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Katedra agroenvironmentální chemie a výživy rostlin

Využití odpadních materiálů při výživě rostlin

doktorská disertační práce

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Prohlášení

Prohlašuji, že jsem disertační práci na téma: **„Využití odpadních materiálů při výživě rostlin“** vypracoval samostatně a použil jen pramenů, které cituji a uvádím v přiloženém seznamu literatury.

V Praze dne Podpis

Poděkování

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1) Úvod

Rychle rostoucí lidská populace s sebou nese významný tlak na udržitelnost a produktivitu zemědělství, které zároveň musí čelit měnícímu se prostředí ovlivněném klimatickou změnou. Udržitelnost a produktivita stávající zemědělské produkce je do značné míry limitována její závislostí na nerostných zdrojích živin, především pak fosforu. Na evropském kontinentu se nenachází významný zdroj fosfátů – hlavního zdroje fosforu pro výrobu minerálních hnojiv. Pro budoucí udržitelnost zemědělství je tedy klíčové fosfor a další minerální živiny recyklovat z odpadních materiálů zpět do zemědělské půdy, uzavřít tak jejich koloběh a současně zvýšit efektivitu jejich příjmu rostlinami.

Významnými zdroji fosforu z odpadních materiálů jsou především čistírenský kal a popely ze spalování biomasy. Chemická forma, ve které je fosfor v těchto materiálech přítomný, se významně liší, avšak v obou případech ho lze považovat za omezeně přístupný rostlinám. Nízká přístupnost negativně ovlivňuje jeho recyklovatelnost a znemožňuje tak uzavření koloběhu fosforu v dostatečně krátkém časovém horizontu. Vedle toho přímá aplikace těchto materiálů na zemědělskou půdu přináší určitá rizika, ať ve formě prašnosti v případě popelů, mikrobiologické závadnosti v případě čistírenských kalů, nevyrovnanému obsahu živin nebo zvýšenému obsahu rizikových prvků a sloučenin. Aby bylo dosaženo úspěšného použití odpadních materiálů v zemědělství, je nutné tyto rizika, ať fyzikální, chemická nebo biologická, eliminovat pomocí dalšího zpracování a zhodnocení.

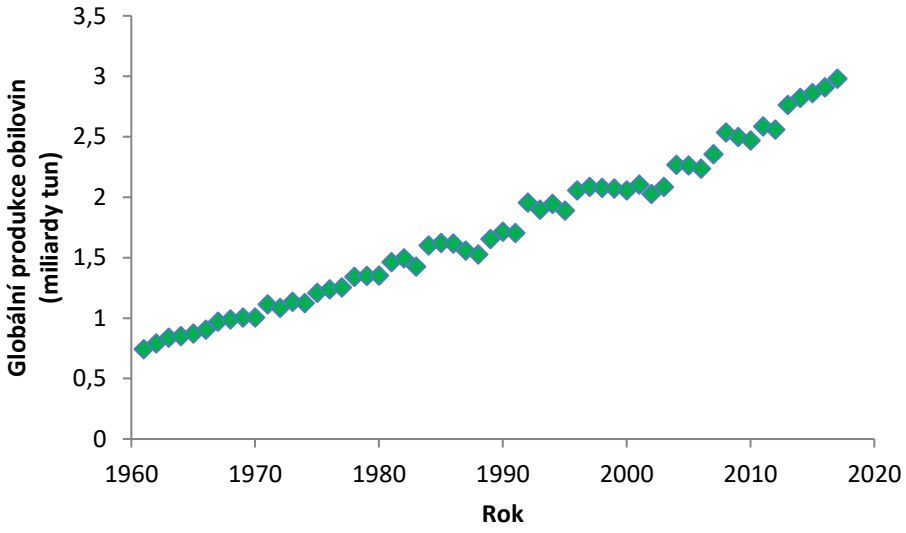
Tato práce se v rešeršní části věnuje aplikaci čistírenského kalu, popelů ze spalování biomasy a termicky zpracované organické hmotě ve formě biocharu na zemědělskou půdu, riziky jejich aplikace a vlivy na růst zemědělských plodin a půdní vlastnosti. Část rešerše je dále zaměřena na popis chemických změn probíhajících během pyrolýzy, jakožto velmi slibné technologie pro zhodnocení odpadních materiálů. Rešerše se dále zabývá hlavními faktory ovlivňujícími přístupnost fosforu rostlinám v půdním prostředí, mechanismy jeho zpřístupnění a možnostmi aplikace fosfor-solubilizujících mikrobiálních inokulantů za účelem zlepšení recyklovatelnosti fosforu z odpadních materiálů. Výsledková část této práce pak shrnuje poznatky z realizovaných experimentů, rozdělených do tří základních okruhů. V prvním jsou identifikovány rozdílné vlivy popela a biocharu na chemické a biologické vlastnosti půdy. Druhý okruh se zabývá odlišným uvolňováním živin z popelů vzniklých spalováním biomasy a možnostmi modifikace jejich složení. Třetí okruh výsledkové části je pak zaměřen na použití mikrobiálních inokulantů za účelem zvýšení přístupnosti živin z odpadních materiálů.

2) Literární přehled

2.1) Udržitelné zemědělství, koloběh živin a zlepšení efektivity využívání živin

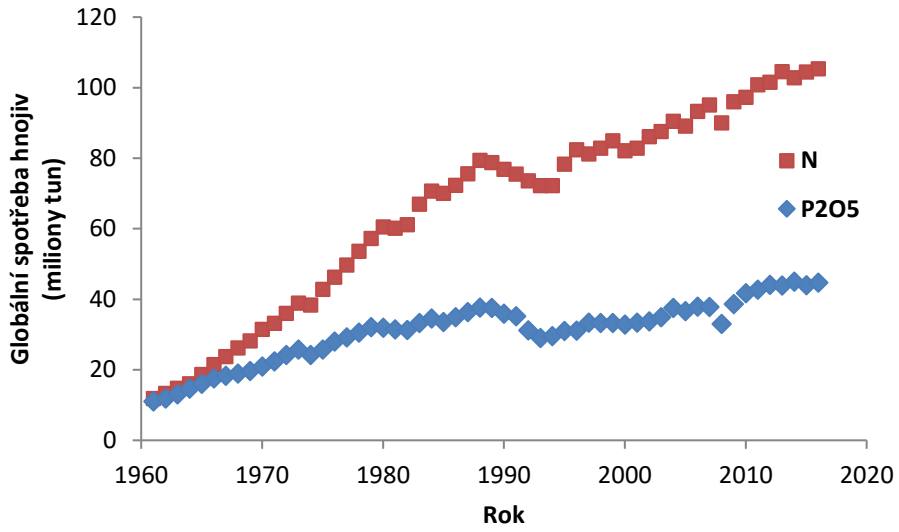
Zemědělství se na naší planetě začalo rozvíjet přibližně před 10 000 lety. Před vznikem zemědělství byla lidská společnost založená na lovu a sběru. Tímto způsobem obživy bylo možné udržet světovou populaci na hranici přibližně 4 milionů lidí (Tilman et al., 2002). Dnešní populace však čítá 7,7 miliardy lidí (Worldometers.info, 2019), přičemž dle odhadů Alexandratos et Bruinsma (2012) světová populace přesáhne v roce 2050 hranici 9 miliard jedinců. To s sebou přinese dle odhadů nutnost dvojnásobně zvýšit globální produkci obilovin oproti stavu v roce 1999 (Cassman, 1999; Alexandratos, 1999). Globální nárůst zemědělské produkce za posledních sto let byl možný zejména díky objevu Haber-Boschovy syntézy amoniaku a tzv. Zelené revoluci. Ta s sebou přinesla především zvýšenou spotřebu minerálních hnojiv, vody a pesticidů. Nárůst produkce obilovin a spotřeby hnojiv v zemědělství mezi roky 1960 - 2018 je zobrazen na Obrázku 1 a 2.

Obrázek 1. Vývoj globální produkce obilovin (1961 – 2017)



(převzato z Tilman et al., 2002; doplněno dle Světová banka, 2019)

Obrázek 2. Vývoj globální spotřeby dusíkatých a fosforečných hnojiv (1961 – 2016)

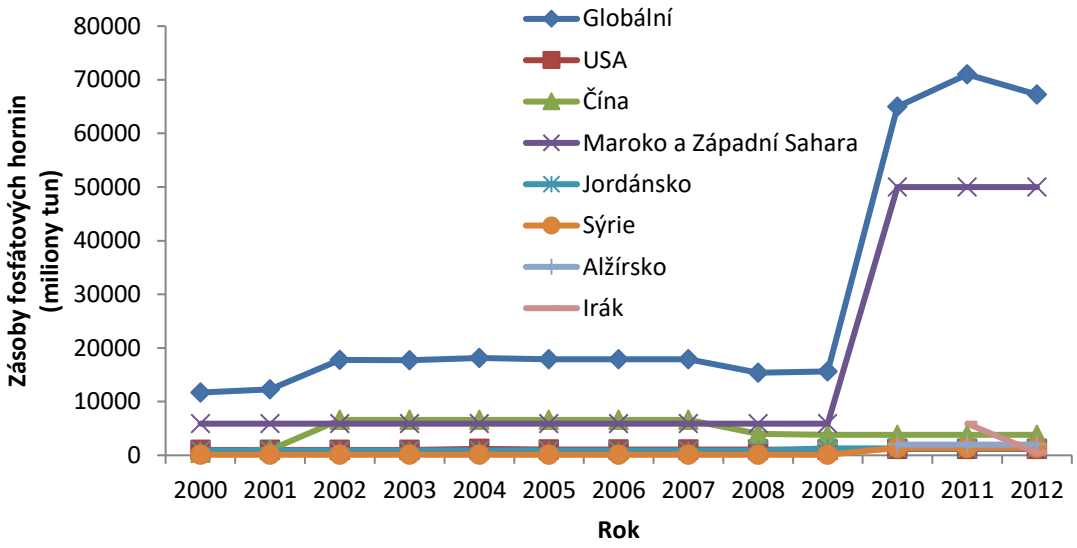


(převzato z Tilman et al., 2002; doplněno dle IFA, 2019)

Je patrné, že stávající zemědělská produkce je závislá především na rostoucí spotřebě fosforečných a dusíkatých hnojiv. Dusíkatá minerální hnojiva lze dlouhodobě vyrábět syntézou ze vzdušného dusíku již zmíněnou Haber-Boschovou syntézou a uzavírat tak jeho globální koloběh. Tento proces je však velice energeticky náročný. Dle Mayer et al. (2016) Haber-Boshova syntéza spotřebovává přibližně 1 % celosvětově produkované energie. Althaus et al. (2007) dále uvádí, že na jeden kg NH_3 je vyprodukováno 1,2 – 2,3 kg CO_2. Tyto skutečnosti tedy vedou k negativnímu vlivu tohoto procesu na změnu klimatu (Sutton et al., 2009). V současnosti se průměrná využitelnost dusíku obilovinami pohybuje v rozmezí 30 – 50 % z celkově aplikovaného dusíku (Cassman et al., 2002). Tato skutečnost vede k přehnojování, což má za následek jednak znečišťování podzemních a povrchových vod, ale také vede ke zvýšeným emisím oxidů dusíku (Delmas et al., 1997) včetně oxidu dusného (Reay et al., 2012). Z tohoto pohledu je tedy klíčové, aby udržitelné zemědělství dosáhlo mnohem vyšší využitelnosti dusíku z minerálních hnojiv rostlinami.

Naproti tomu koloběh fosforu, po dusíku nejvýznamnější živiny, není možné tak snadno uzavřít, jako v případě dusíku. Téměř veškerá fosforečná hnojiva používaná v dnešním intenzivním zemědělství jsou vyráběna z fosfátových hornin. Ložiska těchto neobnovitelných hornin jsou silně nerovnoměrně geograficky rozložená. Rozdělení nalezišť fosfátů mezi jednotlivými zeměmi jsou zobrazena na Obrázku 3.

Obrázek 3. Předpokládané zásoby fosfátů



(převzato z Chowdhury et al., 2017)

Z obrázku je patrné, že na území Evropy se nenachází významný zdroj těchto hornin, a tudíž je většina fosforečných hnojiv do Evropy dovážena. To přináší nestabilitu a velké riziko budoucího strmého růstu cen fosforečných hnojiv. Dle údajů Cordell et al. (2009) se předpokládá nárůst spotřeby fosforečných hnojiv o 50 – 100 % do roku 2050 a tudíž potenciální vyčerpání fosfátových ložisek během následujících 50 – 100 let. Autoři pozdějších studií již však zásoby fosfátů odhadují na 300 – 400 let (Van Kauwenbergh, 2010), ovšem je třeba poznamenat, že veškeré tyto odhady mohou být silně nepřesné kvůli možným novým nalezištím a měnící se spotřebě fosforu. FAO (2017) předpovídá růst poptávky po fosforečných hnojivech mezi roky 2015 a 2020 přibližně o 2,2 % ročně. Růst poptávky po dusíkatých hnojivech pak odhaduje na 1,5 % a pro draselná hnojiva na 2,4 % ročně.

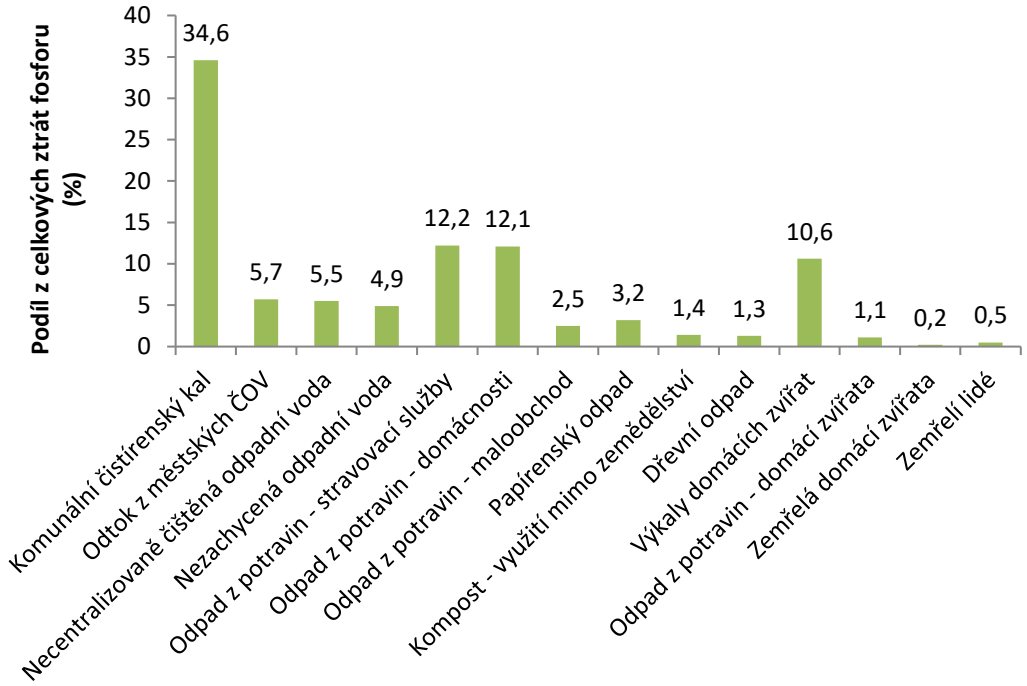
Aktuální využitelnost fosforu obilovinami z aplikovaných hnojiv se celosvětově pohybuje v průměru okolo 45 % (Smil, 2000). To má za následek zvyšování obsahu P v zemědělských půdách. Van Dijk et al. (2016) uvádějí, že v roce 2005 bylo z celkového množství fosforu dovezeného do Evropy 51 % ztraceno formou odpadu, přičemž 39 % z celkového množství zůstalo akumulováno v zemědělských půdách. Zvýšená zásoba fosforu v půdách vede následně díky povrchovým smyvům a erozi k eutrofizaci povrchových vod s dalekosáhlými negativními dopady na globální produkci vodních ekosystémů (Tilman et al., 2002). Vollenweider (1975) uvádí, že eutrofizace vod může nastat už při nízkých koncentracích fosforu (20 – 30 µg P L^{-1}). Proto Fortune et al. (2005) upozorňují i na silný ekologický význam fosforu vymytého ze zemědělských půd gravitační vodou.

