomolekulárních PAU u kostřavy rákosovité (*Festuca arundinacea* Schreb.), proto byla pozorována zejména zvýšená biodegradace jednotlivých PAU ze skupiny NM PAU v půdě.

2.8 Bioremediace PAU pomocí vermikompostování

Vermikompostování představuje alternativní metodu k procesu kompostování, ve které se využívá činnosti některých druhů žížal k transformaci organické hmoty za vzniku organického materiálu s poměrně vysokým obsahem humínových látek označovaného jako vermikompost. Ve srovnání s běžným kompostem může dosahovat až 10 krát vyššího obsahu mikroorganismů. S tím souvisí i vyšší variabilita produkovaných enzymatických látek (Fornes et al., 2012). Hickman et Reid (2008) uvedli, že k vermikompostování se využívají zejména žížaly hnojní (*Eisenia fetida* Savigny), kalifornské hybridy žížal (*Eisenia fetida andrei* Bouché*)*, ale také je možné využít žížalu obecnou (*Dendrobaena veneta* Rosa) nebo žížalu načervenalou (*Lumbricus rubellus* Hoffmeister). Během vermikompostování žížaly ve svém trávicím traktu zpracovávají organický materiál, který využívají přibližně ze 40 % a zbytek vylučují v natrávené formě nebo ve stmelených částech označované jako koprolity (Carrasquero-Durán et Flores, 2009).

Vermikompostování je popisováno jako nízkonákladový, efektivní a rychlý způsob zpracování biologicky rozložitelných odpadů, statkových organických odpadů, kuchyňských biologických odpadů nebo jiných organických materiálů (Garg et Gupta, 2011; Hanč et Chadimová, 2014). Vhodnou potravou pro žížaly jsou snadno rozložitelné organické odpady s poměrem C:N přibližně 20 až 30:1. Pro účinné vermikompostování je třeba zajistit dostatek organického materiálu sloužícího jako potravinový substrát pro žížaly, aerobní podmínky, mesofilní teplotu prostředí, vlhkost materiálu vyšší než 60 % s pH v rozmezí 5 až 8 (Hanč et Plíva, 2013; Hanč et Vašák, 2015). Vermikompostování se podílí na udržitelném hospodaření a podpoře koloběhu živin v prostředí (Pattnaik et Reddy, 2010; Fornes et al., 2012).

Z hlediska remediačních technologií je vermikompostování řazeno mezi bioremediační metody jako možný alternativní způsob bioremediace polutantů k procesu kompostování, který je možný aplikovat na plochy kontaminované organickými polutanty *in situ*, ale i *ex situ* (Martín-Gil et al., 2008; Sinha et al., 2002). Mnozí autoři popisují vermikompostování jako perspektivní, nenákladnou a šetrnou metodu vedoucí k odstraňování PAU z životního prostředí (Samanta et al., 2002; Zhang et al., 2006). To ve své studii potvrzují i Contreras-Ramos et al. (2008), kteří porovnávali změny obsahu fenantrenu, antracenu a benz[a]pyrenu v půdě obohacené o vermikompost. Po 70 dnech experimentu došlo v půdě obohacené o vermikompost až k 99 % poklesu obsahu fenantrenu, 95 % poklesu obsahu antracenu a 16 % poklesu obsahu benz[a]pyrenu. K podobným výsledkům dospěli také Álvarez-Bernal et al. (2006), kteří také sledovali změny obsahu fenantrenu, antracenu a benz[a]pyrenu v půdě po

aplikaci vermikompostu o různých koncentracích. Po 100 dnech trvání experimentu došlo k jejich snížení až o 97, 93 a 78 %, zatímco v neobohacené půdě o vermikompost nebyla pozorována významná změna.

Di Gennaro et al. (2009) se zabývali chováním a genetickými změnami mikroorganismů v půdě kontaminované naftalenem, která byla odebrána z průmyslové oblasti. Přidáním vermikompostu do půdy se zvýšila tvorba genu *nah*Ac půdních mikroorganismů. Uvedený gen byl zodpovědný za zvýšenou expresi enzymu naftalen dehydrogenázy, který umožnil mikrobiální degradaci naftalenu v půdě. Za 30 dnů se obsah naftalenu snížil o více než 90 %.

Dále byl zkoumán například vliv přídavků vermikompostu na zvýšení účinnosti fytoremediace půdy kontaminované PAU (Contreras-Ramos et al., 2008; Chang et al., 2008). Wang et al. (2012) uvedli, že 5 % přídavek vermikompostu vyrobeného z kejdy prasat zvýšil degradaci PAU (fenantrenu, antracenu a pyrenu) v uměle kontaminované půdě společně s rostlinami (*Sedum alfredii* Hance) o 75 až 99 % během 90 dnů. Přídavek vermikompostu pozitivně ovlivnil mikrobiální aktivitu v půdě. Dále zvýšil tvorbu kořenového systému, a tím i produkci exudátů měnících polaritu PAU, která umožnila vyšší dostupnost PAU pro organismy schopných jejich rozkladu.

2.9 Bioaugmentace PAU

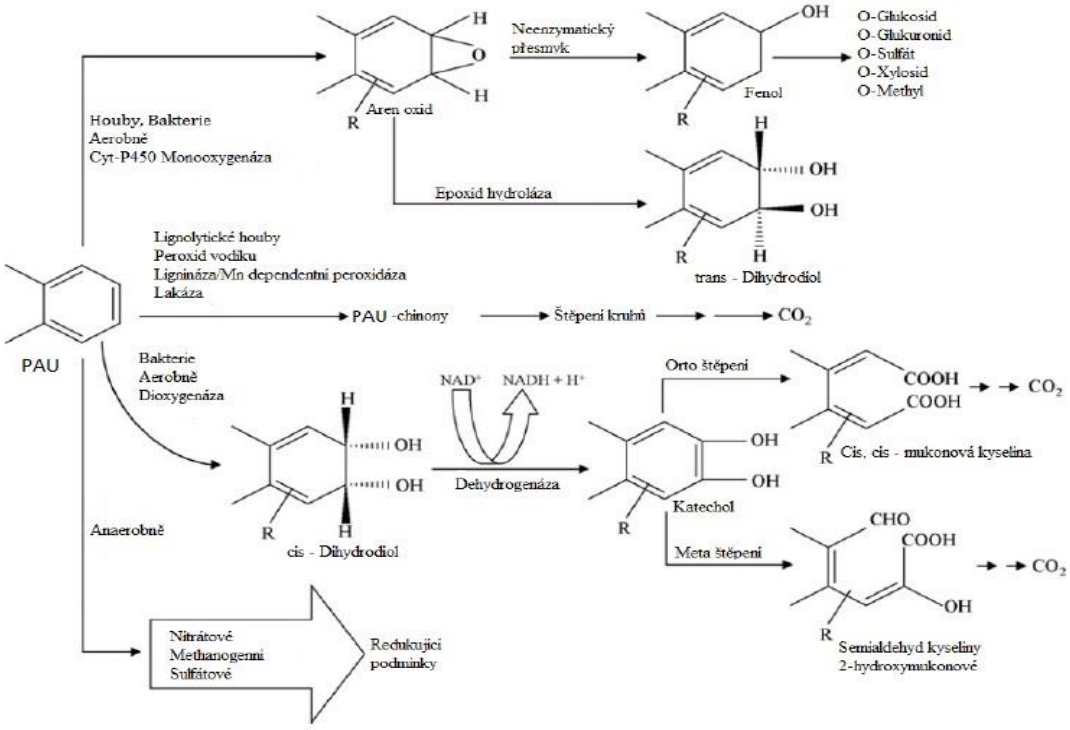
V bioaugmentačních metodách dochází především k aerobnímu odbourávání PAU vlivem činnosti metabolismu různých druhů mikroorganismů (bakterií, hub, plísní nebo jiných) cíleně aplikovaných v kontaminovaném půdním prostředí. Tato metoda může být uplatněna jak *in situ*, tak i *ex situ*. Bioaugmentační metody lze rozdělit dle druhu aplikovaných organismů (Winquist et al., 2014; Gaur et al., 2018).

a) Bakteriální remediace – metoda, ve které se využívají kmeny bakterií schopných degradace PAU. Někdy je tato metoda nazývána i jako bakteriální augmentace a často je zaměňována s obecným označením bioremediace (Kong et al., 2018).

b) Mykoremediace – metoda, ve které se využívají zejména kmeny hub schopných degradace PAU. Někdy je tato metoda nazývána i jako mykoaugmentace nebo funginální remediace (Agnello et al., 2016).

Na Obrázku 2 (strana 25) je uvedeno souhrnné schéma degradace PAU mikroorganismy dle Haritash et Kaushik (2009).

Obrázek 2: Schéma aerobní a anaerobní biodegradace PAU pomocí bakterií a hub dle Haritash et Kaushik (2009).



2.9.1 Bioremediace PAU pomocí bakterií

Bioremediace PAU pomocí bakterií probíhá především za aerobních podmínek působením enzymů dioxygenáz, ale i cytochrom P450 monooxygenáz (Nigam et al., 1998). Jsou známy i mikrobiální degradace PAU za anaerobních podmínek, které jsou ve srovnání s aerobními výrazně pomalejší (Chang et al., 2008). K aerobním bakteriím schopných degradace PAU se řadí například rody: gramnegativní *Pseudomonas*, nesporulující grampozitivní *Mycobacterium*, gramnegativní *Agrobacterium*, nesporulující grampozitivní *Rhodococcus,* grampozitivní sporulující *Bacillus* a mnoho dalších (Cerniglia, 1993).

Innemanová et al. (2018) z dlouhodobě kontaminované půdy izolovali různé kmeny bakterií, které dosud nebyly testovány na bioremediace PAU v půdě. Ty následně použili na bioaugmentaci PAU v půdě. Dle získaných výsledků byla metoda bioaugmentace o 40 % účinnější z hlediska poklesu obsahu PAU než jejich přirozená atenuace kontaminované půdě. Po ukončení bioaugmentačního pokusu se obsah PAU v půdě snížil o 72,9 %. Na základě této studie by mohl být kmen bakterií *Acinetobacter calcoaceticus* využívaný v praxi na bakteriální degradace PAU v dlouhodobě kontaminované půdě.

Při biodegradaci PAU dochází k postupnému štěpení jednotlivých cyklů, ze kterých je složena sloučenina PAU. Například naftalen může být degradován pomocí bakterie *Pseudomonas putida* (Nigam et al., 1998). Naftalen je odbouráván pomocí enzymu naftalendehydrogenázy v *ortho* poloze za vzniku cis-1,2-dihydronaftalendiolu a 1,2-dihydroxynaftalenu. Aromatický systém benzenu se štěpí enzymem extradiol dioxygenázou, kdy vzniká 4-(2-hydroxyfenyl)-2-oxo-3-butenová kyselina. Vlivem aldózy dojde k odštěpení pyruvátu za vzniku salicylaldehydu, který je oxidován až na kyselinu salicylovou. Štěpením v *ortho* poloze vznikne cis-mukonová kyselina. Štěpením v *meta* poloze vznikne pyruvát a acetaldehyd. Degradace většiny PAU probíhá podobným způsobem, tedy postupným štěpením jednotlivých cyklů tvořících PAU. Degradace některých složitějších PAU probíhá s jistými odlišnostmi (Bamforth et Singleton, 2005).

Jauhari et al. (2017) zjistili, že bakterie *Pseudomonas aeruginosa*, je schopna rozkládat antracen pomocí katabolických enzymů katechol 1,2 dioxygenáz za postupného vzniku kyseliny ftalové, která je následně zapojena do metabolických procesů bakterií. Dále Schneider et al. (1996) se zabývali degradacemi pyrenu, benz[a]antracenu a jiných individuálních PAU. Například Moody et al. (2004) popsali bakteriální degradaci benzo[a]pyrenu. Působením bakterie *Mycobacterium vanbaalenii* produkující intracelulární monooxygenázy a dioxygenázy byly pozorovány různé hydroxy, hydromethoxy a methoxy deriváty benzo[a]pyrenu.

Bakteriální degradace PAU zejména v kombinaci se surfaktanty, které zvyšují desorpci PAU z kontaminované matrice, mohou být velice perspektivní metody na degradace PAU v životním prostředí (Liang et al., 2017).

2.9.2 Bioremediace PAU pomocí hub

Houby představují rozsáhlou skupinu eukaryotních heterotrofních organismů, které přijímají živiny ve formě organických látek. Bylo prokázáno, že některé druhy hub jsou schopné oxidovat PAU. Bioremediace PAU pomocí hub se může rozdělit na dva základní mechanismy: neligninolytický a ligninolytický, ve kterých dochází k degradaci PAU pomocí neligninolytických a ligninolytických enzymů (Cerniglia, 1993). Většina neligninolytických hub (například *Aspergillus niger, Cunninghamella elegans*, *Chrysosporium pannorum* nebo jiné) neroste na dřevě. Z tohoto důvodu neprodukují lignin peroxidázové enzymy, které jsou běžně produkované ligninolytickými druhy hub rostoucích na dřevě (například *Bjerkandera adusta, Irpex lacteus)*. Neligninolytické houby produkují enzym cytochrom P450

monooxygenázu, avšak jsou známy takové druhy hub (například *Pleorutus ostreatus*, *Phanerochaete chrysosporium*), které jsou schopny produkce obou druhů enzymů (Bamforth et Singleton, 2005). Rozsáhlý přehled hub využívaných v mykoremediacích uvádí například Fernández-Luqueño et al. (2011).

Metabolismus PAU u neligninolytických druhů hub začíná oxidací aromatického jádra benzenu enzymem cytochrom P450 monooxygenázou, kdy vznikají arenoxidy PAU, ze kterých neenzymatickým přesmykem vznikají fenoly PAU. Tyto sloučeniny slouží jako substráty pro následné reakce za vzniku konjugátů PAU (*ortho*-glukoronidy, *ortho*-glykosidy, *ortho*-sulfáty a další) s nižší toxicitou a vyšší rozpustností. Z reaktivních arenoxidů PAU mohou působením epoxid hydrolázou vznikat *trans*-dihydrodioly PAU a z nich dále mohou vznikat chinony PAU (Haritash et Kaushik, 2009). Metabolismus PAU u ligninolytických hub probíhá působením ligninolytických enzymů rozkládajících lignin a jiné organické materiály. Mezi ligninolytické enzymy schopné degradovat PAU patří peroxidázy (lignin peroxidáza a mangan-dependentní peroxidáza) a lakázy (Baldrian, 2008).

Například Sack et al. (1997) zjistili, že použitím hub *Phanerochaete chrysosporium* nebo *Trametes versicolor* dochází k oxidaci PAU za vzniku chinonů PAU a následně může dojít až k jejich kompletní mineralizaci. Eggen et Majcherczyk (1998) ve své studii s půdou kontaminovanou PAU zjistili, že aplikace hub (*Pleurotus ostreatus*) vedla k 49 % snížení obsahu vysokomolekulárního benzo[a]pyrenu. Nicméně, pouze 1 % benzo[a]pyrenu se kompletně mineralizovalo. Byss et al. (2008) ve své studii zkoumali účinnost degradace PAU v půdě, která byla kontaminovaná dehtovým olejem po impregnaci a konzervaci dřeva. V inkubačním experimentu byla půda s obsahem PAU v hodnotě 194 mg/kg obohacena o substrát s vybranými druhy basidiomycet: *Pleurotus ostreatus* a *Irpex lacteus*. Bylo zjištěno, že v obou případech došlo k úbytku obsahu SM PAU a VM PAU ve srovnání s kontrolní variantou, která obsahovala pouze přirozená mikrobiální společenstva v původní kontaminované půdě. Po ukončení 120 denního experimentu došlo k poklesu obsahu sumy PAU o 55 – 67 % v půdě obohacené o *Pleurotus ostreatus*, zatímco v půdě obohacené o *Irpex lacteus* se obsah sumy PAU snížil o 27 – 36 %.

Mnoho autorů uvádí, že ligninolytické kmeny hub (zejména *Pleurotus* sp., *Phanerochaete* sp., *Irpex* sp., *Bjerkandera* sp. *nebo Trametes* sp.) jsou velice perspektivní pro využití v praxi k odstranění nebo snížení obsahu PAU v různých kontaminovaných materiálech (Pickard et al., 1999; Li et al., 2012; García-Delgado et al., 2015).

3 Vědecké hypotézy a cíle práce

Hypotézy práce

1) V důsledku nedokonalého spalování různých druhů biomasy se mohou v úletovém a roštovém popelu akumulovat vysoké obsahy PAU, které brání jeho využití v zemědělství.

2) Vysoký obsah PAU v popelu by bylo možné odstranit nebo snížit pěstováním rostlin během fytoremediace.

3) Účinnost fytoremediace PAU v půdě by bylo možné zvýšit aplikací kompostu, vermikompostu nebo substrátu obsahujícího ligninolytické houby.

4) Vysoký obsah PAU v popelu by bylo možné odstranit nebo snížit během kompostování a vermikompostování ve směsi s organickými odpady.

Cíle práce

1) Stanovit obsah 16 základních PAU v jednotlivých vzorcích úletového a roštového popelu, které vznikly za reálných podmínek spalování v provozovnách využívajících spalování biomasy k energetickým účelům.

2) Zhodnotit vliv pěstování rostlin na změnu obsahu PAU v půdě po aplikaci popelu během fytoremediace a ověřit možný příjem PAU z půdy do rostlin.

3) Zhodnotit vliv kompostu, vermikompostu a substrátu obsahujícího ligninolytické houby na zvýšení účinnosti fytoremediace PAU v půdě a ověřit možnou zvýšenou extrakci PAU z půdy do rostlin.

4) Zhodnotit vliv kompostování a vermikompostování na změnu obsahu PAU ve směsi organických odpadů s popelem.

4 Publikované práce

4.1 Košnář et al. (2016). Sledování obsahu polycyklických aromatických uhlovodíků v úletovém a roštovém popelu ze zařízení spalujících biomasu ve vztahu k teplotě spalování a obsahu nespáleného uhlíku.

Název: Investigation of polycyclic aromatic hydrocarbon content in fly ash and bottom ash of biomass incineration plants in relation to the operating temperature and unburned carbon content.

Autoři: Zdeněk Košnář, Filip Mercl, Ivana Perná, Pavel Tlustoš.

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Investigation of polycyclic aromatic hydrocarbon content in fly ash and bottom ash of biomass incineration plants in relation to the operating temperature and unburned carbon content

Zdeněk Košnář [a,*], Filip Mercl [a], Ivana Perná [b], Pavel Tlustoš [a]

[a] Department of Agro-Environmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, CZ-165 21 Prague 6, Czech Republic

[b] Institute of Rock Structure and Mechanics, Academy of Sciences of the Czech Republic, V Holešovičkách 41, CZ-180 00, Prague 8, Czech Republic

HIGHLIGHTS

- The 16 EPA PAHs in 96 ash samples of biomass incineration plants were investigated.
- The highest PAH content was observed in phytomass fly ash derived at 500 to 750 °C.
- The LMW, MMW, HMW and total PAHs in ash correlated significantly.
- The total PAHs present in ash increased with increased levels of unburned carbon.
- The ashes suitable for soil applications as an organic amendment were suggested.

GRAPHICAL ABSTRACT

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ABSTRACT

Purpose: The use of biomass fuels in incineration power plants is increasing worldwide. The produced ashes may pose a serious threat to the environment due to the presence of polycyclic aromatic hydrocarbons (PAHs), because some PAHs are potent carcinogens, mutagens and teratogens. The objective of this study was to investigate the content of total and individual PAHs in fly and bottom ash derived from incineration of phytomass and dendromass, because the data on PAH content in biomass ashes is limited. Various operating temperatures of incineration were examined and the relationship between total PAH content and unburned carbon in ashes was also considered.

Methods: The analysis of PAHs was carried out in fly and bottom ash samples collected from various biomass incineration plants. PAH determination was performed using gas chromatography coupled with mass spectrometry. The correlations between the low, medium and high molecular weight PAHs and each other in ashes were conducted. The relationship between PAH content and unburned carbon, determined as a loss on ignition (L.O.I.) in biomass ashes, was performed using regression analysis.

Results and discussion: The PAH content in biomass ashes varied from 41.1 ± 1.8 to 53,800.9 ± 13,818.4 ng/g dw. This variation may be explained by the differences in boiler operating conditions and biomass fuel composition. The correlation coefficients for PAHs in ash ranged from 0.8025 to 0.9790. The regression models were designed and the coefficients of determination varied from 0.908 to 0.980.

Conclusions: The PAH content in ash varied widely with fuel type and the effect of operating temperature on PAH content in ash was evident. Fly ashes contained higher amounts of PAHs than bottom ashes. The low molecular

* Corresponding author at: Department of Agro-Environmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Czech Republic.
E-mail address: kosnarz@af.czu.cz (Z. Košnář).

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weight PAHs prevailed in tested ashes. The exponential relationship between the PAH content and L.O.I. for fly ashes and the linear for bottom ashes was observed.

1. Introduction

Biomass is a type of biological material that can act as a renewable fuel source for energy production. The thermochemical process of conversion of biomass fuels, such as industrial grown plants, fast growing trees, fuel wood, wood chips, forest and harvest residues and many others, is possible to use for the production of useful heat and electricity (McKendry, 2002; James et al., 2012). The energy derived from biomass combustion contributes to the development of environmental, social and economic sustainability. The direct combustion of biomass fuels in incineration plants is considered to be the most promising energy source to mitigate greenhouse gas emissions (Khan et al., 2009; Atkins et al., 2010).

Biomass combustion produces fly ash and bottom ash as a waste–product of the biomass conversion processes (Bridgwater et al., 1999; Johansson and van Bavel, 2003a). Fly ash represents the solid component mixed with unburnt particles of fuel, which is deposited on the precipitator inside the fluidized, circulating fluidized or bubbling fluidized boilers. The bottom ash consists of solid bed particles located in the bottom part of the furnace (Pitman, 2006; Freire et al., 2015).

The ash derived from biomass combustion is rich in macro- and micronutrients and has the potential to be utilized as a mineral fertilizer in agriculture (Ferreiro et al., 2011). The use of this waste material may also reduce landfill disposal (Reijnders, 2005). However, the ash derived from biomass combustion can contain high amounts of hazardous metals that may cause serious health risks (Aronsson and Ekelund, 2004; Pöykiö et al., 2009).

The conditions of biomass combustion can also lead to the formation of polycyclic aromatic hydrocarbons (PAHs) in biomass ash (Chagger et al., 2000; Enell et al., 2008). The most frequent formation mechanism of PAHs is pyrolysis and subsequent pyrosynthesis during the incomplete combustion of biomass. PAH formation via high and low temperature pyrolytic reaction has also been described (McGrath et al., 2001; Morf et al., 2002; Sharma and Hajaligol, 2003; Hays et al., 2005). The formation of PAHs during biomass combustion is strongly dependent on the type of biomass fuels used, their physical and chemical properties and the operating conditions of combustion (Jenkins et al., 1998; Ross et al., 2002).

The PAHs represent persistent organic pollutants (POPs) which are widely distributed in the environment as a consequence of human industrialization (Wheatley and Sadhra, 2004; Bignal et al., 2008). These lipophilic compounds consist of 2 to 7 fused aromatic benzene rings in linear, angular or cluster arrangements. This arrangement predicts their stability in the environment and stable PAHs tend to accumulate mainly in soils and sediments. The physical-chemical properties, distribution and behaviour of PAHs in the environment also vary considerably with molecular weight. The lower molecular weight PAHs (LMW PAHs), containing 2–3 rings, are mobile in the environment, whereas the medium molecular weight PAHs (MMW PAHs) containing 4 rings and higher molecular weight PAHs (HMW PAHs), containing 5–7 rings, are relatively non–mobile. With increasing molecular weight, the melting point, boiling point and lipophilicity of PAHs with respect to log K_{OW} (n–octanol-water partition coefficient) grows and the water solubility decreases, suggesting increased solubility in lipid compounds (Eisler, 2000).

Sixteen basic PAHs are included in the United States Environmental Protection Agency (16 US EPA PAHs) priority pollutant list, because they may pose serious threats to the environment and biota (USEPA, 2016). The interest of researchers in PAH monitoring in ash has increased recently due to the possibility of recycling nutrients from ash to soil, because PAHs could be absorbed and assimilated by plants and subsequently enter into the food chain of animals and humans (Demirbas, 2005; Park et al., 2012). Individual PAH compounds have various toxicities and some PAHs are known to have carcinogenic, teratogenic and mutagenic properties. For instance, naphthalene has been described as the most acutely toxic PAH and benzo[a]pyrene has been identified as carcinogen to humans (Juhasz and Naidu, 2000).

Most previous research has focused on PAHs in ashes derived from municipal solid wastes and wood biomass, and very few studies have characterized the distribution of low, medium and high molecular weight PAHs found in ashes taken from commercial biomass incineration plants operated between 250 and 1000 °C. The data on PAH content in fly and bottom ashes derived from phytomass (including agricultural crop residues) in literature are still limited and the relationship between the unburned combustible carbon and total PAH content in various biomass ashes has not been studied sufficiently.

The main objective of this work was to investigate the total and individual content of the 16 US EPA PAHs in fly ash and bottom ash derived from phytomass and dendromass derived from various operating temperatures of biomass incineration. The relationship between the total PAH content and unburned carbon in ashes was also considered.

2. Experimental

2.1. Ash sampling and preparation

Ninety six ash samples were taken from 48 commercial biomass incineration plants located in the Czech Republic. Fly ash and bottom ash samples were collected from each incineration plant. The fly ashes could be a mixture of ashes collected at the superheater, economizer, and electrostatic precipitator. All bottom ashes were collected from the tank below the fluidized or grate boilers. The approximate amount of 2 kg of each ash sample was taken in four random replications which were further mixed thoroughly. The samples were put into dark glass bottles and transported to the laboratory for further analysis. The tested samples were air–dried to a constant mass in the laboratory, pulverised by a Retch fraction mill (Retch, Germany), passed through a 2.0 mm sieve and subsequently homogenized. A total of 50.0 g of each homogenized ash sample was grounded to a fine powder by a vibration mill with diameter 15–20 μm. If necessary, the samples of ash were stored in a refrigerator at −8/−10 °C in Petri dishes covered with aluminium foil.

Before PAH–specific analysis, each ash sample was diluted in deionized water at a ratio of 5:1 (solid to liquid) and then the pH value was measured after 24 h according to Johansson and van Bavel (2003a). For pH determination, a WTW pH 340i meter with glass ion selective electrode (WTW, Germany) was used. The amount of unburned combustible carbon in each ash sample was determined as a loss on ignition (L.O.I.), when 10.0 g of dry ash sample was heated in a crucible placed in a furnace for 2 h at 105 °C and then the temperature increased to 1000 °C at a rate of 10 °C/min. The L.O.I. was calculated, as follows: L.O.I. (mass %) = $((\text{Weight}_{105} - \text{Weight}_{1000})/\text{Weight}_{105}) \times 100$, where Weight_{105} is the weight of sample after heating at 105 °C and Weight_{1000} is the weight of sample after ignition at 1000 °C. This is a standard method used in many European countries for determination of residual organic matter represented by unburned carbon in ash (Stubington and Wang, 2000). The L.O.I. analyses were performed in the Institute of Rock Structure and Mechanics, Academy of Sciences of the Czech Republic. The analyses of PAHs were performed in the

Department of Agro-Environmental Chemistry and Plant Nutrition of the Czech University of Life Sciences Prague.

2.2. Characterization of ash samples

The ash samples and some operation properties of combustion boilers can be described and characterized as follows:

a) Phytomass fly ash: 24 samples derived from wheat and barley straw based biomass and agricultural crop residues, collected from fixed or fluidized bed boilers with 1.7–20.0 MW reactors, pH = 12.79 ± 0.52, combustion temperature: a) 250–500 °C, 5.2–6.1% of L.O.I.; b) 500–750 °C, 49.6–54.7% of L.O.I.; c) 750–1000 °C, 14.1–26.4% of L.O.I. (eight samples in each group).
b) Phytomass bottom ash: 24 samples derived from wheat and barley straw based biomass and agricultural crop residues, collected from fixed or fluidized bed boilers with 1.7–20.0 MW reactors, pH = 11.52 ± 0.51, combustion temperature: a) 250–500 °C, 2.1–2.9% of L.O.I.; b) 500–750 °C, 5.7–7.6% of L.O.I.; c) 750–1000 °C, 3.1–4.4% of L.O.I. (eight samples in each group).
c) Dendromass fly ash: 24 samples derived from wood chips and pellets and forest and wood residues, collected from fixed or fluidized bed boilers with 0.4–10.0 MW reactors, pH = 12.12 ± 0.62, combustion temperature: a) 250–500 °C, 13.1–39.4% of L.O.I.; b) 500–750 °C, 2.5–33.4% of L.O.I.; c) 750–1000 °C, 3.4–18.8% of L.O.I. (eight samples in each group).
d) Dendromass bottom ash: 24 samples derived from wood chips and pellets and forest and wood residues, collected from fixed or fluidized bed boilers with 0.4–10.0 MW reactors, pH = 12.16 ± 0.41, combustion temperature: a) 250–500 °C, 8.2–42.4% of L.O.I.; b) 500–750 °C, 7.2–40.3% of L.O.I.; c) 750–1000 °C, 5.9–22.2% of L.O.I. (eight samples in each group).

2.3. Chemicals and reagents

Acetone (purchased from Chromservis, Czech Republic), *n*–hexane (95% for GC/ECD residue analysis purchased from Chromservis, Czech Republic), standards of priority 16 US EPA PAHs containing naphthalene (NA), acenaphthylene (ACY), acenaphthene (ACE), fluorene (FL), phenanthrene (PH), anthracene (AN), fluoranthene (FLU), pyrene (PY), benz[*a*]anthracene (BaA), chrysene (CH), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), benzo[*a*]pyrene (BaP), indeno[1,2,3-*cd*]pyrene (IP), dibenz[*a*,*h*]anthracene (DA), and benzo[*g*,*h*,*i*]perylene (BP) in a 100 ng/μl mixture solution of each PAH species in cyclohexane (PAH-mix 9) purchased from Dr. Ehrenstopher GmbH, Germany. Internal standard solution (IS) 1,3,5-triphenylbenzene (3 PB) at 2000 ng/μl in cyclohexane and surrogate standard solution (SS) 2-fluorobiphenyl (2FB) at 2000 ng/μl in cyclohexane were obtained from Dr. Ehrenstopher GmbH, Germany. IS was used for sample quantification and SS was used for the correction of recoveries of PAH concentrations. All working PAH, internal and surrogate standard solutions were diluted with *n*-hexane (*v*/*v*) and stored in a refrigerator at −8 °C. Before the PAH analysis, all glassware was pre-washed with acetone followed by *n*-hexane and dried in an oven at 150 °C for 2 h.

2.4. Sample treatment and extraction

PAH–specific analysis of samples was based on EPA Method 8275A (USEPA, 1996) using the ultrasonic extraction according to EPA Method 3550C (USEPA, 2007). A total of 15.0 g of dry weight (dw) and homogenized ash sample ($n = 4$) was weighed into the titration flasks (100 ml) and 30 ml of *n*-hexane/acetone extraction mixture (2:1, *v*/*v*), and surrogate solution (500 ng/ml) were added into each flask. The samples were extracted with the ultrasonic extraction system (Bandelin Sonorox Digitec DT510/H, Germeny). The samples were ultra-sonicated for 30 min and shaken for 1 h on an orbital shaker (GFL 3017, Germany). The samples were then mixed with 50.0 ml of deionized water. After phase separation, the upper *n*-hexane layer was pipetted out, and subsequently cleaned with Sep-Pak silica cartridges (Chromservis, Czech Republic) and concentrated to 1.0 ml with gentle flow of N_2. The internal solution (500 ng/ml) was added to the extract before further analysis. Blanks ($n = 2$) were prepared following the same procedure without adding the ash sample. The certified reference material EC 2 – river sediment from the National Research Council of Canada (Analytika, Czech Republic) containing 16 US EPA PAHs with concentrations ranging from 22.0 to 558.0 ng/g dw – was used for quality control and prepared following the same procedure ($n = 2$). The ash extracts were analyzed using gas chromatography equipped with mass spectrometry detection (GC/MS).

2.5. GC/MS instrumentation and chromatographic conditions

The analysis of PAHs was performed using an Agilent HP 6890 N gas chromatograph equipped with an Agilent 7683B injector including an Agilent 10.0 μl syringe and connected to an Agilent HP 5975 inert mass selective detector (GC/MSD, 6890N/5975, Agilent Technologies, USA). The separation of PAHs was carried out using a DB-EUPAH (20 m × 0.18 mm inner diameter, 0.14 μm film thickness) capillary column (Agilent J&W Scietific, USA). For the care and protection of the capillary column against contamination from non-volatile residues and interaction of sample components the deactivated fused silica pre-column (5 m × 0.18 mm inner diameter) was used (Agilent Technologies, USA). Pure helium 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, 1 min, purge flow 70 ml/min at 0.75 min, pulsed pressure 25.0 PSI (pound per square inch) and kept at an initial temperature of 300 °C. The temperature of mass selective detector was 300 °C and the quadrupole was at 180 °C. The temperature program of the DB-EUPAH capillary column was initially held at 50 °C for 1 min, then raised to 300 °C/min at a rate of 10 °C/min and held for 10 min at a final temperature of 300 °C. The mass spectrometer was operated using the electron ionization (70 eV) and data acquisition was performed on selective ion monitoring (SIM) mode with characteristic molecular ions of each PAH.

2.6. Identification, quantification and analytical characteristics

The presence of individual PAHs was confirmed by the retention times (9.942–30.285 min) and abundance of quantification/confirmation (q/c) ions (128, 152, 154, 166, 178, 202, 228, 252, 276, 278) in the PAH standard solution. Quantification of individual PAHs was based on the five–point calibration curve (10–1000 ng/ml). The calibration was performed by means of linear regression analysis on a standard mixture of PAHs prepared at the beginning of each run by serial dilutions of the stock solution (1000 ng/ml) to 10, 50, 100, 500, 1000 ng/ml (each point in duplicate). The calibration curve showed acceptable linearity, with correlation coefficients >0.9985 for individual PAHs. All PAHs were quantified using the relative response factors related to IS and concentrations were corrected for the SS recoveries (q/c ions 172 and 306). The accuracy of the determined PAHs was verified by certified EC 2 reference material. The relative standard deviation (RSD) values were estimated from response factors of the calibration curve and the error of individual PAH content in reference material ($n = 4$). The RSDs were below 20%, which was acceptable. Detection limits were estimated from 3 SD/*a* where SD stands for the mean standard deviation of peak areas integrated at the retention time of PAHs of repeated analysis of PAHs at 10 ng/ml ($n = 4$) and *a* for the slope of the calibration curve. Detection limits ranged from 0.5–5.4 ng/g dw. A fresh calibration curve, blanks and reference samples were carried out at the beginning of each run.

2.7. Statistical analyses

The results of PAH content in this study are presented as mean ± standard error of the mean estimated from four replications. If some of individual PAHs were not detected, the half value of their limit of detection was included for the statistical analyses. The evaluation of significant differences in the content of LMW, MMW and HMW PAHs in each group according to the operating temperature and fuel type in Fig. 1 was performed using the non-parametric Kruskal–Wallis test when the homogeneity of variance and normality (Levene and Shapiro–Wilk tests) were not confirmed because the conditions of Analysis of variance (ANOVA) were not met. The differences in the PAH content were detected at the level of significance when p-value was >0.05 ($p < 0.05$). The correlation between the LMW, MMW, HMW, total PAH content (sum of 16 US EPA PAHs) and each other in ash in Table 3 was performed using the Spearman's rank correlation coefficient (r_s) analysis at $p < 0.05$ after the Shapiro–Wilk test was not confirmed. The Pearson product-moment correlation coefficients in Table 4 were determined by multivariate regression analysis, to evaluate the relationship between the LMW, MMW, HMW and total PAHs in fly ash (Y variables) and bottom ash (X variables). The relationship between the total PAH content (Y variables) and unburned carbon, determined as L.O.I. values (X variables) in Fig. 2 was analyzed by stepwise simple linear and non-linear (polynomial) regression analysis. The regression models were verified using the analysis of variance for regression at $p < 0.05$ and the Durbin–Watson test. The statistical analyses and all figures were conducted in Statistica 12 CZ, Statsoft, Tulsa, USA.