Z těchto důvodů je tedy klíčové, aby v zemědělství docházelo jednak k uzavření koloběhu fosforu, ale současně je nutné zvýšit efektivitu jeho příjmu rostlinou. Téměř jedinou možností, jak dosáhnout uzavření cyklu fosforu, je jeho recyklace z odpadních materiálů a následná aplikace na zemědělskou půdu (Schoumans et al., 2015).

2.2) Využití odpadních materiálů v zemědělství

Studie Van Dijk et al. (2016) analyzovala rozložení nevyužitého fosforu v Evropě mezi odpadními materiály. Procentuální zastoupení jednotlivých druhů odpadů na celkovém množství nevyužitého fosforu v EU je zobrazeno na Obrázku 4.

Obrázek 4. Podíl jednotlivých druhů odpadů na celkovém množství nevyužitého fosforu v EU v roce 2005



(převzato z Van Dijk et al., 2016)

Zde je patrné, že hlavní objem nevyužitého fosforu v odpadních materiálech je obsažen v čistírenském kalu. Fosfor obsažený v gastro odpadech je vhodné kompostovat, popř. vermikompostovat nebo anaerobně fermentovat, přičemž fosfor ztracený ze systému ve formě již vyčištěných odpadních vod je nejprve třeba z vody získat a zakoncentrovat pomocí

terciárního čištění a následně využít, popř. tuto vodu použít pro závlahu plodin (Mayer et al., 2016). Z pohledu ekonomické a praktické recyklace fosforu z odpadních materiálů v zemědělství tedy přichází v úvahu využití čistírenského kalu a dřevěného odpadu, resp. popelu ze spalování biomasy.

2.2.1) Čistírenský kal

Čistírenský kal je jedním z konečných produktů procesu čištění odpadních vod a jeho produkce je nedílnou součástí těchto procesů. EUROSTAT (2019) nedisponuje dostatečnými informacemi o celkové produkci čistírenských kalů v EU. Produkci však lze odhadnout z celkového počtu obyvatel EU (508 milionů dle Evropská Komise (2019)) a průměrné roční produkce kalu *per capita* (22,5 kg dle Bianchini et al. (2016)) přibližně na 11 430 000 t sušiny kalu ročně. Produkce kalu v České republice v roce 2018 činila přibližně 202 000 tun sušiny kalu. To při průměrné sušině 25 % představuje přibližně 808 000 t čerstvého kalu. Z tohoto množství bylo přibližně 44 % přímo aplikováno na půdu, 32 % kompostováno, 14 % uloženo na skládky a 10 % bylo spáleno (ČSÚ, 2019).

2.2.1.1) Chemické složení čistírenského kalu

Vlastnosti a složení čistírenských kalů se mohou významně lišit především podle lokality, podle typu stokové sítě a podle technologie úpravy kalu. Typické procesy úpravy kalu shrnují Kacprzak et al. (2017) jako primární zahuštění (gravitační, flotační, sítopásové, centrifugační), stabilizace kapalného kalu (aerobní digesce, anaerobní digesce, přídavek vápna), sekundární zahuštění (gravitační, flotační, sítopásové, centrifugační), úprava vlastností (chemická úprava, termická úprava), odvodnění (sítopásový lis, centrifugace, sušící lože) a finální úprava/aplikace (kompostování, sušení, přídavek vápna, spalování, mokrá oxidace, pyrolýza, dezinfekce, přímá aplikace na půdu).

Suchý čistírenský kal obsahuje v průměru 50 – 70 % organické hmoty a 30 – 50 % minerální složky. Obsah dusíku se v sušině kalu pohybuje v rozmezí 1,6 – 6 % a obsah fosforu v rozmezí 1,5 – 11 %. Z pohledu výživy rostlin je obsah draslíku v kalech většinou nízký (0,5 – 0,7 %) a ve srovnání s obsahem dusíku a fosforu lze obsah draslíku považovat za nedostatečný (Singh et Agrawal, 2008). Obsah dalších makro- a mikroživin, stejně jako rizikových prvků, se významně liší dle jednotlivých čistíren odpadních vod (Kacprzak et al., 2017). Kaly však dále

mohou obsahovat značná množství rizikových a/nebo perzistentních látek, jako např. polycyklické aromatické uhlovodíky (Dai et al., 2007), polychlorované bifenyly, halogenované uhlovodíky (Benabdallah El-Hadj et al., 2007), dibenzodioxiny a dibenzofurany (Urbaniak et al., 2017), pesticidy, přípravky osobní potřeby (Albero et al., 2012; Montes-Grajales et al., 2017), hormonální látky, léčiva (Peysson et Vulliet, 2013), endokrinní disruptory (Chen et al., 2017), mikroplasty (Mahon et al., 2017) nebo nanočástice (Kim et al., 2010). Dále se v kalu mohou vyskytovat různé druhy patogenních organismů, jako jsou bakterie (Clarke et al., 2017), viry nebo prvoci (Kacprzak et al., 2017).

2.2.1.2) Vliv aplikace čistírenského kalu na fyzikálně-chemické a biologické vlastnosti půdy

Aplikace čistírenského kalu na zemědělskou půdu pozitivně ovlivňuje některé půdní vlastnosti, avšak především kvůli obsahu rizikových prvků s sebou nese i určitá rizika. Z důvodu rostoucí mobility Cd, Cu, Ni a Zn s klesajícím pH, se doporučuje kal aplikovat na půdy s pH vyšším než 6,5 (Singh et Agrawal, 2008).

Díky značnému obsahu organické hmoty v kalu lze při vyšších aplikačních dávkách kalu na půdu pozorovat nárůst kationtové výměnné kapacity (Singh et Agrawal, 2008). Aplikace kalu dále zvyšuje obsah humínových a fulvokyselin v půdě (Urbaniak et al., 2017). Albiach et al. (2001) nalezli po aplikaci kalu zvýšené obsahy celkového organického uhlíku a humínových kyselin v půdě. Obsah sacharidů nebyl aplikací kalu ovlivněn. Obecně aplikace kalu zvyšuje retenční vodní kapacitu půdy, snižuje její objemovou hmotnost, zvyšuje stabilitu půdních agregátů a hydraulickou vodivost. Po aplikaci kalu na půdu dochází většinou k snadné mineralizaci organického dusíku v kalu, zvýšení mikrobiální respirace a enzymatické aktivity. Albiach et al. (2001) dále stanovili zvýšené půdní aktivity fosfodiesterázy v půdě po aplikaci kalu, přičemž mikrobiální biomasa a aktivity ureázy, arylsulfatázy a dehydrogenázy nebyly aplikací kalu ovlivněny. Pokud však kal obsahuje zvýšené množství rizikových prvků, může jeho aplikace vést i k celkové inhibici mikrobiální aktivity v půdě (Singh et Agrawal, 2008).

2.2.1.3) Přístupnost živin a agronomický potenciál čistírenského kalu

Aplikace čistírenského kalu na zemědělskou půdu v naprosté většině případů vede k vyšším výnosům biomasy, především díky obsahu fosforu a snadno mineralizovatelného dusíku

(Singh et Agrawal, 2008). Přístupnost fosforu v čistírenských kalech je silně ovlivněna technologií čistírenského procesu, a proto se velice liší mezi jednotlivými provozy. Kaly pocházející z provozů využívající biologické odstraňování fosforu obecně vykazují srovnatelnou přijatelnost fosforu s minerálními hnojivy, avšak pokud je v provozu fosfor odstraňován srážením hlinitými a/nebo železitými solemi, přijatelnost fosforu v kalu výrazně klesá (Maguire et al., 2001). Dle O`Connor et al. (2004) je fosfor v kalech obsahujících více než 50 g železa a hliníku na kg kalu velmi špatně přístupný rostlinám. Studie využívající techniky ^{31}P NMR (nukleární magnetická rezonance) a XANES (X-ray absorption near edge structure) dokazují, že pokud jsou pro odstranění fosforu z odpadní vody použity hlinité a železité soli, fosfor v kalu je následně přítomen ve formě hlinitých a/nebo železitých fosforečnanů, popř. je adsorbován na oxidy železa a hliníku (Huang et Shenker, 2004; Shober et al., 2006). Obdobné výsledky byly potvrzeny pomocí sekvenční extrakce fosforu v kalu, kdy většina fosforu byla přítomná ve frakci extrahovatelné pomocí NaOH (fosfor asociovaný s železem a hliníkem) (Frossard et al., 1994; Ajiboye et al., 2006). Dezinfekce odvodněného kalu vápněním dále mění formu a tudíž i přístupnost fosforu z kalu rostlinám. Dle Schober et al. (2006) vápnění kalu pocházejícího z chemického srážení fosforu solemi železa a hliníku zvýšilo obsah vápenatých fosforečnanů, především pak hydroxyapatitu – minerálu vykazujícího velmi nízkou přístupnost fosforu rostlinám v půdním prostředí. Naproti tomu Huang et al. (2008) popisuje transformaci organického fosforu z NaOH frakce do lépe přístupné $NaHCO_3$ frakce po aplikaci páleného vápna. Obdobně Øgaard et Brod (2016) popisují po aplikaci páleného vápna konverzi fosforu vázaného na oxidy železa a hliníku do více labilních forem, resp. do forem extrahovatelných $NaHCO_3$ a HCl. To je pravděpodobně způsobeno zvýšeným pH po aplikaci vápna, jelikož rozpustnost Fe-/Al-fosforečnanů roste spolu s rostoucím pH (Hinsinger, 2001) a dále tvorbou fosforečnanů vápenatých.

V experimentu Øgaard et Brod (2016) s jílkem mnohokvětým bylo testováno 11 čistírenských kalů z různých provozů lišících se technologií úpravy kalu. Jako růstové médium byla použita pH neutrální směs písku a rašeliny. Relativní hnojivý ekvivalent fosforu se pohyboval v rozmezí 2 – 52 % ve srovnání s $Ca(H_2PO_4)_2$. Obecně nižší hnojivý potenciál kalů byl pozorován v počáteční fázi růstu, což autoři připisují závislosti rostlin na koncentraci snadno přístupného fosforu v půdním roztoku díky nevyvinutém kořenovému systému a nižší intenzitě rhizosférních procesů. To způsobuje nižší osvojovací schopnost fosforu v počátečních fázích vegetace. Autoři dále popisují, že fosfor je rostlinám lépe přístupný v kalech, kde se jako srážecí činidlo používají soli železa, oproti solím hliníku. To lze přisuzovat nižší rozpustnosti

hlinitých fosforečnanů oproti fosforečnanům železitým (Lindsay, 1979). Kaly ošetřené vápnem nevykazovaly ve studii Øgaard et Brod (2016) statisticky odlišnou přístupnost fosforu pro rostliny. Autoři uvádějí, že přístupnost fosforu v kalu je přímo závislá na koncentraci železa a hliníku. Dle Kahiluoto et al. (2016) je z pohledu přístupnosti fosforu rostlinám rozhodující molární poměr Fe/P. Kaly pocházející z biologického odstraňování fosforu a kaly po srážení s nižším poměrem Fe/P než 1,6 dle autorů vykazují ještě lepší přístupnost a recyklovatelnost fosforu než minerální NPK hnojivo. Autoři tuto skutečnost přikládají vlivu organické hmoty přítomné v kalu, která brání sorpci a srážení fosforu v půdě. Kaly s poměrem Fe/P okolo 9,8 nejen že obsahovaly jen velmi špatně přístupné formy fosforu, ale současně vykazovaly vyšší obsahy potenciálně fytotoxických prvků. Tyto výsledky názorně demonstrují význam dávkování srážecích činidel při čištění odpadních vod na recyklovatelnost fosforu. Anaerobní stabilizace kalu dle Kahiluoto et al. (2016) snižuje přístupnost fosforu rostlinám, naproti tomu aerobně stabilizované kaly vykazují přístupnost vyšší (Frossard et al., 1996).

2.2.2) Popel ze spalování biomasy

Popel je minerální zbytek po spalování biomasy. Celosvětová roční produkce popelů ze spalování biomasy je odhadována přibližně na 476 milionů tun (Vassilev et al., 2010). V České republice se dle ISOH (2016) (Informační systém odpadového hospodářství) v roce 2014 vyprodukovalo 90 249 t popelů ze spalování neošetřeného dřeva a rašeliny, přičemž v roce 2013 tato produkce činila 47 196 t. Pro srovnání van Eijk et al. (2012) uvádějí roční produkci popelů pro vybrané státy EU: Rakousko – dřevěný popel – 141 000 t, Finsko – dřevo 100 000 t + 350 000 t rašelina, Německo – dřevo 137 000 t, Irsko – dřevo 1 200 t + 199 000 t rašelina, Nizozemsko – dřevo 26 000 t, Norsko – dřevo 75 000 t, Švédsko – dřevo 155 000 t + 32 000 t rašelina. Bohužel EUROSTAT (2016, osobní komunikace) nesbírá data o produkci popelů z biomasy v rámci EU. Množství vzniklého popela se velmi liší vlivem rozdílného složení spalované biomasy. Dřevo obvykle obsahuje nízké množství popela (méně než 2 %), přičemž znatelně více popelovin nalézáme v kůře, slámě, trávě a obilninách (5 – 10 %) (Biedermann et Obernberger, 2005; James et al., 2012). Obecně množství popelovin v biomase klesá s rostoucím obsahem ligninu (Fahmi et al., 2007).

2.2.2.1) Chemické složení popela z biomasy

Chemické složení popela silně závisí na druhu spalované biomasy, technologii, resp. teplotě spalování a v neposlední řadě na způsobu přepravy a skladování popela (Vassilev et al., 2013b). Ve srovnání s uhelným popelem, obsahuje popel z biomasy vyšší množství K, P, Mg, Ca, Na, Mn, Cl, a znatelně nižší množství S, Fe, Si, Al a Ti. Hlavními minerálními živinami, které nalézáme v popelech z biomasy, jsou K, Mg a Ca.

Průměrné obsahy živin v popelech v ČR po spalování dřeva a slámy uvedené Tlustošem a kol. (2012) jsou zobrazeny v Tabulka 1 a Tabulka 2. Z těchto údajů je patrné, že popely ze spalování dřeva obsahují v porovnání s popely ze slámy vyšší obsahy Mg, Al, Ca, Mn a Fe, přičemž popely ze slámy jsou bohatší na Si, P, S a K.

Tabulka 1. Prvkové složení popelů ze spalování dřevní biomasy

Hm.%	Mg	Al	Si	P	S	K	Ca	Mn	Fe	Zn
průměr	1,71	4,00	16,0	0,92	0,67	5,74	19,4	1,79	3,23	0,19
median	1,68	4,04	17,1	0,83	0,42	5,62	14,3	1,39	3,35	0,07
min	0,75	1,24	3,34	0,21	0,07	4,15	6,10	0,17	1,59	0,03
max	3,00	6,99	27,8	3,95	4,08	9,38	39,5	5,49	4,86	3,58

(*n* = 37; Tlustoš a kol., 2012)

Tabulka 2. Prvkové složení popelů ze spalování obilné slámy

Hm.%	Mg	Al	Si	P	S	K	Ca	Mn	Fe	Zn
průměr	1,03	0,95	21,1	1,26	2,03	16,9	5,08	0,11	0,60	0,03
median	1,01	0,52	21,7	1,21	1,33	15,6	4,77	0,08	0,48	0,03
min	0,14	<0.01	5,52	<0.01	<0.01	6,77	2,54	0,03	0,18	0,01
max	2,16	3,95	36,1	3,08	5,29	32,1	10,0	0,29	1,85	0,07

(*n* = 16; Tlustoš a kol., 2012)

Teplota spalování, nejčastěji ovlivněná především technologií spalování, má také významný vliv na konečné složení popela. Tan et Lagerkvist (2011) uvádějí, že relativní zastoupení P, Fe, Al, Si, Mg, a Ca v popelu ze spalování dřeva roste s rostoucí teplotou spalování v rozmezí 500 – 1500 °C. Podobně pak v rozmezí 500 – 950 °C rostlo zastoupení zmíněných prvků v popelu při spalování pšeničné slámy. To je způsobeno zejména lepším spálením zbytkového uhlíku a tepelným rozkladem uhličitanů, síranů a některých fosforečnanů při vyšších teplotách a dále pak zvýšenou volatilizací mobilních prvků (Br, Cl, C, H, Hg, K, N, Se, S) (Vassilev et al., 2013a).

Při spalování biomasy však vždy dochází ke snížení obsahu K při překročení hraniční teploty 800 °C. V případě olivových zbytků byla tato kritická teplota 600 °C. Je tedy zřejmé,

že pro jednotlivé druhy biomasy se tato hraniční teplota mění v závislosti na jejich chemickém složení. Při překročení kritické teploty může docházet ke vzniku tavenin, a s nimi spojeným problémům se spékáním popela a tvorbě strusek. Draslík přitom vždy hraje klíčovou roli. Vznikající draselné soli (např. KCl) tají už při teplotách 770 °C. Tyto soli (KCl, K_2SO_4, K_2CO_3) mohou dále reagovat s oxidy křemíku za vzniku draselných silikátů. Pokud je v popelu přítomen K_2O a SiO_2 v poměru 1:1, dochází k tavení při teplotách 600 – 700 °C (Wang et al., 2016). Při poměru 2:1 je potom teplota tavení 769 °C (Miles et al., 1996). Vassilev et al. (2014) uvádějí kritický poměr SiO_2/K_2O, pro tvorbu nízkoteplotních tavenin, v rozmezí 1,3 – 4,0. Pokud se v popelu nacházejí minerály jako kalsilit/kaliofilit ($KAlSiO_4$; poměr SiO_2/K_2O = 1,3) nebo leucit ($KAlSi_2O_6$; poměr SiO_2/K_2O = 2,6), indikuje to tvorbu problematických tavenin při spalování (Vassilev et al., 2014). V praxi se problémy spékání a tavení popela řeší především změnou poměru problematických prvků (K, Si, Al, Ca) ve spalované směsi přídavkem mnoha různých materiálů, jako jsou např: kaolinit, mullit, bentonit, K-živec, plagioklas, olivín, křemen, vápno, bauxit, gibbsit, hematit, kalcit, dolomit, magnezit, vápenec, rašelina, nebo uhelný popel (Vassilev et al., 2014).

Možnosti aplikace popela na zemědělskou půdu velmi závisí na obsahu rizikových prvků, popřípadě i organických polutantů v popelu. Většina rizikových prvků je při spalovacím procesu uvolňována v jejich volatilních formách, a tudíž je koncentrována v úletovém popelu mnohonásobně více, než v popelu roštovém (Steenari et al., 1999). Z výsledků Tlustoše a kol. (2012) je patrné, že z rizikových prvků se v úletových popelech nejvíce akumuluje Cd, Pb a As, přičemž v roštových pak převážně Cr. Popely ze slámy, obilí a trávy obsahují přibližně 2 – 10 krát nižší množství rizikových prvků než popely ze dřeva. Důvodem je především nižší obsah popelovin ve dřevě, dlouhé vegetační období lesních porostů a nízká hodnota pH lesních půd, která zvyšuje mobilitu rizikových prvků (Biederman et Obernberger, 2005). Tlustoš a kol. (2012) uvádí směsné popely ze spalování slámy jako většinou nezávadné z pohledu koncentrací rizikových prvků. Dle studie Košnáře et al. (2016) obsahují úletové popele výrazně vyšší množství polycyklických aromatických uhlovodíků (PAU) než popele roštové. Autoři dále uvádějí, že ve smyslu PAU je rizikovější spalování sena/slámy oproti dřevěné biomase, a to zejména z důvodu nedokonalého spalování způsobeného spékáním popela. Nejvyšší obsahy PAU pak byly nalézány v úletových popelech ze slámy a sena. Obdobně pak Freire et al. (2015) poukazují na násobně vyšší koncentrace polychlorovaných dibenzodioxinů a furanů v úletových popelech oproti roštovým.

2.2.2.2) Vliv aplikace popela z biomasy na fyzikálně-chemické a biologické vlastnosti půdy

Obecně aplikace popela z biomasy na půdu vede k výraznému zvýšení reakce pH kvůli vysoké alkalitě samotného popela. S rostoucí teplotou spalování obecně klesá hodnota pH popela. Hodnoty pH jsou vyšší u popelů ze dřeva oproti popelům ze slámy a obilovin. To je způsobeno vyšší koncentrací Ca a nižším obsahem S a Cl v popelech ze dřeva (Vassilev et al., 2013a). Obecně lze očekávat výraznější nárůst hodnoty pH půdy, pokud je popel aplikován na kyselé půdy s nízkým obsahem organické hmoty (Ohno, 1992). Ve srovnání s vápencem navíc popel obsahuje P, Mg, K a další mikroprvky, což je z pohledu výživy rostlin výhodné. Dle studií Clapham et Zibilske (1992) a Muse et Mitchell (1995) reaguje dřevěný popel v půdě mnohem rychleji než vápenec a lze tedy očekávat rychlejší nárůst hodnoty pH, avšak krátkodobější účinek. To je způsobeno přítomností dobře rozpustných oxidů, hydroxidů a uhličitanů draslíku, vápníku a sodíku v popelu zodpovědných za alkalickou reakci (Ulery et al., 1993). Rychlost reakce popela s půdou je také silně závislá na velikosti částic popela. Čím menší částice popela, tím rychlejší lze očekávat reakci (Vance, 1996).

Díky alkalické reakci popelů často dochází ke zvýšené mineralizaci půdní organické hmoty a organického dusíku po jejich aplikaci (Demeyer et al., 2001; Arshad et al., 2012). Hlavními mechanismy vlivu popela na půdní mikrobiální společenstva jsou zvýšené pH a koncentrace rozpustného uhlíku (Jokinen et al., 2006). Vliv popela na půdní mikrobiální biomasu se velmi liší a je především závislý na aplikační dávce, složení a typu popela a půdních vlastnostech. Lupwayi et al. (2009) popisují po aplikaci popela na kyselou zemědělskou půdu změny v diverzitě půdních bakterií, zvýšení mineralizace C a nárůst mikrobiální biomasy ještě čtyři roky po aplikaci popela. Naproti tomu v inkubačním experimentu publikovali Perucci et al. (2006) nárůst mikrobiální biomasy po aplikaci 5 t popela na hektar, přičemž dávka popela 20 t na hektar průkazně snižovala mikrobiální biomasu v zásaditých a neutrálních půdách. Později Perucci et al. (2008) popisují krátkodobý vliv popela na změny v elektrické vodivosti půdy, hydrolytickou aktivitu, aktivitu alkalické fosfatázy, arylsulfatázy a difenoloxidázy v polních podmínkách na alkalické půdě. Autoři uvádějí, že 12 měsíců po aplikaci popela se všechny tyto parametry vrátily na původní hodnoty před aplikací. Odlare et Pell (2009) popisují krátkodobé snížení potenciální míry denitrifikace (90 dní po aplikaci) a zvýšení nitrifikace (7 dní) v půdě po aplikaci dřevěného popela.

Dále aplikace popelů může silně ovlivnit texturu půdy, velikost půdních agregátů, a tím pádem i aeraci a vodní retenční kapacitu půdy (Arshad et al., 2012). Díky obsahu dobře

rozpustných solí může aplikace popelů také vést ke zvýšené salinitě půdy (Demeyer et al., 2001).

2.2.2.3) Přístupnost živin a agronomický potenciál popela z biomasy

Přístupnost živin z popela a jejich následná dynamika uvolňování je ovlivněná třemi základními faktory: 1) fázové složení popela, 2) změny v reakci pH půdy způsobené popelem, 3) změny v půdní mikrobiální aktivitě indukované aplikací popela (Demeyer et al., 2001).

Aplikace dřevěného popela většinou vede ke zvýšení přístupných obsahů P, Ca, Mg a K, ale díky alkalické reakci pH také dochází ke snížení přístupnosti Fe, Mn, Zn a Cu (Demeyer et al., 2001). Množství uvolnitelných živin je především ovlivněno minerální formou dané živiny, přičemž obecně lze obsah ve vodě rozpustných frakcí obsažených v popelech z biomasy považovat za vysoký a může dosahovat až 61 %. Množství a vlastnosti ve vodě rozpustných frakcí popelů jsou silně ovlivněny teplotou spalování. Při teplotách 500 – 800 °C se v popelech vyskytují především uhličitany a hydrogenuhličitany, přičemž při teplotním rozmezí 800 – 1000 °C převažují oxidy a křemičitany (Vassilev et al., 2013b). Rychlost uvolňování živin z popelů z biomasy je uváděna v pořadí K > Ca > Mg > P (Demeyer et al., 2001; Sano et al., 2013). Rozpustné minerály obsažené v popelech jsou většinou sloučeniny alkalických kovů a kovů alkalických zemin. Velmi dobře rozpustné a často zastoupené jsou: sylvín (KCl), halit (NaCl), arcanit (K_2SO_4), syngenit ($K_2Ca(SO_4)_2•H_2O$), ettringit ($Ca_6Al_2(SO_4)_3(OH)_{12}•26H_2O$), sádrovec ($CaSO_4•2H_2O$), vápno (CaO), portlandit ($Ca(OH)_2$). Méně rozpustné: kalcit ($CaCO_3$), některé fosforečnany ($KCaPO_4$, $KMgPO_4$). Špatně rozpustné: apatit ($Ca_5(PO_4)_3(F,Cl,OH)$), křemičitany a živce. (Vassilev et al., 2013b). Boström et al. (2011) identifikovali v popelech ze spalování dřeva dva hlavní minerály fosforu, a to apatit a whitlockit ($Ca_3(PO_4)_2$), což poukazuje na velmi špatnou rozpustnost fosforu z dřevěného popela v půdním prostředí.

Výsledky příjmu jednotlivých makroživin rostlinami se tudíž mezi autory významně liší. Schiemenz et Eichler-Löbermann (2010) uvádějí popele z biomasy jako adekvátní zdroj P srovnatelný s vysoce rozpustnými komerčními hnojivy. Park et al. (2012) uvádějí, že po přidání popela nebyl prokázán zvýšený příjem Ca a Mg rostlinami ovsa a jílku. Naproti tomu Nkana et al. (1998) prokázali zvýšený příjem Ca rostlinami jílku. Přídavek popela pak podle některých autorů zvyšuje příjem K a nemá vliv na příjem P (Nkana et al., 1998; Demeyer et al., 2001; Park et al., 2012). Thind et al. (2012) naopak prokázali zvýšený příjem P rostlinami pšenice a

rýže po přídavku popela. Naproti tomu tito autoři uvádí neprůkazný vliv popela na příjem K rostlinami rýže. Singh et al. (2009) uvádějí neprůkazný vliv aplikace popela na výnos bavlny v prvních dvou letech pěstování. Následně však popisuje nárůst výnosu o 19,6 % v třetím roce experimentu. Někteří autoři uvádějí, že aplikace popela ze spalování biomasy může mimo jiné omezit transfer některých rizikových prvků z půdy do rostlin (Ochecová et al., 2014).

Extrakcí octanem amonným uvolnili Ohno et Erich (1990) při pH 3 z dřevěného popela 48 % celkového Mg, 40 % celkového K a 5,7 % celkového P. Obdobně Meiwes (1995) při pH 4,2 z popelu uvolnil 81 % Ca, 57 % Mg, 34 % K a 20 % celkového P. Podle Callesen et al. (2007) se za dobu 7 let na dvou lesních půdách s rozdílnou úrodností uvolnilo z popelu 35 % Ca, Mg a K, a 19 % P. Srovnávací studie El Make (2000) uvádí uvolnění 20 % K a 10 % Ca za dobu 2 let. Je patrné, že využití živin obsažených v popelech z biomasy, zejména pak fosforu, je stále problematické a je silně ovlivněno druhem popela, cílovou plodinou a půdními vlastnostmi.

2.3) Pyrolýza odpadních materiálů a aplikace biocharu v zemědělství

2.3.1) Princip pyrolýzy

Pyrolýza je termický proces, při kterém je vstupní materiál zahříván za relativně vysokých teplot, zpravidla v rozmezí 300 – 1000 °C, bez přístupu kyslíku. V případě pyrolýzy organické hmoty jsou pak výslednými produkty pyrolýzní plyn, olej a biouhel neboli biochar (Carey et al., 2015).

Pyrolýzní procesy lze rozdělit do tří základních skupin. Dle rychlosti nárůstu teploty se pyrolýza dělí na pomalou, rychlou a bleskovou. Při pomalé pyrolýze je materiál ohříván rychlostí 0,1 – 1 °C/min zpravidla v rozmezí teplot 300 – 700 °C. Za rychlou pyrolýzu je považován nárůst teplot v rozmezí 10 – 200 °C/min, kdy maximální teploty dosahují také přibližně 700 °C. Při tzv. bleskové pyrolýze je pak materiál zahříván rychlostí vyšší než 1000 °C/s při teplotách nejčastěji 750 – 1000 °C (Bridgwater et Bridge, 1991; Barik, 2019).

2.3.2) Produkty a chemismus pyrolýzy

Během pyrolýzy dochází k termochemické dekompozici vstupního materiálu, což vede ke vzniku mnoha nových sloučenin. Jak již bylo řečeno, vzniklé produkty lze rozdělit do tří

základních frakcí: nekondenzovatelná plynná, kondenzovatelná kapalná a pevná. Kapalná složka se dále často dělí na hydrofilní a hydrofóbní frakci.