3. Results and discussion

3.1. Content of individual and total PAHs in ash

The contents of the individual 16 US EPA PAHs and levels of PAHs in fly and bottom ashes derived from phytomass and dendromass are shown in Tables 1 and 2. The PAHs were divided into three groups according to their molecular weight: 1) LMW PAHs (NA, ACE, AC, FL, PH and AN); 2) MMW PAHs (FLU, PY, BaA and CH); 3) HMW PAHs (BbF, BkF, BaP, IP, DA and BP). The group of total PAHs represents the sum of individual 16 US EPA PAHs.

The data of individual PAHs and levels of LMW, MMW, HMW and total PAH content in ashes showed wide variability in this study. The results showed that fly ashes contain higher amounts of PAHs than bottom ashes. Masto et al. (2015) have described that fly ashes usually have a higher specific surface area than bottom ashes and adsorption of more condensed PAHs by fly ash is possible. The amount of individual PAHs in phytomass fly ash considerably varied from 110.8 ± 28.5 ng/g dw for FL to 8287.2 ± 1492.4 ng/g dw for NA. The total PAH content in phytomass fly ash (53,800.9 ± 13,818.4 ng/g dw) was much higher than the 6161 ng/g dw total PAH content reported by Straka and Havelcová (2012). However, Johansson and van Bavel (2003a)

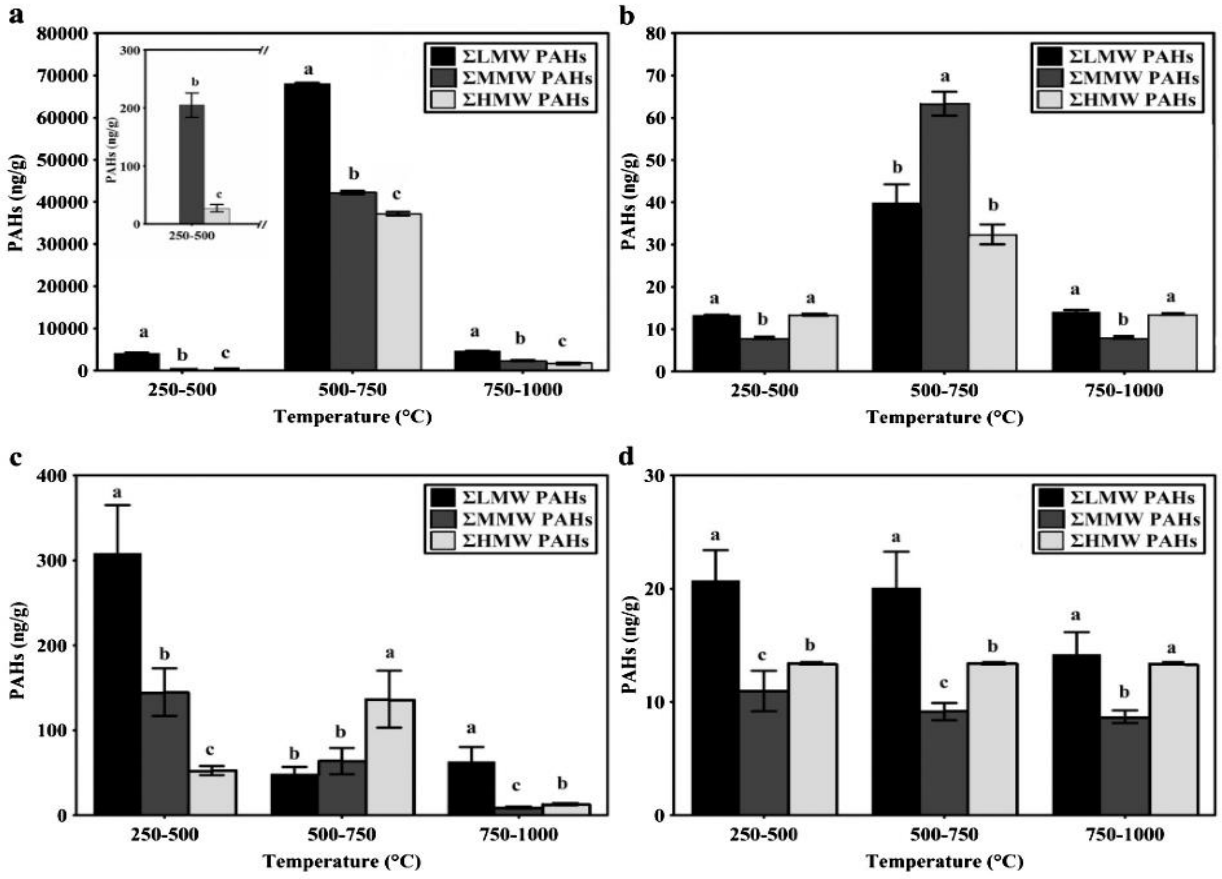

Fig. 1. Influence of the operating temperature of biomass incineration plants on LMW, MMW and HMW PAHs content (ng/g dw) in a) phytomass fly ash, b) phytomass bottom ash, c) dendromass fly ash, and d) dendromass bottom ash ($N = 24$ samples in each group). Error bars indicate standard error of the mean ($n = 4$); columns not sharing the same letter indicate significant differences between means according to the Kruskal–Wallis test at the 95% confidence level.

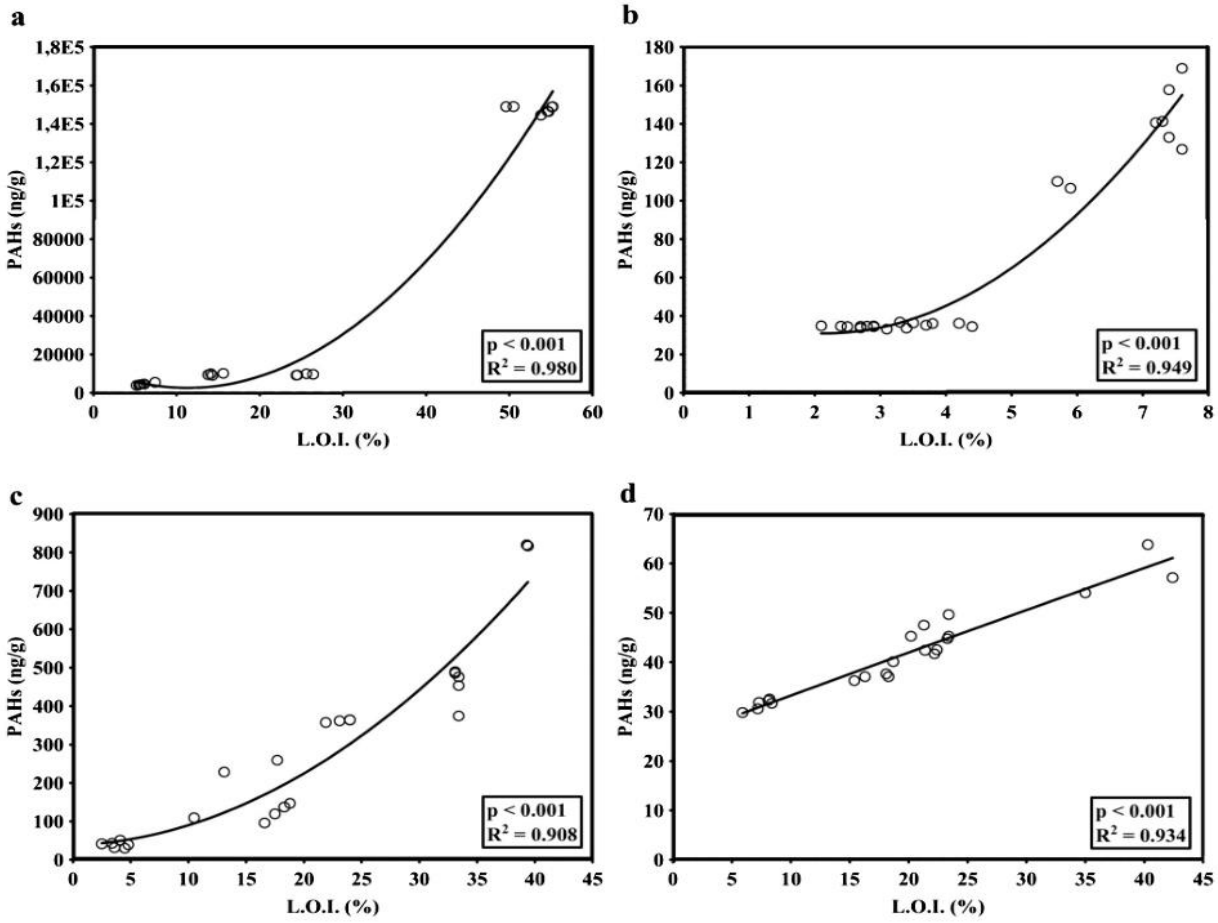

Fig. 2. Relationship between the content of sum of 16 US EPA PAHs (ng/g; $n = 4$) and L.O.I. (%) in a) phytomass fly ash, b) phytomass bottom ash, c) dendromass fly ash, and d) dendromass bottom ash ($N = 24$ samples in each group).

Table 1
Main statistical characteristics of PAHs data (ng/g dw; $n = 4$) in phytomass fly ash and bottom ash ($N = 24$ samples in each group).

	Fly ash						Bottom ash					
PAHs	Mean	SEM[a]	Median	Min	Max	c_V[b]	Mean	SEM	Median	Min	Max	c_V
NA	8287.2	1492.4	3407.2	2673.3	18,542.7	88.2	9.4	0.8	7.7	5.2	17.4	42.5
ACE	4036.8	1039.7	826.3	113.5	11,564.0	126.2	2.4	0.5	0.9	0.9	7.2	95.2
AC	1088.7	320.0	33.6	1.1	3704.1	144.0	1.1	0.0	1.1	1.1	1.1	0.0
FL	110.8	28.5	29.9	1.7	334.2	126.2	1.7	0.0	1.7	1.7	1.7	0.0
PH	5599.4	1520.2	658.0	221.2	16,035.6	133.0	6.5	1.7	1.8	1.8	25.8	131.3
AN	6491.5	1862.4	286.0	18.8	19,320.7	140.6	1.3	0.0	1.3	1.3	1.3	0.0
FLU	4875.2	1249.4	1135.4	75.0	14,273.0	125.5	9.2	2.3	1.7	1.7	31.3	120.9
PY	5607.6	1502.2	943.6	60.2	16,234.4	131.2	11.8	3.1	1.7	1.7	36.6	126.8
BaA	2089.7	599.0	129.2	1.6	6682.6	140.4	9.7	2.0	1.6	1.6	4.8	45.3
CH	2439.0	680.6	262.5	2.8	7084.3	136.7	3.2	0.2	2.8	2.8	6.1	28.9
BbF	4257.1	1208.2	387.9	1.7	13,523.7	139.0	1.7	0.0	1.7	1.7	1.7	0.0
BkF	2539.1	702.6	333.9	0.7	7513.8	135.6	0.7	0.0	0.7	0.7	0.7	0.0
BaP	4189.3	1169.8	458.9	2.7	12,793.3	136.8	5.4	0.8	2.7	2.7	15.2	76.6
IP	517.7	97.4	114.9	2.8	1187.6	124.0	3.8	0.3	2.8	2.8	2.8	0.0
DA	405.8	102.7	413.7	2.7	1169.9	92.6	2.8	0.0	2.7	2.7	7.1	42.7
BP	2307.4	589.9	625.7	2.8	6389.0	125.2	5.3	0.9	2.8	2.8	15.5	86.2
LMW PAHs	25,614.4	6252.9	4712.9	3672.3	68,860.4	119.6	22.4	2.9	14.5	12.0	52.9	64.3
MMW PAHs	14,946.4	4031.0	2343.9	144.6	43,190.9	132.1	26.3	5.5	7.8	7.8	77.1	103.0
HMW PAHs	13,000.8	3575.2	1813.7	13.4	38,374.2	134.7	19.7	2.0	13.4	13.4	40.7	49.9
Total PAHs	53,800.9	13,818.4	9509.8	3864.1	148,991.5	125.8	68.5	10.2	35.7	33.2	168.9	72.9

[a] Standard error of the mean.
[b] Coefficient of variation (%).

Table 2
Main statistical characteristics of PAHs data (ng/g dw; $n = 4$) in dendromass fly ash and bottom ash ($N = 24$ samples in each group).

	Fly ash						Bottom ash					
PAHs	Mean	SEM[a]	Median	Min	Max	c_V[b]	Mean	SEM	Median	Min	Max	c_V
NA	80.7	19.3	48.4	2.2	312.3	117.3	8.1	1.2	6.0	2.3	27.9	75.0
ACE	22.7	8.2	6.5	0.9	145.2	177.0	2.1	0.3	1.7	0.9	7.6	70.5
AC	1.8	0.4	1.1	1.1	8.2	107.9	1.1	0.0	1.1	1.1	1.1	0.0
FL	2.2	0.3	1.7	1.7	7.5	73.3	1.7	0.0	1.7	1.7	1.7	0.3
PH	28.1	7.6	13.0	1.8	136.4	132.1	3.8	0.6	2.0	1.2	10.9	73.3
AN	4.4	0.9	1.3	1.3	13.9	104.2	1.5	0.1	1.3	1.3	3.2	29.3
FLU	20.3	5.3	4.0	1.7	86.7	128.4	2.6	0.4	1.7	1.7	8.3	65.1
PY	23.3	4.9	25.7	1.7	78.8	102.7	2.3	0.3	1.7	1.1	8.8	71.8
BaA	9.4	2.0	1.6	1.6	31.5	105.1	1.6	0.0	1.6	1.6	1.6	0.3
CH	19.4	4.3	21.1	2.8	76.7	108.1	2.8	0.0	2.8	2.8	2.8	0.0
BbF	18.2	3.1	24.1	1.7	36.2	84.3	1.7	0.0	1.7	1.7	1.7	0.0
BkF	7.5	2.4	0.7	0.7	30.5	156.6	0.7	0.0	0.7	0.7	0.7	0.0
BaP	9.2	2.2	2.7	2.7	30.0	117.1	2.7	0.0	2.7	2.7	2.7	0.0
IP	3.1	0.2	2.8	2.8	6.3	29.6	2.8	0.0	2.8	2.8	2.8	0.0
DA	13.9	4.3	2.7	2.7	55.2	152.9	2.7	0.0	2.7	2.7	2.7	0.0
BP	16.8	4.6	2.8	2.8	56.4	134.7	2.8	0.0	2.8	2.8	2.8	0.0
LMW PAHs	139.9	31.3	77.3	9.0	521.3	109.6	18.3	1.6	19.0	8.6	36.8	42.5
MMW PAHs	72.4	15.5	70.8	7.8	262.0	105.1	9.6	0.7	7.9	7.8	21.4	34.5
HMW PAHs	67.5	15.2	40.7	13.4	205.8	110.7	13.4	0.0	13.4	13.4	13.4	0.0
Total PAHs	279.0	47.5	244.0	30.2	819.8	83.5	41.1	1.8	40.9	29.8	63.8	22.0

[a] Standard error of the mean.
[b] Coefficient of variation (%).

published comparable results of total PAHs ranging from 57,822 to 77,086 ng/g dw in fly ashes derived from biofuels.

The content of individual PAHs in phytomass bottom ashes, as well as in dendromass ashes, was much lower than in fly ash and the highest levels were seen for PY (11.8 ± 3.1 ng/g dw). The total sum of PAHs was 68.5 ± 10.2 ng/g dw in phytomass bottom ash. The dendromass fly ash contained a total PAH concentration of 279.0 ± 47.5 ng/g dw and dendromass bottom ash reached a total concentration of 41.1 ± 1.8 ng/g dw.

The PAHs with low molecular weight prevailed in all tested ashes derived from biomass. This corresponds with work reported by Rey-Salgueiro et al. (2016), while Singh et al. (2013) reported that MMW PAHs were predominant in gaseous phases emitted from biomass fuels. García-Falcón et al. (2006) suggested, that the predominance of low and medium molecular weight PAHs reflects the presence of PAHs from combustion products derived from low temperature pyrolytic processes or even from combustion of petrogenic fuels.

Baumard et al. (1998) suggested the following PAH pollutant level classification: 1) low (0–100 ng/g); 2) moderate (100–1000 ng/g); 3) high (1000–5000 ng/g); 4) very high (>5000 ng/g). The phytomass fly ash in our study can be characterized as having a very high pollution level, dendromass fly ash as a moderate pollution level and both bottom ashes as low PAH pollution.

The quality of the biomass ashes in respect to PAH content is assessed by the European Union (EU) legislation described by Johansson and van Bavel (2003b). Nevertheless, the limit amounts of PAH content in biomass ashes intended for field applications, are set in many European countries differently. The Swedish EPA has developed generic guidelines for PAHs in soils. For sensitive land use the limits are set to 300 ng/g for carcinogenic PAHs (the sum of BaA, CH, BbF, BkF, IP, DA) and 20,000 ng/g for the non-carcinogenic PAHs (Johansson and van Bavel, 2003a). Our results in this study indicate that the phytomass fly ash poses a high potential risk to the environment and biota, because the amounts of carcinogenic PAHs ranged from 405.8 ± 102.7 ng/g for DA to 4189.3 ± 1169.8 ng/g dw for BaP.

According to the Czech law No. 377/2013, the limit of total PAH content in biomass ashes is 20,000 ng/g dw (Ministry of Agriculture of the Czech Republic, 2014). Haglund (2008) have described in the NT (Nord Test) Technical Report 613 (NT TR 613), that the limit amount of PAHs in biomass ashes must be lower than 3000 ng/g dw.

The results of this study show that PAH content in fly ash can exceed the limit of 3000 ng/g as well as the limit of 20,000 ng/g dw which can pose serious threats to the environment if the fly ash would be used in agriculture because it may influence several important health effects as described by Nisbet and LaGoy (1992) and inhibit growth/response of soil microbial activity (Feng et al., 2014). Nevertheless, Fabbri et al. (2013) described individual PAH contents up to 19,000 ng/g dw in biochar without any adverse effect on the environment during the agricultural field applications.

The utilization of dendromass ashes and phytomass bottom ashes as a mineral fertilizer or organic amendment in agricultural soil is possible, based on its PAH content. However, based on our results, the utilization of phytomass fly ashes in agriculture is not recommended. The ashes, which are restricted from the utilization in agriculture, should be landfilled or can be used as a construction material in industry or in cement production (Demirbas et al., 2009; Gómez-Barea et al., 2009). In practise, the mixing of fly and bottom ashes is, unfortunately, the most common way to decrease the content of PAHs (James et al., 2012). However, the PAHs, present in ashes, can be also decreased via re-burning (Sarenbo, 2009).

3.2. Correlation and relationship between the LMW, MMW, HMW and total PAH content in ash

Correlations between the content of LMW, MMW, HMW and total PAHs (sum of 16 US EPA PAHs) in 96 ash samples were conducted. Table 3 shows the results of correlation analysis. Positive correlations can be observed between the LMW, MMW, HMW and total PAH content in ash at $p < 0.05$. Spearman's rank correlation coefficient (r_s) varied from 0.8025 to 0.9790. The content of PAHs in ash correlated significantly between each other at the 95% level of confidence. Pearson product-moment correlation coefficients (r) between the LMW, MMW, HMW and total PAH content in fly ash and bottom ash were also determined using multivariate regression analysis, to evaluate the relationship between the distributions of PAHs in 48 fly ash and 48 bottom ash samples. The results in Table 4 demonstrate the existence of positive relationships between the distribution of PAH content in the fly ash and bottom ash. Significant correlations at $p < 0.05$ can be observed between the HMW PAH content in bottom ash and LMW, MMW, HMW, total PAH content in fly ash, with correlation coefficients between 0.9446 and 0.9599. The correlations between the content of LMW,

Table 3
Spearman's rank correlation coefficient (r_s) between the LMW, MMW, HMW and total PAH content in ash ($p < 0.05$).

	LMW PAHs	MMW PAHs	HMW PAHs	Total PAHs
LMW PAHs	–	**0.8932**[a]	**0.8025**	**0.9790**
MMW PAHs	–	–	**0.8862**	**0.9562**
HMW PAHs	–	–	–	**0.8846**
Total PAHs	–	–	–	–

[a] Bold numbers mean significant correlation at the 95% level of confidence.

MMW and total PAHs in bottom ash and LMW, MMW, HMW and total PAH content in fly ash were not significant at $p < 0.05$.

3.3. Effect of temperature on PAH content in ash

The effect of the operating temperature of commercial incineration plants on LMW, MMW and HMW PAH content in ashes was investigated and tested ash samples were sorted in Fig. 1 into three groups according to operating temperature: 1) 250–500 °C, 2) 500–750 °C, and 3) 750–1000 °C (eight samples in each group). Our data demonstrate that PAH content varied widely based on the type of biomass fuel used. Significant differences in PAH content at different temperature groups were detected by Kruskal–Wallis test at the 95% confidence level.

Such strong differences in PAH contents among the different temperature ranges are probably caused by many undesirable reactions, which may occur during the combustion process. Based on the models of Chagger et al. (2000), formation of PAHs occurred mainly during the start-up and quenching processes for the species, which were thermodynamically favoured. Therefore, the variability in combustion temperature and availability of oxygen in furnaces may have strong impact on the formation of PAHs.

The highest total PAH content (147,464.1 ± 1703.8 ng/g dw) was found in phytomass fly ash formed at temperature 500–750 °C. These ashes also contained the highest content of carcinogenic PAHs (46,079.6 ± 947.6 ng/g dw). At temperature 750–1000 °C, the total PAH content in phytomass fly ash (9555.4 ± 139.9 ng/g dw) decreased as well as the content of carcinogenic PAHs (1646.4 ± 58.3 ng/g dw). The total PAH content in phytomass fly ash (4383.2 ± 174.3 ng/g dw) formed at temperature 250–500 °C was also relatively high while the carcinogenic PAHs were below the limits of detection.

The levels of all PAHs investigated varied at the three different temperature intervals. The highest level of LMW PAHs (68,017.7 ± 666.9 ng/g dw) was observed in phytomass fly ash at temperatures between 500 and 750 °C and the PAH content significantly decreased as molecular weight increased. The most abundant MMW PAH content in phytomass bottom ash in the same temperature range was lower than 63.4 ± 8.0 ng/g dw. A different trend was observed in dendromass fly ash where the PAH content significantly decreased as molecular weight increased in group of 250–500 °C. The content of HMW PAHs was predominant between 500 and 750 °C and the levels of MMW and LMW PAHs significantly decreased as molecular weight decreased. The PAH levels in dendromass bottom ash were comparable in all groups of operating temperature.

The literature does not report sufficient examples of LMW, MMW, HMW and total PAH content in biomass ashes formed in our temperature ranges to allow for comparison with other studies. Masto et al. (2015) observed a total PAH content between 200 and 193,000 ng/g dw in fly ash derived from bio-wastes and municipal solid wastes formed at a temperature around 850 °C, and bottom ashes contained <420 ng/g of total PAHs at this temperature. Dai et al. (2014) reported the highest yield of PAHs at 950 °C during pyrolysis of sewage sludge. Several examples of high PAH content in wood fly ash formed during the undefined operating temperature of incineration were published by Enell et al. (2008) and Sarenbo (2009). A much lower PAH content of 2.9 ng/g dw was quantified by Rey-Salgueiro et al. (2016) in bottom ash derived from wood waste and forest residues, which were burned at temperatures between 400 and 500 °C. Levendis et al. (1998) reported the complete decomposition of organic pollutants and a drop in the content of individual PAHs to below the limit of detection when temperatures between 1000 and 1300 °C were used.

Table 4
Pearson product-moment correlation coefficients (r) between the LMW, MMW, HMW and total PAH content in fly ash and bottom ash.

		Fly ash			
		LMW PAHs	MMW PAHs	HMW PAHs	Total PAHs
Bottom ash	LMW PAHs	−0.0784	−0.1326	−0.1357	−0.1089
	MMW PAHs	0.2412	0.1897	0.1881	0.2118
	HMW PAHs	**0.9599**[a]	**0.9448**	**0.9446**	**0.9515**
	Total PAHs	0.2801	0.2279	0.2255	0.2506

[a] Bold numbers mean significant correlation at the 95% level of confidence.

3.4. Relationship between the total PAH content and unburned carbon in ash

The amount of unburned combustible carbon in ash samples was determined as a loss of ignition (L.O.I.). Our results of L.O.I. values varied as follows: a) in phytomass fly ash: 5.2–55.2%; b) in phytomass bottom ash: 2.1–7.6%; c) in dendromass fly ash: 2.5–39.4%; d) in dendromass bottom ash: 5.9–42.4% (eight samples in each group). The total PAH emissions can be associated with the coarsest ash particles released during the combustion. This fact could be explained by the internal surface of coarsest particles with the hydroscopic pore structure offering the surface for PAH condensation and a higher amount of unburned carbon in biomass ashes indicates the presence of highly polycondensed aromatic compounds, which exist as a result of incomplete combustion (Chagger et al., 2000; Janvijitsakul and Kuprianov, 2007). Conversely, the biomass ashes with carbon burnout larger than 99% should not be considered as hazardous (Hustad et al., 1995).

However, biomass ashes are reported to contain substantial amounts of the unburned carbon depending on the type of incineration and combusted fuel. In the study of Gómez-Barea et al. (2009), biomass fly ashes from fluidised-bed gasification pilot plant contained 0.6–20.3% of unburned carbon depending on combustion temperature and the spot of ash collection. Duan et al. (2012) reported L.O.I values of sludge and coal fly ashes from circulating fluidized bed boiler in range of 8.8–14.3%. Similar range (8.3–16.9%) for biomass fly and bottom ashes, was published by Masto et al. (2015). Our results of L.O.I in biomass ashes are comparable with the results of Rey-Salgueiro et al. (2016), who reported wider range of 1–78%.

The utilization of these ashes containing higher amounts of unburned carbon as soil additives may pose serious risks to the environment and Haglund (2008) reported that for unburned organic matter content higher than 5% (wt) the content of PAHs in the ash needs to be determined. The results of Enell et al. (2008) indicate a possible direct relationship between the L.O.I. and PAH content in ashes.

When our results of PAH content – sum of 16 US EPA PAHs (ng/g; Y variables) in ashes were plotted as a function of L.O.I. ($\%_{wt}$; X variables) the regression analyses were established. The relationship between the total PAH content and L.O.I. was analyzed by stepwise simple linear and non-linear (polynomial) regression analysis when the stronger polynomial (quadratic) regression model at $p < 0.001$ was estimated for the following cases: a) phytomass fly ash, b) phytomass bottom ash, and c) dendromass fly ash. The regression lines in each plot in Fig. 2 were designed and regression functions ($y = y_0 + ax + bx^2$) were estimated as follows: a) in phytomass fly ash: $y = 12{,}862.514 - 1815.603x + 80.184x^2$, b) in phytomass bottom ash $y = 50.196 - 17.915x + 4.171x^2$, and c) in dendromass fly ash

$y = 38.097 + 1.046x + 0.415x^2$. The linear regression model was conducted in the case of dendromass bottom ash. The regression line was designed and the regression function ($y = y_0 + ax$) in plot d) in Fig. 2 was described as $y = 24.465 + 0.863x$. The coefficients of determination (r^2) of each regression model were estimated as follows: a) $r^2 = 0.980$ for phytomass fly ash, b) $r^2 = 0.949$ for phytomass bottom ash, c) $r^2 = 0.908$ for dendromass fly ash, and d) $r^2 = 0.934$ for dendromass bottom ash. Proposed regression models were verified using the analysis of variance for regression when the p value was lower than 0.001 and the Durbin–Watson test was confirmed.

The exponential regression model was conducted in case of fly ash from both biomass types together and linear regression model in the case of bottom ashes. The regression functions were estimated as follows: a) in all biomass fly ashes: $y = e^{(0.219x)}$ with $r^2 = 0.860$ and $p < 0.001$; b) in all biomass bottom ashes: $y = 56.1652 - 0.1191x$ with $r^2 = 0.001$ and $p = 0.83$. The regression model of biomass bottom ashes is consider as not relevant, because the p value was higher than 0.05 and the Durbin–Watson test was not confirmed. This was due to the presence of relatively high PAH content vs. low L.O.I. in phytomass bottom ashes incinerated at temperature 500–750 °C. We expected that with increasing temperature of incineration, the total PAH content will decrease along with decreasing values of L.O.I. in ashes. However, the total PAH content in phytomass bottom ashes derived at temperature 500–750 °C increased, even in those ashes, whose L.O.I. was very low (5–10%). These phenomena could be related to undesired reactions during phytomass combustion, such as ash agglomeration and sintering, which may ensure momentary conditions for PAH formation. Vassilev et al. (2013) described low-temperature formation of a melt during combustion due to the low melting points of the formed K, Na, K—Na, K—Ca and K—Mg silicates. According to Risnes et al. (2003), salts composed of alkali-chlorides, carbonates and hydroxides represent high potential for adverse ash sintering. In their study, K-aluminosilicate, with a melting point at 695 °C, was predominated compound in wheat straw bottom ash. Subsequently, Straka and Havelcová (2012) showed that the high amount of PAHs can be preserved easily in the formed porous and mesoporous system of ashes even with low unburned carbon content.

When the regression model was conducted in biomass bottom ashes excluding the phytomass ashes derived at temperature 500–750 °C, the regression function was estimated as $y = 30.713 + 0.622x$ with $r^2 = 0.820$ and $p < 0.001$. The Table 5 shows the regression function of each relationship between the PAH content and unburned carbon estimated as L.O.I. in biomass ashes. The derived regression functions can be used for the estimation of the PAHs mass loading during incineration via unburned carbon in fly and bottom ashes.

Table 5
Statistic equations for the estimation of the total PAH content during biomass incineration via unburned carbon in fly and bottom ashes.

Ash	Biomass	Equation[a]	R^2	p
Fly	Phytomass	$y = 12{,}862.514 - 1815.603x + 80.184x^2$	0.980	<0.001
Fly	Dendromass	$y = 38.097 + 1.046x + 0.415x^2$	0.908	<0.001
Fly	Phytomass and dendromass	$y = e^{(0.219x)}$	0.860	<0.001
Bottom	Phytomass	$y = 50.196 - 17.915x + 4.171x^2$	0.949	<0.001
Bottom	Dendromass	$y = 24.465 + 0.863x$	0.939	<0.001
Bottom	Phytomass and dendromass[b]	$y = 30.713 + 0.622x$	0.820	<0.001

[a] y variable: total PAH content (ng/g dw); x variable: unburned carbon estimated as L.O.I. ($\%_{wt}$).
[b] Excluding biomass bottom ashes derived from phytomass at temperatures 500–750 °C.

4. Conclusions

Analyses of the individual content of 16 US EPA PAHs were carried out in fly and bottom ash samples collected from 48 different commercial biomass incineration plants in the Czech Republic.

The PAH content in ashes varied widely among the types of biomass fuel and operating temperatures. The highest content of total PAHs (147,464.1 ± 1703.8 ng/g dw) was found in phytomass fly ash formed at temperatures 500–750 °C. In general, fly ashes contained much higher levels of PAHs than bottom ashes. With respect to PAH content in ashes, the combustion of phytomass was evaluated as riskier than the combustion of dendromass.

The content of LMW, MMW, HMW and total PAHs in ashes correlated significantly among each other in biomass ashes, with correlation coefficients from 0.8025 to 0.9790. The Pearson product-moment correlation revealed strong correlation between the content of HMW in bottom ashes and LMW, MMW, HMW and total PAH contents in fly ashes. This leads to the suggestion, that the content of HMW in bottom ashes can be used as an indicator of PAH emissions during the combustion process. The quadratic relationship between the total PAH content and L.O.I. was observed for phytomass ashes and dendromass fly ashes while dendromass bottom ashes followed linear relationship. The regression coefficients of determination from 0.908 to 0.980 were estimated.

The statistic equations predicting the PAHs mass loading during incineration via unburned carbon in fly and bottom ashes were estimated as follows: a) in all biomass fly ashes: PAHs [ng/g] $= e^{(0.219*L.O.I[\%wt])}$ and b) in biomass bottom ashes excluding biomass bottom ashes derived from phytomass at temperature from 500 to 750 °C: PAHs [ng/g] $= 30.713 + 0.622^*$L.O.I. [$\%_{wt}$].

Based on our results, both types (fly and bottom) of dendromass ashes and phytomass bottom ashes can be recommended as the environmental-friendly fertilizers in respect to PAH content according to Swedish EPA, Czech law No. 377/2013 and NT TR 613. All our samples (72), belonging to these groups, did not exceed the limit value of 3000 ng/g dw given by NT TR 613. Therefore, these ashes could be applied directly on agricultural soils.

According to the Swedish EPA, we do not recommend the soil application of phytomass fly ashes derived at temperature range of 500–1000 °C. These ashes can exceed the limit content (300 ng/g dw) of carcinogenic PAHs. According to NT TR 613, also the phytomass fly ashes formed at temperature from 250 to 500 °C cannot be recommended for soil application, as they are usually exceeding the limit value of 3000 ng total PAH/g dw.

In our study, the total PAH content in ashes was highly dependent on the unburned carbon content in ash derived from biomass. This unburned carbon content is widely considered as an indicator of efficiency of the combustion process. Therefore, all the technological steps in combustion plants should be aimed to reach high efficiency of the combustion process. Highly effective technologies, such as high-end bubbling fluidized bed or circulating fluidized bed systems, may eliminate the PAH formation in ashes derived from biomass combustion.

Conflict of interests

The authors declare no conflict of interest.

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Název: Ability of natural attenuation and phytoremediation using maize (*Zea mays* L.) to decrease soil contents of polycyclic aromatic hydrocarbons (PAHs) derived from biomass fly ash in comparison with PAHs–spiked soil.

Autoři: Zdeněk Košnář, Filip Mercl, Pavel Tlustoš.

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Ability of natural attenuation and phytoremediation using maize (*Zea mays* L.) to decrease soil contents of polycyclic aromatic hydrocarbons (PAHs) derived from biomass fly ash in comparison with PAHs–spiked soil

Zdeněk Košnář*, Filip Mercl, Pavel Tlustoš

Department of Agro Environmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague 6 - Suchdol, Czech Republic

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ABSTRACT

A 120–day pot experiment was conducted to compare the ability of natural attenuation and phytoremediation approaches to remove polycyclic aromatic hydrocarbons (PAHs) from soil amended with PAHs–contaminated biomass fly ash. The PAH removal from ash–treated soil was compared with PAHs–spiked soil. The removal of 16 individual PAHs from soil ranged between 4.8% and 87.8% within the experiment. The natural attenuation approach led to a negligible total PAH removal. The phytoremediation was the most efficient approach for PAH removal, while the highest removal was observed in the case of ash–treated soil. The content of low molecular weight (LMW) PAHs and the total PAHs in this treatment significantly decreased ($P < .05$) over the whole experiment by 47.6% and 29.4%, respectively. The tested level of PAH soil contamination (~1600 μg PAH/kg soil dry weight) had no adverse effects on maize growth as well on the biomass yield. In addition, the PAHs were detected only in maize roots and their bioaccumulation factors were significantly lower than 1 suggesting negligible PAH uptake from soil by maize roots. The results showed that PAHs of ash origin were similarly susceptible to removal as spiked PAHs. The presence of maize significantly boosted the PAH removal from soil and its aboveground biomass did not represent any environmental risk.