Pyrolýzní plyn, tedy nekondenzovatelná složka produktů pyrolýzy se skládá především z CO, CO_2, H_2 a CH_4. Yanik et al. (2007) při teplotě 500 °C pyrolyzovali zbytky kukuřičných klasů, slámu a bylinné stonky. Vzniklý plyn obsahoval 84 – 90 % obj. oxidů uhlíku, 6 – 8 % obj. CH_4 a stopové množství C_2 – C_4 uhlovodíků. Autoři uvádějí, že složení plynu se významně nelišilo v závislosti na typu vstupní biomasy. Složení pyrolýzního oleje je však silně ovlivněno složením vstupního materiálu, pyrolýzní teplotou, dobou a rychlostí pyrolýzy i samotným technickým řešením pyrolýzního zařízení (Banks et Bridgwater, 2016). Obvykle je olej směsí vody a kyslíkatých organických sloučenin, především alkoholů, furanů, fenolů, kresolů, benzendiolů, aldehydů, organických kyselin, ale dále i polycyklických aromatických uhlovodíků a jejich methylovaných derivátů (Williams et Nugranad, 2000).

Biochar je obecně pevný zbytek procesu pyrolýzy s relativně vysokým podílem C, který dále obsahuje většinu minerální složky vstupního materiálu. Složení a množství vznikajícího biocharu je tedy stejně, jako v případě oleje, ovlivněno složením vstupního materiálu, pyrolýzní teplotou, dobou a rychlostí pyrolýzy. Míra transformace uhlíkaté složky biocharu závisí zejména na teplotě pyrolýzy (Chen et al., 2015).

2.3.2.1) Pyrolýza celulózy

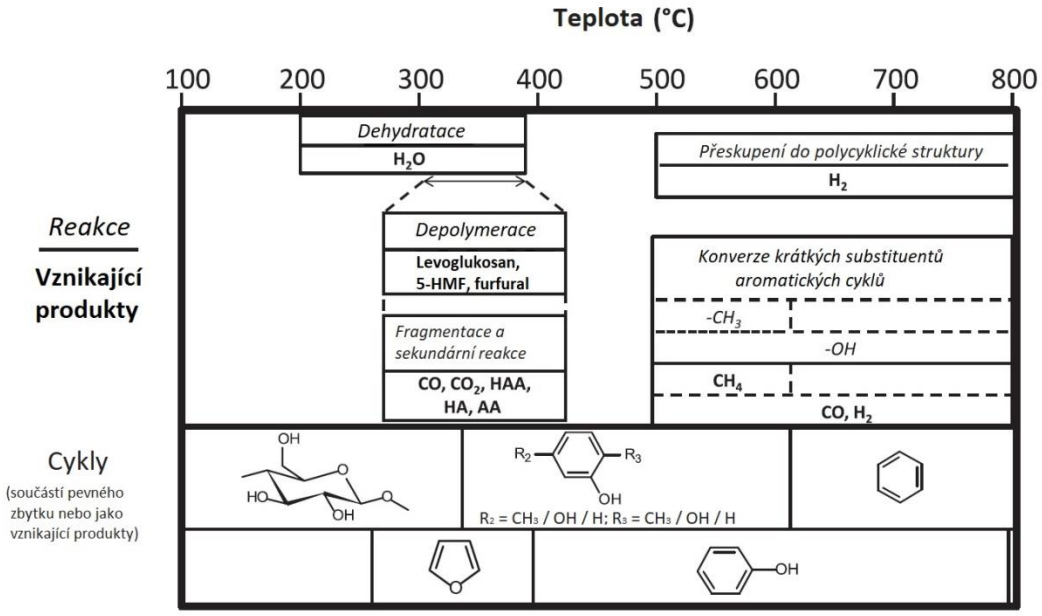
Celulóza je lineární polysacharid složený z monomerů cellobiósy spojených glykosidickou vazbou. Cellobiósa se dále skládá z β-D-glukózy a D-glukózy. Celulóza může být buď amorfní, nebo krystalická. Polymerizované řetězce mohou obsahovat i více než 5000 monomerních jednotek. Rozklad celulózy lze rozdělit do tří hlavních fází. Aktivace a dehydratace celulózy (150 – 300 °C), depolymerizace (300 – 390 °C) a uhelnatění (380 – 800 °C) (Collard et Blin, 2014). Aktivace celulózy při pyrolýze nastává již při teplotách 160 °C. Při těchto teplotách dochází k postupné depolymerizaci. Částečně depolymerizovaná celulóza je někdy nazývána „aktivní“ (Van de Velden et al., 2010). K dehydrataci začíná docházet při teplotě 200 °C, kdy jsou odštěpovány molekuly vody. Takový materiál je znám pod termínem „anhydrocelulóza“ (Collard et Blin, 2014).

Při teplotách 280 – 300 °C začíná následně docházet k intenzivní depolymerizaci kvůli štěpení glykosidických vazeb mezi monomery glukózy. Po odštěpení molekul vody z molekuly

glukózy rychle vzniká levoglukosan a levoglukosenon, hlavní produkty pyrolýzy celulózy při teplotách do 390 °C (Patwardhan et al., 2011; Lu et al., 2011). Díky odštěpení kyslíkatých funkčních skupin z cukerných cyklů a následnému vzniku dvojných vazeb lze ve volatilní frakci dále najít významná množství furanových a fenolových sloučenin, zejména 5-hydroxymethylfuran, 5-methylfurfural, furfural, furfurylalkohol, fenol, methylfenolů a benzendiolů. Mnohé depolymerizační reakce také vedou ke krátkodobému vzniku nestabilních sloučenin s karbonylovými a karboxylovými funkčními skupinami. Jejich rozpadem a dehydratací dále vznikájí CO, CO_2, hydroxyacetaldehyd, hydroxyaceton a acetaldehyd. Pokud je nárůst teplot pomalý, dojde dříve k dehydrataci, než začne depolymerizace. To má za následek vyšší stabilitu matrice a tím pádem i vyšší výnos pevného zbytku. Oproti tomu pokud je nárůst teplot strmý, dojde k depolymerizaci rychle, dehydratace probíhá zároveň a přítomnost molekul vody katalyzuje štěpení glykosidických vazeb. To vede k vyšší produkci volatilních látek a tedy nižší produkci biocharu. Zároveň při teplotách 270 – 300 °C celulóza ztrácí krystalický charakter, díky čemuž se zvyšuje její reaktivita (Pastorova et al., 1994; Yu et al., 2012; Collard et Blin, 2014).

Do teploty 350 °C roste koncentrace furanových sloučenin, avšak od této teploty dále začíná klesat. První benzenová jádra se objevují při teplotě 300 °C, přičemž od hranice 400 °C začínají být benzenová jádra hlavní složkou biocharu. Nejprve benzenová jádra obsahují boční alifatické řetězce a funkční oxoskupiny (hydroxyl, ether). Tyto boční řetězce však postupně mizí v rozmezí teplot 400 – 600 °C. Při teplotách 500 – 800 °C dochází k demethylaci, dehydrogenaci a dehydroxylaci za vzniku CH_4, H_2 a H_2O a spojování benzenových jader do polycyklických struktur. Přeměny při pyrolýze celulózy jsou zjednodušeny na Obrázek 5.

Obrázek 5. Shrnutí chemických přeměn při pyrolýze celulózy



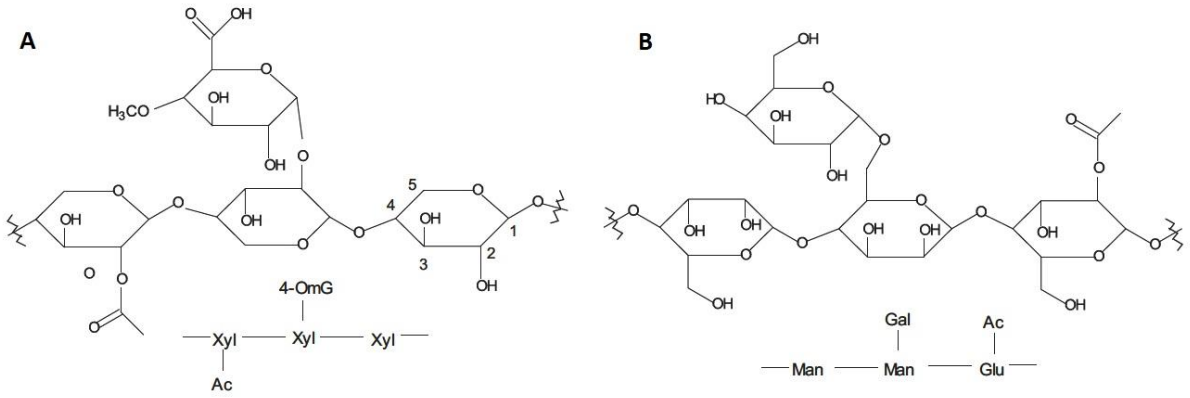
(převzato z Collard et Blin, 2014)

5-HMF: 5-hydroxymethylfurfural; HAA: hydroxyacetaldehyd; HA:hydroxyaceton; AA: acetaldehyd

2.3.2.2) Pyrolýza hemicelulózy

Hemicelulóza je polysacharid, jehož složení se liší dle rostlinných druhů. Hemicelulóza krytosemenných rostlin obsahuje především xylany, přičemž hemicelulóza nahosemenných je složena hlavně z glukomananu. Jejich struktura je uvedena na Obrázku 6. Hemicelulóza je vždy amorfního charakteru a řetězce nepřesahují 200 monomerních jednotek. Podobně jako u celulózy, degradace hemicelulózy probíhá ve třech fázích. Dehydratace a porušování méně stabilních vazeb (xylan: 150 – 240 °C; glukomanan: 150 – 270 °C), depolymerace (xylan: 240 – 320 °C; glukomanan: 270 – 350 °C) a uhelnatění (xylan: 320 – 800 °C; glukomanan: 350 – 800 °C).

Obrázek 6. Příklad typických struktur hemicelulózy, xylan (A), glukomanan (B),



(převzato z Collard et Blin, 2014)

Xyl: xylóza; 4-Omg: 4-methylglukoronová kyselina; Ac: acetyl; Man: manóza; Gal: galaktóza; Glu: glukóza

Dehydratace hemicelulózy nastává již od 150 °C, avšak až při teplotě 200 °C začíná mít odštěpení molekul vody významnou intenzitu (Collard et Blin, 2014). Při této teplotě dochází i k porušování méně stabilních chemických vazeb, především na substituentech hlavního řetězce. Z methoxy skupin přítomných ve struktuře xylanu vzniká methanol, z karboxylových funkčních skupin pak kyselina mravenčí, ale také CO_2. Acetylované substituenty mohou u hemicelulózy představovat až 10 hm.%. Jejich fragmentací vzniká kyselina octová, která tak představuje významný produkt této fáze rozkladu (Prins et al., 2006). Při teplotách 220 °C již lze v produktech detekovat stopy furfuralových sloučenin, což značí počátky depolymerizace (Shen et al., 2010).

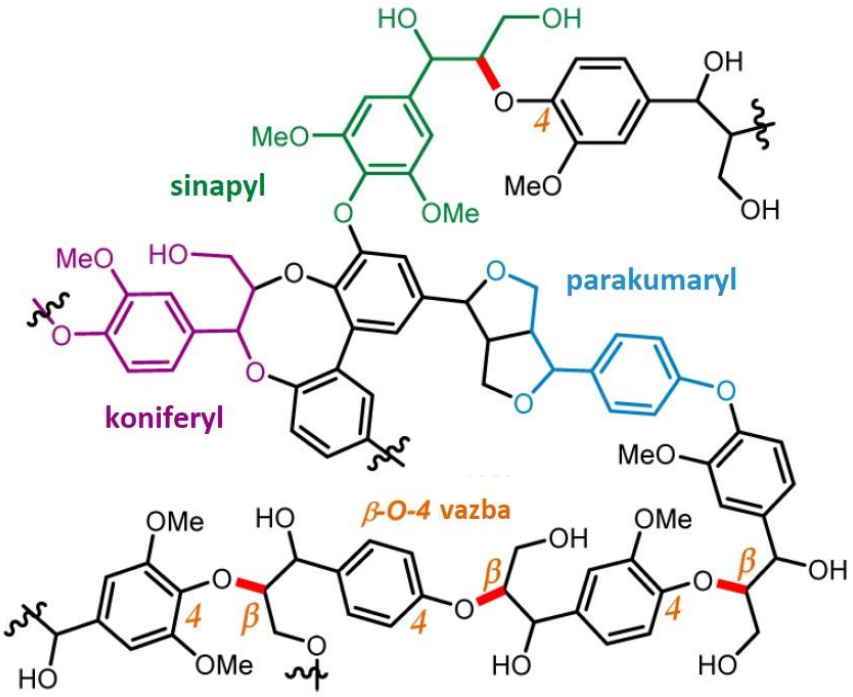
Při teplotách 240 °C v případě xylanu a 270 °C v případě glukomananu se začínají intenzivně štěpit glykosidické vazby mezi jednotlivými monomery polysacharidů a dochází k depolymerizaci. Tyto reakce vedou k tvorbě různých anhydrocukrů. V pyrolýzním oleji vzniklém při těchto teplotách se většinou nacházejí především sloučeniny levoglukosan, levomanosan a levogalaktosan (Hosoya et al., 2007; Alén et al., 1996) vzniklé obdobně, jako v případě celulózy. Depolymerizací glukomananu dále vznikají 5-hydroxymethylfurfural, 5-methylfurfural a furfural. Při depolymerizaci xylanu je pak hlavní složkou pyrolýzního oleje pouze furfural (Shen et al., 2010). V pyrolýzním oleji při těchto teplotách dále nacházíme hydroxyacetaldehyd, hydroxyaceton, hydroxybutanon, kyselinu octovou a cyklopentenony. Rychlá depolymerizace hemicelulózy způsobuje vznik dále velkého množství nestabilních meziproduktů. Tyto meziprodukty okamžitě dehydratují, fragmentují, popř. druhotně reagují. To vše má za následek vznik značného množství H_2O, CO_2 a CO (Collard et Blin, 2014).

Pokud teploty pyrolýzy stoupnou nad 300 °C, struktura residuí začíná být více a více aromatická. Některá benzenová jádra obsahují methylové a oxo-substituenty. Peng et Wu (2010) identifikovali několik methylfenolů při pyrolýze hemicelulózy při teplotách 320 – 350 °C. Při teplotě 550 °C dochází k maximální produkci CH_4, což nasvědčuje především o průběhu demethylačních reakcí. Obdobně pak produkci CO při teplotách nad 500 °C lze vysvětlit především odštěpením oxo skupin. Při teplotách 480 – 800 °C dochází k produkci hlavně H_2, což má za následek více kondenzovanou chemickou strukturu biocharu. V případě hemicelulózy začíná být H_2 produkován při teplotě 480 °C, přičemž u ligninu nebo celulózy toto nadchází až při 500 °C. Tato nižší teplota produkce H_2 je přisuzována katalytickému efektu minerálních sloučenin, které jsou v hemicelulóze přítomné v mnohem vyšším množství (Couhert et al., 2009; Collard et Blin, 2014).

2.3.2.3) Pyrolýza ligninu

Lignin je složitý, trojrozměrný amorfní polymer složený především ze tří hlavních fenylpropenových jednotek: parakumarylalkohol, jehož hlavní jednotkou je p-hydroxyfenyl, koniferylalkohol, kde je aromatický cyklus nazýván guajakol a sinapylalkohol s jednotkou zvanou syringyl. Poměr jednotlivých monomerních jednotek je velmi proměnlivý a je určen především druhem rostliny. p-hydroxyfenyl neobsahuje methoxy funkční skupinu, guaiacyl obsahuje jednu a syringyl obsahuje dvě methoxy skupiny. Schematická struktura části řetězce ligninu je zobrazena na Obrázek 7. Alkylové řetězce však mohou mimo hydroxylových skupin obsahovat také karbonylové a karboxylové skupiny. Jednotlivé monomerní jednotky jsou ve struktuře ligninu vzájemně propojeny etherickými a C-C vazbami (Lapierre et al., 1995). Pyrolýzní degradaci ligninu lze obecně rozdělit do dvou hlavních fází: konverze alkylových řetězců monomerních jednotek s porušením vazeb spojujících jednotlivé monomery (150 – 420 °C) a konverze krátkých substituentů benzenových jader a uhelnatění (380 – 800 °C) (Obrázek 8) (Collard et Blin, 2014).

Obrázek 7. Schématická struktura ligninu



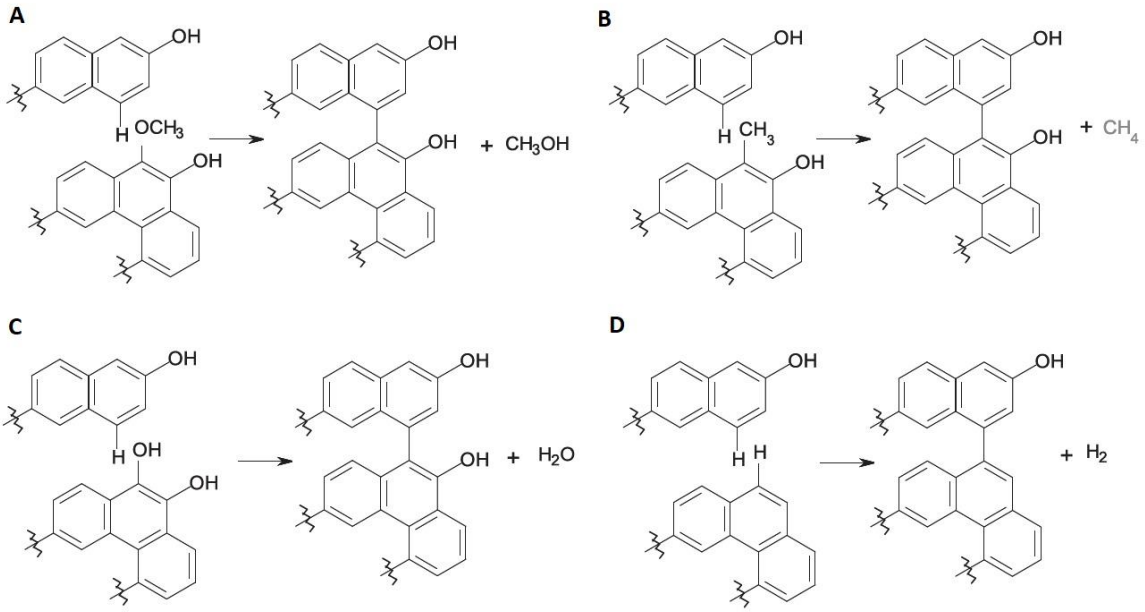
(převzato z Bosque et al., 2017)

Dehydratace propylénových bočních řetězců začíná při teplotách již okolo 180 °C, kdy dochází k dehydrataci hydroxylové skupiny na konci těchto řetězců (Jakab et al., 1995). Při tomto může také dojít k porušení C-C vazby mezi posledními dvěma uhlíky alifatického řetězce s následným vznikem formaldehydu. Pokud je místo hydroxylové funkční skupiny přítomen karboxyl nebo karbonyl, dochází při mírně vyšších teplotách ke vzniku CO a CO_2. Etherické chemické vazby spojující jednotlivé monomerní jednotky se začínají porušovat již při teplotách 200 °C, přičemž nejběžnější vazba spojující monomery v ligninu se štěpí při teplotě 245 °C (Collard et Blin, 2014). Při porušení těchto etherických vazeb vznikají jednak CO, CO_2 a H_2O, ale také dochází k uvolnění fenolických zbytků monomerních, popř. oligomerních jednotek. Mezi tyto sloučeniny se pak řadí např. 4-vinylguajakol nebo eugenol (Huang et al., 2011). Při teplotách nad 300 °C se stává většina C-C vazeb uvnitř i mezi alkylovými řetězci nestabilní a reaktivní. Díky tomu nad touto teplotou dochází ke vzniku jedno- až tříuhlíkatých sloučenin právě z těchto alifatických řetězců. Mezi tyto sloučeniny se řadí především CH_4, acetaldehyd a kyselina octová (Collard et Blin, 2014). Zbylé části monomerů pak odcházejí ve formě více, či méně methylovaných fenolických sloučenin. Obecně fenoly jsou hlavní složkou pyrolýzy ligninu vznikající při teplotách 360 – 400 °C (Huang et al., 2011; Wang et al., 2009).

Pokud je methoxy funkční skupina v ortho pozici s hydroxylovou, stává se reaktivní od teplot 380 - 400 °C (Asmadi et al., 2011; Collard et Blin, 2014). Různé typy fragmentačních

reakcí způsobují nahrazení methoxyskupin skupinami –OH, -CH_3 nebo –H. Odštěpení methoxy skupin je zodpovědné za vznik methanolu při 400 °C (Obrázek 8a), přičemž při 430 °C z methoxyskupin vzniká CH_4. Při teplotě 450 °C, většina vazeb mezi monomerními jednotkami ligninu je již rozštěpena a hlavními substituenty benzenových jader jsou –CH_3 a –OH (Obrázek 8b a Obrázek 8c). Pevný pyrolytický zbytek se stává více a více aromatickým, přičemž naprostá většina vznikajících plynů je nekondenzovatelných. Mezi 500 a 600 °C dochází k vymizení methylovaných substituentů (Collard et Blin, 2014). Mnoho autorů udává zvýšenou evoluci CH_4 při teplotách 550 - 580 °C (Huang et al., 2011; Jakab et al., 1995; Wang et al., 2009). Princip těchto demethylačních a dehydrogenačních reakcí je zobrazen na Obrázek 8b a Obrázek 8d a vysvětluje zvyšující se polycyklizaci biocharu s rostoucí teplotou.

Obrázek 8. Princip aromatizace biocharu a vznik plynných produktů při teplotách nad 380 °C



(převzato z Collard et Blin, 2014)

2.3.2.4) Pyrolýza triacylglycerolů

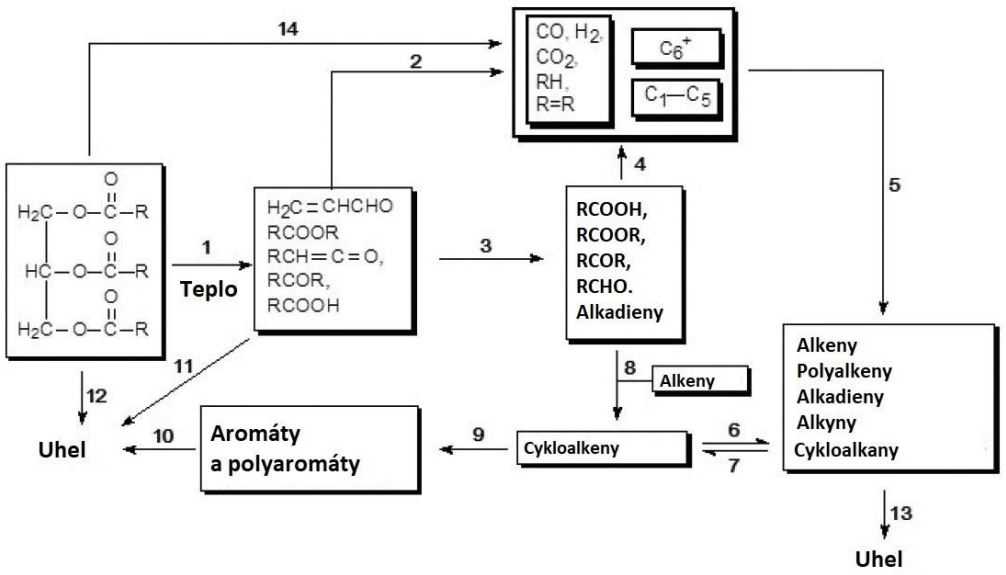
Triacylglyceroly, nebo také triglyceridy, jsou hlavní složkou rostlinných olejů a živočišných tuků. Molekuly triacylglycerolů jsou tvořeny molekulou glycerolu, kde na každou OH skupinu je estericky vázána mastná kyselina. Výzkum pyrolýzy triacylglycerolů má dlouhou historii a započal již v 30. letech minulého století za účelem krakování alifatických

řetězců mastných kyselin pro výrobu paliv jako potenciální řešení nedostatku ropy (Egloff and Morrell, 1932; Egloff and Nelson, 1933). V té době se jednalo především o nekatalytickou pyrolýzu, přičemž dnešní výzkum se takřka bez výjimek zaměřuje na studium katalytických rozkladů. Pyrolýzní degradaci triglyceridů lze zjednodušeně rozdělit do tří hlavních fází: porušení esterické vazby mezi mastnými kyselinami a glycerolem (300 °C), dekarboxylace/dekarbonylace mastných kyselin (390 °C) a štěpení alifatických řetězců mastných kyselin (360 – 500 °C) a uhelnatění (> 350 °C) (Maher et Bressler, 2007; Ito et al., 2012; Moldoveanu, 2010).

Rozštepení esterické vazby mezi glycerolem a mastnými kyselinami nastává již při teplotách okolo 300 °C. Výsledkem je v konečném důsledku vznik molekul mastných kyselin a akroleinu (Maher et Bressler, 2007; Ito et al., 2012). Dle Cheng et al. (2004) může docházet k porušování esterické vazby již při teplotě 288 °C, což vede ke vzniku meziproduktů tohoto rozpadu, diacylglycerolu a monoacylglycerolu.

Pokud je molekula mastné kyseliny nasycená, dochází dále při teplotách okolo 400 °C k dekarboxylaci/dekarbonylaci této kyseliny za vzniku CO_2, H_2O a alifatického nasyceného, nebo nenasyceného uhlovodíku. Pokud však je molekula mastné kyseliny nenasycená a obsahuje dvojné vazby, může se ještě před dekarboxylací nejprve začít štěpit uhlíkatý řetězec, jelikož dvojná vazba v řetězci mastné kyseliny podporuje štěpení sousedních C-C vazeb (pozice α a β) (Maher et Bressler, 2007; Ito et al., 2012). To může mít za následek vznik širokého spektra látek. Mohou tímto vznikat C1 – C5 alifatické i větvené uhlovodíky, kratší mastné kyseliny, různé aldehydy, ketony, estery a alkoholy. Z těchto následně vzniká široké spektrum alkenů, polyalkenů, alkadienů a cykloalkanů (Maher et Bressler, 2007). Rostoucí teplota pyrolýzy pak vede k cyklizaci za vzniku cykloalkenů. Typicky je tato cyklizace vysvětlována Dield-Alderovou reakcí, kdy reaguje alkadien s alkenem za vzniku substituovaného cyklohexenu, ovšem probíhají i další mechanismy cyklizace, např. mezi radikály alkenylu a alkadienylu (Kubátová et al., 2012). Proces uhelnatění a vznik aromatických a polyaromatických struktur pak probíhá podle shodného schématu jako v případě celulózy nebo hemicelulózy. Zjednodušené schéma pyrolýzního rozkladu triacylglycerolů je uvedeno na Obrázek 9.

Obrázek 9. Schématické znázornění chemických reakcí probíhajících při pyrolýze triacylglycerolů



(převzato z Wiggers et al., 2017)

1) počáteční štěpení, termolýza esterické vazby; 2) dekarboxylace/dekarbonylace kyslíkatých uhlovodíků s dlouhým řetězcem; 3) štěpení C-C vazby nenasycených kyslíkatých uhlovodíků; 4) dekarboxylace/dekarbonylace kyslíkatých uhlovodíků s krátkým řetězcem; 5) izomerizace, polymerizace/dehydrogenace, cyklizace; 6) hydrogenace cykloalkenů za vzniku cykloalkánů; 7) dehydrogenace cykloalkánů za vzniku cylkoalkenů; 8) Diels-Alderova adice konjugovaných alkadienů se substituovanými alkeny za vzniku substituovaných cyklohexenů; 9) aromatizace cykloalkenů za vzniku aromatických a polyaromatických uhlovodíků; 10) uhelnatění polyaromátů; 11) uhelnatění polykondenzací kyslíkatých uhlovodíků; 12) uhelnatění polykondenzací molekul triacylglycerolů; 13) uhelnatění polymerizací alkenů; 14) přímý vznik C1 – C5 uhlovodíků z triacylglycerolů

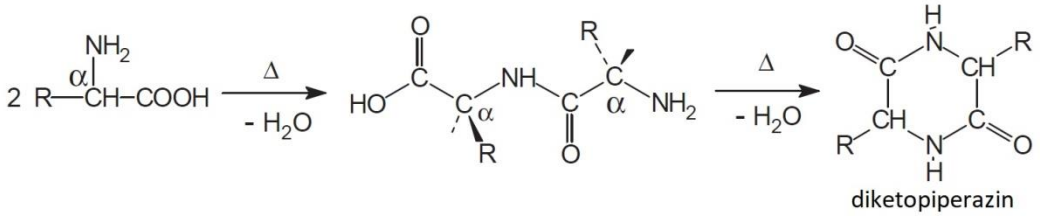
2.3.2.5) Pyrolýza proteinů

Proteiny jsou velké makromolekuly složené z jednoho nebo více řetězců aminokyselin spojených peptidickou vazbou. Počet molekul aminokyselin v peptidu může dosahovat i počtu 10000. Lineární řetězec aminokyselin se nazývá polypeptid. Krátké polypeptidy obsahující 20 – 30 jednotlivých molekul aminokyselin se nazývají oligopeptidy. Proteiny jsou v naprosté většině organismů tvořeny 20ti různými α-aminokyselinami, které se výrazně strukturně liší. Tento fakt společně s extrémní různorodostí proteinů způsobuje značnou složitost procesů probíhajících během pyrolýzy (Moldoveanu, 2010). Chemické reakce probíhající při pyrolýze proteinů zahrnují hydrolýzu, dehydratace, deaminace, cyklizace, dimerizace atd. (Wang et al., 2017a; Moldoveanu, 1998)

Při teplotách 220 - 250 °C dochází jednak k hydrolýze peptidických vazeb a rozpadu proteinů na jednotlivé aminokyseliny, ale zároveň k vyšší provázanosti mezi peptidy a k tzv. síťování peptidů. K síťování dochází obecně v teplotním rozmezí 180 – 300 °C. Oba procesy, hydrolýza peptidických vazeb a síťování, probíhají paralelně a jejich maximální intenzita leží mezi 240 a 260 °C. Dosud nebyla určena hraniční teplota, kdy by jeden proces vykazoval vyšší intenzitu než ten druhý. Teplota porušení peptidické vazby se liší v závislosti na konkrétních aminokyselinách (Liu et Basile, 2011). Při teplotě 220 °C již dále dochází k dehydrataci a odštěpování molekul amoniaku.