1. Introduction

The thermal conversion of bio–fuels in power plants for generating heat and electricity is proposed as one of the most promising way to mitigate the greenhouse gas emissions (Abbasi and Abbasi, 2010). Beyond the main products i.e. CO_2 and H_2O, flue gas emissions from biomass combustion may include some pollutants associated with incomplete combustion. These emissions are referred to the primary stage furnace effluents and include CO, volatile and semi–volatile hydrocarbons, and particulate matter (Tarelho et al., 2011). The released unburned hydrocarbons include primary traces of organic contaminants, especially of polycyclic aromatic hydrocarbons (PAHs) which are formed during the volatile combustion phase of the primary stage furnace effluents (Bragato et al., 2012). According to Palma (2013), PAH compounds are formed by a direct combination of fused aromatic rings. Kaisalo et al. (2015) suggested the role of radical addition reactions of ethylene and acetylene with aromatic rings in the formation and sequential growth of PAHs.

The PAHs are ubiquitous organic contaminants which consist of two or more benzene rings with toxic, mutagenic, teratogenic and carcinogenic properties (IARC, 2010). Sixteen basic PAHs are included in the United States Environmental Protection Agency (16 US EPA PAHs) priority pollutant list because they may pose serious threats to the environment and biota. Due to their lipophilic character PAHs tend to accumulate in soils, sediments, and sewage sludge (Vácha et al., 2005; Dvořák et al., 2017; García-Sánchez et al., 2018). The sorption of PAHs onto organic matter results in the low bioavailability of PAHs and therefore recalcitrance of PAHs to biodegradation. This process is known as the environmental "aging" of PAHs (Boopathy, 2000).

Besides the gas emissions, biomass ashes are generated as waste products from biomass incineration in power plants. The ashes can be considered as chemically and physically variable products because of different inputs to the combustion process. Nevertheless, biomass ashes are characterized by strongly alkaline pH values and usually contain high amounts of mineral nutrients such as Ca, K, P, and Mg. Therefore, there is an effort to recycle biomass ashes as soil amendments and/or fertilizers (Mercl et al., 2016; Ochecová et al., 2017). However, the unfavourable conditions of burning process, mainly incomplete combustion, can lead to the accumulation of PAHs in biomass ashes (Chagger et al., 1998). The yields of PAHs vary considerably between

* Corresponding author.
E-mail address: kosnarz@af.czu.cz (Z. Košnář).

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bottom and fly ashes (Košnář et al., 2016) and are strongly affected by the physico–chemical properties of biomass fuel input, incineration temperature and other operating conditions of combustion (Chagger et al., 2000). Masto et al. (2015) observed the total PAH content in biomass ashes up to 193 mg/kg at temperatures around 850 °C. The possible PAH content in ashes applied in soil have not received considerable attention. Therefore, PAHs–polluted biomass ashes could be a possible source of agricultural soil contamination (Enell et al., 2008).

Phytoremediation is designed as the possible appropriate bioremediation approach to remove the PAHs from contaminated soils. The presence of plants in PAH contaminated soil is important due to the increasing PAH accessibility in soil. Organic acids and phenols released by roots may increase the PAH solubility in soil solution and PAHs may become more extractable by plants. Nevertheless, plants should be considered as a means to control PAH bioavailability in soil, and to stimulate the PAH biodegradation and irreversible trapping processes of PAHs in soil (Ouvrard et al., 2014). Many works were carried out to study only the potential of varied plants for the extraction of trace elements such as As, Cd, Cr, and Pb in fly ash–contaminated soils (Gupta and Sinha, 2008; Jambhulkar and Juwarkar, 2009). Nevertheless, a study focused on removal of PAHs from biomass ash in soil conditions is still missing. The main aim of this paper was: i) to compare the removal of PAHs from soil amended with PAHs–contaminated biomass fly ash between non–planted (natural attenuation) and planted (phytoremediation using *Zea mays* L.) treatment, and ii) to compare the PAH removal in ash–treated soil with PAHs–spiked soil.

2. Material and methods

2.1. Chemicals and reagents

Acetone and hexane (both for the GC/ECD residue analysis) were purchased from Chromservis, Czech Republic. Anhydrous sodium sulfate (p.a., activated at 400 °C for 4 h, cooled and stored at desiccator before use) was purchased from Lachner, Czech Republic. Standards of 16 US EPA PAHs in a 2000 mg/L mixture solution of each PAH species (SV Mix 5, Restek, USA) was purchased from Chromservis, Czech Republic. The 16 individual PAH names and their abbreviations are listed in Table 1. Internal standard mixture (IS) in a 2000 mg/L mixture solution of each deuterated PAH species (Restek, USA) and surrogate standards (SS) of 2-Fluorobiphenyl (2-FBP) and *p*-Terphenyl-d_{14} (*p*-TER-*d14*) each in a 2000 mg/L solution (Restek, USA) was purchased from Chromservis, Czech Republic. All working PAH, IS and SS solutions were diluted with hexane (v/v). The certified reference material (CZ 7006) containing 16 individual PAHs with concentrations ranging from 40 to 3840 µg/kg dry weight (dw) was obtained from Analytika, Czech Republic.

2.2. Characterization of experimental soil

The experimental soil was collected from a long–term field trial site close to the city of Humpolec (49°33'16"N, 15°21'2"E, Czech Republic). The site characteristics are: altitude, 525 m above sea level; mean annual temperature, 7.0 °C; mean annual precipitation, 665 mm; soil type, Cambisol; soil texture, sandy loam (clay, 5.8%$_{w/w}$; silt, 43.6%$_{w/w}$; sand, 50.6%$_{w/w}$). The experimental soil was prepared by the thoroughly mixing of different sub–samples collected from different zones of the field area at a depth of 0–20 cm. Before establishment of the experiment, the collected non–sterilized soil was homogenized, air–dried at room temperature and passed through a 5 mm stainless steel sieve. The main physical–chemical properties of the experimental soil were: pH ($CaCl_2$), 5.3; CEC, 159 mmol/kg; C_{tot}, 18 g/kg; N_{tot}, 1.7 g/kg; $P_{pseudotot}$, 0.90 g/kg; $K_{pseudotot}$, 9.63 g/kg; $Ca_{pseudotot}$, 1.83 g/kg; $Mg_{pseudotot}$, 8.47 g/kg. The soil was checked for the PAH background contamination and the 16 individual PAHs were below the quantifiable limit (< 5.6 µg/kg dw).

2.3. Characterization of PAHs–contaminated fly ash

The fly ash from commercial biomass power plant operated in the Czech Republic was used in this study. The tested ash originated from the combustion of wheat straw in a grate–fired 20 MW boiler with estimated production of more than 2000 t of ash per year. The incineration temperature declared by the producer was in the range 600–700 °C. The amount of 5 kg of ash sample was taken in four random replications from a container with ash from the electrostatic precipitator. Before the

Table 1
Initial PAH content (µg/kg dw) in collected biomass fly ash (average; n = 4) and experimental ash–amended and PAHs–spiked soil (average; n = 8) at 0 days of the experiment. Means within the same row followed by different lower case letters indicate significant difference ($P < .05$) between the ash–amended and PAHs–spiked soil treatments as determined by Tukey´s test. Treatment abbreviations: SA–ash amended soil; PSA–planted ash amended soil; SS–spiked soil; PSS–planted spiked soil.

	Abbreviation	Fly ash (mg/kg dw)	SA and PSA (µg/kg dw)	SS and PSS (µg/kg dw)
Low molecular weight PAHs:				
Naphthalene	NAP	19.1 ± 0.3	313.4 ± 22^a	99.4 ± 6.7^b
Acenapthylene	ACY	12.6 ± 0.5	55.1 ± 6.0^b	96.4 ± 3.7^a
Acenaphtene	ACE	3.9 ± 0.1	33.8 ± 4.5^a	106.5 ± 13^b
Fluorene	FLU	0.2 ± 0.1	4.1 ± 0.4^b	99.1 ± 4.5^a
Phenanthrene	PHE	17.9 ± 0.5	150.9 ± 8.3^a	97.5 ± 2.2^b
Anthracene	ANT	21.7 ± 0.1	132.4 ± 8.5^a	97.3 ± 6.1^b
Medium molecular weight PAHs:				
Fluoranthene	FLUO	16.3 ± 0.2	146.1 ± 13^a	97.3 ± 4.9^b
Pyrene	PYR	6.5 ± 0.2	159.3 ± 14^a	96.5 ± 4.2^b
Benzo[*a*]anthracene	BaA	7.3 ± 0.3	61.9 ± 3.9^b	99.0 ± 5.1^a
Chrysene	CHR	6.5 ± 0.3	89.3 ± 7.7^a	93.9 ± 6.0^a
High molecular weight PAHs:				
Benzo[*b*]fluoranthene	BbF	13.8 ± 0.5	124.1 ± 11^a	103.9 ± 7.5^a
Benzo[*k*]fluoranthene	BkF	7.6 ± 0.2	79.3 ± 3.7^b	98.4 ± 3.7^a
Benzo[*a*]pyrene	BaP	15.0 ± 0.1	183.1 ± 8.5^a	103.6 ± 4.1^b
Indeno[*l*,2,3-*c*,*d*]pyrene	IPY	1.2 ± 0.1	45.7 ± 2.5^b	99.9 ± 7.2^a
Dibenz[*a*,*h*]anthracene	DBA	3.9 ± 0.3	34.8 ± 2.4^b	108.8 ± 6.4^a
Benzo[*g*,*h*,*i*]perylene	BghiP	6.7 ± 0.4	63.4 ± 5.1^b	103.5 ± 5.8^a
ΣLow molecular weight PAHs	LMW PAHs	75.4 ± 0.7	688.8 ± 17^a	596.2 ± 14^b
ΣMedium molecular weight PAHs	MMW PAHs	36.7 ± 0.6	456.5 ± 28^a	393.8 ± 15^b
ΣHigh molecular weight PAHs	HMW PAHs	48.1 ± 0.7	530.3 ± 13^b	617.4 ± 18^a
Σ16 individual PAHs	Total PAHs	160.2 ± 1.6	1675.6 ± 43^a	1607.4 ± 26^a

PAH analysis the collected ash was air–dried and thoroughly homogenized in the laboratory. The main characteristics and inorganic content determined according to Száková et al. (2013) were: particle size fraction < 0.25 mm, 67.8%$_{w/w}$; size fraction 0.25–1.6 mm, 32.2%$_{w/w}$; pH (H_2O), 10.3; EC, 9.9 mS/cm; loss on ignition, 52.6%; $P_{pseudotot}$, 9.9 g/kg; $K_{pseudotot}$, 95.1 g/kg; $Ca_{pseudotot}$, 17.9 g/kg; $Mg_{pseudotot}$, 5.7 g/kg. The PAHs in biomass fly ash were analysed in fly ash extracts obtained from one–cycle ultrasonic extraction according to the methodology described by Košnář et al. (2016). The 16 individual and total PAHs in analysed experimental fly ash (μg/kg dw) are shown in Table 1.

2.4. Experimental design setup

The 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 (open top = 21.0 cm, base = 18.0 cm, and height 20.5 cm). Each pot contained 5 kg dry weight (dw) of experimental soil. The pot experiment was set up in 6 treatments as follows: i) control soil (CS); ii) soil amended with PAHs–contaminated fly ash (SA); iii) PAHs–spiked soil (SS); iv) planted control soil (PCS); v) planted soil amended with PAHs–contaminated fly ash (PSA); and vi) planted PAHs–spiked soil (PSS). Non–planted soil of SA and SS treatments simulated the natural attenuation of soil PAHs and planted soil of PSA and PSS treatments represented the phytoremediation of soil PAHs. The amount of applied ash in SA and PSA treatments was 50 g per each pot, representing 1% (w/w) of ash per pot in all respective treatments. The soil–ash mixture was mixed thoroughly. Each treatment was carried out in four replications. Before the experiment was established a synthetic mixture of 16 PAHs (SV Mix 5, Restesk, USA) was added to the soil of SS and PSS treatments to provide the content of 100 μg/kg dw of each PAH species in soil. The soil was spiked with PAHs following the procedure described by Smith et al. (2006). Briefly, each soil of respective pot was laid out thinly and the PAHs dissolved in hexane were added to the soil using a pipette at a rate of 100 mL of PAH solution per pot. The soil was turned over repeatedly during the additions. The soil was then left for three days to ensure the evaporation of hexane. The soil was then stored for 4 weeks to age and stabilise in the dark. During this period, the PAH spiked soil was thoroughly mixed to obtain a homogeneous distribution of the spiked PAHs. The initial content of PAHs in experimental ash–amended soil and PAHs–spiked soil are listed in Table 1. The control (CS) and planted control (PCS) treatments are not included in the Table 1 because the PAHs were not detected in them initially.

At the beginning of experiment the soil of each pot was also fertilized as follows: 100 mg N (NH_4NO_3 water solution); 32 mg P and 80 mg K (K_2HPO_4 water solution) per 1 kg of soil dw. The experimental plant tested was maize (*Zea mays* L. cv. Colisee, purchased from KWS, Germany). Maize was chosen because its reported ability to grow on PAH contaminated sites (Lin et al., 2007; Dupuy et al., 2015). Maize seeds were surface–disinfected according to Smith et al. (2006). The maize seeds were sown directly in soil at a 2–3 cm depth at a rate of 8 seeds per pot. The plants were thinned to 3 per pot at the age of the third leaf emergence. Each pot consisted of two plastic open pots while the inner one was perforated in the bottom. The outer, not perforated one served to catch possible excess percolate, which was transferred back to soil if appeared. The pots were manually watered with demineralized water in order to keep soil moisture at 60% of the maximum water holding capacity (MWHC) controlled gravimetrically prior to each irrigation event. From 60 days after emergence the planted treatments were kept at 70% MWHC because of intensive transpiration of maize plants. The MWHC of each treatment was determined according the procedure described by Mercl et al. (2016). The pots were randomized once a week. During the growth of maize, weeds were removed to avoid interplant competition.

Soil samples were collected at the end of the 120–days experiment. Each soil sample was obtained from each respective pot by thoroughly mixing five sub–samples randomly collected from the whole soil profile of the pot. A total of 24 soil samples were obtained and a stainless steel tool was used for the collection. The soil samples were freeze–dried, ground with a mortar and subsequently sieved through 2 mm stainless steel mesh. The plant biomass was harvested at the end of the experiment and plant samples: roots and shoots (aboveground biomass including stems and leaves) were separated. Roots were washed with distilled water to remove attached soil particles. Each plant sample (roots and shoots separately) was composite of three maize plants from each respective pot. A total of 12 root and 12 shoot samples were cut into small pieces separately, homogenized, dried at 35 °C in an oven for 7 days, then weighed and pulverised to a fine powder using cutting mill (Retsch SM100, Germany). Each soil and plant sample was divided in two technical replicates for the further analysis of PAHs.

2.5. Analysis of PAHs

The extraction of PAHs in soil samples were carried out in the frame of US EPA Method (3550)C (2007) using the ultrasonic bath extraction procedure with a continuous three re–extraction cycle. Briefly, for the determination of soil PAHs, an aliquot sample of 5 g (accuracy ± 0.001 g) was weighed into the glass flask and thoroughly mixed with 5 g of activated anhydrous sodium sulfate. Ultrasonic extraction of the sample in the glass–capped flask was performed with 25 mL of hexane–acetone mixture (1:1, v/v) with an ultrasonic bath system (Bandelin Sonorox Digitec DT510/H, Germany) and sonicated for 30 min of irradiation at a bath temperature of 35 °C. Before the ultrasonic extraction the standard solution (SS) at 500 μg/L was added into each sample. The reaction mixture was shaken on the orbital shaker (GFL 3017, Germany) for 30 min at 170 rpm. The reaction mixture was then filtered through the filtrate paper (Filpap, KA–2, Czech Republic). The sample was re–extracted twice with 30 mL of hexane–acetone mixture (1:1, v/v). All sample extracts were pooled together and then evaporated on a rotatory evaporator (Büchi rotavapor R–300, Switzerland) at 40 °C to 1 mL for further purification and cleanup process. The sample extracts were purified and cleaned up according to US EPA Method (3630)C (1996) with modifications described by Feng et al. (2014). Final soil extract (1 mL) was analysed after the addition of the IS solution at 500 μg/L by gas chromatography coupled with mass spectrometric detector (GC/MS) for PAHs. In the case of plant samples for the PAH analysis the plant samples were extracted and treated as was described above for the soil samples.

The chromatographic analysis of individual PAH compounds was based on a GC–MS methodology US EPA Method (8270)D (2014) with modification described below. Briefly, the PAHs were analysed in an Agilent HP 6890 N gas chromatograph equipped with an Agilent 7683B injector including an Agilent 10.0 μL syringe and connected to an Agilent HP 5975 inert mass selective detector (GC/MSD, 6890 N/5975, Agilent Technologies, USA). The separation of PAHs was carried out using a DBEUPAH (20 m × 0.18 mm inner diameter, 0.14 μm film thickness) capillary column (Agilent J&W Scientific, USA). Pure helium (6.0, Linde, Czech Republic) was used as the carrier gas at a constant ramped flow rate of 1.0 mL/min. The extracts were injected under the pulsed splitless condition mode (1 μL, 1 min, purge flow 70 mL/min at 0.75 min), pulsed pressure 25.0 PSI and kept at an initial temperature of 300 °C. The temperature of mass selective detector was 300 °C and the quadrupole was at 180 °C. More detailed mass spectrometer regime is described in an article by Košnář et al. (2016).

The quantitation of PAHs in sample extracts was done by comparing their target ion signals intensities to multilevel concentration curves for the target compounds. The PAHs were further quantified using the response factors related to the respective internal standards based on an external five–point calibration curve for each individual compounds (10–1000 μg/L). The calibration curves showed acceptable linearity ($R > 0.9987$) for each of the PAHs. The relative standard deviation

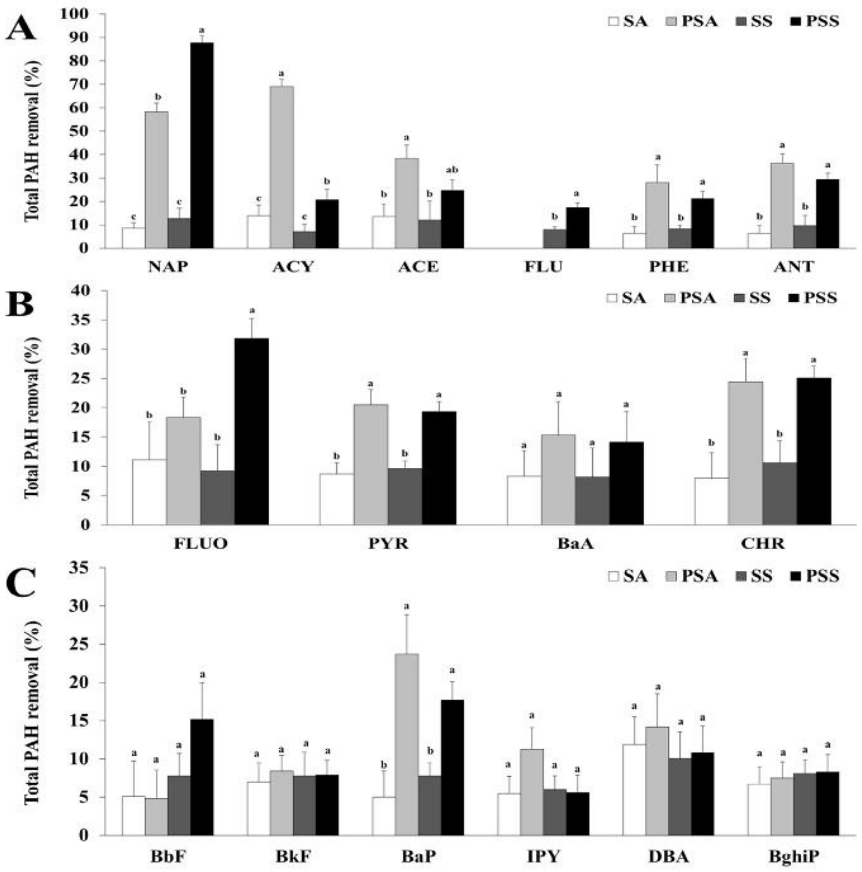

Fig. 1. The total removal (%) of (A) individual LMW PAHs, (B) individual MMW PAHs, and (C) individual HMW PAHs from soil, at the end of the 120–day experiment. The columns of each respective PAH removal (means; n = 4) sharing different lower case letters indicate significant differences ($P < .05$) between the treatments as determined by Tukey´s test. Error bars indicate SD values of n = 4. Treatment abbreviations: SA–ash amended soil; PSA–planted ash amended soil; SS–spiked soil; PSS–planted spiked soil.

(RSD) values estimated from the response factors based on the calibration curve were below 20%. The blank sample (without the sample addition; n = 2) and spiked blank (check standard at 1.0 mg/mL; n = 2) were treated and analysed simultaneously with the sample extract duplicates. The PAH quantification limits were calculated in the range of 1.8 (ACE) and 5.6 (BghiP) μg/kg dw. The recoveries of 16 PAHs in spiked blank ranged from 84% to 116%. The SS recoveries of 2-FBP and *p*-TER-d_{14} were 98.1% and 117.5% for soil samples, and for the plant samples 92.5% and 89.7%, respectively. There was a good agreement among the results of the PAH content analysed in CRM extracts within the acceptable error < 20% for individual PAHs.

2.6. Data processing and statistical analysis

In this study, the group of total PAHs represents the sum of the 16 individual PAHs. The total PAHs were divided into three fractions according to their molecular weight and number of rings as follows: i) LMW PAHs (the sum of NAP, ACY, ACE, FLU, PHE, and ANT); ii) MMW PAHs (the sum of FLUO, PYR, BaA, and CHR); iii) HMW PAHs (the sum of BbF, BkF, BaP, IPY, DBA, and BghiP). The total PAH removal (%; w/w) of PAHs from soil (Figs. 1 and 2) was calculated as follows:

$$100 \times \left[\frac{PAH_{initial} \times PAH_{residual}}{PAH_{initial}} \right],$$

where the $PAH_{initial}$ refers to the initial content of PAHs (μg/kg dw) at 0 days (in Table 1) and $PAH_{residual}$ refers to the residual content of PAHs (μg/kg dw) at 120 days of the experiment (Table S1, Appendix A). The PAH removal by maize roots from soil (%; w/w) in Table 2 was calculated as follows:

$$100 \times \left[\frac{roots\ yield \times PAH roots\ concentration}{PAH_{initial}\ per\ pot} \right],$$

where the roots yield refers to the maize *roots yield* (kg/pot) at the end of the experiment (Table S3, Appendix A), *PAH roots concentration* refers to the PAH concentration detected in maize roots (μg PAH/kg roots dw) at the end of the experiment (Table 2), and $PAH_{initial}$ *per pot* refers to the initial PAH content at 0 days of the experiment per pot containing 5 kg soil dw (μg PAH/pot). To estimate the PAH accumulation by maize, the bioaccumulation factor (BAF) was used. The BAF in Table 2 was calculated as a ratio of the PAH concentration in roots (μg PAH/kg roots dw) to its respective initial PAH content in soil (μg PAH/kg soil dw). A pair–wise comparison (one–way analysis of variance – ANOVA at $P < .05$) followed by Tukey´s test *post–hoc* analysis ($\alpha = 0.05$) was performed to evaluate the statistical differences between the recorded data. The assumptions of one–way ANOVA were confirmed using the tests of normality and homogeneity of variance (Shapiro–Wilk and Levene tests). All statistical analyses and figures were conducted in Microsoft Excel 2010 and Statistica 12.0 CZ software (StatSoft, Tulsa, USA).

3. Results and discussion

3.1. Total removal of individual PAHs from fly ash–amended soil and spiked–soil by natural attenuation and phytoremediation

The total removal of each individual PAH from soil (%) is shown in Fig. 1A–C. The removal of FLU from soil of ash–amended treatments (SA and PSA) was not considered in our study due to its very low initial soil content. The control soil (CS) and planted control soil (PCS) were

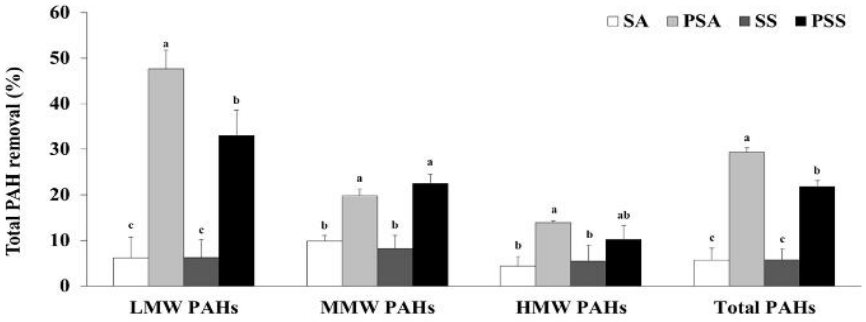

Fig. 2. The total removal (%) of LMW, MMW, and HMW PAHs, and total PAHs from soil, at the end of the 120–day experiment. The columns of each respective PAH group removal (means; n = 4) sharing different lower case letters indicate significant differences ($P < .05$) between the treatments as determined by Tukey´s test. Error bars indicate SD values of n = 4. Treatment abbreviations: SA–ash amended soil; PSA–planted ash amended soil; SS–spiked soil; PSS–planted spiked soil.

excluded from Figs. 1 and 2 because the PAHs were not detected in them initially, as well in the end of the experiment. Even though Larsson et al. (2013) described individual LMW and MMW PAHs as more prone to volatilization, photo–oxidation and biodegradable, in our study, the removal of individual LMW and MMW PAHs through the natural attenuation (SA and SS treatments) was negligible after 120 days of the experiment. According to Fig. 1 the removal of most individual PAHs in PSA indicated that the phytoremediation could be more efficient strategy for the bioremediation of PAHs in soil contaminated by fly ash than the natural attenuation. This was also confirmed in the case of planted PAHs–spiked soil in PSS.

The highest removal of individual PAHs in ash–amended soil (PSA) was reported in the case of NAP which decreased after the phytoremediation by 58.2% (Fig. 1A). The NAP removal in PSA was statistically lower ($P < .05$) than the removal of spiked NAP in PSS (87.8%). Conversely, the spiked ACY removal (20.7%) in PSS was statistically lower ($P < .05$) than the removal of ash–ACY in PSA (58.2%). The rest of individual PAHs in the LMW and MMW groups (Fig. 1A and B) which were the most susceptible to be removed in the phytoremediated soil rather than in natural attenuated soil were PHE, ANT, PYR, CHR (in PSA and PSS), ACE (in PSA only), and FLU, FLUO (in PSS only). The removal of these PAHs ranged from 17.4% (FLU in PSS) to 36.3% (ANT in PSA) over all of the treatments. The suitability of using maize in phytoremediation of these soil PAHs is in line with Xu et al. (2006).

Unfortunately the removal of BaA (Fig. 1B) in our study was not significantly different ($P < .05$) in any treatments nor was BkF, IPY, DBA, and BghiP of HMW PAHs group (Fig. 1C). This finding is consistent with research by Guo et al. (2016) where individual PAHs with more than 4 rings exhibit higher hydrophobicity and were more likely to be adsorbed by the soil matrix, which leads to lower bioavailability for plants and soil PAH–degraders. In the present study, majority of HMW PAHs showed insignificant removal differences between the treatments. However, the possible effect of maize to enhance the HMW PAH removal in soil was confirmed in case of the BaP (Fig. 1C). The BaP was removed significantly ($P < .05$) by 23.7% in ash–treated soil (PSA) compared to SA. The similar trend was true for the spiked BaP removal in PSS. However, there was no significant difference between PSA and PSS treatments in the BaP removal from soil.

The higher removal of most low and medium molecular weight PAHs in phytoremediation treatment indicated that the maize plants could enhance the rhizodegradation of these PAHs by stimulating the growth and activity of microbial biomass as a result of released root exudates as was suggested by Segura and Ramos (2013). An approximate explanation for the removal of individual PAHs with 2–4 rings could be that the maize during the phytoremediation has a capacity to increase the activity of native rhizosphere microorganisms in the production of extracellular enzymes involved in soil biodegradation of these PAHs. This hypothesis needs a further investigation but can be supported by the study of Wu et al. (2011).

3.2. Total removal of LMW, MMW, HMW, and total PAHs from fly ash–amended soil and spiked–soil by natural attenuation and phytoremediation

Treatments of natural attenuation (SA and SS) showed markedly lower removal of LMW PAHs from soil than phytoremediated treatments (PSA and PSS) in Fig. 2. The highest removal of LMW PAHs was found in PSA treatment (47.6%) followed by PSS (33.0%). LMW PAHs removal in naturally attenuated treatments was lower than 6.3% and without significant difference between SA and SS treatments. Removal of MMW PAHs by 22.5% in PSA and 19.8% in PSS followed a similar trend to LMW PAH removal. Significantly higher ($P < .05$) removal of

Table 2
Concentration of PAHs in maize roots (μg PAH/kg roots dw), bioaccumulation factor values of PAHs (BAF), and PAH removal by maize roots (%) at the end of the 120–day experiment. Means ± SD (n = 4) within the same row followed by different lower case letters indicate significant differences (P < .05) between the PSA and PSS treatments in PAH concentration, and row followed by different upper case letters indicate significant differences (P < .05) between the PSA and PSS treatments in PAH removal by maize roots as determined by Tukey´s test. Treatment abbreviations: PSA–planted ash amended soil; PSS–planted spiked soil.

	PSA			**PSS**		
	Concentration (μg PAH/kg roots dw)	BAF	Removal by roots (%)	Concentration (μg PAH/kg roots dw)	BAF	Removal by roots (%)
NAP	46.4 ± 5.1^{a}	0.14	0.04^{B}	30.4 ± 4.7^{b}	0.31	0.09^{A}
ACY	2.5 ± 0.3^{a}	0.05	0.01^{A}	2.5 ± 0.5^{a}	0.03	0.01^{A}
ACE	3.0 ± 0.1^{a}	0.09	0.03^{A}	4.3 ± 0.7^{b}	0.04	0.01^{A}
FLU	[1]n.d.	[2]n.d.	[2]n.d.	3.9 ± 0.5	0.04	0.01
PHE	10.1 ± 4.1^{a}	0.07	0.02^{A}	9.3 ± 1.5^{a}	0.09	0.03^{A}
ANT	9.6 ± 1.0^{a}	0.07	0.02^{A}	5.8 ± 1.5^{b}	0.06	0.02^{A}
FLUO	11.1 ± 1.8^{a}	0.08	0.03^{A}	12.2 ± 1.1^{a}	0.12	0.04^{A}
PYR	8.9 ± 0.9^{a}	0.06	0.01^{A}	8.4 ± 0.7^{a}	0.09	0.02^{A}
LMW PAHs	70.1 ± 8.6^{a}	0.10	0.03^{A}	56.1 ± 5.8^{b}	0.09	0.03^{A}
MMW PAHs	20.0 ± 1.7^{a}	0.04	0.01^{A}	20.6 ± 1.5^{a}	0.05	0.02^{A}
Total PAHs	90.1 ± 8.1^{a}	0.05	0.01^{A}	76.7 ± 5.5^{a}	0.05	0.01^{A}

[1] n.d. – not detected.
[2] n.d. – not defined.

HMW PAHs (13.9%) was found only in planted ash–amended soil (PSA) compared to the respective treatment without plants (SA).

The higher removal of LMW PAHs than MMW and HMW PAHs in the same treatment of soil phytoremediation is in agreement with previous studies of Lee et al. (2008) and Feng et al. (2014). Parrish et al. (2005) described LMW PAHs as more volatile, water soluble and less lipophilic which make them biodegradable more easily while the other PAHs tended to be more recalcitrant to microbial attack and thus hydrophobic MMW and HMW PAHs prone to be absorbed and immobilized by lipophilic soil organic matter as suggested Cofield et al. (2007).

In our experiment the initial total PAH content significantly decreased ($P < .05$) in all treatments after 120 days. This study shows that the PAHs dissipation in ash–amended soil as well in PAHs–spiked soil could be accelerated by the presence of vegetation which is in line with previous study by Maila and Cloete (2002). This is caused by increased microbial density and activity in the rhizosphere in comparison to non–planted soil. For example, the extracellular hydrolytic enzymes such as peroxidases, lipases, and proteases released as plant exudates or produced by mycorrhizal fungi increase the bioavailability of PAHs. These are subsequently more prone to co–metabolic degradation and/or biotransformation (Nanekar et al., 2015). Whereas the removal of PAHs from natural attenuated soils is mainly caused by autochthonous microorganisms and abiotic losses of LMW PAHs (Declercq et al., 2012).

Therefore at the end of our experiment the different removal efficiencies of total PAHs were determined as follows: PSA > PSS > SA ~ SS. The highest removal of total PAHs (29.4%) in our experiment was observed in ash–amended soil cultivated with maize (PSA) and followed by PSS (21.8%). The resulting residual soil content of total PAHs in PSA (1184 μg/kg dw in Table S1, Appendix A) was still higher than the preventative limit of total PAHs (1000 μg/kg dw) according to the Public Notice No. 153 (2016) for soils in the Czech Republic. To break through this limit, further PAHs removal may be possible with the continuation of maize cultivation for a second season as proposed by Chirakkara et al. (2016).

3.3. Influence of PAHs in fly ash–amended soil and spiked–soil on maize growth and dry matter yields in phytoremediation approach

The growth response of maize (*Zea mays* L.) to PAH contaminated soil was assessed by the measurement of plant heights (Table S2, Appendix A) during the vegetation period and by the determination of root and shoot dry biomass yield at the end of the 120–day experiment. Harvested biomass yield of maize roots and shoots is listed in Table S3 (Appendix A). Our results are comparable with another 120–day experiment presented by Liao et al. (2015), who cultivated maize in PAH contaminated soil treated with biosurfactants. In our experiment, ash–PAHs and spiked PAHs in soil had no adverse effects on maize growth or biomass yield during the phytoremediation as there was no significant difference ($P < .05$) between the planted treatments (PSA and PSS) including the control planted treatment (PCS). Similarly, Wu et al. (2011) reported no direct evidence of any changes in morphology of maize, even if the initial content of spiked PHE and PYR was 12.0 and 7.4 mg/kg, respectively. Xu et al. (2006) suggested the maize as a suitable plant for phytoremediation of highly contaminated soils. Nevertheless, Dupuy et al. (2015) showed biomass reduction and metabolism perturbations of maize exposed to PHE levels above 50 mg PHE/kg.

3.4. Accumulation of PAHs in maize and PAH removal by maize in phytoremediation approach

Phytoremediation of PAH–polluted soil commonly involves accumulation of PAHs by plants and other basic mechanisms described by Alagić et al. (2015). In our study, the content of PAHs was found only in maize roots of PSA and PSS treatments. Therefore, the PAH content in maize shoots cultivated in control planted soil (PCS), ash–amended soil (PSA), and PAHs–spiked soil are not included in Table 2. The FLU in roots of PSA treatment was not detected due to its very low initial content in soil. The individual PAHs with more than 4 aromatic rings are more hydrophobic and prone to be physically less compatible with passing through cell membranes (Lin et al., 2007). This could explain why BaA, CHR, and all individual HMW PAHs were not detected in maize roots in our study. Nevertheless, individual PAHs in the roots of PSA treatment (Table 2) ranged between 2.5 and 44.9 μg/kg roots dw which resulted in the maize roots of PSA containing significantly higher ($P < .05$) content of LMW PAHs (69.6 μg/kg roots dw) than the roots of PAHs–spiked soil. However the difference between the total PAHs (89.5 μg/kg roots dw) of PSA and PSS (76.9 μg/kg roots dw) in maize roots was not significant at $P < .05$. In our study, any of the PAHs detected in roots were not detected in shoots, which indicates that the transfer of PAHs from roots to the maize shoots was not observed. This is in line with studies by Wild et al. (2005) and Gao et al. (2011), who reported PAHs to be accumulated and transformed within root cortex rather than be translocated to aboveground biomass.