Termální stabilita jednotlivých aminokyselin se výrazně liší, např. kys. glutamová je kompletně zpyrolyzována při 400 °C, avšak prolín zůstává z 1 % nerozložen při 500 °C, obdobně pak glycin při 600 °C a valín a leucin při 700 °C (Moldoveanu, 2010). Rozpad molekul aminokyselin se děje několika způsoby. Někdy je celá molekula fragmentována za vzniku CO_2, NH_3 a uhlovodíku, nejčastěji alkenu. Dále také může docházet k dekarboxylaci za vzniku amínu, který následně ztrácí vodík, až vzniká nitril. Vzniklý amín však může také reagovat s druhou molekulou amínu, kdy za odštěpení vodíku a amoniaku dochází ke spojení obou molekul přes atom dusíku. Toto se děje především při pyrolýze alaninu, valinu, leucinu a isoleucinu. Dále je pro pyrolýzu aminokyselin díky dehydrataci typický vznik cyklických dipeptidů, diketopiperazinů. Vznik těchto látek je schematicky zobrazen na Obrázek 10. Li et al. (2007) studovali průběh pyrolýzy glycinu. První plynné produkty se objevili při 260 °C (NH_3, H_2O a CO_2), přičemž produkce NH_3 a H_2O dosáhla vrcholu při 282 °C a CO_2 při 308 °C. Při 400 °C byl hlavním produktem HNCO společně s CO a HCN. HCN pak byl hlavním plynným produktem při teplotě 700 °C. Vznik HNCO a HCN autoři přisuzují štěpení sekundárně vznikajícího cyklického amidu 2,5-diketopiperazinu. Autoři dále uvádějí, že pyrolýzní produkty glycinu a jeho dipeptidu glycylglycinu byly rozdílné do teploty 350 °C, přičemž nad touto teplotou se již nelišily, což nasvědčuje rozpad dipeptidu a obdobný průběh štěpení jako v případě samotné aminokyseliny. Autoři nenalezli NO ani N_20 v plynných produktech.

Obrázek 10. Vznik diketopiperazinů ze dvou molekul aminokyselin během pyrolýzy



(převzato z Moldoveanu, 2010)

2.3.3) Biochar a jeho aplikace na zemědělskou půdu

2.3.3.1) Složení a vlastnosti biocharu

Biochar je pevný uhlíkatý zbytek získaný pyrolýzou biomasy za relativně nízkých teplot (< 700 °C) (Lehman et Joseph, 2009). Někdy je jako biochar označován materiál, resp. pyrolyzovaná biomasa, která je aplikována do půdy za účelem zlepšení jejich vlastností a úrodnosti (Lehmann et al., 2006). Vzhledem k rostoucí lidské populaci a s tím spojeným rostoucím množstvím produkovaných odpadů, stává se aplikace biocharu velmi intenzivně studovaným tématem především kvůli možnosti zpracování širokého spektra odpadů. Pyrolýzou těchto odpadů lze docílit odstranění některých nežádoucích vlastností a následnou aplikací biocharu do půdy lze sekvestrovat uhlík a současně zvyšovat půdní úrodnost (Ding et al., 2016; Zhu et al., 2017).

Hodnota pH biocharu, porozita a velikost specifického povrchu jsou silně ovlivněny podmínkami pyrolýzy (Zhao et al., 2013). Biochar má obecně zásaditý charakter s pH reakcí v rozmezí 8 – 10 (Gaskin et al., 2008), biochar je mikroporézní materiál, přičemž úroveň porozity stoupá s rostoucí teplotou pyrolýzy. Jimenez-Cordero et al. (2013) nalezli nejvyšší zastoupení mikropórů (0,1 – 0,4 nm) u biocharu z hroznových semen pyrolyzovaných při teplotě 700 – 800 °C. Specifický povrch biocharu je silně ovlivněn vstupním materiálem a s rostoucí teplotou pyrolýzy stoupá (Keiluweit et al., 2010).

Obsah uhlíku a ostatních prvků v biocharu je nejvíce ovlivněn složením vstupní biomasy. S rostoucí teplotou obecně roste celkový obsah uhlíku, přičemž klesá zastoupení kyslíku a vodíku. Jak již bylo naznačeno v předchozích kapitolách, je to způsobeno rostoucí aromatizací uhlíkatých sloučenin a snižujícím se počtem kyslíkatých funkčních skupin. Pomocí molárního poměru H:C nebo O:C tak lze velmi dobře odhadnout celkovou polaritu biocharu. Obecně platí, že čím vyšší je teplota pyrolýzy, tím méně polární a více stabilní je produkovaný

biochar (Novak et al., 2009). Stabilita biocharu v půdě a tudíž jeho sekvestrační potenciál závisí zejména na stupni aromatické kondenzace a obsahu alifatických a volatilních látek (Novotny et al., 2015). Brassard et al. (2016) uvádějí, že pro sekvestraci uhlíku jsou výhodné biochary připravené za vyšších pyrolytických teplot s $O:C_{tot}$ poměrem < 0.2 a $H:C_{org}$ < 0,4. Pro biochary s $O:C_{tot}$ < 0.2 Spokas (2010) odhaduje poločas rozpadu v půdě přibližně 1000 let.

2.3.3.2) Vliv biocharu na půdní vlastnosti

Aplikace biocharu může zvýšit vodní retenční kapacitu půd. Dle Sun et Lu (2014) lze aplikací biocharu zvýšit množství rostlinám dostupné vody v jílovitých půdách. Wang et al. (2019) uvádějí, že navýšení vodní retenční kapacity půd, stejně jako množství rostlinám dostupné vody, lze očekávat jen u půd s hrubou texturou, např. písčitých, a pouze po aplikaci biocharu s vysokým objemem pórů. Navíc vliv takové aplikace je dle autorů pouze dočasný, jelikož v půdě dochází k vyplňování pórů biocharu částicemi jílu a organickou hmotou. Mollinedo et al. (2015) uvádí, že aplikací 4 hm. % biocharu do půdy lze v optimálním případě prodloužit dobu transpirace kukuřice nebo sóji v suchém období o 1,4 – 2,6 dne. Aplikací biocharu lze také modifikovat kationtovou výměnnou kapacitu (KVK) půd. KVK biocharu je většinou nižší, než KVK půdní organické hmoty díky existenci pozitivně i negativně nabitých míst na povrchu biocharu (Lehmann, 2007). Pokud je biochar aplikován do půdy s nižším KVK než je KVK biocharu, dochází k celkovému zvýšení a naopak. Vyšší hodnotu KVK lze obecně očekávat u biocharů s vyšším podílem minerální složky. Dle Novak et al. (2009) je KVK vyšší u biocharů produkovaných do teplot 500 °C. Obecně se zdá, že míra vlivu aplikace biocharu na KVK půdy je především určena množstvím vyměnitelného Ca v půdě a biocharu (Hailegnaw et al., 2019a).

V kyselých podmínkách se mohou prvky jako Ca, P nebo K z matrice biocharu vyluhovat do půdního roztoku a mnoho autorů tak uvádí zvýšení rostlinám dostupných obsahů P, K, Mg a Ca v půdě po aplikaci biocharu (Chan et al., 2007; Sun and Lu, 2014; Hailegnaw et al., 2019a; Novak et al., 2009). Oproti tomu dusík je v biocharu vázán velmi pevně, obvykle není z biocharu vyluhován, ale biochar může naopak sloužit jako výrazný sorbent amonného i nitrátové dusíku v půdách a zamezovat tak jejich vyplavení (Hailegnaw et al., 2019b). Díky sorpci minerálního dusíku v půdě může však aplikace biocharu vést k nižším výnosům plodin, popř. ke zvýšené spotřebě hnojiv (Borchard et al., 2014). Díky změnám koloběhu půdního dusíku po aplikaci biocharu dochází dále ke snížení, ale i zvýšení emisí N_2O z půd (Sánchez-

García et al., 2014). Obdobně se pak liší výsledky emisí NH_3 a CO_2, kdy některé studie hlásí pokles a jiné naopak nárůst emisí těchto plynů po aplikaci biocharu (Mandal et al., 2016; He et al., 2018; Chang et al., 2016; Wang et al., 2017b).

Díky vysokému specifickému povrchu, aromatickému charakteru a mikroporositě je také biochar zmiňován jako vynikající sorbent pesticidů a jiných agrochemikálií. Na druhou stranu je v literatuře zmiňována výrazně nižší účinnost preemergentních pesticidů na půdách po aplikaci biocharu (Kookana et al., 2011). Dále díky vyšší sorpci na částice biocharu v půdě dochází k výrazně pomalejší mikrobiální degradaci některých pesticidů (Spokas et al., 2009; Loganathan et al., 2009).

2.4) Půdní fosfor a mechanismy jeho zpřístupnění

2.4.1) Formy a chemismus fosforu v půdě

Fosfor je v porovnání s ostatními živinami považován za nejméně mobilní a dostupnou (Gaume, 2000) a v půdě se vyskytuje ve dvou formách – organické a anorganické. Koncentrace anorganického fosforu v půdním roztoku je ve většině půd poměrně nízká, přičemž jeho značný podíl je v půdě vázaný ve formě minerálů. Fosforečnanové ionty mohou být v půdě adsorbovány na kladně nabité minerály, nejčastěji oxidy železa a hliníku, nebo samy tvoří množství sraženin/minerálů, nejčastěji v kombinaci s Ca, Fe a Al (Lindsay, 1979). V zásaditých a vápenatých půdách je fosfor imobilizován především srážením s vápníkem, přičemž v kyselých půdách je fosfor sorbován na oxidy/hydroxidy železa a hliníku, komplexován s humínovými kyselinami ve formě fosforečnanů hlinitých/železitých, popř. přímo vysrážen jako fosforečnan hlinitý/železitý (Neuman et Römheld, 2007). Gérard (2016) však upozorňuje, že relativní zastoupení jílových minerálů v sorpci fosforu může být nesprávně dlouhodobě ignorováno, a že v některých případech mohou jílové minerály vykazovat i vyšší sorpční schopnost než oxidy železa/hliníku. Rovnováhy mezi adsorpcí/desorpcí a srážením/rozpouštěním určují koncentraci minerálního P v půdním roztoku a tudíž i jeho mobilitu a biodostupnost. Rovnovážný stav těchto reakcí je určen třemi hlavními faktory: 1) pH, 2) koncentrací dalších aniontů, které soupeří s fosforečnanovými ionty při reakcích výměny ligand, 3) koncentrace kovů (Ca, Fe, Al), které se mohou srážet s fosforečnanovými anionty. Chemické podmínky rhizosféry jsou díky aktivitě kořenů a mikroorganismů velice odlišné od samotné půdy a tudíž i samotná dostupnost fosforu v rhizosféře se velice liší. Koncentrace P v rhizosférním půdním roztoku je nejvíce ovlivněna samotnou aktivitou příjmu kořene. Další

vliv pak mají procesy ovlivňující pH rhizosféry, jako jsou rovnováhy mezi H^+/HCO_3^- a O_2/CO_2 a exsudace organických aniontů kořenem. Relativní význam těchto faktorů se však významně liší podle rostlinného druhu, stavu výživy rostliny a půdních podmínek (Hinsinger, 2001).

Podíl organického fosforu se v půdách značně liší. V minerálních půdách organický fosfor představuje v průměru 30 – 65 % z celkového fosforu, přičemž v půdách organických může jeho obsah přesahovat až 90 % (Jones et Oburger, 2011). Organický fosfor je tvořen především třemi formami: inositol fosfáty, fosfolipidy a nukleovými kyselinami (Quiquampoix et Mousain, 2005; Turner et al., 2002). Obsah inositol fosfátů je velice proměnlivý, avšak často představuje až 80 % z celkového organického fosforu v půdě (Dalal, 1977). Inositol fosfáty jsou v půdě zastoupeny řadou fosfoesterů inositolu, tj. inositol monofosfátem až inositol hexafosfátem, který dále tvoří několik stereoisomerů (myo, scyllo, neo, D-chiro) (Celi et Barberis, 2005). Množství fosfátových skupin určuje stabilitu těchto sloučenin, přičemž čím více jich je přítomno, tím je sloučenina stabilnější a následně vykazuje v půdě vyšší obsah. Nejběžnějším isomerem v půdě je myo-inositol hexafosfát, neboli kyselina fytová či fytát. Inositol fosfáty jsou charakteristické svou vysokou kyselostí, a často tvoří v půdě polymery, nebo nerozpustné komplexy s bílkovinami a lipidy (Jones et Oburger, 2011).

Celkově tedy existují dvě základní strategie zvýšení přístupnosti fosforu v půdě, a to: 1) zvýšení rozpustnosti minerálního fosforu a, 2) enzymatický rozklad sloučenin organického fosforu (Jones et Oburger, 2011).

2.4.2) Vliv rhizosférního pH na přístupnost fosforu

Kořeny rostlin způsobují výrazné změny v rhizosférním pH, což je způsobeno především exkrecí H^+ pomocí transmembránového proteinu H^+ ATPázy nebo OH^-/HCO_3^- iontů. Tato exkrece je nezbytná pro udržení vnitřní rovnováhy mezi kationty a anionty v kořenech (Neumann et Römheld, 2007). Z tohoto pohledu hraje klíčovou roli dusík, jelikož je většinou rostlinných druhů přijímán v největším množství. Rostliny mohou přijímat dusík ve dvou základních formách, kationtové (NH_4^+) a aniontové (NO_3^-), popř. v případě leguminóz lze ještě uvažovat příjem neutrální formy (N_2). Příjem NH_4^+ je současně provázen exkrecí H^+ vedoucí k acidifikaci rhizosféry, přičemž příjem NO_3^- je provázen příjmem dvou H^+ (Brimecombe et al., 2007; Mistrik et Ullrich, 1996) a případně exkrecí OH^- nebo HCO_3^- způsobující zvýšení rhizosférního pH (Marschner, 1995; Hawkesford et al., 2012).

Rhizosférní pH je dále ovlivněno exsudací organických kyselin. Organické kyseliny jsou však exsudovány z kořene ve formě aniontu, který sám o sobě nemá vliv na změnu pH. Pro udržení rovnováhy mezi anionty a kationty v kořenech může být aniont organické kyseliny exsudován společně s H^+ (Jones 1998, Hinsinger 1998), ale také s kationtem kovu, např. K^+ (Ryan et al., 1995). V druhém případě tedy exsudace organických aniontů nevede ke snížení rhizosférního pH.

Změna pH v rhizosféře může dále nastat díky indukované změně redox potenciálu kořenem nebo respirací kořene a rhizosférních mikroorganismů. Tím vzniká značné množství CO_2, který následně okyseluje půdní roztok (Hinsinger, 2001). Význam respirace na okyselení rhizosféry má však pouze malý význam v dobře provzdušněných půdách, jelikož CO_2 rychle odchází zavzdušněnými půdními póry (Marschner, 2012).

Okyselení rhizosféry pomocí exsudace H^+ je u dvouděložných rostlin také způsobeno nedostatkem P. V těchto případech dochází k omezení příjmu NO_3^- a následné nerovnováze mezi anionty a kationty v kořenové tkáni, která je následně udržována právě exsudací H^+. V těchto případech jsou H^+ exsudovány v blízkosti kořenových špiček (Marschner, 2012; Neumann et Römheld, 1999).

Pokud je fosfor v půdě přítomný ve formě vápenatých minerálů, jako např. dihydrát hydrogenfosforečnanu vápenatého ($Ca_2HPO_4 \cdot 2H_2O$), pentahydrát dihydrogenfosforečnanu oktavápenatého ($Ca_8H_2(PO_4)_6 \cdot 5H_2O$) nebo hydroxyapatitu ($Ca_5(PO_4)_3OH$), lze po okyselení rhizosféry očekávat jejich zvýšenou rozpustnost, a tudíž i přístupnost fosforu rostlinám (Andersson et al., 2016). Pokud však je fosfor přítomný v minerálech Fe a Al, jako např. variscit ($AlPO_4 \cdot 2H_2O$), strengit ($FePO_4 \cdot 2H_2O$) nebo wavellit [$Al_3(OH)_3(PO_4)_2 \cdot 5H_2O$] okyselení rhizosféry nevede ke zvýšení přístupnosti fosforu díky klesající rozpustnosti těchto minerálů s klesajícím pH (Lindsay, 1979). Navíc s klesajícím pH roste množství pozitivních nábojů na povrchu Fe/Al oxidů, což dále zvyšuje jejich schopnost adsorbovat fosfor (Hinsinger, 2001).

2.4.3) Exsudace organických kyselin

Zvýšená exsudace organických kyselin při nedostatku fosforu se vyskytuje především u dvouděložných rostlin (Neuman et Römheld, 1999). Zpřístupnění fosforu pomocí organických kyselin je způsobeno především výměnou ligand, ligandickým rozpouštěním minerálů fosforu

a kompeticí o sorpční místa (Neuman et Römheld, 2007; Oburger et al., 2011). Rozdíl mezi výměnou ligandu a ligandickým rozpouštěním je schematicky uveden v Rovnici 1 a 2.

Rovnice 1. Výměna ligandu

$$\text{Fe/Al oxid-P} + \text{L} \rightarrow \text{Fe/Al oxid-L} + \text{P}_i$$

(Johnson et Loeppert, 2006)

Rovnice 2. Ligandické rozpouštění

$$\text{Fe/Al oxid-P} + \text{L} \rightarrow \text{Fe/Al oxid} + \text{Al}^{3+}/\text{Fe}^{3+}\text{-L} + \text{P}_i$$

(Johnson et Loeppert, 2006)

L = komplexotvorná organická liganda

Za nejvíce efektivní organické anionty ve smyslu zpřístupnění fosforu v půdě jsou považovány citráty a šťavelany, popř. jablečnany, díky jejich stabilním komplexům s Fe, Al a Ca (Jones, 1998). Efektivita zpřístupnění fosforu anionty organických kyselin se však značně liší v závislosti na typu půdy (Jones et al., 2003; Oburger et al., 2011). Zatím nebylo objasněno, proč tomu tak je. V experimentu Jones et al. (2003) množství mobilizovaného fosforu z jednotlivých půd nekorelovalo s celkovým obsahem fosforu v půdě, s reakcí pH půdy, se sorpční kapacitou, ani s celkovým obsahem železa v půdě. Oburger et al. (2011) uvádí, že při nižším stupni nasycení sorpčních míst fosforem, bylo ligandické rozpouštění P-minerálů hlavním mechanismem zodpovědným za zpřístupnění fosforu. V půdách s nízkou aniontovou sorpční schopností nebo při vyšším nasycení půdy fosforem je pak hlavním mechanismem výměna ligand, jelikož dochází celkově ke zvýšené kompetici mezi anionty o kladně nabitá sorpční místa. Celkově nejúčinnější zpřístupnění fosforu pomocí aniontů organických kyselin autoři pozorovali na půdách se středním až vysokým obsahem aniontových vazebných míst při středním nasycení těchto míst fosforem.

Organické anionty mohou také mimo přímého rozpouštění anorganického fosforu zlepšovat rozpustnost některých sloučenin organického fosforu, např. solí fytátu, a tím ho zpřístupňovat pro následnou enzymatickou hydrolýzu (Hayes et al., 2000). Tang et al. (2006) uvádí efektivitu v rozpouštění fytátových solí pro organické kyseliny v pořadí citronová > šťavelová > jablečná. Giles et al. (2012) publikovali zpřístupnění fytátových solí sorbovaných na goethit organickými anionty v pořadí: askorbát > citrát > oxalát > pyruvát > acetát. Jako hlavní mechanismy zpřístupnění fytátu autoři uvádějí chelataci nebo reduktivní rozpouštění. Anionty organických kyselin dále mohou mobilizovat fosfor vázaný v komplexech humínových látek s kovem (Richardson et al., 2009).

Půdní mikroorganismy produkují široké spektrum organických kyselin, především kyselinu glukonovou, 2-ketoglukonovou, citrónovou, jablečnou, šťavelovou, jantarovou, mléčnou, vinnou, nebo glykolovou. Kyselina glukonová a 2-ketoglukonová jsou často uváděny ve spojitosti s bakteriální produkcí, naproti tomu kyseliny citrónová a šťavelová jsou často produkovány houbami (Jones et Oburger, 2011). Neumann et Römheld (2007) uvádí, že pro účinnou mobilizaci fosforu z půdy jsou zapotřebí koncentrace vyšší než 5 - 10 μmol aniontu na g půdy. Takové koncentrace organických kyselin v rhizosféře byly doposud nalezeny pouze u omezeného množství rostlinných druhů. Dobře zdokumentovaný příklad představují tzv. „cluster roots" vyskytující se u lupiny bílé (*Lupinus albus* L.) a téměř všech zástupců čeledi *Proteaceae* (Skene, 1998; Neumann et Martinoia, 2002). Není tedy stále jisté, zda exsudace organických aniontů u ostatních druhů rostlin má opravdu přímý vliv na zpřístupnění fosforu v rhizosféře. Pearse et al. (2007) uvádějí, že samotná kořenová exsudace organických aniontů u hrachu setého (*Pisum sativum* L.) a cizrny beraní (*Cicer aeretivum* L.) nezajišťuje těmto druhům schopnost přijímat fosfor z málo rozpustných minerálů ($AlPO_4$, $FePO_4$, $Ca_5(PO_4)_3OH$) a pokládají hypotézu, že efektivní kořenová exsudace organických aniontů musí probíhat v součinnosti se správným pH a morfologií kořenů tak, aby organické anionty mohly dosáhnout účinné koncentrace a zároveň, aby jimi uvolněný fosfor mohly kořeny okamžitě přijmout. To potvrzují Lyu et al. (2016), kteří uvádí že hlavní odezva pšenice (*Triticum aestivum* L.), kukuřice (*Zea mays* L.) a řepky olejky (*Brassica napus* L.) na nedostatek fosforu spočívá spíše ve změně morfologie kořenů, než na zvýšené exsudaci organických kyselin nebo fosfatáz. Naproti tomu lupina bílá (*Lupinus albus* L.) a cizrna beraní (*Cicer aeretivum* L.) reagují především změnou exsudace organických kyselin a fosfatáz. Sója luštinatá (*Glycine max* L.) a bob obecný (*Vicia faba* L.) pak vykazují jak změnu morfologie kořenů, tak fyziologické změny v exsudaci.

2.4.4) Fosfatázy

Fosfatázy, nebo také fosfohydrolázy představují široké spektrum enzymů katalyzujících hydrolýzu esterů a anhydridů kyseliny trihydrogenfosforečné (Nannipieri et al., 2011). V zásadě se dělí na dvě skupiny, kyselé a zásadité, podle pH optimálního jejich aktivitě (Richardson et al., 2005). Dle IUBMB (2017) mezi fosfatázy řadíme hydrolázy monoesterů kyseliny trihydrogenfosforečné (fosfomonoesterázy), hydrolázy diesterů kyseliny fosforečné (fosfodiesterázy), hydrolázy monoesterů kyseliny trifosforečné (trifosfomonoesterázy) a dále

enzymy působící na anhydridy obsahující fosforylovou funkční skupinu a enzymy hydrolyzující P–N vazby (Nannipieri et al., 2011).

Ze všech těchto skupin mají ve výživě rostlin nejvyšší význam fosfomonoesterázy (Jones et Oburger, 2011) a fosfodiesterázy (Richardson et al., 2005). Skupina fosfomonoesteráz zahrnuje fosfoproteinfosfatázu (hydrolyzující esterické vazby fosfátové skupiny fosfoserinu, fosfothreoninu a fosfotyrosinu), fytázy hydrolyzující všech šest fosfátových skupin z kyseliny fytové (myo-inositol hexafosfát), nukleotidázy a dále kyselou a zásaditou fosfomonoesterázu (hydrolyzující monoesterické vazby, mimo jiné obsažené např. v mononukleotidech nukleových kyselin nebo fosfoesterech sacharidů, nehydrolyzuje však fosfátové skupiny z kyseliny fytové, ale může hydrolyzovat její deriváty s nižším počtem fosfátových skupin) (Nannipieri et al., 2011).

Rozdělení mezi kyselou a alkalickou extracelulární fosfomonoesterázou spočívá v optimálním pH jejich aktivity. Vyšší obsahy kyselé fosfomonoesterázy jsou tudíž většinou nalézány v kyselých půdách a naopak, alkalická fosfomonoesteráza je více přítomná v půdách alkalických. Obě tyto fosfatázy mohou být produkovány půdními mikroorganismy, přičemž některé bakterie mohou produkovat obě fosfomonoesterázy i fytázu současně (Jorquera et al., 2008). Kořeny rostlin produkují především kyselou fosfomonoesterázu (Jones et Oburger, 2011), přičemž tyto extracelulární enzymy mohou být buď vázány na buněčnou stěnu, nebo se mohou vyskytovat volně v půdním prostředí (Richardson et al., 2005). Produkce fosfatáz kořenem je považována za obecnou reakci rostlin na nedostatek fosforu (Richardson et al., 2005), avšak jejich relativní podíl na hydrolýze fosforu v rhizosféře není dosud znám. To je především způsobeno vysokým množstvím a aktivitou rhizosférních bakterií a hub, díky čemuž je rozlišení enzymů pocházejících z kořene velice problematické (George et al., 2011; Richardson et al., 2005). V hydroponickém experimentu studovali Seeling et Jungk (1996) příjem organického fosforu rostlinami ječmene (*Hordeum vulgare* L.). Z jejich výsledků je patrné, že pouze 55 % rozpuštěného organického fosforu bylo přístupné rostlinám. To indikuje fakt, že rostlinné fosfatázy nemají schopnost hydrolyzovat veškerý organický fosfor přítomný v půdním roztoku. Tarafdar et al. (2001) uvádí, že fosfatázy produkované houbami mají vyšší afinitu ke sloučeninám organického fosforu než fosfatázy produkované rostlinami.

Aktivita fosfatáz a fytáz je obecně inhibována přítomností orto-fosfátu (výsledný produkt reakce), F^-, přítomností vícemocných aniontů (MoO_4^{2-}, AsO_4^{3-}), vyššími koncentracemi kovů (Zn, Hg, Cu, Mn^{2+}, Fe^{2+}) a chelatačními činidly (EDTA, 8-

hydroxychinolin, vinan, šťavelan) (Quiquampoix et Mousain, 2005). Naproti tomu nižší koncentrace dvojmocných kationtů (Ca^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}) působí jako aktivátory těchto enzymů. Aktivita fosfatáz může být dále silně ovlivněna jejich adsorpcí na půdní minerály nebo organominerální povrchy. Typicky jsou fosfatázy nejsilněji vázány na jílové částice. Tyto výsledky tedy celkově demonstrují, že aktivita fosfatáz v půdě není závislá pouze na míře jejich exkrece kořenem rostliny nebo půdními mikroorganismy, ale je silně ovlivněná půdními vlastnostmi jako je pH, minerální složení, obsah a kvalita půdní organické hmoty (Jones et Oburger, 2011). V experimentu Spohn et Kuzyakov (2013) byla aktivita kyselé fosfatázy v rhizosféře nalézána v těsné blízkosti kořenů, přičemž aktivita alkalické fosfatázy se nacházela i v delší vzdálenosti od kořene. Naproti tomu Nannipieri et al. (2011) zmiňuje, že zvýšená aktivita kyselé fosfatázy dosahuje v rhizosféře do vzdálenosti 2 – 3,1 mm, přičemž aktivita alkalické jen do 1,2 – 1,6 mm. Mergalef et al. (2017) uvádějí, že pro celkový odhad aktivity kyselé fosfatázy v půdách je určující obsah organického fosforu, na rozdíl od podílu přístupného minerálního P.

2.5) Mikrobiální inokulanty - Bioefektory

Bioefektory jsou obecně definovány jako životaschopné mikroorganismy nebo aktivní přírodní látky, které přímo nebo nepřímo pozitivně ovlivňují růst a výživu rostlin bez významného přídavku živin do půdy. Z této definice vyplývá, že bioefektory zahrnují široké spektrum látek a organismů, které mohou fungovat jako rostlinné biostimulanty, biopesticidy nebo biohnojiva (du Jardin, 2015). Vzhledem ke zaměření této práce se následující kapitoly zabývají užší skupinou bioefektorů, a to tzv. růst rostlin podporujícími mikroorganismy (PGPM – plant growth-promoting microorganisms) schopnými zpřístupňovat minerální živiny, především pak fosfor, v půdním prostředí.

2.5.1) Mechanismy působení bioefektorů a jejich výběr pro praktické využití

Rhizosférní mikroorganismy mohou zvýšit schopnost rostlin přijímat živiny mnoha způsoby. Tyto mechanismy zahrnují především: 1) přímé zvětšení plochy kořenového systému (mykorhiza), 2) zvýšení růstu kořenů, jejich větvení nebo podporu tvorby kořenového vlášení, 3) přímý podíl na zvýšení dostupnosti živin v půdě nebo stimulace metabolických procesů, které zvyšují přístupnost živin (např. zvýšená exsudace protonů a organických aniontů), 4) posun v sorpčních rovnováhách, který následně způsobuje vyšší koncentraci živin v půdním roztoku nebo změnu zastoupení určité živiny mezi organickou a anorganickou frakcí, 5) zvýšení přeměny a metabolismu mikrobiální biomasy v rhizosféře (Richardson et al., 2009). Závislost mezi složením rhizosférní mikrobioty a aktivitou, resp. její funkcionalitou je většinou velmi slabá, jelikož každá funkce v rhizosféře je zastoupena širokou řadou různých druhů mikroorganismů. Díky tomu změna jednoho druhu mikroorganismu v rhizosféře má pouze malý vliv na danou funkci (Miethling et al., 2003; Hinsinger et al., 2011).