Some studies have suggested that the accumulation of PAHs by plant species occurs due to the phytoextraction process when the bioaccumulation factor (BAF) and translocation factor (TF) values of PAHs are higher than 1 (Alagić et al., 2015). Therefore, in order to estimate if the PAHs in maize tissues (roots and shoots) may be associated with a phytoextraction process, the BAF and TF of PAHs were calculated. The BAF values of individual PAHs (Table 2) were significantly lower than 1 in all planted treatments and ranged from 0.01 (FLU) to 0.31 (NAP). In our study, the TF values of PAHs (a ratio of PAH concentration in maize shoots to the PAH concentration in maize roots) were not defined because the PAHs in maize shoots were not detected. This indicated that the ability of PAH uptake by maize shoots from the soil amended with fly ash contaminated by PAHs as well from PAHs–spiked soil through the maize roots was not observed in this experiment. Our BAF values of total PAHs in PSA and PSS treatments (both 0.05) were similar to the BAF of total PAHs (0.09) of maize grown in soil with low organic matter content obtained by Kacálková and Tlustoš (2011). This similar trend could indicate that PAHs during the phytoremediation of ash amended soil, as well the PAHs–spiked soil in our experiment tended to be removed in the maize rhizosphere and stabilized via maize root surfaces rather than to be extracted from soil which is in accordance with Binet et al. (2000).

Moreover, the results of PAH removal by maize roots (Table 2) showed a negligible ability of maize roots to remove PAHs from soil of PSA and PSS in the range between 0.01% and 0.09%. This could indicate that the maize used in our phytoremediation approach significantly boosted the PAH microbial degradation in soil and the contribution of PAH accumulated in maize roots on the total PAH removal from soil (Figs. 1 and 2) is minimal. Considering the fact that the 16 individual PAHs in maize shoots were not detected in our study, the harvested aboveground biomass does not have to be landfilled, because the content of total PAHs was < 10 μg/kg dw according to the Public Notice No. 53 (2012) in the Czech Republic.

4. Conclusion

The removal of total PAHs present in biomass fly ash–amended soil was more effective with the aid of phytoremediation using maize (*Zea mays* L., var. Colisee) than natural attenuation. This trend was also confirmed in the case of PAHs–spiked soil.

The adverse impact of PAHs on maize growth during this experiment was not observed. After the harvest, the PAHs were found only in maize roots and their transport to shoots remained unconfirmed. The bioaccumulation factors of all individual PAHs were significantly lower than 1 suggesting rhizodegradation of PAHs in soil and phytostabilization of PAHs via root surfaces. In this study, the contribution of PAH removal by maize roots on the total PAH removal from soil was negligible. Therefore, maize plants significantly boosted the total PAH

removal in soil. Harvested aboveground maize biomass did not represent any environmental risk.

Our results showed that maize plants could be used to enhance the bioremediation of PAHs in agricultural soils, and that PAHs of biomass ash origin were similarly susceptible to removal as PAHs in spiked soil. Cultivation of maize may be used as an in situ PAH phytoremediation approach representing a low cost and environmentally friendly strategy.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2018.01.049.

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Removal of soil polycyclic aromatic hydrocarbons derived from biomass fly ash by plants and organic amendments

Zdeněk KOŠNÁŘ*, Pavel TLUSTOŠ

Department of Agroenvironmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic

**Corresponding author: kosnarz@af.czu.cz*

ABSTRACT

Košnář Z., Tlustoš P. (2018): Removal of soil polycyclic aromatic hydrocarbons derived from biomass fly ash by plants and organic amendments. Plant Soil Environ., 64: 88–94.

Phytoremediation using maize (*Zea mays* L.) assisted by the compost or vermicompost amendments was the most appropriate strategy for bioremediation of soil contaminated by polycyclic aromatic hydrocarbons (PAHs) derived from biomass fly ash. Higher removal of low molecular weight PAHs than medium and high molecular weight PAHs within the same treatment were observed. The total PAH content in planted soil with compost or vermicompost was decreased in a range between 62.9–64.9%. There were no significant differences ($P < 0.05$) between the compost and vermicompost amendments on the total removal of ash-PAHs. The content of PAH derived by ash did not have adverse effect on maize cultivation and biomass yield. The contribution of PAH reduction by maize roots on the soil total PAH removal was negligible. Therefore, maize significantly boosted the PAH removal in soil. The harvested maize shoots did not represent any environmental risk.

Keywords: carcinogenic compound; combustion residues; contamination; degradation; soil amendments

Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic organic compounds with two or more benzene aromatic rings. Some PAHs are considered as potentially carcinogenic compounds to humans; they can be formed mainly during the incomplete combustion (Paris et al. 2018). The released PAHs tend to be persistent e.g. in soils, sediments and sewage sludge (Vácha et al. 2005, Dvořák et al. 2017, García-Sánchez et al. 2018). The unfavourable conditions of biomass combustion in power plants can also lead to the accumulation of PAHs in resulting ashes (Masto et al. 2015). The biomass ashes usually also contain high amounts of mineral nutrients such as Ca, K, P and Mg, and therefore there is an effort to recycle them as soil amendments and/or fertilizers (Ochecová et al. 2017). The possible PAH content in ashes applied in soil have not received considerable attention. The increased content of PAHs in ashes can limit the soil ash application and elevate the agricultural soil contamination (Enell et al. 2008). Phytoremediation using maize (*Zea mays* L.) could be suitable strategy for clean-up of ash-PAHs contaminated soils because maize significantly enhanced the degradation of aged PAHs in soil from a wastewater-irrigated area (Guo et al. 2017). Moreover, the compost or vermicompost application into a soil amended by PAH-contaminated ashes could improve the removal similarly as in the case of bioremediation of artificially and aged PAHs contaminated soil described by Wang et al. (2012) and Feng et al. (2014).

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The main aims of this study were: (i) to determine the removal of PAHs derived from biomass fly ash in non-planted/planted ash-soil in comparison to non-planted/planted ash-soil amended with compost or vermicompost; (ii) to determine the contribution of maize on the PAH removal from soil.

MATERIAL AND METHODS

PAHs. In this study, 16 individual US EPA priority PAHs were investigated. The PAHs were sorted into four groups as follows: LMW PAHs – low molecular weight PAHs (the sum of naphthalene, acenapthylene, acenaphtene, fluorene, phenanthrene, anthracene); MMW PAHs – medium molecular weight PAHs (the sum of fluoranthene, pyrene, benzo[*a*]anthracene, chrysene); HMW PAHs – high molecular weight PAHs (the sum of benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[1,2,3-*c,d*]pyrene, dibenz[*a,h*]anthracene, benzo[*g,h,i*]perylene); total PAHs – the sum of all 16 individual PAHs.

Soil. The experimental soil originated from a long-term trial site close to the city of Humpolec in the Czech Republic (49°33'15''N, 15°21'00''E). The soil was obtained by mixing different subsamples collected from the field site at a depth of 0–20 cm. The non-sterilized soil was homogenized, air-dried at room temperature and passed through a 5 mm stainless steel sieve. The main physico-chemical characteristics of the experimental soil (Cambisol – sandy loam according to the FAO soil classification) are listed in Table 1.

Compost and vermicompost. The compost was obtained from the composting of biowaste mixture following the methodology described by Habart et al. (2010). Briefly, the compost production was carried out in a 70 L plastic laboratory fermenter. The mixture was prepared from livestock manure, fresh grass, straw and waste paper in a ratio of 9:9:1:1 (w/w). The fermenter was placed in a laboratory at 25°C and the composting process was carried out with forced aeration. After 180 days, the compost was considered as mature and sufficiently stabilized to be used. The vermicompost was obtained from the vermicomposting of the same biowaste mixture mentioned above following the methodology described by Hanč et al. (2017). Briefly, vermicomposting was conducted in a plastic vermicompost reactors (Ekodomov, Prague, Czech Republic) placed in a laboratory. 5 kg of the biowaste mixture was inoculated with 0.5 kg of a substrate containing earthworms of the genus *Eisenia*. This mixture was placed into a 12 L plastic bowl of vermi-reactor and left 180 days for vermicomposting. The main physico-chemical characteristics of the 'ready to use' compost and vermicompost are shown in Table 1.

Ash. The experimental biomass fly ash was obtained from a commercial biomass power plant operated in the Czech Republic using a 20 MW grate boiler. The tested ash was derived from the

Table 1. Physico-chemical characteristics of soil, compost and vermicompost

Parameter	Soil	Compost	Vermicompost
pH_{CaCl_2}	5.2 ± 0.0	8.4 ± 0.0	8.7 ± 0.1
C_{tot} (g/kg)	18.3 ± 2.5	316 ± 2.0	317 ± 1.8
N_{tot} (g/kg)	1.35 ± 0.1	25.8 ± 0.3	29.9 ± 0.1
P_{avail} (mg/kg)[1]/$_{tot}$ (g/kg)[2]	80.2 ± 4.4	2.02 ± 0.3	3.12 ± 0.4
K_{avail} (mg/kg)/$_{tot}$ (g/kg)	190 ± 8.9	29.2 ± 0.6	19.2 ± 0.6
Ca_{avail} (mg/kg)/$_{tot}$ (g/kg)	1586 ± 72	6.95 ± 0.5	9.36 ± 0.7
Mg_{avail} (mg/kg)/$_{tot}$ (g/kg)	153 ± 10	1.99 ± 0.1	2.46 ± 0.2
Total PAHs (µg/kg)	nd	nd	nd

[1]Avail. – available element contents (P, K, Ca and Mg) in soil (mg/kg) were determined using Mehlich 3 extraction; [2]tot. – total element contents (P, K, Ca and Mg) in compost and vermicompost (g/kg) were determined according to the method used by Hanč et al. (2017). nd – not detected (individual polycyclic aromatic hydrocarbons (PAHs) were lower than the detection limit in the range between 1.8–5.6 µg/kg dry weight)

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combustion of wheat straw in the temperature range 600–700°C. The collected ash was a composite of four random sub-samples taken from a container with fly ash from electrostatic precipitator. The ash was air-dried at room temperature and thoroughly mixed in a laboratory. The main physico-chemical characteristics of the experimental ash determined according to Mercl et al. (2016) and Košnář et al. (2016) were: particle size – fraction < 0.25 mm, 67.8%; fraction 0.25–1.6 mm, 32.2%; pH_{H_2O}, 10.3; electrical conductivity, 9.9 mS/cm; loss on ignition, 52.6%; P_{tot}, 0.1%; K_{tot}, 9.5%; Ca_{tot}, 1.8%; Mg_{tot}, 0.57%; total PAHs, 160.2 mg/kg DW (dry weight).

Pot experiment. The experiment was conducted in an outdoor, atmospheric precipitation-controlled, vegetation hall with natural temperature and light using a series of 6 L polypropylene pots: open top, 21 cm; base, 18 cm; height, 20 cm. The pot experiment was carried out in 9 treatments each in four separated pots for replication as follows: S – soil (control); C – compost-soil; V – vermicompost-soil; A – ash-soil; CA – compost-ash-soil; VA – vermicompost-ash-soil; PS – planted soil (control for plants); PC – planted compost-soil; PV – planted vermicompost-soil; PA – planted ash-soil; PCA – planted compost-ash-soil; PVA – planted vermicompost-ash-soil. Each treatment (S and PS) contained 5 kg soil DW per pot and rest of the treatments contained 5 kg of amended soil per pot. The ash, compost and vermicompost in amended soil of the respective treatment represented 1% (w/w), 10% (w/w) and 10% (w/w), respectively. The initial PAH contents of experimental treatments are shown in Table 2 excluding the treatments without the ash addition because the PAHs were not detected in them initially. As the experimental plant was tested maize (*Zea mays* L. var. Colisee). The maize seeds were gained from the KWS (Einbeck, Germany). Before sowing, each pot received 500 mg N in NH_4NO_3 water solution, 32 mg P and 80 mg K in K_2HPO_4 water solution. The maize seeds were sown directly in soil at a depth of 2–3 cm, at a rate of 8 seeds per pot. The plants were thinned to 3 per pot at the age of the third leaf emergence. The pots were manually watered with demineralized water regularly in order to keep soil moisture at 60–70% of the maximum water holding capacity. Soil samples were collected at the end of the 120-days experiment. Each soil sample was a composite of five sub-samples from different zones of each pot. The plant samples (roots and shoots separately) were obtained after the harvest. Roots were washed with distilled water to remove the attached soil particles. Before the PAH analysis the samples were separately air-dried at laboratory temperature, homogenized and plant samples were pulverized to a fine powder with a mill (Retsch, Haan, Germany).

PAH analysis. The extraction of PAHs from soil and plant samples was carried out according to the US EPA (2007) using an ultrasonic bath (Bandelin electronic, Berlin, Germany) with a continuous re-extraction cycles followed by the silica gel clean-up process in concordance to the US EPA (1996). The PAH identification followed by the PAH quantification was based on a gas

Table 2. Initial polycyclic aromatic hydrocarbon (PAH) contents (μg/kg dry weight) in experimental treatments

Treatment	LMW PAHs	MMW PAHs	HMW PAHs	Total PAHs
A	745.4	371.5	484.8	1601.7
CA	750.1	376.7	477.3	1604.0
VA	739.2	387.4	493.8	1611.4
PA	730.8	417.8	526.6	1675.2
PCA	732.9	369.1	480.0	1582.0
PVA	725.8	401.0	483.3	1610.1

All values represent means (n = 4). There were no significant differences (P < 0.05) in initial PAH contents between the treatments: A – ash-soil; CA – compost-ash-soil; VA – vermicompost-ash-soil; PA – planted ash-soil; PCA – planted compost-ash-soil; PVA – planted vermicompost-ash-soil; LMW PAHs – low molecular weight PAHs; MMW PAHs – medium molecular weight PAHs; HMW PAHs – high molecular weight PAHs; total PAHs – the sum of all 16 individual PAHs

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chromatography/mass spectrometry method described by US EPA (2014) using a 6890N-gas chromatograph with 5975-mass detector (Agilent Technologies, Santa Clara, USA). The separation of PAHs was carried out using a capillary column (20 m × 0.18 mm inner diameter, 0.14 µm film thickness) (Agilent J&W Scientific, Santa Clara, USA). The detailed chromatographic regime and analytical precision of the method were described elsewhere by Košnář et al. (2016).

Data processing and statistical analysis. The total soil PAH removal (%) in Figures 1 and 2 was calculated as follows:

$$\text{Total soil PAH removal} = 100 \times (PAH_{residual} - PAH_{initial})/PAH_{initial}$$

Where: $PAH_{residual}$ – residual content of PAHs in soil (µg/kg DW) at the end of the 120-days experiment; $PAH_{initial}$ – initial content of PAHs in soil (µg/kg DW) at 0 days.

The plant PAH removal (%) in Table 3 was calculated as follows:

$$\text{Plant PAH removal} = 100 \times \left[\frac{\text{plant yield} \times PAH_{concentration}}{PAH_{initial}/\text{pot}}\right]$$

Where: plant yield – maize roots yield (kg roots DW/pot); $PAH_{concentration}$ – PAH concentration in maize roots (µg PAH/kg plant DW); $PAH_{initial}$/pot – initial PAH content in soil at 0 days per each pot (µg PAH/pot).

The one-way ANOVA at $P < 0.05$ followed by the Tukey's test was performed to evaluate the statistical differences between the treatments. The data were evaluated using the Microsoft Excel 2010 (Microsoft Corporation, Redmond, USA) and Statistica 12.0 (StatSoft, Tulsa, USA).

RESULTS AND DISCUSSION

The influence of ash-PAHs in the development of bioremediation approaches was assessed by comparing of maize biomass yield in Table 3. The compost and vermicompost amendments in PC and PV treatments significantly increased ($P < 0.05$) the yield of maize roots in comparison to the planted control treatment (PS). The maize shoots yields were the same in all the treatments. The soil contaminated by ash-PAHs had no adverse effects on maize biomass (roots and shoots) yield as there were no significant differences ($P < 0.05$) between the respective treatments. Our results of biomass yield were comparable with 120 days experiment with maize grown in soil contaminated by pyrene and phenanthrene artificially (Liao et al. 2015). In our study, the PAHs were found only in maize roots cultivated on ash-amended soil (Table 3). The compost and vermicompost amendments could enhance

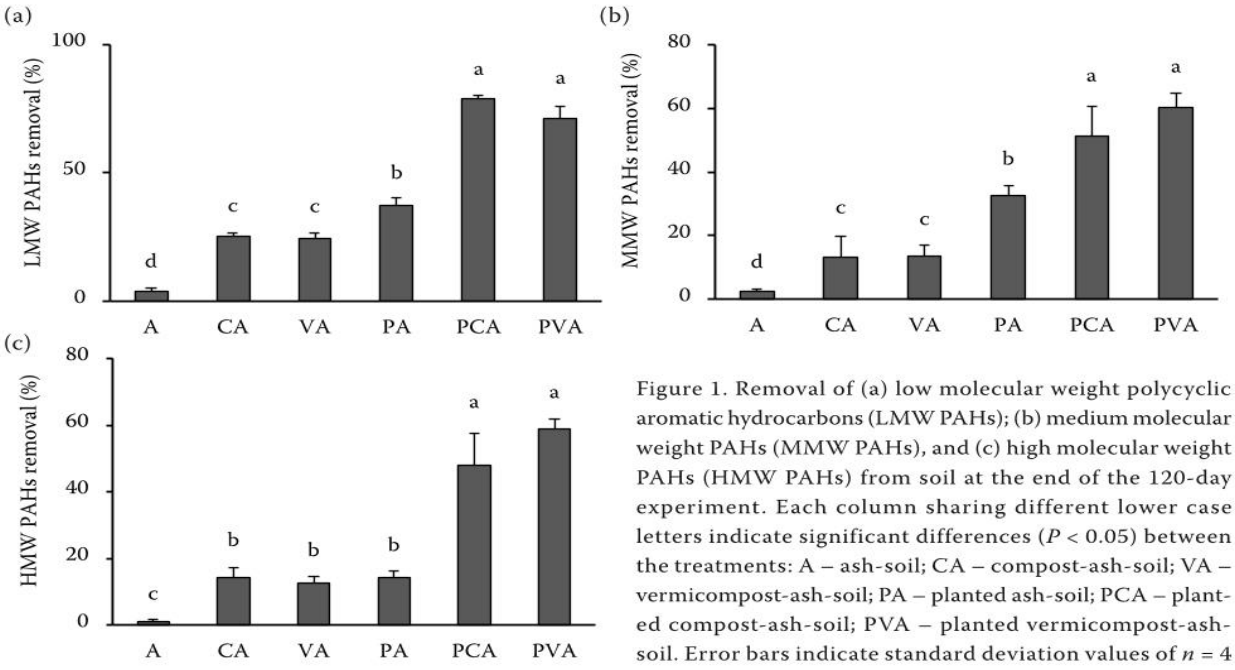

Figure 1. Removal of (a) low molecular weight polycyclic aromatic hydrocarbons (LMW PAHs); (b) medium molecular weight PAHs (MMW PAHs), and (c) high molecular weight PAHs (HMW PAHs) from soil at the end of the 120-day experiment. Each column sharing different lower case letters indicate significant differences ($P < 0.05$) between the treatments: A – ash-soil; CA – compost-ash-soil; VA – vermicompost-ash-soil; PA – planted ash-soil; PCA – planted compost-ash-soil; PVA – planted vermicompost-ash-soil. Error bars indicate standard deviation values of $n = 4$

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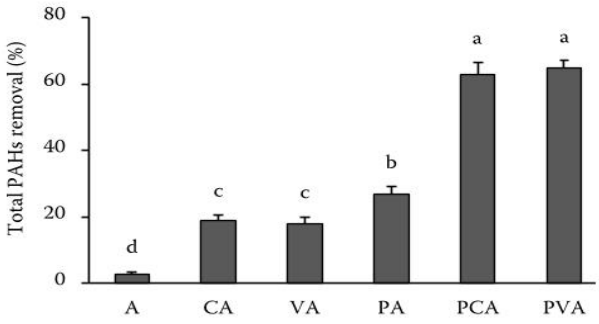

Figure 2. Removal of total polycyclic aromatic hydrocarbons (PAHs) from soil at the end of the 120-day experiment. Each column sharing different lower case letters indicate significant differences ($P < 0.05$) between the treatments: A – ash-soil; CA – compost-ash-soil; VA – vermicompost-ash-soil; PA – planted ash-soil; PCA – planted compost-ash-soil; PVA – planted vermicompost-ash-soil. Error bars indicate standard deviation values of $n = 4$

the bioavailability of soil ash-PAHs because significantly increased PAH content was observed in roots of PCA and PVA treatments than in PA treatment. The PAH content in maize shoots was not detected and translocation of ash-PAHs found in roots to the shoots remained unconfirmed. This was in line with the study by Gao et al. (2011) who indicated that the spiked PAHs are accumulated in roots rather than transported through the xylem flow to the shoots.

In this study, the PAH removal of ash origin from soil by maize roots was negligible in a range between 0.02–0.04% (Table 3). This could indicate that the contribution of PAH accumulated in maize roots on the total PAH removal from soil of PCA and PVA treatments was minimal. This finding is consistent to the study by Kacálková and Tlustoš (2011) who reported significantly lower than 1% accumulation of aged PAHs by maize from soil.

The removal of individual PAH groups is shown in Figures 1 and 2 excluding the treatments without the addition of ash because the PAHs were detected in them neither at the beginning nor in the end of the experiment. Different LMW, MMW and total PAH removal from soil of all investigated treatments was determined as follows: PCA ~ PVA > PS > CA ~ VA > A, and followed by PCA ~ PVA > PS ~ CA ~ VA > A for the HMW PAH removal. The phytoremediation of ash-soil assisted by compost or vermicompost (PCA and PVA) was the most appropriate strategy in the PAH removal. Cultivation of maize on ash-soil in combination with compost amendment in PCA treatment removed 79.0% of LMW PAHs, 51.5% of MMW PAHs and 48.1% of HMW PAHs from soil. These PAH removals were not statistically ($P < 0.05$) different to those reached in the PVA treatment using maize on vermicompost amended ash-soil. The higher LMW PAH removal than the MMW and HMW PAH removal in the same treatment indicated that the LMW PAHs of ash origin are susceptible to be biodegradable similarly to the aged PAHs as was described by Feng et al. (2014).

The results of total PAH removal in Figure 2 showed that the total PAHs derived from biomass

Table 3. Yield of maize roots and shoots, polycyclic aromatic hydrocarbon (PAH) concentration in roots and PAH removal by roots

Treatment	Root (g/pot DW)	Shoot (g/pot DW)	Total PAHs in roots (μg PAH/kg roots DW)	Plant PAH removal (%)
PS	15.8^{b}	106.9^{a}	nd	nd
PC	22.4^{a}	111.5^{a}	nd	nd
PV	22.1^{a}	105.5^{a}	nd	nd
PA	15.5^{b}	109.5^{a}	83.8^{b}	0.02^{b}
PCA	22.7^{a}	105.1^{a}	143.9^{a}	0.04^{a}
PVA	22.8^{b}	106.0^{a}	161.2^{a}	0.04^{a}

nd – not detected (individual PAHs were lower than the detection limit in the range between 1.8–5.6 μg/kg dry weight (DW)). All values represent means ($n = 4$). Different lower case letters within the same column indicate significant differences ($P < 0.05$) between the treatments: PS – planted soil (control for plants); PC – planted compost-soil; PV – planted vermicompost-soil; PA – planted ash-soil; PCA – planted compost-ash-soil; PVA – planted vermicompost-ash-soil

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fly ash in non-amended bare soil (A treatment) were removed negligibly. The compost or vermicompost which were applied into the soil of non-planted treatments (CA and VA) separately decreased the total PAH content in soil significantly in comparison to the non-amended treatment (A) in the range between 15.1–17.8%. The phytoremediation of PAHs in ash-soil (PA treatment) showed that the maize has a significantly higher ability to remove ash-PAHs than the CA and VA treatments because the sum of total PAH content was removed by 26.7%. The total PAH content in the planted soil amended with compost and vermicompost (PCA and PVA treatments) decreased by 62.9% and 64.9%, respectively. However, there were no significant differences ($P < 0.05$) between the compost and vermicompost amendment on the removal of total ash-PAHs. The higher PAH removal in PCA and PVA treatments than in other treatments indicated that the maize plants could stimulate the growth and activity of soil autochthonous microorganism involved in PAH degradation due to the production of exudates released in maize rhizosphere as was reported by Nanekar et al. (2015). Moreover, the compost or vermicompost used together with maize could stimulate the activity of soil autochthonous PAH degraders which could be supported by PAH degraders augmented to soil from the amendments. Furthermore, the incorporation of these organic materials could support the PAH removal by the irreversible trapping processes of PAHs in soil as was described by Ouvrard et al. (2014).

This study showed that the maize cultivation on a PAH contaminated soil amended with the compost and vermicompost as separately applied amendments were the most efficient bioremediation approaches of soil contaminated by PAHs of ash origin. The resulted residual PAH contents in PCA (587.4 µg/kg DW) and PVA (565.6 µg/kg DW) were significantly lower than the limit of total PAHs (1000 µg/kg DW) for soils required by the Ministry of the Environment of the Czech Republic (2016). Moreover, the harvested aboveground biomass of maize did not represent any environmental risk.

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

Autoři: Zdeněk Košnář, Tereza Částková, Lucie Wiesnerová, Lukáš Praus, Ivan Jablonský, Martin Koudela, Pavel Tlustoš.

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

Zdeněk Košnář[1,*], *Tereza Částková*[1], *Lucie Wiesnerová*[2], *Lukáš Praus*[1], *Ivan Jablonský*[2], *Martin Koudela*[2], *Pavel Tlustoš*[1]

1. *Department of Agroenvironmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague 6 - Suchdol, Czech Republic*

2. *Department of Horticulture, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague 6 - Suchdol, Czech Republic*

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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

* *Corresponding author. E-mail:* kosnarz@af.czu.cz (Zdeněk Košnář).

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(Dvořák et al., 2017), and sewage sludge (Vácha et al., 2005). The 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.

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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.

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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

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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

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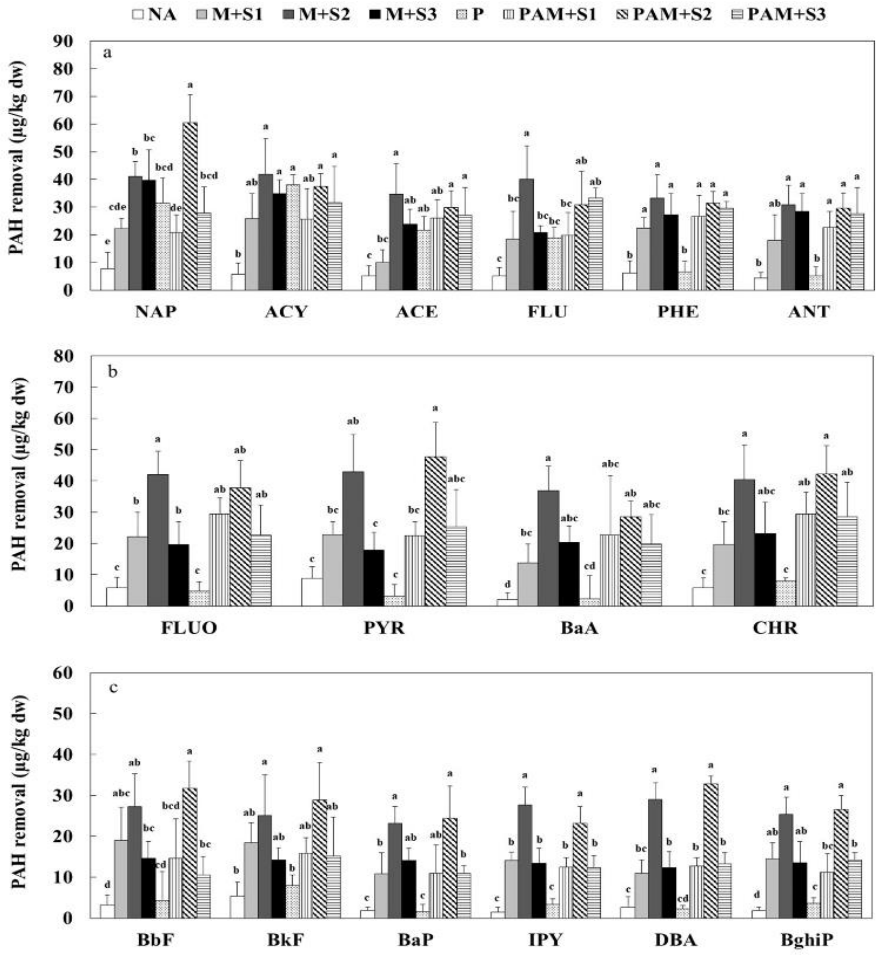

Fig. 1 – The PAH removal of (a) individual LMW PAHs, (b) individual MMW PAHs, and (c) individual HMW PAHs from soil, at the end of the 120-day experiment. The columns of each respective PAH removal (means; $n = 4$) sharing different lowercase letters indicate significant differences ($p < 0.05$) between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

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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

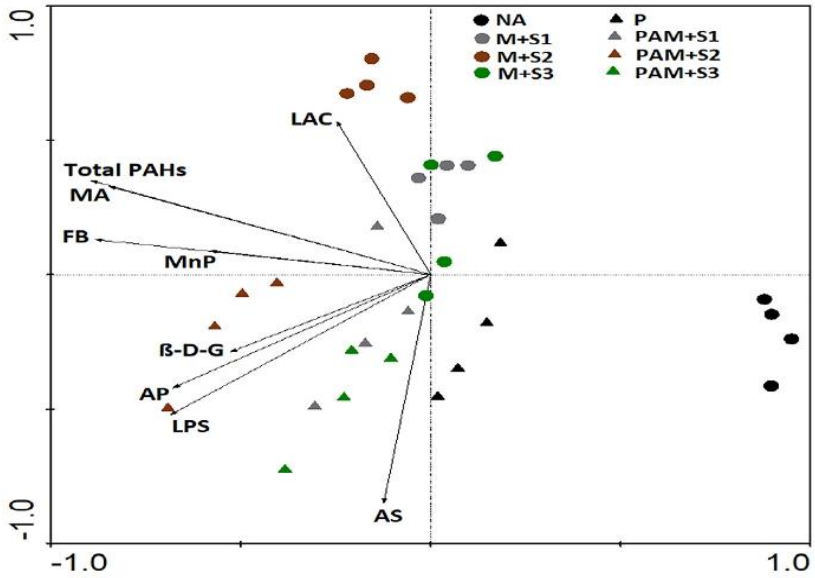

Fig. 2 – Ordination diagram showing the results of a principal component analysis (PCA) to evaluate the multivariate data of total PAH removal, microbial activity, fungal biomass, and extracellular enzyme activities in PAH–contaminated soil at the end of 120–days experiment. The first axis of the PCA 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.

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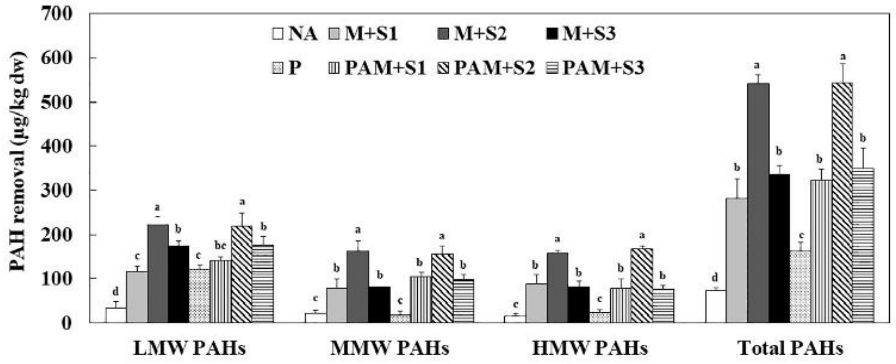

Fig. 3 – The PAH removal of the sum of LMW, MMW, and HMW PAHs and total PAHs from soil, at the end of the 120–day experiment. The columns of each respective PAH group removal (means; $n = 4$) sharing different lowercase letters indicate significant differences ($p < 0.05$) between the treatments as determined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

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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.

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4.5 García-Sánchez, M., Kořnář, Z., et al. (2018). Srovnávací studie na vyhodnocení metod přirozeného útlumu, mykoaugmentace, fytoremediace a mikrobiálně podporované fytoremediace na bioremediace PAU v dlouhodobě kontaminované půdě.

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

Autoři: Mercedes García-Sánchez, Zdeněk Košnář, Filip Mercl, Elisabet Aranda, Pavel Tlustoš.

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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

Mercedes García-Sánchez[a,*,1], Zdeněk Košnář[a], Filip Mercl[a], Elisabet Aranda[b], Pavel Tlustoš[a]

[a] Department of Agro-Environmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences, Kamýcká 129, 165 00 Prague 6-Suchdol, Czech Republic

[b] Department of Microbiology, Institute for Water Research, University of Granada, Ramón y Cajal 4, E-18071 Granada, Spain

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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

* Corresponding author.
E-mail address: garcia.sanchez.mercedes@gmail.com (M. García-Sánchez).
[1] Current address: Institut National de Recherche Agronomique– UMR Eco & Sols, 2 Place Viala, 34060 Montpellier, France.

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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,

and the location of the posts was randomly changed once a week. The experiment was conducted in a roofed, outdoor, atmospheric precipitation-controlled, vegetation hall with natural temperature and light. In the case of vegetated pots five seeds of maize were sown in each pot. After germination, three 15-days-old maize seedlings of uniform size were selected and cultivated during the experiment. Soil samples were collected by mixing five sub-samples randomly collected from whole soil profile using a stainless steel tool after 0, 60, 120, 150, and 180 days of experiment. Thereafter, soils from each pot were homogenized and sieved (2 mm mesh) and subdivided into three subsamples, one of which was air-dried for PAH analysis; a second, which was kept at 4 °C for enzymatic analysis; and a third, which was kept at −80 °C for ergosterol analysis. The plant biomass was harvested at full maturity of maize (180 days), and roots and shoots were separated. Roots were washed with distilled water to remove attached soil particles, and a portion of material (roots and shoots) was kept at 4 °C for further analysis of PAH content.

2.5. Sample extraction and analysis of PAHs

PAH-specific analyses of soil and plant samples were done using the ultrasonic extraction method, as previously described elsewhere (Košnář et al., 2016). Briefly, dry soil and plant samples (15 g; 5 g, respectively) were mixed with 30 mL *n*–hexane/acetone mixture (2:1 v/v). Samples were extracted with the ultrasonic extraction system (Bandelin Sonorox Digitec DT510/H, Germany) for 30 min and shaken for 1 h in an orbital shaker (GFL 3017, Germany). Thereafter, the samples were mixed with 50 mL of deionized water, and after phase separation, part of the upper *n*–hexane layer was pipetted out, cleaned with Sep-Pak silica cartridges (Chromservis, Czech Republic), and then concentrated to 1.0 mL by rotatory vacuum evaporation at 40 °C (Büchi Rotavapor R–300, Switzerland). The IS solution at a concentration of 500 ng mL^{-1} was added to the extract before further analysis. Blanks were prepared following the same procedure without adding the sample. The certified reference material EC 2 - river sediment (Analytika, Czech Republic) was used for quality control.

The sample extracts were analyzed using an Agilent HP 6890 N gas chromatograph equipped with an Agilent 7683B injector including an Agilent 10.0 µL syringe and connected to an Agilent HP 5975 inert mass selective detector (GC/MSD, 6890 N/5975, Agilent Technologies, USA). Quality control and assurance of gas chromatography analysis were described by Košnář et al. (2016).

2.6. Soil microbial biomass and activity

The soil microbial biomass was assayed by the quantification of the dehydrogenase activity (EC 1.1) according to García and Hernández (1997). One g of soil was incubated with 1 mL of 1.2% of triphenyl tetrazolium chloride (TTC) dissolved in 0.1 M Tris–HCl pH 7.6 for 24 h at 30 °C. After incubation, the triphenylformazan (TPF) produced was extracted with acetone in a ratio of 1:4 [soil extract: acetone (v/v)] and measured spectrophotometrically at 490 nm. The results were calculated using a standard curve and expressed as µg TPF g^{-1} dry soil.

Overall microbial activity was recorded through the hydrolysis of the fluorescein diacetate (FDA) following the procedure described by Adam and Duncan (2001). One g of soil sample was incubated with 0.2 mL of FDA dissolved in acetone and 7.5 mL of 60 mM K-phosphate pH 7.6 for 60 min at room temperature. The reaction was stopped after the addition of 7.5 mL of acetone and the suspension was centrifuged at 3500*g* for 150 min. The fluorescein produced was spectrophotometrically measured at 490 nm and the results were expressed as µg fluorescein g^{-1} dry soil.

2.7. Ergosterol content

Soil fungal biomass was quantified by extracting ergosterol using the method of Djajakirana et al. (1996). Two grams of soil were suspended in 50 mL of ethanol in amber bottles and shaken for 30 min at 250 rpm followed by centrifugation at 4400 rpm for 30 min. An aliquot of 10 mL was transferred into a test tube and evaporated in a vacuum rotary evaporator at 50 °C. The dry extract was dissolved in 1 mL methanol and percolated through a syringe filter (cellulose-acetate, 0.45 µm pore size) into an amber HPLC glass vial. Extracts were measured using an HPLC system (1260 Infinity, Agilent Technologies, USA) equipped with a diode array detector and a Phenomenex Luna C18 column (250 mm × 4.60 mm; particle size 5 µm; pore size 100 Å) equilibrated with pure methanol at a flow rate of 0.75 mL min^{-1}. The sample injection volume was 10 µL. Ergosterol was detected at 282 nm and quantified using a calibration curve with the pure standard.

2.8. Statistical analysis

A pair-wise comparison (one-way ANOVA) of individual PAHs data in each treatment was performed using the Tukey test ($p < 0.05$) with SPSS software version 17.0. The degradation percentage of each individual PAHs (%) in each treatment was calculated following the equation: [100 −(Ct*100)/Ct0], where C_t refers to the content of each PAHs at different times of sampling (60, 120, 150, and 180 days) and C_{t0} refers to the content PAHs at the initial time (0 days). A pair-wise comparison of individual PAHs degradation including the sum of PAHs fractions among different treatments at 180 days was done by one-way ANOVA. Data of each PAH degradation were subjected to principal component analysis (PCA) using PAST software package. One-way ANOVA was used to compare individual PAHs content among different treatments in roots and shoots at 180 days. To estimate the accumulation rates of PAHs in plant tissues (roots and shoots), the bioconcentration (BF) and translocation (TF) factors were used. BF was calculated as a ratio of the compound content in the roots to its content in the soil, whereas the TF was done according to the relationship between the PAHs content in shoots and roots. Statistical analysis to analyze differences in BF and TF factors between treatments were calculated by one-way ANOVA. A multiple pair-wise comparison (two-way ANOVA) of soil microbial activities and ergosterol content was carried out to compare differences among means within the same treatment type as a function of time and among different treatments.

3. Results and discussion

3.1. Effect of bioremediation treatments in the degradation of PAH in an aged polluted soil

3.1.1. Natural attenuation (NA)

The NA produced a decrease after 180 days of the initial PAH soil content, reaching values from 1132 to 658.2 µg kg^{-1} (Table 1). Overall, the degradation of individual PAHs indicated that the most susceptible PAHs compounds to degrade within 60 days of experiment were the ACEN, and DBA reaching percentages of removal ranged from 32.3%, and 52.4% respectively (Table S2). The highest percentages of degradation, in terms of 2–3 − C rings, were found in FLU, PHE and ANT, with values amounting to 57%, 61.4% and 57.7%, respectively, at 180 days. The removal efficiency of the initial PAH content could be possibly related to the chemical structure, and thus, PAHs with 2–3 rings are more readily biodegradable by the autochthonous soil microbiota, but they are mostly prone to volatilization and photoxidation as recently observed (Nanekar et al., 2015). The degradation of 4 ring hydrocarbons was also favored by the NA reaching higher rates of degradation in the case of FLUO (57.2%) and PYR (45%) after 180 days of experiment (Table S2). Biodegradation through the functional adaptation of indigenous microorganisms seems to be the main pathway for pollutant removal, as previously suggested by Declercq et al. (2012). However, in this study, the removal of 5–6 rings under NA conditions

Table 1
Content of the individual PAHs (μg kg^{-1} DW) in an aged polluted soil under natural attenuation after 0, 60, 120, 150, and 180 days. The data represent means (± SD) of four replicate measurements. Statistical pair-wise of data (one way ANOVA) were carried out according to the Tukey test. The same lowercase letters indicate a lack of statistically significant differences ($p < 0.05$) among means within the same row.

	Time of soil incubation				
Compound	0 days	60 days	120 days	150 days	180 days
NAP	87 ± 1.7b	79.82 ± 3.7b	78.82 ± 2.6ab	72.82 ± 3.9ab	65.5 ± 3.7a
ACE	13.4 ± 0.6b	12.2 ± 0.7b	12.8 ± 0.5b	9.2 ± 0.3a	9.2 ± 0.7a
ACEN	10.1 ± 0.7c	6.8 ± 0.1b	4.7 ± 0.2a	5.6 ± 0.3ab	5.5 ± 0.4ab
FLU	12.7 ± 0.9b	7.2 ± 0.5a	6.9 ± 0.1a	5.7 ± 0.2a	5.5 ± 0.3a
PHE	71.3 ± 2.2b	67 ± 6.4b	35.8 ± 2.3a	31.1 ± 2.6a	27.4 ± 3a
ANT	70.1 ± 1.4b	74 ± 12.9b	37.1 ± 2.8a	33.1 ± 3.3a	29.7 ± 2.1a
Sum 2–3 rings	264.3 ± 4.4c	237.2 ± 13.2c	160.9 ± 2.4b	142.6 ± 3.7ab	138.2 ± 3.7a
FLUO	153 ± 2.2b	128.7 ± 12.8b	92.8 ± 6.1a	68.8 ± 6.3a	65.2 ± 5.9a
PYR	109.7 ± 3b	98.3 ± 6.5b	69.9 ± 2.7a	60.6 ± 5.4a	59.8 ± 6.4a
BaA	94 ± 2b	79.2 ± 10.5ab	68.3 ± 5.1ab	58.2 ± 9.7ab	56.1 ± 9.8a
CHR	88.9 ± 0.9b	69 ± 5.4ab	65.4 ± 3.6a	50.6 ± 6.7a	50.3 ± 6.9a
Sum 4 rings	445 ± 7.5c	375.2 ± 29.3bc	296.1 ± 9.4ab	238.3 ± 25.3a	234.5 ± 27.2a
BbF	83.4 ± 2.8a	79.4 ± 7.4a	73 ± 5a	64 ± 6a	61.5 ± 6a
BkF	94.1 ± 1.1b	75.1 ± 9.2ab	67 ± 6.1a	58.9 ± 3.2a	56.2 ± 2.7a
BaP	103 ± 5b	98.2 ± 11.9b	68.2 ± 5.7a	65 ± 2.1a	63 ± 1.5a
DBA	6 ± 0.1b	2.8 ± 0a	2.8 ± 0a	2.8 ± 0a	2.8 ± 0a
IPY	70 ± 2.3a	66.8 ± 6.3a	54.5 ± 5.3a	48 ± 7.2a	45.3 ± 7.1a
BghiP	67.9 ± 3a	71.1 ± 10.7a	56.9 ± 6.7a	56.9 ± 5.6a	55.1 ± 5.5a
Sum 5–6 rings	423 ± 13.2c	393.4 ± 39.8bc	322.3 ± 23.1abc	295.6 ± 19ab	284 ± 18a
Σ 16 PAH	1132 ± 20.8b	1016.4 ± 80.8b	794.6 ± 28.8a	691.3 ± 42.3a	658.2 ± 43.8a

was relatively low, compared with 2–3, 4 ring compounds (Table 1, S2). In fact, significant percentages of degradation were only achieved in the case of BaP reaching values up to 38% at 180 days. This finding is consistent with the fact that higher ring number and alkyl substitution exhibit higher hydrophobicity and are more likely to be adsorbed by the soil matrix, which leads to lower bioavailability towards biodegradation (Guo et al., 2010).

3.1.2. *Mycoaugmentation assisted by Crucibulum laeve (M)*

The inoculation of *C. laeve* into the aged PAH-polluted soil resulted in a reduction of Σ16 PAHs content, which amounted to 695.5 μg kg^{-1} (Table 2). The individual 2–3 ring compounds such as ACEN, FLU, PHE, and ANT were degraded throughout the frame time of the experiment, reaching the highest percentages of degradation values: up to 47.9%, 52.5%, 54.6%, and 55.6%, respectively, at 180 days (Table S3). Meanwhile, NAP reached a percentage of removal amounting to 37.4% which was significantly higher compared to NA (Table S6). This result may suggest that the removal of NAP could be achieved enzymatically by LME. However, in this study, the values of LME in soil samples (laccases and Mn peroxidase) were very low and under the limit of detection (data not shown). This fact is consistent with the previous finding reported by Lladó et al. (2013), who observed a lack of laccase and/or Mn peroxidase during the degradation of hydrocarbon compounds in a bioaugmented microcosm; therefore, the degradation may also be achieved by means of a high physical adsorption into the mycelium or by intracellular enzymatic systems. The efficiency of M treatment, in terms of 4 rings degradation, was lower compared with those found during the NA of this soil (Fig. S1). However, in regards to

Table 2
Content of the individual PAHs (μg kg^{-1} DW) in an aged polluted soil under mycoaugmentation treatment after 60, 120, 150, and 180 days of incubation. The data represent means (± SD) of four replicate measurements. Statistical pair-wise of data (one way ANOVA) were carried out according to the Tukey test. The same lowercase letters indicate a lack of statistically significant differences ($p < 0.05$) among means within the same row.

	Time of soil incubation				
Compound	0 days	60 days	120 days	150 days	180 days
NAP	87 ± 1.7b	82.7 ± 3.6b	60.3 ± 0.9a	54.7 ± 6a	54.3 ± 6.3a
ACE	13.4 ± 0.6b	11.1 ± 0.7ab	11.4 ± 0.7ab	9.6 ± 0.4a	9.2 ± 0.2a
ACEN	10.1 ± 0.7c	8.6 ± 0.5b	5.4 ± 0.4a	5.2 ± 0.4a	5.3 ± 0.5a
FLU	12.7 ± 0.9b	10 ± 1.7b	7.1 ± 0.1ab	6.1 ± 0.5ab	6.0 ± 0.5a
PHE	71.3 ± 2.2b	61.9 ± 4.8b	37.7 ± 1.9a	34.3 ± 3.4a	32.3 ± 2.7a
ANT	70.1 ± 1.4b	63 ± 7.9b	39 ± 2.2a	32.7 ± 3a	31.1 ± 2.7a
Sum 2–3 rings	264.3 ± 4.4c	237.2 ± 8.5b	160.9 ± 4.3a	142.6 ± 11.7a	138.2 ± 12a
FLUO	153 ± 2.2b	136.8 ± 8.7c	106.5 ± 3.3b	93.2 ± 10.5ab	73.5 ± 4.6a
PYR	109.7 ± 3b	101 ± 5.9bc	81.1 ± 2.4ab	72.8 ± 7.7a	68.9 ± 7.1a
BaA	94 ± 2b	87.9 ± 8.6ab	71.9 ± 5.1ab	62.2 ± 10ab	57.2 ± 8.4a
CHR	88.9 ± 0.9b	89.9 ± 7.9a	79 ± 3.2a	77.1 ± 3.4a	77.1 ± 3.4a
Sum 4 rings	445 ± 7.5c	415.7 ± 26bc	338.4 ± 8.4ab	305.4 ± 24.5a	276.8 ± 17.2a
BbF	83.4 ± 2.8a	71.6 ± 2.7ab	67.8 ± 1.9ab	58.3 ± 6a	58.2 ± 6a
BkF	94.1 ± 1.1b	80.9 ± 4.7a	80.3 ± 5.6a	69.6 ± 11a	67.5 ± 10.5a
BaP	103 ± 5b	95.4 ± 10b	66.5 ± 2.5a	60.6 ± 3.1a	59.1 ± 2.9a
DBA	6 ± 0.1b	2.8 ± 0a	2.8 ± 0a	2.8 ± 0a	2.8 ± 0a
IPY	70 ± 2.3a	61.9 ± 5.4a	59.2 ± 4.6a	53.8 ± 6.5a	52.9 ± 6.4a
BghiP	67.9 ± 3a	73.8 ± 4.9b	44.2 ± 2a	41.4 ± 1.1a	40.1 ± 1a
Sum 5–6 rings	423 ± 13.2c	386.4 ± 12.2bc	320.8 ± 5ab	286.4 ± 23.5a	280.6 ± 23.2a
Σ 16 PAH	1132 ± 20.8b	1039.3 ± 45.2b	820.1 ± 17.4a	734.4 ± 59.3a	695.5 ± 52.3a

Table 3
Content of the individual PAHs (μg kg^{-1} DW) in an aged polluted soil under phytoremediation after 60, 120, 150, and 180 days. The data represent means (± SD) of four replicate measurements. Statistical pair-wise of data (one way ANOVA) were carried out according to the Tukey test. The same lowercase letters indicate a lack of statistically significant differences ($p < 0.05$) among means within the same row.

	Time of soil incubation				
Compound	0 days	60 days	120 days	150 days	180 days
NAP	87 ± 1.7b	85.2 ± 2.5c	70 ± 1.02b	47.8 ± 3.8a	42.9 ± 3.6a
ACE	13.4 ± 0.6b	14.5 ± 0.6b	13.7 ± 0.6b	11.02 ± 0.5a	10.1 ± 0.4a
ACEN	10.1 ± 0.7c	7.7 ± 0.3bc	4.4 ± 0.3ab	5.7 ± 0.6ab	6.2 ± 1a
FLU	12.7 ± 0.9b	11.3 ± 1.4b	7 ± 0.7a	6.2 ± 0.2a	6.3 ± 0.2a
PHE	71.3 ± 2.2b	65.4 ± 1.6b	26.7 ± 0.7a	24.9 ± 0.9a	25.1 ± 1.1a
ANT	70.1 ± 1.4b	68.1 ± 1.8b	32.9 ± 2.1a	29 ± 2.5a	27.8 ± 2.4a
Sum 2–3 rings	264.3 ± 4.4c	252.2 ± 1.7c	154.8 ± 4.3b	124.7 ± 8.1a	118.2 ± 8.2a
FLUO	153 ± 2.2b	141.9 ± 5.5b	79.9 ± 2.7a	70 ± 2.9a	69.1 ± 2.9a
PYR	109.7 ± 3b	107.3 ± 7.3b	55 ± 2.6a	54.2 ± 1.9a	54.3 ± 2.4a
BaA	94 ± 2b	76.4 ± 4.8b	62.4 ± 4.01ab	56.9 ± 5.9a	55.1 ± 5.3a
CHR	88.9 ± 0.9b	82.9 ± 4.2b	66.6 ± 2.1ab	57.3 ± 4.8a	55.4 ± 4.8a
Sum 4 rings	445 ± 7.5c	408.3 ± 4.7b	257.7 ± 8.8a	238.4 ± 13.2a	234 ± 13.2a
BbF	83.4 ± 2.8a	76.5 ± 3.6b	67.2 ± 1.4ab	58.8 ± 6.3a	57 ± 5.9a
BkF	94.1 ± 1.1b	86.3 ± 2.7a	77.8 ± 5.1a	71.1 ± 8.7a	69 ± 9.6a
BaP	103 ± 5b	95.5 ± 10b	65.5 ± 3.02a	58.6 ± 5.7a	56.7 ± 6.1a
DBA	6 ± 0.1b	2.8 ± 0a	2.8 ± 0a	2.8 ± 0a	2.8 ± 0a
IPY	70 ± 2.3a	71.4 ± 1.5b	38.2 ± 2.5a	37.1 ± 1.7a	36.2 ± 1.8a
BghiP	67.9 ± 3a	63.3 ± 2.1b	48.1 ± 1.1a	44.4 ± 3.6a	43 ± 3.5a
Sum 5–6 rings	423 ± 13.2c	395.8 ± 12.3b	299.7 ± 9.1a	272.8 ± 23.9a	264.5 ± 24.9a
Σ 16 PAH	1132 ± 20.8b	1056.3 ± 15.7b	712.1 ± 21.7a	635.8 ± 44.5a	616.6 ± 45.8a

5–6 ring compound degradation, a remarkable percentage of degradation was significantly achieved in the case of BghiP, in an opposite trend to NA treatment (Table S6) reaching percentages of degradation amounting to 40% after 180 days of experiment. This finding may suggest the potential ability of *C. laeve* to diffuse into the soil matrix and potentially degrade this aromatic − C compound with a low bioavailability through the monooxygenase system of cytochrom P-450, as previously reviewed (Haritash and Kaushik, 2009). Overall, the PAH degradation during the mycoaugmented treatment was not different in relation to NA treatment, suggesting that the colonization of the upper soil layer by *C. laeve* was hindered by the exceptional growth capabilities of indigenous soil microbiota. This is in concordance with the previous results found by Lladó et al. (2013), in which a markedly antagonist effect towards *T. versicolor* and *L. tigrinus* was observed by the resident microbial populations.

3.1.3. Phytoremediation (P)

The Σ 16 PAHs achieved values up to 616.6 μg kg^{-1} after 180 days of phytoremediation treatment (P) (Table 3). The degradation of the individual 2–3 rings reached percentages of degradation up to 50.5% (NAP), 50% (FLU), 64.6% (PHE), and 60.2% (ANT), after 180 days of experiment (Table S4). Maize plants could enhance the biodegradation processes by stimulating the indigenous microbial biomass and/or activity in the rhizosphere (Segura and Ramos, 2013). This finding may suggest that autochthonous microbial communities of the present aged PAH-polluted soil appear to be adapted and functional for degrading most of the 2 rings, mainly in the case of the NAP which was found to be the most degradable 2,3 ring compound when compared with NA and M treatments. The degradation of the individual 4 rings was also significantly increased during the soil phytoremediation, reaching percentages of degradation higher to those found in M treatments, especially in the case of PYR, BaA, and CHR (Table S6). However, the removal of the individual 5–6 rings followed similar trend as it was found in the case of NA and M treatments at 180 days. This treatment reached percentages of degradation up to 47.1% and 36.7% for IPY and BghiP, respectively, which significantly differed to those found in the NA and M treatments (Table S6). Clearly, P treatment led to higher 2–3 and 4 rings compound degradation than NA and M treatments, respectively, indicating the potential characteristic of maize plants to clean up sites contaminated with aromatic compound during phytoremediation approaches (Fig. S1). There are two possible explanations for these results. Firstly, it can be suggested that maize plants had an active role in the rhizospheric degradation of PAHs as the result of the action of plant enzymes released in root exudates. This finding may be supported by previous studies reported by Tejeda-Agredano et al. (2013) and Agnello et al. (2016), but in the rhizosphere of alfalfa plants. Secondly, the desorption of PAHs from soil particles could be stimulate through root exudates (abiotic factor) which could favor their bioavailability and consequent degradation through indigenous microorganisms (Sun et al., 2012; Tejeda-Agredano et al., 2013).

3.1.4. Microbe-assisted phytoremediation (MAP)

The joint action of plants and microbes has been suggested as a profitable bioremediation approach to deal with all possible problems caused by PAH contamination (Huguenot et al., 2015). In fact, the Σ 16 PAHs in the MAP treatment after 180 days revealed a significant decrease reaching values up to 475.1 μg kg^{-1} at 180 days (Table 4). In regards to individual PAHs degradation, the most significant changes were observed in the NAP, PHE and ANT removal reaching values up to 64.1%, 64.6% and 62.2%, respectively after 180 days of experimentation (Table S5), being significant higher in the case of NAP to those found in the case of NA, and M (Table S6). The interaction between maize roots and *C. laeve* appeared to be effective in terms of individual 4 rings degradation (Table 4). Thus, the highest percentages of degradation up to 63.6%, 59.3% and 63.4% were achieved in the case of FLUO, PYR and BaA, respectively, which significantly differed to those found in other treatments (Table S6). However, the most significant changes were detected concerning 5–6 ring compounds, which have been reported to be the most potentially carcinogenic to humans or animals (IARC, 2010; EPA, 2015). In these findings, BbF, BkF, BaP, and BghiP were shown to be the most degraded 5–6 ring compounds, reaching significant percentages of degradation up to 48.2%, 56.3%, 62.4%, and 54.3%, respectively (Table S5). The maize plant-*C. laeve* association clearly suggests a synergistic contribution, and therefore this treatment was the most effective approach in terms of 4, 5–6 rings degradation in comparison with other treatments (Fig. S1). It should be pointed out that the interaction between *C. laeve* and maize plants could make several noteworthy further contributions, for example: it increases the surface adsorption area through fungal hyphae, which can

Table 4
Content of the individual PAHs (μg kg^{-1} DW) in an aged polluted soil under microbe-assisted phytoremediation after 60, 120, 150, and 180 days of incubation. The data represent means (± SD) of four replicate measurements. Statistical pair-wise of data (one way ANOVA) were carried out according to the Tukey test. The same lowercase letters indicate a lack of statistically significant differences ($p < 0.05$) among means within the same row.

	Time of soil incubation				
Compound	0 days	60 days	120 days	150 days	180 days
NAP	87 ± 1.7b	84.6 ± 2.2c	46.02 ± 2.8b	41.1 ± 1.6b	31.1 ± 1.2a
ACE	13.4 ± 0.6b	12.1 ± 0.2c	11.7 ± 0.7bc	9.8 ± 0.2ab	8.8 ± 0.1a
ACEN	10.1 ± 0.7c	8.9 ± 1.6b	4.1 ± 0.1a	4.6 ± 0.3a	4.8 ± 0.4a
FLU	12.7 ± 0.9b	13.6 ± 0.9b	7.2 ± 0.2a	6.8 ± 0.3a	6.7 ± 0.3a
PHE	71.3 ± 2.2b	70.9 ± 3.9b	33.7 ± 1.9a	27.9 ± 1.6a	25.1 ± 1.4a
ANT	70.1 ± 1.4b	68.4 ± 3.6c	37.4 ± 0.9b	31.4 ± 2.9ab	26.4 ± 1.6a
Sum 2–3 rings	264.3 ± 4.4c	258.5 ± 6.3c	140.2 ± 5.9b	121.6 ± 3ab	102.8 ± 2.9a
FLUO	153 ± 2.2b	140.4 ± 6.9b	76.5 ± 9.1a	65 ± 2.7a	55.6 ± 2.4a
PYR	109.7 ± 3b	92.4 ± 4.7c	69.9 ± 1.4b	59.1 ± 4.6ab	44.7 ± 4.5a
BaA	94 ± 2b	79.5 ± 5.7c	56.9 ± 5.3b	46.9 ± 1.3ab	34.3 ± 1.2a
CHR	88.9 ± 0.9b	83 ± 5.9c	63.9 ± 1.9b	56 ± 2.2ab	46.7 ± 2.1a
Sum 4 rings	445 ± 7.5c	395.2 ± 17.4c	267.15 ± 17b	226.9 ± 6.7ab	181.3 ± 7.3a
BbF	83.4 ± 2.8a	74.9 ± 4.4c	58.1 ± 4.2b	49.4 ± 1.4ab	43 ± 1.1a
BkF	94.1 ± 1.1b	84.6 ± 2.6b	54.4 ± 6a	45.4 ± 2a	41 ± 0.9a
BaP	103 ± 5b	92.5 ± 9b	49.8 ± 3.7a	42.4 ± 1.4a	38.2 ± 0.7a
DBA	6 ± 0.1b	2.8 ± 0a	2.8 ± 0a	2.8 ± 0a	2.8 ± 0a
IPY	70 ± 2.3a	63.9 ± 4.6b	40.2 ± 4.5a	37.3 ± 3.2a	35.3 ± 2.3a
BghiP	67.9 ± 3a	57.1 ± 5.4b	39.1 ± 1.4a	32.9 ± 1.9a	30.7 ± 1.9a
Sum 5–6 rings	423 ± 13.2c	375.9 ± 21.4b	242.4 ± 18.2a	210.3 ± 1.5a	191.1 ± 1.5a
Σ 16 PAH	1132 ± 20.8b	1029.6 ± 40.2c	649.7 ± 40.4b	558.8 ± 10ab	475.1 ± 10.4a

also serve as support for bacterial transport through the soil; the alteration of root exudates increasing bioavailability and biodegradability of PAHs; and/or trophic contribution as previously reported by Kanaly and Bartha (2009).

3.2. Overall assessment of PAH degradation (integrated multivariate analysis)

A multivariate study based on principal component analysis was used to obtain insight into similarities or differences among treatments and the degradation of individual PAHs and their respective interaction (Fig. S2). To address this purpose, sixteen individual PAHs degradation were analyzed in each soil treatment (NA, M, P, and MAP) after 60, 120, 150, and 180 days. The analysis revealed that approximately 74% of the total variance of the data was explained by the first of two principal components (63.8% and 10.4%, respectively) (Fig. S2a). Clearly, the position of loadings plot found in the case of DBA indicated that this PAH was not modified by any component, whilst the 2 and 6 ring compounds, NAP and BghiP, were strongly separated by both components (Fig. S2b). 150-d, 180-d NA, M and P treatments and 120-d, 150-d, and 180-d MAP treatments were separate from the other treatments by the first component (Fig. S2a), indicating that the principal grouping factor in this study was the time of treatment. The main PAH removal was reached in most of the individual compounds, as indicated by the respective vector lengths (Fig. S2b). Interestingly, in this study the 150-d and 180-d NA and P treatments, which showed similar ratios of degradation for 4 rings within 150 days of experiment, were located in the right-upper quadrant corresponding to FLUO, IPY, PHE. Meanwhile, 150-d and 180-d M treatments, which were shown to reach the higher values of degradation for NAP and BghiP, were placed in the same quadrant (right-lower). The gradually degradation of ACE, BbF, BaP, BghiP and NAP during the microbe-assisted degradation was evidenced in this study, as indicated the position of 120-d, 150-d, and 180-d MAP treatments in the right-lower quadrant (Fig. S2a). The PCA analysis, moreover, indicated that 60-d M, P, and MAP treatments and 120-d M treatment were shown to be the less effective treatments degrading ANT, BkF, CHR, FLU, IPY, PHE, and PYR, as revealed their respective position in the left-lower quadrant. Similarly, 60-d and 120-d NA, and 120-d P treatments that were shown to lead a low ratio of degradation of these compounds (NAP, ACE, BbF and BghiP), which were located in the opposite quadrant to those variables (Fig. S2a, b).

3.3. Effect of phytoremediation and microbe-assisted phytoremediation on maize plants PAH accumulation

Maize plants were harvested at the end of the experiment (180 days), and data on plant biomass (roots and shoots) of both P and MAP treatments were recorded (data not shown). No significant changes in root (10 ± 0.4; 10 ± 1 g) or shoot (55.6 ± 9; 47.2 ± 3.6 g) dry weight were found between P and MAP treatments, respectively. Phytoremediation of PAH-polluted soil commonly involves four mechanisms: absorption by plants, volatilization, transformation by plant exudates and enzymes, and the rhizodegradation through soil microbial communities (Alagić et al., 2015). The analysis of the individual PAH content in maize samples revealed their presence in roots, except in the case of FLU and DBA, which were under the limit of detection. The content of Σ16 PAHs reached values up to 109.6 μg kg^{-1} in MAP treatment, whereas in P treatment it was up to 73.9 μg kg^{-1} (Table S7). Interestingly, NAP, ACEN, PHE, and ANT content were significantly increased in the MAP treatment when compared to P treatment. This fact might indicate an alteration of root exudates which are increasing the bioavailability of PAHs owing to the assisted rhizodegradation stimulated by *C. laeve* inoculation. However, this hypothesis requires a further research. Meanwhile, no changes in regards to 4, 5-, 6 ring compounds were found between both treatments, except in the case of FLUO (Fig. 1). Similarly, maize shoots showed a similar trend as it was found in the roots, thus the sum of 16 PAHs reached higher values in MAP treatments (44.8 μg kg^{-1}) than in P treatment (29 μg kg^{-1}) (Table S7). In this regard, MAP treatment resulted in higher accumulation of NAP, PHE, ANT, FLUO, and PYR compared with P treatment (Fig. 1). Conversely, 5- and 6 ring compounds were not detected in the aboveground biomass of maize plants. This finding suggests that PAHs with more than 4 aromatic rings are more hydrophobic and physically less compatible with passing through cell membranes. In order to estimate if the content of individual PAHs in maize tissues (roots and shoots) may be associated with a phytoextraction process, the BF and TF were analyzed (Table 5). In our study, the BF values indicated high accumulation in NAP, ACEN, PHE, ANT, and FLUO compounds, in both treatments (Table 5). However, the presence of *C. laeve* in the rhizosphere of maize roots had a remarkable effect that significantly

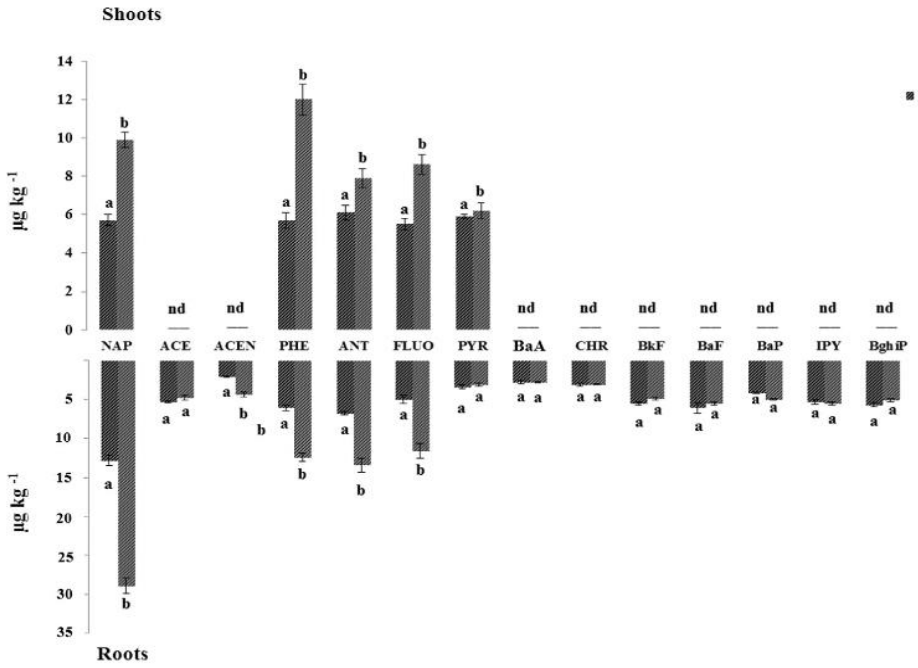

Fig. 1. Individual PAH content in roots and shoots at 180 days of experiment in phytoremediation (P) and microbe-assisted phytoremediation (MAP). Data are the mean (± SD) of four replicates. The same lowercase letters above bars indicate a lack of statistically ($p < 0.05$) significant difference between P and MAP treatments. nd = not detected.

Table 5
Bioconversion (BF) and translocation (TF) factors after 180 days of experiment under P phytoremediation (P), and microbe-assisted phytoremediation (MAP.) BF and TF were calculated as the ratio of concentration of each individual PAH in roots to its concentration in soil and between each individual PAH content in roots and shoots, respectively. The data represent means (± SD) of four replicate measurements. Statistical pair-wise of data (one way ANOVA) were carried out according to the Tukey test. The same lowercase letters indicate a lack of statistically significant differences ($p < 0.05$) among means within the same row.

	Treatments			
	BF		TF	
Compounds	P	MAP	P	MAP
NAP	0.1 ± 0.02a	0.3 ± 0.02b	0.45 ± 0.06a	0.35 ± 0.04a
ACE	0.4 ± 0.03a	0.4 ± 0.01a	0	0
ACEN	0.2 ± 0.01a	0.4 ± 0.08b	0	0
FLU	n.d	n.d	n.d	n.d
PHE	0.1 ± 0a	0.2 ± 0.02b	0.95 ± 0.12a	0.98 ± 0.05a
ANT	0.1 ± 0.01a	0.2 ± 0.02b	0.91 ± 0.09b	0.64 ± 0.06a
FLUO	0.1 ± 0a	0.1 ± 0.01b	1.16 ± 0.27b	0.75 ± 0.11a
PYR	0.03 ± 0.01a	0.03 ± 0.01a	1.73 ± 0.17a	2 ± 0.37a
BaA	0.03 ± 0.01a	0.03 ± 0a	0	0
CHR	0.03 ± 0a	0.03 ± 0a	0	0
BbF	0.01 ± 0a	0.1 ± 0a	0	0
BkF	0.01 ± 0 a	0.1 ± 0a	0	0
BaP	0.04 ± 0a	0.05 ± 0.01a	0	0
DBA	n.d	n.d	n.d	n.d
IPY	0.01 ± 0.01a	0.01 ± 0a	0	0
BghiP	0.01 ± 0.01a	0.01 ± 0a	0	0

enhanced the accumulation of the 2–3 ring compounds compared with P treatments. On the other hand, the TF indicated that NAP, PHE, ANT, FLUO, and PYR were found to be the only PAH compounds translocated and accumulated in maize shoots, in both treatments (Table 5). Besides, this factor indicated that the transfer of ANT and FLUO from roots to shoots was significantly higher in P treatment compared to MAP treatment. This result is consistent with the previous findings reported by Agnello et al. (2016), who found that during microbe-assisted phytoextraction processes, TF values generally are decreased. Overall, it has been reported that the 2–3 ring compounds are easily uptaken by root plants through the cell wall and subsequently translocated to shoots (Gao et al., 2011). Some studies have suggested the potential capacity of plant species rather for phytoextraction than phytostabilization processes when BF and TF values are higher than 1 (Alagić et al., 2015; Agnello et al., 2016). However, in our study, BF ranged in value from 0.01 to 0.4 in both P and MAP treatments. Meanwhile, TF reached values up to 2, but only in the case of PYR, which could indicate that the association of maize plants and *C. laeve* may be more effective for phytostabilization/rhizodegradation rather phytoextraction processes.

3.4. Impact of treatments on soil microbial biomass and functionality

Dehydrogenase activity and FDA hydrolysis have been widely used in soil as an accurate and simple index for overall microbial biomass present only in viable cells and to determinate amounts of enzymatically active soil microorganisms, respectively (Green et al., 2006; Wolińska and Stępniewska, 2012). In the context of remediation of PAH-polluted soils, both dehydrogenase and FDA hydrolysis represent useful indicators for a better understanding of the impact of biological approaches on the autochthonous microbial activity. In this study, NA was the treatment in which the lowest values of dehydrogenase activity and FDA hydrolysis were recorded in the time frame of the experiment as well as when compared with other treatments (Fig. 2a, b). Interestingly, this treatment showed a significant decline in the Σ 16 PAHs after 180 days (Table 1), which may indicate that the indigenous microbial communities are adapted and functional to the PAH degradation. In spite of this fact, it has to be considered that abiotic losses such as the volatilization of low volatile fractions of PAHs and/or photooxidation can also be responsible for PAH degradation (Nanekar et al., 2015). The level of dehydrogenase activity and FDA hydrolysis was notably and predictably stimulated by the application of viable fungal inocula in the M and MAP treatment at the beginning of the experiment (Fig. 2a, b). In some cases, the M treatment showed higher levels of dehydrogenase activity and FDA hydrolysis compared with NA at any time of the experiment. However, the dehydrogenase activity in M treatment had similar values to those found in P treatment at 60, 150, and 180 days,

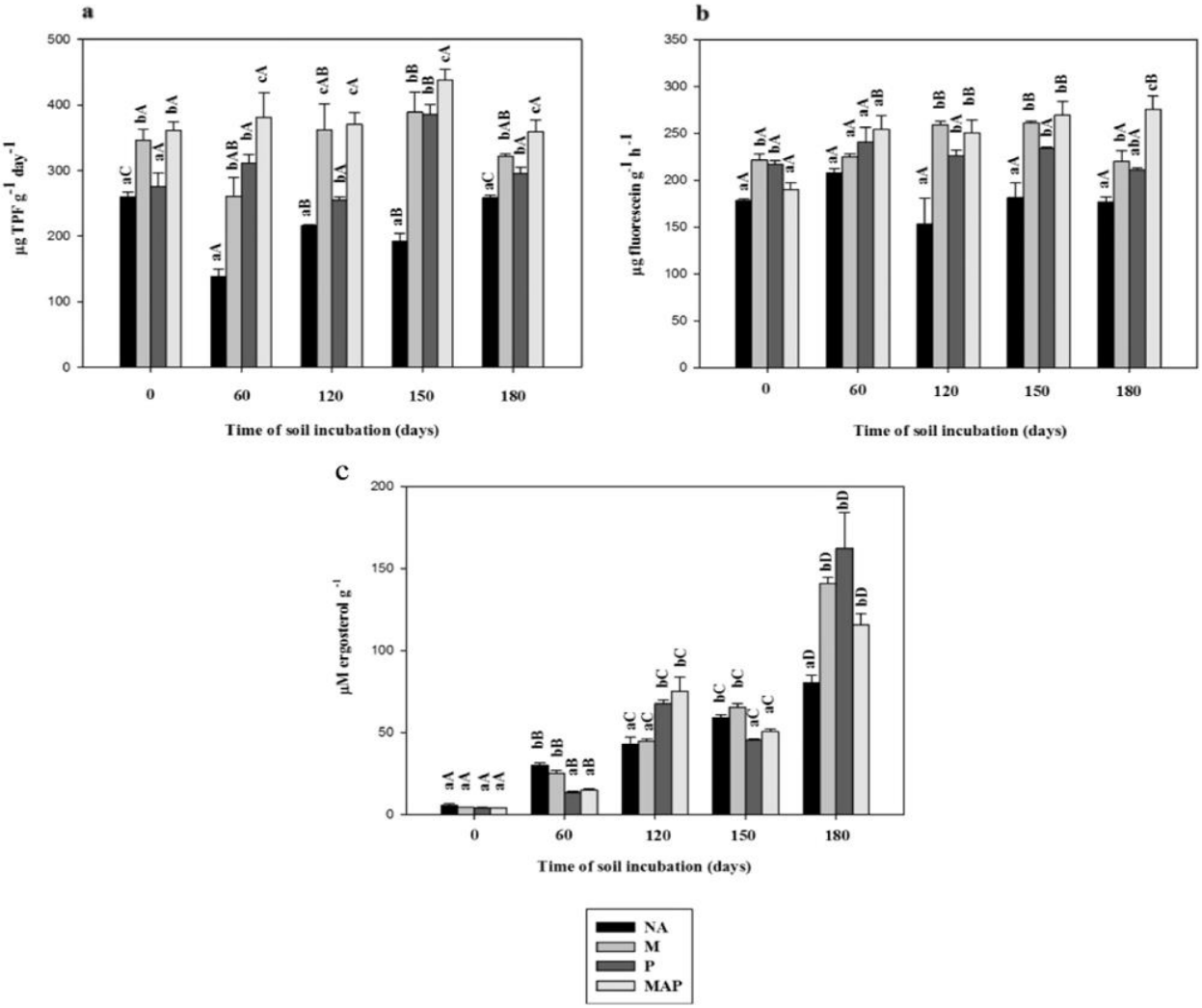

Fig. 2. Dehydrogenase activity (a), FDA hydrolysis (b) and (c) ergosterol content in an aged PAH-polluted soil at 0, 60, 120, 150, and 180 days under natural attenuation (NA), mycoaugmentation (M), phytoremediation (P), and microbe-assisted phytoremediation (MAP). Data are the mean ± (± SD) of four replicates. The same lowercase letters above bars indicate that differences among treatments were not significant. The same uppercase letters indicate a lack of statistically ($p < 0.05$) significant difference within each treatment throughout the frame time of the experiment.

and respect to MAP treatment after 120 days (Fig. 2a). Meanwhile, the M treatment did not significantly modify the FDA hydrolysis in comparison to P and MAP treatments at any time of experiment, but it was lower when compared with MAP treatment at 180 days (Fig. 2b). This result might not be associated with the PAH degradation during M treatment, but rather to an increase of the biomass and functionality of resident soil microbial population, as the result of the presence of viable inocula of *C. laeve* and/or the trophic contribution of the carrier used (barley seeds) which contains great amounts of potential growth substrates, such as cellulose and hemicellulose (Vane et al., 2006). Root exudates are commonly composed of organic acids and/or sugars that can be used as carbon sources by microbes (Liu et al., 2013). This fact could explain the enhancement in the dehydrogenase activity and FDA hydrolysis found in the P treatment during the experiment when compared with the NA (Fig. 2a, b). Plants can stimulate the growth of specific microbial communities in soil or induce enzyme systems of existing bacterial population and accelerate bioremediation in soil (Liu et al., 2013). This is in concordance with previous findings described by Chen et al. (2016), in which it was markedly noted a strong rhizospheric effect on degradation of PAHs through the stimulation of ryegrass exudates. It was noteworthy that P treatment showed similar trends as were previously observed in the M treatment, but the dehydrogenase activity was significantly lower compared with MAP treatment at all the times tested (Fig. 2a). It has to be assumed that this enhancement in the dehydrogenase activity, in the MAP treatment, could be associated with an increase in the resident soil microbiota as a consequence of an additive contribution of root exudates and *C. laeve*, rather than a synergistic effect. Conversely, the soil microbial functionality in the MAP treatment was significantly increased compared to the NA, but it reached similar values to those found in M and P treatments, except at 180 days, at which time the FDA hydrolysis was highly enhanced (Fig. 2b). In this treatment, the highest PAH degradation values (58%) were found, which may suggest the combination of root exudates and inoculation of *C. laeve* provided for favorable conditions that assisted growth of hydrocarbon degraders.

3.5. Impact of treatments on soil fungal biota

To assess the influence of the inoculation of *C. laeve* and maize plants on the soil fungal communities during the PAH degradation, the ergosterol content in soil was assayed (Fig. 2c). Ergosterol content was increased throughout the incubation period under the NA treatment. Significant enhancement were found in the ergosterol content after 60 and 150 days compared with P and MAP treatments, whereas a decline was detected at 180 days (Fig. 2c). M treatment showed a similar trend as previously was found during the NA, with the exception that ergosterol was significantly increased at 180 days compared to NA. Besides, time-dependent changes were also found in this treatment (Fig. 2c). This may indicate that the growth and survival of *C. laeve* were relatively low. Some studies have observed that immobilized mycelia through natural lignocellulosic carriers reduce the growth inhibition by contaminants more than free mycelia (Covino et al., 2010a, 2010b). However, in our study, it is probable that the soil colonization by *C. laeve* mycelia was very fast in the early days of incubation. The addition of exogenous fungal inocula was markedly hindered by the autochthonous fungal biota; the significant enhancement found at 180 days could be explained by a profuse growth of autochthonous fungal populations as a result of trophic effect. Moreover, a study has also found that the exogenous microbes decreased quickly after having been introduced into soil as a consequence of factors such as moisture, temperature, pH, and/or limited nutrients (Dueholm et al., 2015). The ergosterol content was significantly increased during the experiment in P and MAP treatment, reaching maximum values at 120 and 180 days, but decreased at 60 and 150 days compared to NA and M treatments (Fig. 2c). The fluctuations in the ergosterol content in both P and MAP treatments are contradictory with the evidence that plant roots, through exudates, are able to stimulate the soil fungal biomass. Curiously, a study has compared the ergosterol content in the rhizosphere of potato plants and in the bulk soils, observing higher soil fungal biomass in planted soil compared with unplanted (Hannula et al., 2010). In contrast, Chen et al. (2008) did not observe any difference in fungal biomass, as estimated by phospholipid fatty acid analysis among soils planted and unplanted with various legumes and grasses. So far, it is not clear to what extent these different results could be caused by the use of different methodologies. However, the stronger enhancement in the ergosterol content at 180 days in both, P and MAP treatments respect to NA (Fig. 2c) which could be the consequence of the positive influence of plant roots on soil microbial communities refers to the "*rhizosphere effect*" (Mukerji et al., 2006). Contrary to expectation, our study did not find clear differences in the ergosterol content between P and MAP treatments. It could have been expected that root exudates may have provided C sources for stimulating the growth of *C. laeve*; however nutritional requirements of *C. laeve*, as a ligninolytic fungi, are more complex than simple organic C compounds released by roots. Nevertheless, it is also noteworthy that the degradation of PAHs reached by MAP treatment was significantly higher compared with other treatments, and, moreover, dehydrogenase and FDA activities were highly enhanced. This finding may suggest that barley seeds were probably a worthy trophic substrate for the autochthonous fungal microbial communities, and specific microbial communities involved in PAH degradation, as previously reported in another study (García-Delgado et al., 2015). In our study, the application of new-generation sequencing tools (NGS) and/or analysis of phospholipid fatty acids (PLFA) and/or quantitative PCR would lead to a better understanding of the complex potential interactions among autochthonous microbial communities, exogenous fungi applications, and plant rhizosphere in a PAH-polluted soil.

4. Conclusions

The present study compares different approaches for bioremediation of an aged PAH-polluted soil through natural attenuation, mycoaugmentation, phytoremediation, and microbe-assisted phytoremediation. Among all of them, the microbe-assisted phytoremediation was found to be the most profitable approach reaching higher rates of degradation close up 60% in relation to other strategies. Bioconversion and translocation factors did not indicate higher capacities of maize plants for phytoextraction of PAHs, but as a result, the microbe-assisted phytoremediation showed a high potential for their stabilization/degradation mediated by an active role of the autochthonous microbial population. This study provided novel evidence with respect to microbe-assisted phytoremediation demonstrating that maize-*C. laeve* association could be an environmentally sound management approach for the treatment of aged PAH-polluted soils.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.08.012.

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4.6 Košnář et al. (2018). Bioremediace polycyklických aromatických uhlovodíků (PAU) přítomných v úletovém popelu z biomasy začleněného do společného kompostování a vermikompostování s organickými odpady.

Název: Bioremediation of polycyclic aromatic hydrocarbons (PAHs) present in biomass fly ash incorporated to the co–composting and co–vermicomposting with organic wastes.

Autoři: Zdeněk Košnář, Tereza Částková, Lucie Wiesnerová, Kroulíková Stanislava, Jiří Bouček, Filip Mercl, Pavel Tlustoš.

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Bioremediation of polycyclic aromatic hydrocarbons (PAHs) present in biomass fly ash incorporated to the co–composting and co–vermicomposting with organic wastes

Zdeněk Košnář[a,*], Tereza Částková[a], Lucie Wiesnerová[b], Stanislava Kroulíková[a], Jiří Bouček[a], Filip Mercl[a], Pavel Tlustoš[a]

[a]Department of Agroenvironmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague 6 - Suchdol, Czech Republic

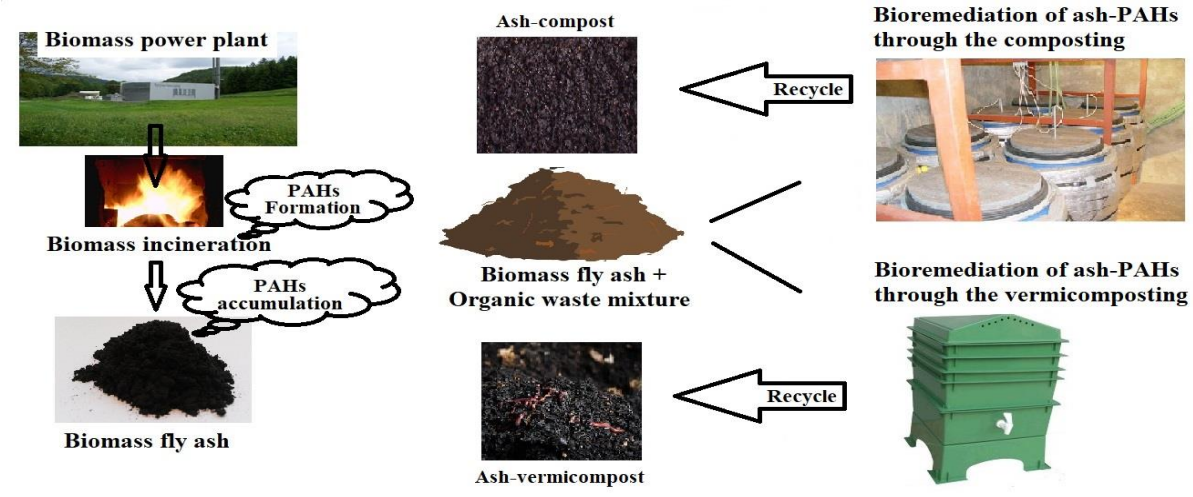
[b]Department of Horticulture, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague 6 - Suchdol, Czech Republic

[*]Corresponding author. E-mail: kosnarz@af.czu.cz (Zdeněk Košnář)

HIGHLIGHTS

- Composting was more efficient in the removal of ash PAHs than vermicomposting
- Ash PAHs showed a similar ability to be removed as the spiked PAHs
- 3D paraboloid PAH removal equations of each bioremediation treatment were estimated
- PAH first–order kinetics in composting and vermicomposting treatments were derived
- Resulted compost and vermicompost could be reuse e.g. as soil amendments

GRAPHICAL ABSTRACT



Abstract

An experiment was established for the comparison of composting and vermicomposting treatments to decrease the content of polycyclic aromatic hydrocarbons (PAHs) in biomass fly ash incorporated to the organic waste mixture. The removal of PAHs from the ash–organic waste mixture was compared to the same mixture spiked with PAHs. The removal of 16 individual ash PAHs ranged between 28.7 and 98.5% within the 240–day experiment. The higher dissipation of total PAH content of ash origin was observed at the end of composting (84.5%) than after the vermicomposting (61.6%). The results showed that most of ash PAHs were removed similarly as spiked PAHs with the aid of composting and vermicomposting. The boosted manganese peroxidase in composting treatment indicated the increased activity of ligninolytic PAH–degraders. The 3D model for the total PAH removal prediction was conducted using the polarity index and organic matter and paraboloid equations in each treatment were estimated ($R^2 > 0.91$). The two–phase model of pseudo–first order kinetics analysis showed faster PAH removal by the increased first rate constants in the first 120 days of the experiment. The produced compost and vermicompost from the bioremediation treatments could be reused e.g. as soil organic amendments.

Keywords: PAHs; Elemental analysis; Enzyme activities; Biodegradation; First–order kinetics

Capsule summary

Composting and vermicomposting approaches have a positive effect on the PAH dissipation from the biomass fly ash and obtained compost and vermicompost could be used in agriculture.

1. Introduction

The polycyclic aromatic hydrocarbons (PAHs) belong to the group of persistent pollutants with two or more fused benzene rings, which are ubiquitous in the environment. Some of the PAHs are known as mutagens, teratogens, or carcinogens. Therefore may pose a serious threat to the surrounding environment and human health (Lamichhane et al., 2017). The PAH formation is mainly associated with the incomplete combustion of fuels (Růžičková, 2018). Due to their properties, the released PAHs can be accumulated mainly in soils, sediments, and sewage sludge (Vácha et al., 2005; Dvořák et al., 2017; García-Sánchez et al., 2018). The unfavourable conditions of the burning process in biomass combustion plants lead to the PAH formation followed by their accumulation in fly ashes derived from biomass. The relatively high content up to 193 mg PAH/kg of ash dry weight (dw) was found in incinerated biomass at 850 °C (Masto et al., 2015). However, the resulted ashes as waste products from biomass combustion could be re–used in agriculture due to the relatively high amounts of nutrient (Ochecová et al., 2017). The maximum content of PAHs in biomass ashes which could be applied as a soil organic amendment and/or fertilizer is set to 20 mg/kg dw in the Czech Republic (Public Notice No. 113, 2014). Before the possible reuse, an appropriate bioremediation approach of highly PAH–contaminated biomass ash is needed (Reijnders, 2005). On the basis of our previous studies, the dissipation of ash PAHs in natural attenuated soil was negligible and the phytoremediation decreased the PAH content nearly by 30%. When the phytoremediation of ash PAHs was assisted with organic amendments the PAH removal increased up to 65% (Košnář et al., 2018b; Košnář and Tlustoš, 2018). The bioremediation of ash PAHs incorporated to the co–composting with organic wastes could be a more effective strategy as was indicated by the PAH removal from contaminated soil, sewage sludge, or sediments (Cai et al., 2007; Mattei et al., 2016; Mizwar et al., 2016). The vermiremediation of PAHs (composting using earthworms e.g. *Eisenia andrei*, *Eisenia fetida* etc.) is also used to enhance the PAH removal from highly contaminated environmental matrixes (Kuppusamy et al., 2017). The PAH biodegradation using these approaches occurs mainly due to the high ability to increase the PAH bioavailability and stimulate the growth of native microorganisms involved in PAH degradation (Poluszyńska et al., 2017). The autochthonous ligninolytic fungi present in obtained compost or vermicompost are known for the production of extracellular ligninolytic enzymes, which are able to modify or completely mineralize the PAHs (Kadri et al., 2017). Therefore, autochthonous fungi in obtained compost or vermicompost could be promising in the bioremediation of PAHs. Moreover, earthworms

with the aid of vermicomposting could be able to transform the PAHs by ingestion and rendering them harmless (Sinha et al, 2002). The main objective of this work was to compare the removal of ash PAHs through the co–composting and co–vermicomposting with organic wastes over a period of 240 days.

2. Materials and methods

2.1. Characterization of experimental biomass fly ash and organic waste mixture

The experimental PAH–contaminated fly ash derived from wheat straw incineration was collected at 20 MW combustion plant in the Czech Republic. Detailed description of the ash, and its collection and preparation is mentioned in our previous study by Košnář et al. (2018b). The experimental organic waste mixture used in this study was prepared by mixing livestock manure, grass clippings, waste paper and wheat straw in the portion of 9:9:1:1 of each respective waste material (*w*/*w*; fresh weight basis). This ratio was chosen to achieve the optimal properties as described Ali et al. (2015). The main physico–chemical characteristics, elemental analysis and PAH content in experimental biomass fly ash and organic waste mixture are shown in Tables S1 and S2.

2.2. Experimental design setup

For the bioremediation of PAHs of biomass fly ash origin two different bioremediation approaches were tested: composting and vermicomposting of ash incorporated to the organic waste mixture as was described above. The bioremediation of spiked PAHs added into the same organic waste mixture was used as a control treatment. The experiment was carried out using 4 different treatments and each respective treatment was done in 4 replications as follows: (1) Bioremediation of ash PAHs through the composting of organic waste mixture amended with ash (COW+A); (2) Bioremediation of spiked PAHs through the composting of organic waste mixture spiked with PAHs (COW+S); (3) Bioremediation of ash PAHs through the vermicomposting of organic waste mixture amended with ash (VOW+A); (4) Bioremediation of spiked PAHs through the vermicomposting of organic waste mixture spiked with PAHs (VOW+S). The 20 kg or 5 kg of fresh organic waste mixture was used in each composting and vermicomposting treatment, respectively. The dose of applied ash (5%, *w*/*w*) in respective treatments (COW+A and VOW+A) was based on the recommendation by

Ali et al. (2015). Before the experiment was set up, the organic waste mixture in COW+S and VOW+S treatments were contaminated artificially by a PAH mixture including US EPA priority PAHs (SV Calibration Mix 5, Restek, Bellefonte, USA) in *n*–hexane (GC/MS grade, Chromservis, Prague, Czech Republic) to reach the 500 μg of each individual PAH compound per one kg of material dw according to the methodology by Smith et al. (2006). The material of each treatment at 0 days of experiment was described by the elemental analysis in Fig. 1. The resulted initial PAH content in each treatment is shown in Table 1.

The composting treatments (COW+A and COW+S) were carried out in the 120 L plastic laboratory fermenters (8 in total) with a thermometer equipped with plastic insulation to prevent heat losses (Graphical abstract). The forced aeration during the composting process was provided by the aeration device (Atmos, Plzeň, Czech Republic) equipped with a flowmeter. Each organic waste mixture was aerated for 5 min out of each 60 min from the bottom during the composting. The hoses from outlets on the top of all fermenters led into a condensing flask to avoid the condensing of excess air inside the fermenter. This air flow was set according to the experiences described by Habart et al. (2010) to achieve the optimal conditions.

The vermicomposting treatments (VOW+A and VOW+S) using earthworms (*Eisenia andrei* from Jakub Filip company, Lužice u Hodonína, Czech Republic) were conducted in the plastic vertical vermireactors (VermuHut5, VermiTek Corporation, Portland, USA) (Graphical abstract). Each vermireactor (8 in total) consisted of plastic trays (16 L) with a perforated bottom. The base of the vermireactor equipped with an outlet was used as a keeper of a possible leachate. The 2.5 kg of fresh cow manure substrate containing about 800 earthworms was placed into the first tray above the vermireactor base to provide an initial environment for the earthworms. The raw material of VOW+A and VOW+S treatments was put into the second tray. The new trays with organic waste mixture without the PAHs as a fresh feeding was placed after every 60th of experiment day gradually above the second tray to increase the earthworm activity in the treated tray. Due to the perforated bottom earthworms could move from one tray to another during the whole experiment.

The composting and vermicomposting experiments were hold for 240 days in two separate laboratories at 25 °C. The temperature inside the fermenters during composting and through the vermicomposting process is shown in Fig. S1. The material in each treatment was manually homogenized every 14th day of experiment. The moisture of all materials was regularly controlled during experiment and kept in a range of 60–70%. The samples from four

treatments in four replications were collected after 0, 30, 60, 90, 120, 150, 180, 210, and 240 days in duplicates as a technical replication. The collected samples were dried at 35 °C in an oven and ground with a mortar to a fine powder before the analysis of PAHs.

2.3. Analytical procedures and enzyme analysis

The changes in elemental analysis (total C, H, N, and S) of treatments during the 240–day experiment was performed using an elemental analyser (CHNS Vario MACRO cube, Elementar Analysensysteme, Hanau, Germany). Oxygen (Fig. 1e) was calculated by difference between the ash–free organic matter (OM) and the sum of C, H, N, and S. Organic matter (Fig. 1f) was determined using a procedure described elsewhere by Antizar-Ladislao et al. (2006). The molar atomic ratios (H/C, O/C, C/N) and polarity index (PI) (Fig. 1g–j) was calculated on moist and ash–free basis. The basic physico–chemical characteristics (Table S1) were proceed using the methods described in our previous studies (Košnář et al., 2018b; Mercl et al., 2018). Extracellular enzyme activities (Fig. 2) of selected hydrolytic enzymes (ß-D-glukosidase, acid phosphatase, lipase), and selected ligninolytic enzymes (manganese–dependent peroxidase, lignin peroxidase, laccase) were measured in sample extracts using the method described elsewhere in Košnář et al. (2018a) using the Tecan Infinite M200 multi–plate reader (Infinite M200, Tecan Trading AG, Männedorf, Switzerland). Each enzyme activity was expressed as U/g soil dw. The one unit (U) stands for the respective enzyme producing 1 μmol of each reaction product.

2.4 Analysis of PAHs

A five gram of each compost and vermicompost sample was extracted for PAHs using the same procedure as was described in our recent study by Košnář et al. (2018a). Briefly, each sample was extracted with 30 ml mixture (1:1, *v/v*) of acetone and hexane (Chromservis, Prague, Czech Republic). The PAH extraction was carried out using the ultrasonic extraction method described by US EPA (2007). The samples were sonicated in a bath (Sonorox Digitec DT510/H, Bandelin, Berlin, Germany) at 35 °C for 30 min after the addition of 2-flurobiphenyl at 2000 mg/L as the surrogate (Restek, Bellefonte, USA). Each sample extract was then filtrated (Filtrate paper Grade 5B, Advantec, Tokyo, Japan) a re–extracted twice using the same procedure. The respective sample filtrates were collected, evaporated to dryness under vacuum at 40 °C (Rotavapor R–300, Büchi Labortechnik AG, Postfach,

Switzerland), dissolved in hexane (1 mL) and purified using the SPE cartridges (Strata SI–1 Silica, Phenomenex, Torrance, USA) according the procedure described by US EPA (1996). The concentrated eluate (1 mL) together with *p*-terphenyl-d_{14} as the internal standard at 500 µg/L (Restek, Bellefonte, USA) was then analysed for PAHs following the method described by US EPA (2014). The analysis of PAHs was carried out using an Agilent gas chromatograph/mass selective detector (6890N/5975) with an Agilent autosampler (7683B) (Agilent Technologies, Santa Clara, USA). The separation of PAHs was performed on a DB–EUPAH (20 m length, 0.18 mm inner diameter, 0.14 µm film) capillary column (Agilent J&W Scientific, Folsom, USA) at a constant helium (6.0) flow rate (1.0 mL/min). The 1.0 µL of a sample was injected in splitless regime (purge flow 70 mL/min at 0.75 min). The more detailed chromatographic conditions and temperature regimes during the PAH analysis was characterized in our previous study (Košnář et al., 2016). The PAH identification/quantification was carried out using the calibration curve of a PAH standard (SV Calibration Mix 5, Restek, Bellefonte, USA) in a range from 10 to 1000 µg/L with the PAH linearity higher than 0.9981. The PAH limit of quantification ranged 1.7–5.8 µg/kg dw. The surrogate recoveries ranged from 88% to 117% in the analysed samples.

2.7 Statistical and data analysis

In this study, the 16 priority US EPA PAHs are excluded by the ACE, FLU, IPY, and DBA due to their non–detected or low content in initial experimental treatments. The sum of 12 selected individual PAHs (total PAHs) were sorted into three groups: (1) Low molecular weight PAHs (LMW PAHs) – the sum of naphthalene (NAP), acenapthylene (ACY), phenanthrene (PHE), and anthracene (ANT); (2) Medium molecular weight (MMW PAHs) – the sum of fluoranthene (FLUO), pyrene (PYR), benzo[a]anthracene (BaA), and chrysene (CHR); (3) High molecular weight PAHs (HMW PAHs) – the sum of benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), and benzo[g,h,i]perylene (BghiP). The removal of PAHs (%) during the 240–day experiment (Fig. 3.) was calculated as follows:

$$R_{\mathbf{PAH}}\ (\%) = 100 \times \left[\frac{C_i - C_r}{C_i}\right],$$

The removal rate constants of PAHs were obtained from the pseudo–order first kinetic approximation using the linear integrated form of

$$ln\left[\frac{C_r}{C_i}\right] = -kt,$$

where C_i (µg/kg dw) stands for the initial PAH content at 0 days (Table 1) and C_r (µg/kg dw) stands for the residual PAH content at the respective time of sample collection (30, 60, 90, 120, 150, 180, 210, or 240 days) (Tables S3–S6), k (1/day) is the first–order constant of PAH removal (obtained by linear regression), and t is the time at the respective time of sample collection.

All statistical analyses, such as data normality (Shapiro–Wilk test), homogeneity of variance (Levene test), one–way analysis of variance at $p < 0.05$ (ANOVA) followed by post–hoc Tukey´s test ($\alpha = 0.05$), and Pearson correlation analysis (Table S7–S8) were performed in Statistica 12.0 (StatSoft, Tulsa, USA). Microsoft Excel 2010 (Microsoft Corporation, Redmond, USA) and SigmaPlot 11.0 (Systat Software, San Jose, USA) were used for the preparation of figures.

3. Results and discussion

3.1 Changes in elemental analysis of treatments during the bioremediation

The changes in material composition and parameters derived from the elemental analysis of material used in each composting (COW+A and COW+S) and vermicomposting (VOW+A and VOW+S) treatments were investigated during the 240–day experiment (Figs. 1a–j). The residual amounts of total elements (C, H, N, O), organic matter (OM), and molar atomic ratios (H/C and C/N) in all treatments were significantly lower ($p < 0.05$) at the end of the experiment than their respective initial amounts. The highest element reduction of 22.0 % was observed in the case of total C (Fig. 1a) through the composting of organic wastes and ash (COW+A) and the resulted residual amount of total C (18.6 %) was significantly ($p < 0.05$) lower than in other treatments. As a result of decomposition of biomass, the similar trend was observed also in the case of OM (Fig. 1f) which was decreased from 76.1% to 45.2% in the same treatment. This change of total C was in line with the results derived from the composting of organic waste mixture presented by Francou et al. (2008). The reduce of OM in ash amended treatments was in agreement with the composting of PAH contaminated coal–tar studied by Antizar-Ladislao et al. (2006). The total amount of H, N, and S (Figs. 1b–d) decreased gradually during the composting and vermicomposting treatments with the addition

of ash (COW+A and VOW+A). These changes in the most of collection times tended to be statistically different ($p < 0.05$) when compared to the treatments without the ash (COW+S and VOW+S). The decrease of these elements during composting of ash and organic waste mixture could be associated with the abiotic/biotic losses under aerobic conditions, as was indicated by Mahimairaja et al. (1994), which could be increased by the ash addition due to its strong alkaline reaction. The total amount of O (Figs. 1e) and the H/C ratio (Fig. 1g) also tended to be lower at the end of treatments with the ash addition than those in PAH spiked treatments indicating a higher transformation process.

The C/N ratio (Fig. 1i) showed the opposite trend by the end of tested ash amended treatments. The C/N ratio was decreased through the composting of organic waste mixture and ash (COW+A) from 23.1 to 15.5 which was statistically higher ($p < 0.05$) in comparison to the composting of PAH spiked organic waste mixture (COW+S). The changes of C/N ratio during the vermicomposting experiment copied the trend observed in composting treatments. Our changes of C/N ratio were comparable to those obtained by Amir et al. (2004). The O/C ratio (0.90) and polarity index (PI) defined as (O+N)/C ratio (0.96) were significantly higher ($p < 0.05$) during the composting of organic waste mixture with ash (COW+A) (Figs. 1h and 1j) than those (0.69 and 0.75, respectively) in vermicomposting treatment (VOW+A). However, there was no statistical difference ($p < 0.05$) between the COW+A and COW+S treatments in the O/C ratio and PI in the end of composting treatments and the same was true for the vermicomposting treatments. The PI was used to determine the approximate polar/nonpolar characteristics of a sample for the prediction of PAH behaviour during the composting and vermicomposting experiments according to the suggestions described by Xing et al. (1994). The changes of PI in our study showed the increasing polarity of a sample during the experiments. This could cause a higher desorption of PAHs from an environmental matrix and simultaneously could increase the solubility of aromatic rings, as was studied by Rutherford et al. (1992). This trend could be also same for the increasing O/C ratio during our bioremediation experiments because Huang and Weber (1997) observed the higher desorption of phenanthrene from organic matter with the increasing of O/C ratio in soil and sediment samples.

3.2 Enzyme activities in composting and vermicomposting treatments

In our study, the selected extracellular enzyme activities were measured at 0 days (initial time) and at the end of experiment (240 days). The results of enzyme activities are shown in

Fig. 2 excluded by the activities of lignin peroxidase and laccase as they were not detected at the initial time, likewise by the end which is in concordance with the study by Novotný et al. (2004). The ß-D-glukosidase (ß-D-G), acid phosphatase (AP) in all treatments and lipase (LPS) only in vermicomosting treatments were significantly decreased ($p < 0.05$) in the end of the experiment in comparison to their respective initial time. Simultaneously these enzyme activities were also significantly reduced by the addition of ash into the organic waste mixture when compared to the respective treatments without the ash. Decreased hydrolytic enzymes activities in the end of composting and vermicomposting were in agreement with previous study by García-Sánchez et al. (2017) who reported the highest enzyme activities at 120 days and after the 180 days of the experiment were gradually reduced to the initial values. The LPS and manganese–dependent peroxidase (MnP) found in the end of composting and vermicomposting treatments could be involved in the PAH removal during the whole experiment as they were increased or not statistically different ($p < 0.05$) to their respective initial amounts. Moreover, the MnP was significantly boosted in the end of each composting treatment which could indicate a good ability of autochthonous MnP–producers to recolonize the organic waste mixtures after the thermophilic phase of composting. The higher amounts of LPS and MnP in composting treatments could provide good conditions for the removal of PAHs of ash origin as there were strong correlations of LPS and MnP with the total PAH removal which were observed in our previous study by Košnář at al. (2018a).

3.2 Removal of PAHs during the composting and vermicomposting treatments

The removal of 12 selected individual US EPA PAHs (%) during the composting and vermicomposting is shown in Figs. 3a–l. The individual low molecular weight PAHs (LMW PAHs) of ash origin (Figs 3a–d) were removed from 89.3% to 96.9% by composting treatment (COW+A) and the vermicomposting treatment (VOW+A) decreased the ash PAHs in a range of 65.0–86.3% by the end (240 days) of experiment. The total sum of ash LMW PAHs using the composting treatment (Fig. 3m) was removed by 93.6%. This resulted in the statistically highest ($p < 0.05$) removal from all treatments. The removal of individual ash PAHs from medium molecular weight PAHs group (Figs 3e–h) and high molecular weight PAHs group (Figs 3i–l) were comparable in the end of each bioremediation treatment. The composting was a more efficient bioremediation approach than the vermicomposting in the dissipation of individual PAHs of ash origin from the MMW PAHs and HMW PAHs groups. Therefore, the total MMW PAHs were decreased from ash by 80.1% (Fig. 3n) and HMW PAHs by 73.1%

(Fig. 3o) which was statistically higher ($p < 0.05$) after the composting than in the end of vermicomposting (45.3 % and 53.6%, respectively) treatment. Only in the case of spiked MMW PAHs and HMW PAHs in vermicomposting treatment there was a slightly higher PAH removal in comparison to ash PAH removal using the same treatment. These differences could be caused by significantly lower ($p < 0.05$) hydrolytic enzyme activities (ß-D-G, AP, and LPS) in ash amended vermicomposting treatment (VOW+A) as was indicated in Fig. 2. The LMW PAHs were removed more than MMW PAHs and HMW PAHs within the same bioremediation approach. This was in line with the study of PAH phytoremediation in soil presented by Feng et al. (2014).

The total PAH removal from ash by 84.5% at the end of co–composting of the organic waste mixture with biomass fly ash was significantly higher ($p < 0.05$) than the total ash PAH removal (61.6%) by the vermicomposting after 240 days of experiment (Fig. 3p). The higher removal of total ash PAHs by composting than vermicomposting could be caused by a significantly higher ($p < 0.05$) polarity index (0.96) in composting (Fig. 1j) than in vermicomposting (0.75) as a strong correlation was observed between the polarity index and the total PAH removal ($r > 0.65$) by the Pearson correlation analysis (Table S7 and Table S8). The same was true for the decrease of total organic matter which is also linked to the decrease of total C during the bioremediation treatments within the Pearson correlation coefficients higher than 0.94 (Table S7 and Table S8).

Therefore, the PAH removal (%) model using the changes of organic matter and polarity index of each composting and vermicomposting treatment was conducted (Fig. 4) and the 3D paraboloid equations were estimated as follows:

$$r_{\mathrm{Comp}} = -2.803\exp(2) + 9.104x + 2.519\exp(2)y - 8.799\exp(-2)x^2 - 1.205\exp(2)y^2,$$

and

$$r_{\mathrm{Vermi}} = -1.685\exp(2) + 7.349x + 5.270\exp(1)y - 7.281\exp(-2)x^2 - 7.463\exp(0)y^2,$$

where, r_{Comp} refers to the removal of PAHs during the composting (%), r_{Vermi} refers to the removal of PAHs during the vermicomposting (%), x refers to the organic matter (%), and y refers to the polarity index value. The PAH removal equation for the composting was fitted

with a sufficient coefficient of determination (R^2 = 0.91) as well for the vermicomposting (R^2 = 0.92).

Our suggested bioremediation treatments showed the ability to decrease the PAHs of ash origin as well the spiked PAHs. In most of the investigated sampling times the composting treatment was a more efficient strategy than the vermicomposting in the removal of PAHs from ash and spiked PAHs as well. In most cases the removal of individual spiked PAHs tended to copy the removal of PAHs derived from ash which is in concordance with the work presented by Košnář et al. (2018b). The removal of total PAHs by 80.2% at day 120 of composting treatment with ash PAHs in organic wastes mixture was higher than the total PAH removal by natural attenuation (5.7%), phytoremediation (29.4%), and plant assisted phytoremediation (64.9%) in our previous 120–day experiments studied by Košnář et al. (2018b) and Košnář and Tlustoš (2018). The removal of total ash PAHs (84.5%) in the end of our composting approach was higher than the 75.2% removal of total PAHs by composting of aged PAH–contaminated soil by Antizar-Ladislao et al. (2005) or could be comparable with the 82.9–88.1% PAH removal from a sewage sludge using the composting studied by Oleszczuk (2006). The removal of total ash PAHs at day 120 of vermicomposting (54.1%) was lower than in the assisted phytoremediaton described above. However, the removal of total ash PAHs (61.6%) in the end of our vermicomposting treatment was almost double than the PAH removal from vermicomposting of soil presented by Hickman and Reid (2008).

The PAHs in our study were gradually decreased during the first 120 days of tested bioremediation treatments and after that time the changes in PAH removal started to be negligible. This could be caused by the reduction of microorganisms involved in PAH degradation, as was indicated by decreasing microbial activities after the 120th day of a treatment as was reported in the study by García-Sánchez et al. (2017). This is contradictory to a high activity of MnP and LPS (Figs. 2c–d) in the end of experiment suggesting the possible PAH stabilization within new substances formed during the maturation process of composting or vermicomposting. The remaining PAHs could start to be more recalcitrant to a further biodegradation due to the lesser bioavailability to the autochthonous PAH–degraders. Nevertheless, the residual content of ash PAHs in the end of composting (1128 μg/kg dw) and vermicomposting (2957 μg/kg dw) suggested that the obtained compost and vermicompost could be reused as soil organic amendments (Public Notice No. 341, 2008).

3.4 Kinetics of PAH removal in composting and vermicomposting treatments

The pseudo–first order kinetic analysis was performed to obtain the phase rate constant (k) to compare the removal rate of PAHs in tested composting and vermicomposting treatments as was described by Antizar-Ladislao et al. (2005). The rate constants of individual ash and spiked PAHs after the composting and vermicomposting approaches are shown in Table 2. The PAH removal rate constants derived from one–phase pseudo–first order kinetics model in each composting and vermicomposting treatment ranged from 0.0024 to 0.01 per day (R^2 = 0.71–0.99) and 0.002 to 0.0075 per day (R^2 = 0.69–0.97), respectively.

When the two–phase pseudo–first order kinetics model using two separate regression analyses in the two separate phases (first phase was for the 0–120 days and the second for 121–240 days of each treatment) was applied the difference between the first rate constant (k_1) and the second rate constant (k_2) was observed. Using the two–phase model showed a 2–3 times greater first rate constant (k_1) than second rate constant (k_2) for most of the individual PAHs from the low and medium molecular weight PAHs group in the whole experiment. The two–phase model improved the fitting of pseudo–first order kinetics by the increase of R^2 values than in the one–phase model, which is in line with study by Thiele-Bruhn and Brümmer (2005). Removal rates of LMW PAHs, MMW PAHs, and HMW PAHs were also investigated. The results indicated that the fastest PAH removal rates (0.0091–0.018) were observed in the case of ash LMW PAHs during the first 120 days of composting (COW+A). The k_1 constant of total ash PAHs was 0.0093 per day (R^2 = 0.96) in the first period of that composting treatment and after that time the k_2 constant was only 0.002 per day (R^2 = 0.99). Our rate constants of total PAHs in the first period of composting were comparable with the study by Antizar-Ladislao et al. (2005) who published that the PAH loss rate constants from soil were in range of 0.009–0.013 per day in composted soil–green waste mixtures. The k_1 constants in vermicomposting treatments could be comparable with the rate constants in soil amended with a sewage sludge compost studied by Feng at al. (2014).

In this study, the pseudo–first order kinetics analysis indicated that there were only a slightly differences between the phase rate constants of ash and spiked PAHs according to the results of the two–phase model in tested bioremediation treatments. However, the two–phase model also showed that the k_1 constants of total ash PAHs in composting treatment were almost 2 times higher than the k_1 constant (0.0046 per day, R^2 = 0.99) in vermicomposting

treatment, but the k_2 in both treatments were comparable. This trend was also confirmed for the spiked PAHs.

4. Conclusion

In the present study the bioremediation of PAHs derived from biomass fly ash through the 240–day experiment of co–composting and co–vermicomposting with organic waste mixture was investigated in comparison to the spiked PAH treatments. The composting and vermicomposting were very effective in the removal of ash PAHs. The differences between the removal of ash and spiked PAHs were negligible. The polarity index of PAH–contaminated organic waste mixtures has increased with the decrease of organic matter during the bioremediation and there was very strong correlation with the removal of total PAHs. Manganese peroxidase and lipase activities indicated the presence of autochthonous PAH–degraders during the bioremediation.

The significantly higher ($p < 0.05$) removal of PAHs by 84.5% was observed in the end of composting than the 61.6% total PAH removal by vermicomposting. The paraboloid equations were estimated for the prediction of total PAH removal in relation to the organic matter and polarity index in each tested treatment. The two–phase model of pseudo–first order kinetics analysis showed a faster PAH removal by the increased first rate constants in the first 120 days of the experiment. The compost and vermicompost obtained from the bioremediation treatments could be reused, e.g. as soil organic amendments according to the Czech legislation.

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Fig. 1. Changes of total C, H, N, S, O (a–e) (%; moist free and ash free dw basis), organic matter (f) (%; dw basis), and atomic ratios of H/C, O/C, C/N, PI–polarity index: (O+N)/C (g–j) derived from elemental analysis of treatments during the 240–days experiment. Error bars indicate standard deviation of n = 4. Treatment abbreviations: COW+A (composting of organic wastes and ash); COW+S (composting of organic wastes and spiked PAHs) treatment; VOW+A (vermicomposting of organic wastes and ash); VOW+S (vermicomposting of organic wastes and spiked PAHs)

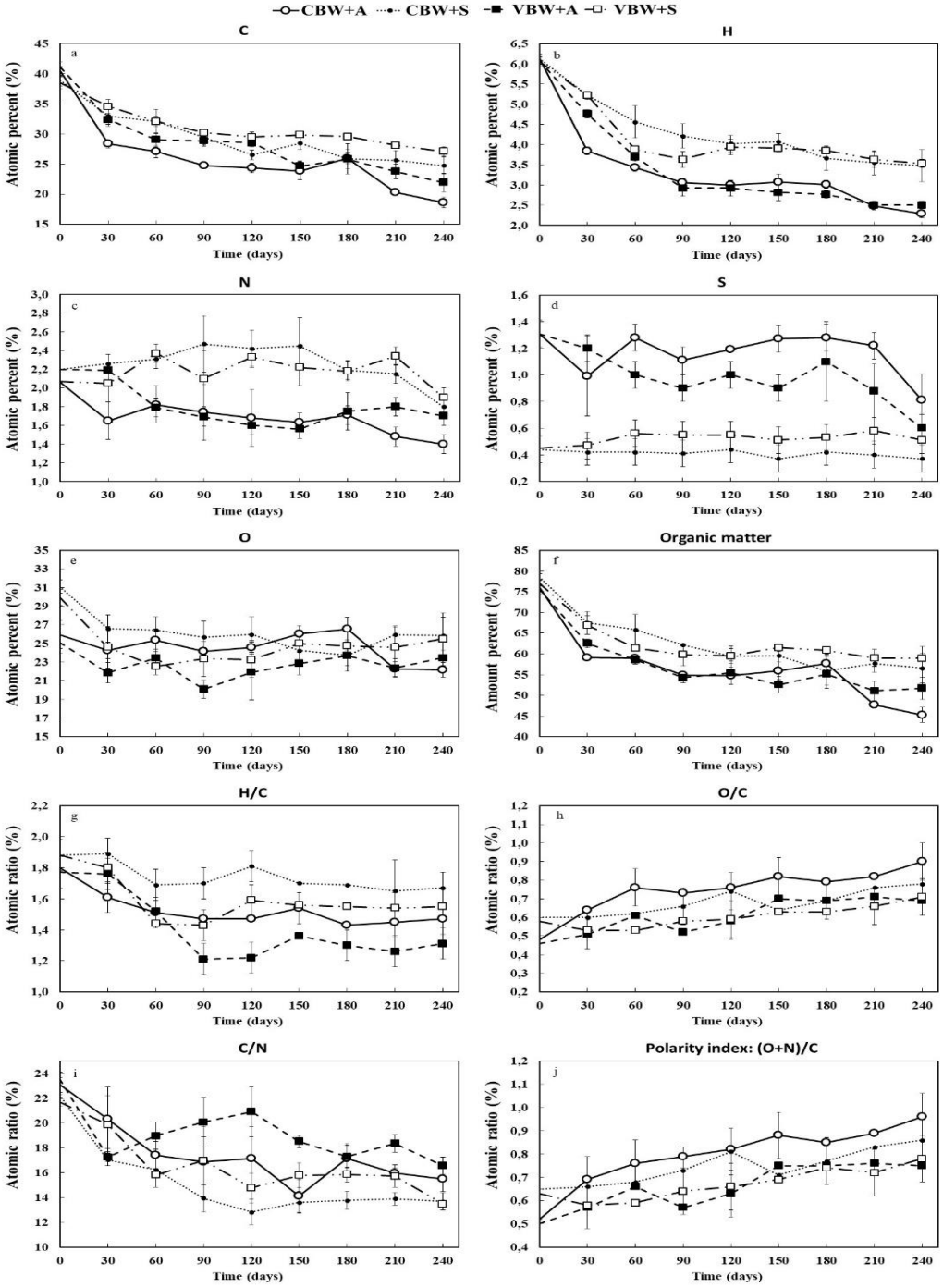

Fig. 2. Selected extracellular enzymes: a) ß-D-G (ß-D-glukosidase); b) AP (acid phosphatase; c) LPS (lipase); d) MnP (manganese peroxidase) activities (average ± SD; n = 4) at initial time (0 days) and in the end of the experiment (240 days). Error bars indicate standard deviation of n = 4. Means within the same row followed by different lowercase letters indicate significant differences ($p < 0.05$) between the treatments and uppercase letters indicate significant differences ($p < 0.05$) between the time of collection (0 and 240 days) in each experimental treatment as determined by Tukey´s test. Treatment abbreviations: COW+A (composting of organic wastes and ash); COW+S (composting of organic wastes and spiked PAHs) treatment; VOW+A (vermicomposting of organic wastes and ash); VOW+S (vermicomposting of organic wastes and spiked PAHs)

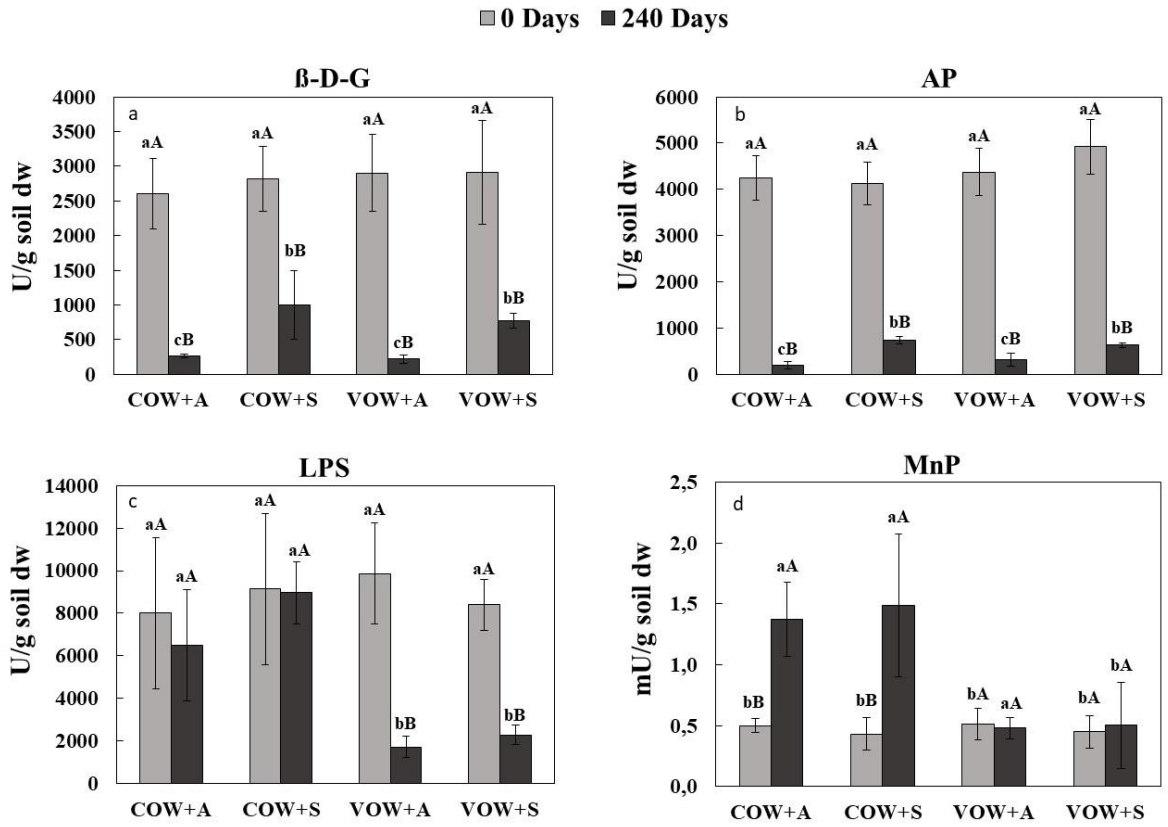

Fig. 3. Removal (%) of individual PAHs (a–l), LMW PAHs (m), MMW PAHs (n), HMW PAHs (o), and total PAHs (p) in each treatment (average ± SD; n = 4) during the 240–days experiment. Error bars indicate standard deviation of n = 4. Treatment abbreviations: COW+A (composting of organic wastes and ash); COW+S (composting of organic wastes and spiked PAHs) treatment; VOW+A (vermicomposting of organic wastes and ash); VOW+S (vermicomposting of organic wastes and spiked PAHs)

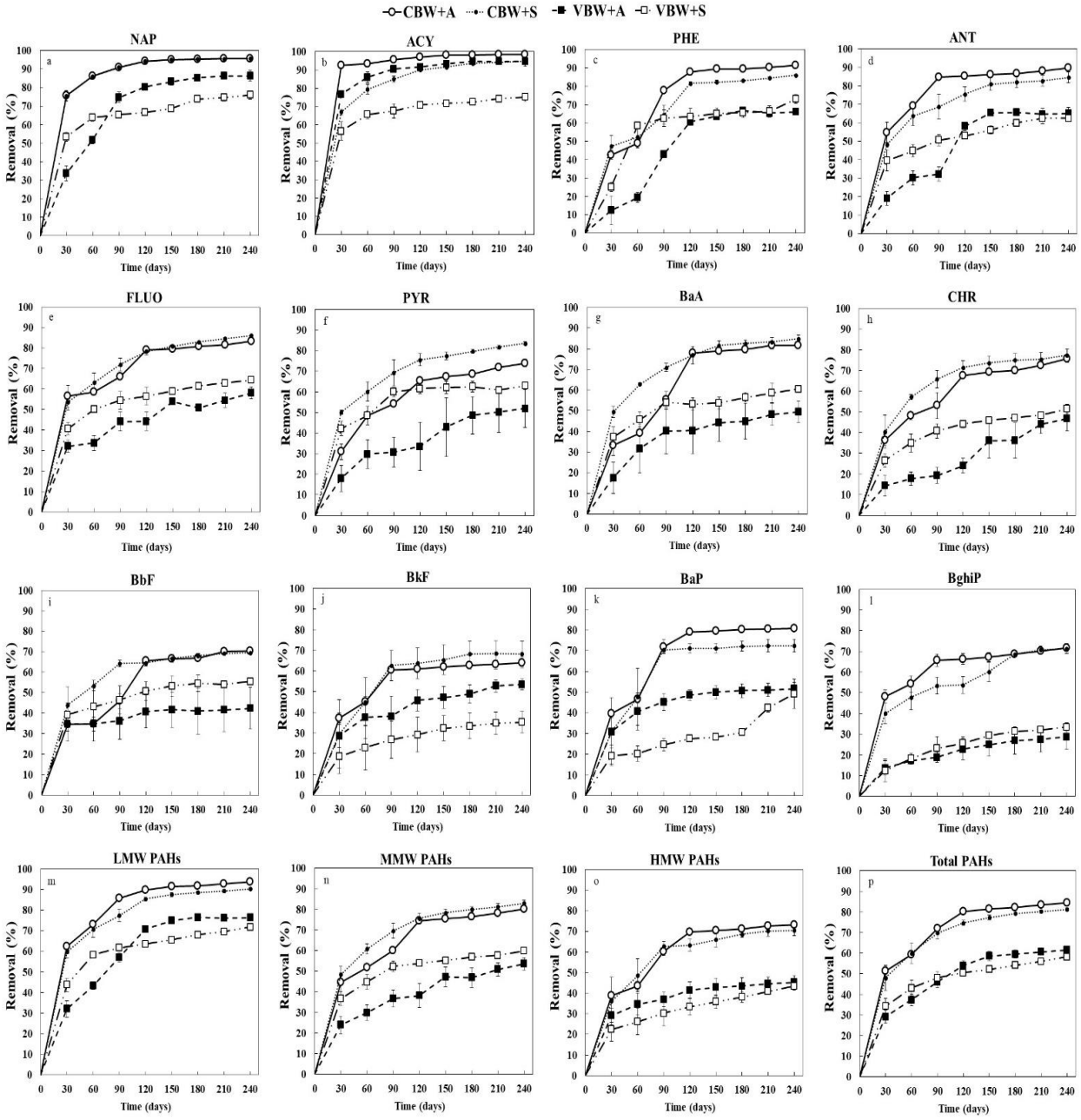

Fig. 4. Model of total PAH removal (%; z - axis) designed using the changes of OM–organic matter (%; x - axis) and PI– polarity index (%; y - axis) during the 240–days experiment

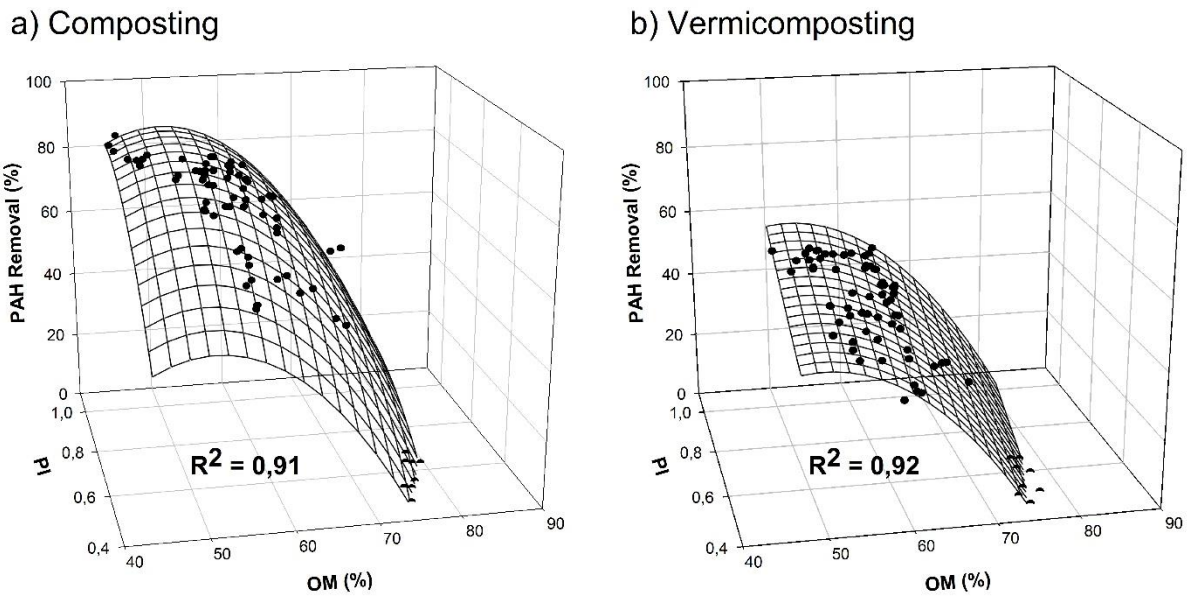

Table 1. Initial PAH content (µg/kg dw) at 0 days in each treatment (average ± SD; n = 4) of the 240–days experiment. Means within the same row followed by different lowercase letters indicate significant differences ($p < 0.05$) between the treatments as determined by Tukey´s test. Treatment abbreviations: COW+A (composting of organic wastes and ash); COW+S (composting of organic wastes and spiked PAHs) treatment; VOW+A (vermicomposting of organic wastes and ash); VOW+S (vermicomposting of organic wastes and spiked PAHs).

Polycyclic aromatic hydrocarbons (PAHs)	**Abbreviation**	**COW+A**	**COW+S**	**VOW+A**	**VOW+S**
		(µg/kg dw)			
Low molecular weight PAHs:					
Naphthalene	NAP	957.2 ± 6.3^a	501.8 ± 4.2^b	951.7 ± 8.1^a	500.2 ± 5.7^b
Acenapthylene	ACY	632.7 ± 6.3^a	506.3 ± 4.4^b	630.3 ± 3.8^a	505.3 ± 3.5^b
Phenanthrene	PHE	950.8 ± 13^a	495.0 ± 1.3^b	937.2 ± 6.7^a	495.6 ± 2.8^b
Anthracene	ANT	1057 ± 12^a	495.9 ± 6.8^b	1002 ± 36^a	495.6 ± 7.1^b
Medium molecular weight PAHs:					
Fluoranthene	FLUO	915.2 ± 14^a	491.1 ± 2.8^b	929.7 ± 26^a	493.9 ± 1.6^b
Pyrene	PYR	328.6 ± 7.5^b	500.9 ± 8.1^a	353.8 ± 19^b	501.8 ± 7.6^a
Benzo[*a*]anthracene	BaA	326.5 ± 6.8^b	502.8 ± 2.7^a	344.6 ± 19^b	504.8 ± 2.6^a
Chrysene	CHR	354.8 ± 10^b	496.5 ± 9.3^a	348.4 ± 1.8^b	498.5 ± 9.3^a
High molecular weight PAHs:					
Benzo[*b*]fluoranthene	BbF	763.8 ± 11^a	506.7 ± 4.3^b	734.3 ± 17^a	508.5 ± 4.3^b
Benzo[*k*]fluoranthene	BkF	335.0 ± 12^b	496.8 ± 6.9^a	346.3 ± 5.8^b	498.8 ± 6.9^a
Benzo[*a*]pyrene	BaP	726.2 ± 16^a	503.8 ± 2.6^b	772.0 ± 19^a	505.4 ± 2.2^b
Benzo[*g,h,i*]perylene	BghiP	334.7 ± 12^b	506.0 ± 5.1^a	350.1 ± 8.8^b	506.0 ± 6.5^a
∑Low molecular weight PAHs	LMW PAHs	3598 ± 13^a	1999 ± 7.5^b	3522 ± 44^a	1994 ± 14^b
∑Medium molecular weight PAHs	MMW PAHs	1925 ± 4.3^b	1991 ± 10^a	1976 ± 17^a	1999 ± 10^a
∑High molecular weight PAHs	HMW PAHs	2160 ± 25^b	2013 ± 6.9^a	2202 ± 31^b	2013 ± 6.8^a
^{1}∑ Individual US EPA PAHs	Total PAHs	7682 ± 35^a	6003 ± 12^b	7701 ± 11^a	6002 ± 13^b

Table 2. Removal rate constants of PAHs obtained from the pseudo–first kinetics using regression analysis in one–phase and two–phase models in each treatment of the 240–days experiment. Treatment abbreviations: COW+A (composting of organic wastes and ash); COW+S (composting of organic wastes and spiked PAHs) treatment; VOW+A (vermicomposting of organic wastes and ash); VOW+S (vermicomposting of organic wastes and spiked PAHs).

PAH Compound	COW+A			COW+S			VOW+A			VOW+S		
	Rate constant ($\times10^{-3}$/day)			Rate constant ($\times10^{-3}$/day)			Rate constant ($\times10^{-3}$/day)			Rate constant ($\times10^{-3}$/day)		
	k (R^2)	k_1 (R^2)	k_2 (R^2)	k (R^2)	k_1 (R^2)	k_2 (R^2)	k (R^2)	k_1 (R^2)	k_2 (R^2)	k (R^2)	k_1 (R^2)	k_2 (R^2)
NAP	10 (0.94)	15 (0.96)	8.2 (0.76)	7.9 (0.82)	16 (0.99)	2.2 (0.81)	7.5 (0.86)	15 (0.96)	3.1 (0.90)	2.9 (0.94)	15 (0.96)	2.9 (0.93)
ACY	8.5 (0.92)	9.1 (0.99)	5.1 (0.76)	8.8 (0.95)	13 (0.99)	5.6 (0.97)	6.7 (0.88)	4.9 (0.99)	3.7 (0.79)	2.3 (0.89)	4.9 (0.89)	1.4 (0.97)
PHE	9.5 (0.83)	16 (0.84)	2.6 (0.86)	6.8 (0.87)	6.6 (0.93)	2.3 (0.96)	5.0 (0.83)	7.2 (0.89)	1.1 (0.63)	3.4 (0.69)	7.6 (0.85)	2.2 (0.74)
ANT	6.2 (0.80)	18 (0.97)	3.0 (0.93)	5.6 (0.93)	8.4 (0.95)	3.4 (0.88)	4.6 (0.82)	2.9 (0.87)	1.1 (0.41)	2.4 (0.97)	3.4 (0.99)	2.0 (0.93)
FLUO	5.0 (0.88)	4.2 (0.94)	1.9 (0.94)	5.7 (0.96)	8.4 (0.98)	3.7 (0.99)	2.3 (0.84)	1.9 (0.75)	1.9 (0.75)	2.2 (0.94)	4.4 (0.96)	1.7 (0.99)
PYR	4.4 (0.92)	6.9 (0.95)	2.4 (0.98)	5.2 (0.95)	8.1 (0.99)	3.3 (0.99)	2.6 (0.96)	2.7 (0.81)	2.6 (0.89)	2.1 (0.69)	6.1 (0.96)	0.3 (0.98)
BaA	6.9 (0.84)	6.7 (0.91)	1.7 (0.95)	5.6 (0.92)	9.3 (0.99)	3.1 (0.89)	2.0 (0.85)	5.4 (0.99)	1.3 (0.96)	1.9 (0.86)	5.2 (0.99)	1.5 (0.97)
CHR	4.5 (0.92)	5.1 (0.96)	2.3 (0.92)	4.2 (0.86)	9.2 (0.99)	1.8 (0.97)	2.4 (0.95)	1.0 (0.95)	2.8 (0.93)	1.8 (0.91)	3.6 (0.99)	1.1 (0.95)
BbF	4.3 (0.86)	3.2 (0.78)	1.4 (0.89)	2.7 (0.81)	7.5 (0.99)	1.3 (0.95)	0.7 (0.84)	0.4 (0.78)	0.2 (0.77)	1.5 (0.91)	2.1 (0.99)	0.7 (0.82)
BkF	2.4 (0.71)	7.8 (0.94)	0.6 (0.99)	3.5 (0.76)	11 (0.98)	1.2 (0.81)	2.0 (0.95)	2.4 (0.79)	1.4 (0.95)	1.1 (0.94)	1.8 (0.99)	0.7 (0.92)
BaP	5.6 (0.75)	13 (0.88)	0.7 (0.95)	3.9 (0.64)	14 (0.96)	0.5 (0.85)	1.5 (0.78)	3.9 (0.97)	0.5 (0.94)	2.0 (0.85)	1.1 (0.88)	3.1 (0.87)
BghiP	2.7 (0.84)	6.9 (0.95)	1.5 (0.99)	3.7 (0.96)	4.2 (0.99)	4.2 (0.91)	0.9 (0.97)	1.1 (0.96)	0.7 (0.96)	1.3 (0.95)	2.2 (0.99)	0.9 (0.93)
LMW PAHs	8.3 (0.87)	16 (0.97)	3.8 (0.97)	6.8 (0.92)	9.7 (0.99)	3.2 (0.97)	5.4 (0.86)	7.6 (0.98)	1.6 (0.68)	2.8 (0.89)	6.4 (0.90)	2.1 (0.99)
MMW PAHs	5.1 (0.90)	5.4 (0.99)	2.1 (0.97)	5.1 (0.93)	8.8 (0.99)	2.9 (0.99)	2.3 (0.97)	3.1 (0.99)	2.1 (0.90)	1.9 (0.87)	4.7 (0.99)	1.1 (0.98)
HMW PAHs	4.1 (0.83)	7.1 (0.88)	1.1 (0.97)	3.5 (0.84)	9.0 (0.99)	1.8 (0.87)	1.2 (0.90)	2.0 (0.96)	0.5 (0.99)	1.5 (0.99)	1.8 (0.99)	1.4 (0.98)
Total PAHs	5.5 (0.87)	9.3 (0.96)	2.0 (0.99)	4.7 (0.90)	9.1 (0.99)	2.4 (0.96)	3.0 (0.90)	4.6 (0.99)	1.4 (0.88)	1.9 (0.94)	3.9 (0.98)	1.5 (0.99)

k ($\times10^{-3}$/day) represents phase rate constant from one–phase model, k_1 ($\times10^{-3}$/day), and k_2 ($\times10^{-3}$/day) represent the first and second phase rate constants from two–phase model, R^2 refers to the coefficient of determination derived from Pearson correlation analysis.

5 Sumární diskuse

Tématem disertační práce bylo zjistit vliv vybraných bioremediačních metod na změny obsahu PAU přítomných v popelu po spalování biomasy.

V první části sumární diskuse (podkapitola 5.1) jsou shrnuty poznatky z vědeckého článku (podkapitola 4.1), ve kterém byly diskutovány obsahy PAU v úletových a roštových popelech po spalování fytomasy (slámové popele) a dendromasy (dřevní popele). Obsah PAU byl sledován v 96 vzorcích popelu (24 úletových a 24 roštových z fytomasy, stejný počet byl i pro popel z dendromasy) odebraných v provozovnách, které spalují biomasu pro energetické účely. Jednotlivé obsahy PAU v popelech, které vznikly za reálných podmínek spalování, byly diskutovány ve vztahu k teplotě spalování a ve vztahu k obsahu nespáleného organického uhlíku. Vzorky popelů byly charakterizovány základními fyzikálně – chemickými metodami (detail viz podkapitola 4.1).

V druhé části sumární diskuse (podkapitola 5.2) jsou shrnuty poznatky z vědeckých článků (podkapitoly 4.2 až 4.5), ve kterých byly diskutovány vybrané metody bioremediace PAU v půdě.

V podkapitole 4.2 byl zkoumán vliv přirozené atenuace a fytoremediace na změnu obsahu PAU v půdě (vždy 5 kg na nádobu) po aplikaci popelu (1 % *w/w*, dále jsou uvedena vždy jen procenta) ve vztahu k syntetickým PAU (0,1 mg/kg pro každý jednotlivý PAU). Jako experimentální popel z biomasy byl vybrán úletový popel po spalování slámy, ve kterém byl stanoven nejvyšší obsah PAU (detail viz podkapitola 4.2).

V podkapitole 4.3 byly v nádobovém experimentu sledovány změny obsahu PAU v půdě pomocí bioaugmentace (10 % přídavek kompostu nebo vermikompostu v půdě s 1 % přídavkem popelu) a fytobioaugmentace (pěstování rostlin na půdě se stejnými dávkami popelu, kompostu nebo vermikompostu).

V podkapitole 4.4 byly v rámci nádobového experimentu sledovány změny obsahu syntetických PAU v uměle kontaminované půdě (0,1 mg/kg pro každý jednotlivý PAU) pomocí mykoremediace: a) mykoaugmentace (5 % přídavek substrátu s hlívou ústřičnou v půdě), b) fytomykoaugmentace (pěstování rostlin na půdě se stejnou dávkou substrátu).

V podkapitole 4.5 byly v rámci nádobového experimentu sledovány změny obsahu PAU v dlouhodobě kontaminované zemědělské půdě pomocí mykoremediace: a)

mykoaugmentace (6 % přídavek substrátu s penízovkou obecnou v půdě), b) fytomykoaugmentace (pěstování rostlin na půdě se stejnou dávkou substrátu).

V rámci nádobových experimentů s rostlinami kukuřice seté (*Zea mays* L.) na půdě kontaminované PAU (podkapitoly 4.2 až 4.4) byla využita kambizem modální (Humpolec) a v experimentu s půdou dlouhodobě kontaminovanou PAU (podkapitola 4.5) byla využita fluvizem glejová (Ostrava). Základní agrochemické charakteristiky využitých půd jsou uvedeny ve zmíněných podkapitolách. V rámci výše uvedených bioremediačních metod s rostlinami byl posuzován vliv PAU v půdě na výnos biomasy rostlin. Byla také zkoumána schopnost pěstovaných rostlin extrahovat PAU z půdy. Na základě stanovených relativních odběrů PAU rostlinami byl posouzen vliv rostlin na změny obsahu PAU v půdě.

V třetí části sumární diskuse (podkapitola 5.3) jsou shrnuty poznatky z vědeckého článku (podkapitola 4.6), ve kterém byly diskutovány bioremediace PAU pomocí kompostování a vermikompostování.

V podkapitole 4.6 byly sledovány změny obsahu PAU ve směsi biologicky rozložitelných odpadů (směs hnoje skotu, zahradní travní seče, odpadní papírové lepenky a obilné slámy v poměru 9:9:1:1 čerstvé hmoty) po aplikaci popelu (5 %) a syntetických PAU (0,5 mg/kg pro každý jednotlivý PAU) v průběhu kompostování a vermikompostování po dobu 240 dnů.

Ve čtvrté části sumární diskuse (podkapitola 5.4) je uveden závěrečný přehled účinnosti jednotlivých bioremediačních metod z hlediska poklesu celkového obsahu PAU za stejné časové období (podkapitoly 4.2 až 4.6).

5.1 Sledování obsahu PAU v popelu po spalování biomasy

Obsahy jednotlivých 16 PAU v úletových a roštových popelech po spalování fytomasy nebo dendromasy dosahovaly značné variability. S tím souvisí i značná variabilita mezi obsahy sumy nízkomolekulárních PAU (NM PAU), středněmolekulárních PAU (SM PAU), vysokomolekulárních PAU (VM PAU) a celkové sumy 16 PAU (PAU). Z výsledků bylo zřejmé, že úletové popele dosahovaly vyššího obsahu PAU než roštové popele. Masto et al. (2015) popsali, že úletový popel dosahuje většího specifického povrchu než roštový, a proto lze očekávat vyšší schopnost akumulace PAU. V předložené studii obsahy individuálních 16 PAU v úletových popelech se lišily značně v průměru od 0,1 do 8,3 mg/kg. Průměrný obsah

celkových PAU v úletovém popelu z fytomasy (53,8 mg/kg) byl mnohem vyšší než suma 6,11 mg/kg PAU, kterou stanovili Straka et Havelcová (2012). Nicméně, Johansson et van Bavel (2003a) stanovili daleko vyšší obsahy PAU v úletových popelech. Roštové popely z fytomasy, stejně jako popely z dendromasy dosahovaly průměrné sumy PAU nižší než 0,3 mg/kg. Ve všech testovaných popelech z biomasy převládaly NM PAU nad SM PAU nebo VM PAU. To bylo se shodně s výsledky, které publikovali Rey-Salgueiro et al. (2016). Vyšší obsah NM PAU než ostatních PAU v popelech mohl být způsoben pyrolýzou probíhající během nízkých teplot spalování, jak ve své práci uvedli García-Falcón et al. (2006).

Na základě provedené Spearmanovy korelační analýzy byl zjištěn velmi silný statisticky průkazný ($p < 0,05$) pozitivní vztah ($r = 0,80$ až $0,97$) mezi obsahem NM PAU, SM PAU, VM PAU a celkové sumy PAU. Pearsonova korelační analýza ukázala velmi silný a statisticky průkazný ($p < 0,05$) vztah mezi distribucí VM PAU v roštových popelech a celkových PAU v úletových popelech ($r > 0,94$). Z tohoto důvodu by se obsah VM PAU v roštových popelech mohl používat jako indikátor kontaminace úletových popelů PAU. S tím souvisí také riziko emisí PAU v úletových popelech, v případě jejich nezachycení na filtrech spalovacího zařízení.

V předkládané práci celkové obsahy PAU v popelech dosahovaly značné heterogenity ve vztahu k teplotě spalování. To je ve shodě s Chagger et al. (2000), kteří popsali vznik PAU v popelech v prvotní fázi spalování, kdy není ustálená teplota spalování a dostatečný přísun kyslíku, což silně ovlivňuje tvorbu PAU spojenou s jejich následnou akumulací ve vznikajících popelech. V předložené práci nejvyšší obsah PAU byl stanoven v úletovém popelu z fytomasy (až 147 mg/kg) při teplotě 500 – 750 °C. Tento popel navíc dosahoval nejvyšší sumy potenciálně karcinogenních PAU (až 46 mg/kg). Dále v této studii bylo zjištěno, že stejné druhy popelů z fytomasy vzniklé při vyšších teplotách spalování (750 – 1000 °C) dosahovaly rapidně nižších obsahů PAU (přibližně 9,5 mg/kg). Tento trend platil i pro popely, které vznikly při teplotách nižších než 500 °C. Při těchto teplotách byly potenciálně karcinogenní PAU v úletových popelech z fytomasy pod mezí detekce.

Obsah organického nedopalu v popelech závisí jak druhu a vlastnostech spalované biomasy, tak i na kvalitě spalovacího procesu (Gómez-Barea et al., 2009). Proto obsah nespáleného organického uhlíku v testovaných popelech dosahoval široké variability. Nejvyšší obsah 55,2 % byl stanoven v úletovém popelu z fytomasy. V roštových popelech z fytomasy

byl maximálně 7,6 %. Podobný trend byl sledován i v popelech z dendromasy. V popelech z fytomasy a úletovém popelu z dendromasy byla navržena kvadratická regrese ($r > 0,91$) a v roštových popelech z dendromasy lineární regrese ($r = 0,93$) mezi nespáleným uhlíkem a obsahem celkové sumy PAU. Na tomto základě byly pro jednotlivé druhy popelů odvozeny rovnice k predikci celkového obsahu PAU na základě obsahu nespáleného organického uhlíku v popelech.

Kvalita popela z biomasy je hodnocena v mnoha státech odlišně. Johansson et van Bavel (2003b) popsali, že ve Švédsku se nesmí v zemědělství využívat popel, který obsahuje sumu potenciálně karcinogenních PAU vyšší než 0,3 mg/kg. Podle tohoto hlediska by se v této studii nemohl využívat pouze popel z fytomasy, protože obsahoval sumu potenciálně karcinogenních PAU v rozmezí od 0,4 do 4,2 mg/kg. Podobně by tomu bylo i v Norsku dle Haglund (2008), který v popelech z biomasy uvedl maximální sumu celkových PAU 3 mg/kg. V ČR by se některé popely z fytomasy mohly využívat, jelikož dle MZe ČR (2014) je maximální suma PAU 20 mg/kg, avšak půda nesmí překročit preventivní sumu PAU (1 mg/kg) dle MŽp ČR (2016). V ČR se může zdát maximální obsah PAU v popelech určených k využití v zemědělství jako příliš vysoký, ale například Nisbet et LaGoy (1992) při tomto obsahu nezaznamenali žádný vliv PAU na životní prostředí. Na základě získaných výsledků stanovení obsahu PAU v popelech lze popele ze spalování dendromasy a roštové popele z fytomasy doporučit k využití v zemědělství. Možné využití úletových popelů z fytomasy v zemědělství je nutné posuzovat vždy individuálně na základě provedeného rozboru obsahu PAU, ale i jiných rizikových látek daných legislativou (MZe ČR, 2014). Pro možné využití úletových popelů z fytomasy s vysokým obsahem PAU v zemědělství z důvodu omezení skládkování popela a navrácení živin zpět do půdy bylo nutné zjistit vliv vybraných bioremediačních metod na změny obsahu PAU v půdě po aplikaci popelu.

5.2 Bioremediace PAU v půdě

5.2.1 Přirozená atenuace

V případě, že experimentální úletový popel z biomasy (slámy) s obsahem PAU (přibližně 160 mg/kg) byl aplikován do půdy samostatně bez aditiv a půda byla ponechána bez rostlin, tak přirozená schopnost půdy snížit obsah PAU v půdě byla za 120 dnů zanedbatelná. V půdě obohacené o popel v nádobových experimentech se obsah PAU metodou přirozené

atenuace snížil do 5,7 %. Podobně tomu bylo i v případě syntetických PAU, jejichž celkový obsah se snížil o 5,3 % (průměr ze dvou experimentů). Vyšší tendence poklesu obsahu PAU byla zaznamenána již za 60 dnů v případě dlouhodobě kontaminované půdy odebrané z oblasti se silnou antropogenní činností. Rozdíly v odstranění PAU metodou přirozené atenuace v půdě mohou být především způsobeny rozdílným zastoupením jednotlivých prioritních PAU, rozdílnými charakteristikami experimentálních půd a vlivem ročníku. Residuální obsah PAU v přirozeně atenuované půdě nebyl v některých případech statisticky významně rozdílný ($p < 0,05$) s příslušným počátečním obsahem PAU. V této práci bylo zjištěno, že přirozená atenuace PAU v půdě nebyla příliš vhodnou bioremediační metodou PAU, i když Declercq et al. (2012) publikovali, že většina jednotlivých PAU ze skupin NM PAU a SM PAU je dobře volatilní, fotodegradabilní a snáze degradovatelná původními mikroorganismy v půdě.

5.2.2 Fytoremediace

V rámci fytoremediace PAU v půdě po aplikaci kontaminovaného popelu z biomasy jako experimentální rostlina byla používaná kukuřice setá (*Zea mays* L.), protože je vhodná pro pěstování na půdách s vysokým obsahem PAU jak publikovali Lin et al. (2007). V předložené práci bylo zaznamenáno, že k nejvyššímu odstranění individuálních PAU o 58 % z půdy došlo v případě naftalenu ze skupiny NM PAU. V půdě s rostlinami a přidanými syntetickými PAU se nejvíce snížil acenaftylen o 87 % a v dlouhodobě kontaminované půdě bylo odstranění jednotlivých PAU ze skupiny NM PAU velmi podobné. Ve srovnání s přirozeným útlumem došlo v průběhu fytoremediace ke statisticky průkazně ($p < 0,05$) vyššímu odstranění většiny jednotlivých PAU z popela kromě benz[a]antracenu, benzo[k]fluorantenu, indeno[1,2,3-c,d]pyrenu, dibenz[a,h]antracenu a benzo[g,h,i]perylenu ze skupiny VM PAU. Podobně tomu bylo i v případě syntetických PAU, ale i PAU v dlouhodobě kontaminované půdě. V provedených experimentech byla prokázána vhodnost použití kukuřice zejména na fytoremediace dvou až čtyř cyklických PAU a benz[a]pyrenu, jako jediného ze skupiny VM PAU, v půdě odvozených z popela po spalování biomasy. Vhodnost použití kukuřice ve fytoremediačních technologií je ve shodě s prací, kterou publikovali Xu et al. (2006).

Celková suma NM PAU v půdě po aplikaci popelu se pěstováním rostlin snížila v průměru o 47,6 %, dále suma SM PAU o 19,8 % a suma VM PAU o 13,9 %. Pěstováním rostlin se celkový obsah syntetických NM PAU snížil podobně o 33 %, SM PAU o 22,5 % a VM PAU o 10,2 %. V případě změn obsahů PAU v dlouhodobě kontaminované půdě se jednotlivé skupiny PAU snížily v rozmezí od 28,7 % do 42,0 %. V případě PAU původem z popela bylo pozorováno vyšší odstranění NM PAU než SM PAU a VM PAU v půdě s rostlinami. To bylo ve shodě s výzkumy, které publikovali Feng et al. (2008) a Lee et al. (2008). Možným důvodem vyššího odstranění NM PAU je schopnost kukuřice podpořit růst a aktivitu nativních mikroorganismů v půdě zapojených do degradace PAU. Vlivem exudace v oblasti rhizosféry se podporuje vyšší biodostupnost zejména NM PAU pro rhizosférní mikroorganismy schopných odbourávat tyto polutanty (Segura et Ramos, 2013). Parrish et al. (2005) uvedli, že NM PAU jsou více volatilní, vodorozpustné a méně lipofilní, což naznačuje vyšší náchylnost k biodegradaci než PAU s vyšší molekulovou hmotností. PAU s vyšší molekulovou hmotností tíhnou k větší stabilizaci v půdě vlivem sorpce na půdní organickou hmotu (Cofield et al., 2007).

Na rozdíl od půdy bez rostlin, přítomnost vegetace v rámci bioremediace PAU v půdě je velmi důležitá, protože schopnost podpořit biodegradaci PAU v půdě *ex planta,* je prokazatelná (Maila et Cloete, 2002). Rostliny v půdě kontaminované PAU jsou schopny produkce extracelulárních hydrolytických enzymů, které jsou obecně spojovány se zvyšováním biodostupnosti PAU. Biodostupné PAU mohou být následně v rámci metabolismu půdních organismů degradovány nebo transformovány (Nanekar et al., 2015). Guo et al. (2017) ve své studii publikovali, že kukuřice během fytoremediace měla schopnost vlivem rostlinné exudace měnit zastoupení půdních mikroorganismů a v půdě silně kontaminované pyrenem podpořila růst celkové biomasy mikroorganismů a zvýšila jejich aktivitu v půdě. Následně bylo zjištěno, že kmeny bakterií například *Bacillus* sp., *Sphingomonas* sp. a *Pseudomonas* sp. byly zapojeny do biodegradace PAU v půdě za přítomnosti kukuřice. Nicméně, Gao et al. (2011) ve své práci uvedli, že i arbuskulární mykorrhiza, která se vytváří při pěstování rostlin v půdě, podpořila půdní mikrobiotu k degradaci vybraných PAU v půdě.

Výše zmíněné důvody mohly přispět k tomu, že po ukončení jednotlivých experimentů s kukuřicí, která rostla na půdě s popelem, se celkový obsah PAU v půdě prokazatelně snížil v

rozmezí od 26,7 % do 29,4 %. V půdě kontaminované syntetickými PAU se jejich obsah snížil podobně až o 21,8 %, nejvíce pak v douhodobě kontaminované půdě až o 36,9 %. Provedené experimenty (podkapitoly 4.2 až 4.5) ukázaly, že fytoremediace PAU v půdě po aplikaci popelu probíhá podobně jako fytoremediace PAU v půdě kontaminované uměle nebo antropogenní činností. Ve srovnání s přirozenou atenuací vegetační experimenty dosáhly pozitivních rozdílů ve schopnosti rostlin snížit obsah PAU v půdě.

5.2.3 Bioaugmentace a fytobioaugmentace

Bioaugmentační a fytobioaugmentační metody, ve kterých se aplikoval kompost nebo vermikompost, byly testovány pouze na půdě obsahující PAU původem z popela. Bylo zjištěno (podkapitola 4.3), že bioaugmentace kompostu nebo vermikompostu vyrobených ze stejných směsí organických odpadů v půdě, měla pozitivní vliv na změnu obsahu PAU v půdě s popelem. Nebyl zaznamenán statisticky průkazný ($p < 0,05$) rozdíl mezi kompostem a vermikompostem na změnu obsahu PAU v půdě. Ve srovnání s fytoremediací PAU v půdě s popelem byla bioaugmentace méně účinná, avšak byla více účinná ve snížení obsahu PAU než v případě přirozeného útlumu PAU v půdě po aplikaci popela. Kombinace rostlin s kompostem nebo vermikompostem (fytobioaugmentace) byla ještě účinnější ve snížení obsahu PAU v půdě než jejich přirozená atenuace, samostatná fytoremediace nebo bioaugmentace. V půdě obohacené popelem a kompostem nebo vermikompostem po sklizni kukuřice bylo zaznamenáno snížení obsahu PAU v rozmezí od 62,9 do 64,9 % za 120 dnů. Podobně jako v případě bioaugmentace i zde v rámci fytoaugmentace nebyl zaznamenán statisticky průkazný rozdíl mezi aplikovanými materiály na bioremediace PAU. Přídavky organických materiálů v půdě společně s rostlinami podporují autochtonní mikroorganismy, mění polaritu polutantů a tím zvyšují jejich biodostupnost. Zároveň zmíněné přídavky obohacují půdu o nové druhy mikroorganismů, které mohou degradovat PAU v půdě (Ouvrard, et al. 2014).

5.2.4 Mykoremediace (mykoaugmentace a fytomykoaugmentace)

Lignocelulózní substrát vyrobený z 30 – 50 mm jabloňové štěpky obsahující mycelium ligninolytické houby hlívy ústřičné (*Pleurotus* ostreatus (Jacq.) P. Kumm.) (dále jen *P. ostreatus*) byl nejúčinnější ze všech testovaných mykoremediačních substrátů (podkapitola

4.4) na snížení obsahu většiny jednotlivých syntetických PAU v půdě. Tento substrát s *P. ostreatus* statisticky průkazně ($p < 0,05$) snížil i obsah jednotlivých vysokomolekulárních PAU až o 42 % ve srovnání s přirozeným útlumem a fytoremediací PAU v půdě, kde se jednotlivé vysokomolekulární PAU snížily v maximu o 23 %. Aplikací tohoto substrátu se zároveň snížila suma NM PAU o 37,8 %, suma SM PAU o 42,3 %, suma VM PAU o 28,7 % a suma celkových PAU o 36 %. Jestliže byl stejný substrát použit v kombinaci s kukuřicí, tak bioremediační efekt rostlin na konci experimentu (120 dnů) nebyl pozorován, protože pokles obsahu celkových syntetických PAU v půdě nebyl od sebe statisticky rozdílný ($p < 0,05$). Je možné, že k výraznějšímu odstranění PAU v půdě by došlo v delším časovém období než ve zkoumaných 120 dnech, protože metoda fytomykoaugmentace dosahovala statisticky průkazně vyšších hodnot celkové půdní biomasy hub, mikrobiální a mangan peroxidázové aktivity než metody přirozené atenuace, samostatné fytoremediace a mykoaugmentace. Zároveň bylo zjištěno, že zmíněná biomasa hub a enzymatické aktivity v půdě velmi silně korelovaly s celkovým odstraněním PAU z půdy.

Na mykoremediaci zemědělské půdy kontaminované PAU antropogenní činností byly využity substráty (zrno ječmene) prorostlé myceliem houby pohárovky obecné (*Crucibulum leave* Huds. dále jen *C. leave*). Substrát s *C. leave* snížil obsah PAU v půdě o 27,6 % a pokud byl substrát aplikován v kombinaci s rostlinami, tak se obsah PAU v půdě snížil o 42,7 %. Po 120 dnech byly pozorovány podobné trendy jako při využití *P. ostreatus*. Avšak, po 180 dnech samostatná mykoaugmentace pomocí *C. leave* snížila obsah PAU v půdě o 48 % a fytomykoaugmentace o 58 %. Mnozí autoři uvedli, že bioremediace pomocí hub by mohly být perspektivní pro využití na bioremediace PAU v praxi (Li et al., 2012; García-Delgado et al., 2015). V této práci mykoaugmentace pomocí *P. ostreatus* dosáhla zhruba o 30 % vyššího odstranění syntetických PAU, než tomu bylo v případě přirozeného útlumu a ve srovnání s fytoremediací přibližně o 10 %. Avšak, mykoaugmentace pomocí *P. ostreatus* dosáhla téměř o 7 % nižšího odstranění syntetických PAU v půdě než fytomykoaugmentace PAU v dlouhodobě kontaminované zemědělské půdě pomocí *C. leave* za dobu 120 dnů.

5.2.5 Vliv PAU v půdě na růst rostlin a obsah PAU v rostlinách

Téměř ve všech diskutovaných bioremediacích PAU v půdě se již samostatným pěstováním rostlin docílilo nižšího celkového obsahu residuálních PAU než je preventivní

obsah PAU daný legislativou (MŽp ČR, 2016). Pokud byl obsah residuálních PAU v půdě po sklizni rostlin stále vysoký, doporučuje se jejich pěstování i v dalším vegetačním období (Chirakkara et al., 2016).

V případě růstu rostlin kukuřice na půdě s popelem kontaminovaným PAU a na půdě se syntetickými PAU, tak nebyly pozorovány žádné statisticky významné rozdíly ($p < 0,05$) ve výnosu biomasy ve srovnání s rostlinami na půdě s obsahem PAU pod mezí detekce. To bylo ve shodě s prací Wu et al. (2011). Dupuy et al. (2015) publikovali, že k redukci biomasy a poruchám metabolismu rostlin došlo až při růstu kukuřice na půdě s obsahem PAU vyšším než 50 mg/kg. V této předložené práci byl stanoven obsah PAU zejména v kořenech kukuřice, který byl nižší než 0,1 mg/kg. Stanovené bioakumulační faktory (BAF) PAU v kořenech byly výrazně nižší než 1. Translokační faktory (TF) PAU zde nebyly stanoveny, protože obsah PAU v nadzemní biomase byl pod mezí detekce. Toto zjištění je ve shodě s předchozími pracemi Wild et al. (2005) a Gao et al. (2011), kteří uvedli, že PAU jsou spíše akumulovány v kořenovém kortexu, než aby byly transportovány xylémem do nadzemní biomasy.

Dále v této předkládané práci byl stanoven procentuální podíl, kterým rostliny kukuřice přispěly k celkovému odstranění PAU z půdy. Tyto hodnoty byly však výrazně nižší než 1 % z celkového původního obsahu PAU v půdě (~1,6 mg/kg). Z toho vyplynulo, že kukuřice pravděpodobně podpořily pouze mikrobiální odstranění PAU v oblasti rhizosféry vlivem rostlinné exudace, tedy že dochází spíše k bioremediaci PAU *ex planta* jak uvedli Binet et al. (2000). Podobné trendy byly pozorovány i v případě kombinovaného využití rostlin pěstovaných na půdě s popelem a kompostem nebo vermikompostem, ale i v případě mykoremediací půdy pomocí substrátů s *P. ostreatus*. V experimentech, kde se aplikovaly substráty obsahující *C. leave* do půdy dlouhodobě kontaminované PAU, byly stanoveny PAU i v nadzemní biomase, avšak jejich obsah byl téměř zanedbatelný vzhledem k celkovému odstranění PAU z půdy. Stanovené BAF a TF byly tudíž významně nižší než 1 a procentuální podíl rostlin na celkovém odstranění PAU z půdy byl také výrazně nižší než 1 %.

Pokud přijmeme tezi, že stanovené NM PAU a SM PAU se extrahovaly z půdy až do nadzemní biomasy přes kořenový systém, prokázalo se, že pohyb PAU rostlinou kukuřice je možný, avšak velmi omezený. Nicméně, ve všech experimentech s rostlinami, daná nadzemní biomasa nepředstavovala žádné riziko pro životní prostředí, jelikož obsahovala celkovou sumu PAU menší než 10 násobek přípustného množství (0,01 mg/kg) dle MZ ČR (2002) nebo

méně než 0,05 mg/kg v sušené biomase dle Nařízení Komise (ES) č. 1881 (2006). Pokud by sklizená nadzemní biomasa po ukončení fytoremediačního opatření byla z hlediska vysokého obsahu PAU vyhodnocena jako riziková pro životní prostředí, musela by se skládkovat jako nebezpečný odpad obsahující PAU nebo by se mohla bioremediovat například kompostováním jak publikovali Mizwar et al. (2016).

5.3 Bioremediace PAU kompostováním a vermikompostováním

Kompostování a vermikompostování úletového popela z biomasy ve směsi s biologicky rozložitelnými odpady byly velmi účinné bioremediační metody z hlediska odstranění PAU z popela. Pokles obsahu PAU ve směsi organických odpadů byl porovnán s poklesem obsahu syntetických PAU ve stejné směsi organických odpadů. PAU původem z popela byly podobně náchylné k odstranění jako v případě syntetických PAU. To bylo ve shodě i s předchozím experimentem zaměřeným na fytoremediace půdy po aplikaci popelu kontaminovaného PAU. Během kompostování a vermikompostování bylo zaznamenáno, že se obsah jednotlivých PAU snížil v rozmezí od 28,7 % až 98,5 % v rámci 240 denního experimentu. Bioremediace PAU kompostováním vedla k 93,6 % poklesu NM PAU, avšak tento pokles nebyl statisticky průkazně rozdílný (p < 0,05) ve srovnání s vermikompostováním. Naopak, během kompostování byl zjištěn průkazný rozdíl v poklesu sumy SM PAU (80,1 %) a sumy VM PAU (73,1 %) ve srovnání s vermikompostovním, ve kterém se suma SM PAU snížila o 45,3 % a suma VM PAU o 53,6 %. To mohlo být způsobeno průkazně (p < 0,05) nižší aktivitou extracelulárních enzymů zaznamenaných ve směsi popelu s organickými odpady po ukončení vermikompostování. Během kompostování, ale i vermikompostování byla zaznamenána vyšší schopnost degradace NM PAU než SM PAU a VM PAU z popela, podobně jako tomu bylo v případě fytoremediačního opatření. Celkový obsah PAU ve směsi s organickými odpady s popelem se po ukončení kompostování snížil statisticky významně (p < 0,05) o 84,5 % v porovnání s vermikompostováním, kde se celkový obsah PAU snížil o 64,5 %.

Celkový pokles obsahu PAU ve směsi organických odpadů s popelem nebo syntetickými PAU během kompostování byl v této práci vyšší (84,5 %) než pokles obsahu PAU (75,2 %) v kontaminované půdě pomocí kompostování, jak uvedli Antizar-Ladislao et al. (2005). Pokles obsahu PAU (84,5 %) během kompostování byl v této práci srovnatelný s

82,1 až 88,1 % poklesem obsahu PAU v kompostovaném kalu z odpadních vod ve studii, kterou publikoval Oleszczuk (2006). Pokles obsahu PAU během vermikompostování (50,5 až 54,1 %) byl v této předložené práci téměř 2 krát vyšší než pokles obsahu PAU v půdě s organickými materiály, jak uvedli Hickman et Reid (2008). Avšak, pokles obsahu PAU po ukončení vermikompostování byl v této předložené práci nižší než v experimentu na bioaugmentace PAU v půdě s popelem pomocí kompostu nebo vermikompostu, kde se celkový obsah PAU snížil o 62,9 až 64,9 %. Vyšší pokles obsahu PAU kompostováním než vermikompostováním mohl být způsoben tím, že během kompostování bylo dosaženo průkazně vyššího ($p < 0,05$) indexu polarity (0,96) než při vermikompostování (0,75), jelikož byla zjištěna silná korelace ($r > 0,65$) mezi indexem polarity a celkovým poklesem obsahu PAU. K podobnému zjištění se došlo i v případě vyššího poklesu celkové organické hmoty během kompostování, který koreloval velmi silně ($r > 0,94$) s celkovým odstraněním PAU. Na tomto základě byly navrženy 3D modely celkového poklesu PAU ve vztahu k růstu indexu polarity a poklesu organické hmoty během kompostování a vermikompostování s koeficienty determinace (R^2) vyšších než 0,91. Z jednotlivých modelů byly odvozeny parabolické rovnice k predikci poklesu celkového obsahu PAU v závislosti na změnách indexu polarity a organické hmoty. Z analýzy dvoufázového reakčního modelu bylo zjištěno, že reakční konstanty popisující rychlost poklesu celkových PAU během prvních 120 dnů dosahují daleko vyšších hodnot (max. 0,0093 za den) než reakční konstanty popisující rychlost poklesu celkových PAU během 121 až 240 dnů (max. 0,0024 za den). To naznačovalo, že PAU během kompostování a vermikompostování se degradovaly zejména během prvních 120 dnů experimentu.

Získané komposty a vermikomposty obsahující PAU původem z popela nebo syntetické PAU nepředstavovaly žádné riziko pro životní prostředí, protože obsahovaly sumu residuálních PAU nižší než 2 mg/kg danou legislativou (MŽp ČR, 2008). Z tohoto důvodu, by se komposty a vermikomposty po ukončení bioremediačního opatření mohly využívat například jako půdní organická aditiva.

5.4 Souhrnný přehled vlivu zkoumaných bioremediačních metod na změny obsahu PAU

Na základě souhrnného přehledu v Tabulce 1 lze učinit závěr, že kompostování bylo nejúčinnější bioremediační metodou vedoucí k nejvyššímu poklesu celkových PAU, které se akumulovaly v popelu po spalování biomasy. Podobně tomu bylo i v případě syntetických PAU. V případě PAU v dlouhodobě kontaminované půdě se nejvíce tyto PAU snížily v rámci metody fytoaugmentace půdy se substrátem, který obsahoval *C. leave*.

Tabulka 1. Pokles celkového obsahu PAU (%; $\bar{x}$ ± SD) v půdě v rámci jednotlivých bioremediačních metod za stejné časové období (120 dnů). Stejná velká písmena ve stejném sloupci a stejná malá písmena ve stejném řádku znační statisticky neprůkazné rozdíly mezi variantami dle *post-hoc* Tukeyeho testu.

	Pokles obsahu PAU v půdě (%)		
Bioremediační metoda	**Popel**	**Syntetické**	**Antropogenní**
Přirozená atenuace	$4{,}3 \pm 2{,}1^{Ea*}$	$5{,}4 \pm 0{,}7^{Ea*}$	$8{,}0 \pm 2{,}5^{Da}$
Fytoremediace	$28{,}1 \pm 1{,}9^{Cb*}$	$21{,}8 \pm 1{,}8^{Db*}$	$36.9 \pm 1{,}1^{Ba}$
Bioaugmentace (kompost)	$18{,}8 \pm 1{,}8^{D}$	[1]–	–
Bioaugmentace (vermikompost)	$17{,}8 \pm 2{,}1^{D}$	–	–
Fytobioaugmentace (kompost)	$62{,}9 \pm 3{,}5^{B}$	–	–
Fytobioaugmentace (vermikompost)	$64{,}9 \pm 2{,}3^{B}$	–	–
Mykoaugmentace (*P. ostreatus* na 10 – 30 mm substrátě)	–	$18{,}9 \pm 2{,}5^{D}$	–
Mykoaugmentace (*P. ostreatus* na 30 – 50 mm substrátě)	–	$36{,}0 \pm 1{,}3^{C}$	–
Mykoaugmentace (*P. ostreatus* na 10 – 50 mm substrátě)	–	$22{,}2 \pm 0{,}7^{D}$	–
Fytomykoaugmentace (*P. ostreatus* na 10 – 30 mm substrátě)	–	$21{,}3 \pm 1{,}8^{D}$	–
Fytomykoaugmentace (*P. ostreatus* na 30 – 50 mm substrátě)	–	$36{,}2 \pm 2{,}5^{C}$	–
Fytomykoaugmentace (*P. ostreatus* na 10 – 50 mm substrátě)	–	$23{,}3 \pm 2{,}3^{D}$	–
Mykoaugmentace (*C. leave*)	–	–	$27{,}4 \pm 1{,}1^{C}$
Fytomykoaugmentace (*C. leave*)	–	–	$42{,}7 \pm 1{,}0^{A}$
Kompostování	$80{,}2 \pm 0{,}1^{Aa}$	$74{,}7 \pm 1{,}3^{Ab}$	–
Vermikompostování	$54{,}1 \pm 2{,}3^{Ca}$	$50{,}5 \pm 0{,}8^{Ba}$	–

*Průměr ze dvou experimentů; [1]Nebylo zkoumáno.

6 Závěr

Předkládaná disertační práce je souborem komentovaných již publikovaných 5 impaktovaných vědeckých prací a jedné vědecké práce připravené k odeslání do časopisu s impakt faktorem k recenznímu řízení. Dosavadní přehled o přítomnosti polycyklických aromatických uhlovodíků (PAU) v úletových a roštových popelech po spalování biomasy, které vznikly za reálných podmínek spalování, je v současné době ve vědecké literatuře stále velmi omezený. Výzkum založený na recyklaci popelů s vysokým obsahem PAU v rámci environmentálně šetrných a ekonomicky dostupných bioremediačních metod nebyl před touto prací na základě dosavadních znalostí vědeckých publikací proveden.

V první části experimentální práce byla věnována značná pozornost sledování obsahu PAU v popelech po spalování biomasy ve vztahu k teplotě spalování a nespálenému organickému uhlíku. Druhá část experimentální práce se zabývala vlivem vybraných bioremediačních metod na změny obsahu PAU v půdě nebo ve směsi s organickými odpady.

Na základě provedeného monitorování obsahu PAU v popelech bylo zjištěno, že z hlediska tvorby PAU s jejich následnou akumulací ve vznikajících popelech bylo nejrizikovější spalování slámy při teplotě v rozmezí 500 až 750 °C. Popely po spalování dendromasy (dřevní popele) a roštové popely ze slámy (mimo popelů vzniklých v rozmezí 500 – 750 °C) byly vyhodnoceny jako bezrizikové z hlediska nízkého obsahu PAU a mohly by se využívat v zemědělství. Úletové popely dosahovaly významně vyšších obsahů PAU než roštové. Ve většině popelů převažoval obsah nízkomolekulárních PAU než obsah středně a vysokomolekulárních PAU. Na základě vztahu mezi nespáleným organickým uhlíkem a celkovým obsahem PAU v popelech byly odvozeny rovnice, které by se v praxi mohly využívat k predikci obsahu PAU v popelech. Získané výsledky z monitorování obsahu PAU v popelech v této práci byly použity MZe ČR jako podklad pro nastavení legislativního obsahu PAU v popelech po spalování biomasy určených pro využití v zemědělství.

Ve fytoremediačních metodách byla použita rostlina kukuřice setá (*Zea mays* L.). Fytoremediace PAU v půdě po aplikaci popelu měla prokazatelně vyšší vliv na pokles obsahu celkových PAU než přirozená atenuace PAU v půdě. PAU původem z popela byly podobně náchylné k biodegradaci jako syntetické PAU v uměle kontaminované půdě. Metoda fytobioagumentace PAU v půdě po aplikaci popelu, tedy pomocí kompostu nebo vermikompostu v půdě s rostlinami, byla z hlediska poklesu obsahu PAU v půdě prokazatelně

účinnější než samostatná fytoremediace nebo samostatná bioaugmentace. V rámci mykoremediace půdy kontaminované syntetickými PAU bylo z hlediska vlivu na pokles obsahu PAU v půdě nejvýhodnější aplikovat 30 – 50 mm substrát ze štěpky obsahující hlívu ústřičnou (*Pleurotus ostreatus*). V tomto experimentu nebyl zaznamenán statisticky průkazný rozdíl mezi samostatnou aplikací substrátu v půdě a jeho kombinovaným využitím s rostlinami. Ve srovnání s fytobioaugmentací PAU v popelu byla tato mykoremediace méně účinná. Avšak, mykoremediace PAU v dlouhodobě kontaminované půdě pomocí kombinovaného využití rostlin a substrátu s penízovkou obecnou (*Crucibulum leave*) prokázala, že i mykoremediační substráty mají velkou schopnost degradovat PAU v půdě. Z důvodu produkce ligninolytických enzymů v půdě schopných degradovat PAU je aplikace mykoremediačních substrátů do půdy kontaminované PAU velice perspektivní metodou. Vermikompostování směsi organických odpadů s popelem bylo poměrně efektivní metodou bioremediace PAU, avšak srovnatelnou s metodou fytobioagumentace PAU v půdě obohacené o popel. Nejefektivnější metodou bioremediace PAU z popela bylo kompostování ve směsi s organickými odpady. PAU ve směsi organických odpadů po aplikaci popelu byly podobně degradovatelné jako syntetické PAU. Kompostování poskytlo vyšší růst indexu polarity, významnější pokles organické hmoty a zvýšenou aktivitu mangan peroxidázy. Tyto parametry korelovaly velmi silně s celkovým poklesem obsahu PAU. Dvoufázová analýza kinetiky prvního řádu ukázala, že se PAU degradovaly zejména během prvních 120 dnů pokusu.

V rámci experimentů s rostlinami kukuřice seté (*Zea mays* L.) na půdě kontaminované PAU bylo zjištěno, že PAU v půdě neměly významný vliv na redukci výnosu biomasy rostlin. V případě růstu rostlin na půdě s popelem byly PAU nalezeny zejména v kořenech rostlin. V nadzemní biomase byly PAU stanoveny pouze v případě rostlin, které rostly na půdě dlouhodobě kontaminované PAU. Na základě stanoveného relativního podílu rostlin na celkovém poklesu obsahu PAU v půdě bylo zjištěno, že rostliny kukuřice pravděpodobně podporují pouze bioremediaci PAU v půdě. Téměř ve všech případech byl po fytoremediaci půdy residuální obsah PAU nižší než je preventivní obsah PAU v půdě daný legislativou. Sklizená nadzemní biomasa z hlediska nízkého obsahu PAU nepředstavovala žádné riziko pro životní prostředí. Z důvodu sumy PAU nižší než 2 mg/kg dané legislativou by se získané komposty a vermikomposty po bioremediaci PAU mohly využívat například jako půdní organická aditiva.

7 Seznam literatury použité v literárním přehledu a diskusi

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