Široké spektrum půdních mikroorganismů tedy vykazuje schopnost exkrece H^+, organických aniontů a extracelulárních enzymů, díky čemuž mají schopnosti přímo zpřístupňovat fosfor v půdě. Obecně jsou tyto mikroorganismy nazývány jako fosfát-mobilizující mikroorganismy (PSM - phosphate-solubilizing microorganisms). Jejich hlavní charakteristikou je schopnost rozpouštět anorganické sloučeniny fosforu. Tato skupina zahrnuje široké množství symbiotických i nesymbiotických mikroorganismů, např. z rodů *Pseudomonas*, *Bacillus*, *Aspergillus* nebo *Penicillium* (Gyaneshwar et al., 2002; Khan et al., 2014). Výběr a izolace těchto mikroorganismů je rutinně založená na jejich kultivaci

v laboratorním prostředí, kde se sleduje jejich schopnost rozpouštět nejčastěji fosforečnany vápenaté, typicky potom $Ca_3(PO_4)_2$ (Richardson et al., 2009). Dále lze schopnost mikroorganismů testovat pomocí fluoro- a hydroxy-apatitů ($Ca_{10}(PO_4)_6F_2$; $Ca_5(PO_4)_3OH$), nebo fosforečnanů hlinitých a železitých. Tyto se však v praxi využívají jen zřídka. Testování mikroorganismů pouze na rozpouštění $Ca_3(PO_4)_2$ však nezaručuje účinné zpřístupnění fosforu v půdě díky heterogenitě půdních forem fosforu (Bashan et al., 2013). Bashan et al. (2013) doporučují testovat nově izolované mikroorganismy na směsi různých forem fosforu a následně vybrat vhodné kmeny podle druhu cílové půdy, tedy kmeny schopné rozpouštět fosforečnany hlinité a železité pro kyselé půdy, kmeny rozpouštějící fosforečnany vápenaté pro zásadité půdy a kmeny zpřístupňující fosfor z fytátů pro půdy organické. Ve všech případech autoři kladou důraz na zvýšenou produkci organických kyselin (Bashan et al., 2013).

Vessey (2003) uvádí, že samotná schopnost mikroorganismu zpřístupňovat fosfor ještě nezaručuje jeho pozitivní vliv na růst rostlin. Dle mnoha autorů (van Veen et al., 1997; Gyaneshwar et al., 2002; Richardson et al., 2011) hraje v účinnosti inokulovaného mikroorganismu velkou roli jeho schopnost a kapacita kolonizovat, přežít a rozmnožit se ve velmi kompetitivním prostředí rhizosféry. Naproti tomu v aktuální studii Mosimann et al. (2017), byl i přes perzistenci inokulovaného kmenu *Pseudomonas* sp. DSMZ 13134 v rhizosféře kukuřice nalezen jeho pozitivní vliv na výnos pouze na jedné ze tří testovaných půd. To implikuje skutečnost, že ani úspěšná kolonizace rhizosféry dobře známým a fosfor-solubilizujícím kmenem nezaručuje pozitivní vliv na výnos rostlin.

2.5.2) Praktické aplikace zpřístupnění živin pomocí bioefektorů

Houbové inokulanty rodů *Trichoderma* a *Penicillium* jsou jedny z nejčastěji studovaných bioefektorů (Harman et al., 2004; Takeda et Knight, 2006). Altomare et al. (1999) studovali schopnost kmene *Trichoderma harzianum* T-22 zpřístupňovat živiny z CuO, Fe_2O_3, MnO_2, kovového Zn, a $Ca_5(PO_4)_3OH$ *in vitro*. Testovaný kmen vykázal schopnost zpřístupňovat Fe, Zn a P z těchto sloučenin. Autoři dále uvádějí, že hlavními mechanismy nebyla produkce H^+, tedy okyselení media, ani produkce organických kyselin, ale produkce jiných, blíže neurčených chelatačních a redukčních činidel. Později byl pro tento kmen demonstrován jeho pozitivní vliv na růst vrby (Adams et al., 2007) a kukuřice (Akladious et Abbas, 2014). Nedávná studie Saravanakumar et al. (2017) uvádí, že aplikace *T. harzianum*

zvýšila počet růst-podporujících acidobakterií v rhizosféře kukuřice, avšak jako hlavní mechanismus působení uvádí indukovanou resistenci proti houbovým patogenům.

Takeda et Knight (2006) publikovali schopnost kmene *Penicillium bilaii* (ATCC 20851) uvolňovat fosfor z mletého fosfátu *in vitro*. Během experimentu autoři pozorovali významné snížení hodnoty pH růstového media a následně mechanismus zpřístupnění fosforu přisuzují produkci kyselin citrónové a šťavelové. Později Wakelin et al. (2007) prokázali zvýšenou produkci nadzemní biomasy u čočky, tolice dětelové a pšenice po aplikaci *P. bilaii*. Ve všech případech byla zvýšená produkce biomasy spojená s vyšším příjmem fosforu z půdy, avšak vliv inokulantů se významně lišil mezi testovanými půdami. Naproti tomu Karamanos et al. (2010) publikovali, že inokulace *P. bilaii* vedla k nárůstu biomasy pšenice pouze v 5 ti ze 47 mi polních experimentů realizovaných během let 1989 – 1995. Avšak autoři Leggett et al. (2015) uvádějí, že inokulace kukuřice houbou *P. bilaii* vedla k nárůstu výnosu v 361 případech z 461 uskutečněných polních experimentů během let 2005 – 2011, přičemž vliv inokulace se lišil podle zásobenosti půdy fosforem.

Žádná z výše uvedených studií se však nezabývala zpřístupněním živin z odpadních materiálů. Takových prací bylo dosud publikováno jen velmi omezené množství. Jedním z příkladů je studie Basak et Biswas (2009). V této práci se autoři zabývali zpřístupněním draslíku z odpadních slíd. Po aplikaci gram positivní bakterie *Paenibacillus mucilaginosus* autoři zaznamenali zvýšený obsah draslíku v půdním roztoku a současně vyšší výnos biomasy a odběr draslíku touto biomasou u čiroku (*Sorgum vulgare* Pers.). Obdobně pak Singh et al. (2010) demonstrovali schopnost bakterií *P. mucilaginosus* a *Azotobacter chrononococcum* zpřístupňovat draslík rostlinám pšenice a kukuřice z odpadních slíd, avšak pouze v podmínkách hydroponického experimentu. Nedávná práce autorů Lekfeldt et al. (2016) s pšenicí jarní testovala 5 mikrobiálních inokulantů (*T. harzianum* T22, *Pseudomonas* sp. DSMZ 13134, *Bacillus amyloliquefaciens*, *Penicillium* sp. A směs *T. harzianum* s pěti druhy bakterií rodu *Bacillus*) v kombinaci s několika odpadními materiály (čistírenský kal, popel ze spalování čistírenského kalu, popel ze spalování slámy, popel ze spalování dřevní štěpky, Thomasova moučka a struska z výroby oceli). V této práci však nebyla prokázána schopnost ani jednoho z testovaných inokulantů zpřístupnit fosfor a nebyl pozorován ani průkazně vyšší výnos pšenice po aplikaci inokulantů. Je tedy zřejmé, že pro budoucí účinnější využití živin z odpadních materiálů je třeba nadále hledat vhodné kombinace systému půda - rostlina – bioefektor – odpadní materiál.

3) Hypotézy a cíle práce

Pro tuto práci byly stanoveny tři hypotézy a k nim odpovídající tři hlavní cíle práce. Tyto cíle se snaží reflektovat některé překážky v širším využití odpadních materiálů v zemědělství, zejména tedy neznámý vliv materiálů na půdní vlastnosti, jejich těžko předvídatelný hnojivý účinek a potom také nízkou přístupnost fosforu z odpadních materiálů rostlinám.

Hypotéza 1: Aplikací odpadních materiálů lze zlepšit půdní vlastnosti, avšak vliv jednotlivých materiálů na půdu se liší dle způsobu jejich zpracování.

> **Cíl:** Zhodnotit vliv aplikace popela po spalování dřeva a biocharu ze dřeva na chemické a biologické vlastnosti půdy.

Hypotéza 2: Původ a zpracování odpadních materiálů ovlivňují celkový obsah prvků v těchto materiálech a jejich přístupnost rostlinám. Aplikace odpadních materiálů s různým původem biomasy povede k odlišným změnám ve složení půdního roztoku a následně k rozdílnému odběru živin rostlinami.

> **Cíl:** Stanovit rozdíly ve složení a hnojivém účinku popela po spalování dřeva a popela po spalování slámy, a dále sledovat vliv jejich aplikace na složení půdního roztoku.

Hypotéza 3: Půdní mikroorganismy mají schopnost napomáhat k uvolňování fosforu z odpadních materiálů a zvýšit tak jeho příjem rostlinami.

> **Cíl:** Nalézt vhodnou kombinaci odpadního materiálu a mikroorganismu, která povede k vyššímu hnojivému účinku odpadního materiálu.

4) Publikované práce

4.1) The improvement of multi-contaminated sandy loam soil chemical and biological properties by the biochar, wood ash, and humic substances amendments

Autoři: Pukalchik, M., Mercl, F., Panova, M., Břendová, K., Terekhova, V. A., Tlustoš, P.

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The improvement of multi-contaminated sandy loam soil chemical and biological properties by the biochar, wood ash, and humic substances amendments☆

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ABSTRACT

Nowadays trace metal contamination of soils represents an important environmental hazard. Nevertheless, the use of some secondary waste products as amendments may restore the common soil functions. This paper focuses on the chemical and biological influence of wood biochar (BC), wood ash (WA) and humic substances (HS), alone and in the mixtures, on a heavily multi-contaminated sandy loam soil. The soil was amended by above-mentioned materials to follow a pH-increasing design (pH_{Ca} from 6.0 to 6.5, 7.0 and 7.5); soil samples were analyzed after 3, 30, and 60 days using a set of variables, namely the plant-available trace element concentrations (Cu, Cd, and Zn), microbial biomass carbon (Cmic), and microbial quotient (qCO_2), as well as toxicity to *Sinapis alba* and *Daphnia magna*. Wood ash and WA + HS were the most efficient treatments to decrease mobile Cd and Zn concentrations in the soil, while HS, BC, and BC + HS combinations were the most effective in reducing the Cu mobility. The effect of BC and WA on the Cmic and qCO_2 was mostly negative, whereas adding HS markedly increased Cmic and reduced qCO_2 in soil. After amendment applications, the root elongation of mustard was significantly increased in HS and combined treatments (BC + HS, WA + HS). Additionally, BC + HS, WA + HS and WA 8.4% significantly decreased the toxicity of leachates to *D. magna* to the low-, or non-toxic levels. Our results suggest that the combination of amendments with HS can be a suitable remediation strategy for heavily contaminated soils.

1. Introduction

Trace elements (TE's) are considered to be the priority pollutants of environmental concern, but unlike organic pollutants, they cannot be degraded and thus constitute a persistent environmental hazard. *In situ* remediation techniques therefore attract growing attention because they have been proved to be a promising green and low cost alternative for landfilling, as well as soil washing and have been tested in pilot and full-scale field studies. However, there are a lot of complications and unresolved issues on the way to their application for improving the soil quality.

On the one hand, the use of amendments from renewable natural resources and wastes is among the most prospective trends in soil remediation, although it has been still widely debated what amendments should be used and what should not (EPA, 2007; Bolan et al., 2014). A large body of experimental data and experience gained in soil studies suggest that biochar (BC), wood ash (WA) as well as commercial humic substances (HS) can modify soil physicochemical properties (Pitman, 2006), decrease mobility of pollutants (Conte et al., 2005; Beesley and Marmiroli, 2011; Ochecová et al., 2014), and induce changes in the structure of soil microbial communities (Saarsalmi et al., 2012; Yakimenko et al., 2013; Chintala et al., 2014); all these benefits are eminently achievable and worthwhile.

On the other hand, we are facing the challenge that the determination of pollutant contents is not sufficient parameter for a

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comprehensive soil quality evaluation in terms of ecotoxicological hazard (Chapman et al., 2013). For this reason, microbiological and standardized toxicological assays are widely used for soil quality assessment in soil remediation studies since they signalize soil health recovery. This approach was applied to evaluate the efficiency of selected amendments in different studies (Tejada et al., 2008; Pardo et al., 2014); nevertheless, the influence of BC, WA, and HS on soil biological properties has been underexplored so far.

Little attention has been directed at the effectiveness of their combination with humic products compared to a growing body of physico-chemical, toxicological, and environmentally relevant data regarding for individual amendment. Up to our knowledge, only limited number of studies has focused on adding of humic substances along with biochar or wood ash into polluted soils. Chirenje et al. (2002) showed decreased Cr and increased As leaching from ash flushed with HS versus water in column leaching experiment. Wang et al. (2008) described promotion of metal adsorption on coal fly ash by the presence of humic acids in surface and waste water systems. According to Zhang et al. (2014), the best growth of *Calathea insignis* was found when compost-based growth medium was amended with 20% BC and 7% HA. This information suggests that the combination of biochar or wood ash with HS may improve soil quality, fertility or restore degraded soil functions due to interaction between these amendments. Hence, we hypothesized that soil microbiology and ecotoxicity quality also could benefit from applying amendment mixtures in comparison with such amendments applied separately.

The aim of this study was, therefore, to compare the short-term (0–60 days) effects of biochar, wood ash and humic substances alone and in mixtures, on: i) plant-available Cd, Cu and Zn concentrations in soil, ii) soil respiration activities, and, iii) bioassay endpoints.

2. Material and methods

2.1. Experimental design

The soil used in this study was collected from the alluvium of the Litavka River in the village of Trhové Dušníky (60 km south of Prague). This area is characterized by multi-contaminated soils resulting from mining and smelting activities. Further information on the pollution dispersion across the area can be found in Vyslouzilova et al. (2003). The soil was collected from the 4 m^2 (topsoil layer of the 0–20 cm depth), homogenized, air dried, and passed through a 2 mm mesh prior to the incubation experiment. The physicochemical properties of this soil were: Clay 6%, Silt 49%, Sand 45%, pH 6.0 ± 0.1, TOC 3.60 ± 0.00%, CEC 149 ± 5.90 mmol kg-1, P_{tot} 0.02 ± 0.00%, Ktot 0.25 ± 0.00%, Mg_{tot} 0.12 ± 0.00%, Ca_{tot} 0.14 ± 0.01%, S_{tot} 0.03 ± 0.00%, Fe_{tot} 11.76 ± 0.62%, Mn_{tot} 0.14 ± 0.00%, Zn_{tot} 7595.65 ± 194.76 ppm, Cd_{tot} 80.82 ± 4.58 ppm, Cu_{tot} 62.51 ± 6.45 ppm, Pb_{tot} 4346.13 ± 140.08 ppm, Ni_{tot} 5.61 ± 0.25 ppm.

The effect of amendments on soil chemical and microbiological properties was studied in incubation experiment. Three different amendments were tested independently and in a mixture with commercial potassium humic substances (HS) Lignohumate (Amagro, Czech Republic) produced by alkaline extraction from lignin and supplied in the form of solution containing ca. 33.4% of humic and fulvic acid in total. Wood ash (WA) was collected from a fluidized bed reactor (15 MWt) for wood chips burning at a commercial biomass power plant. Biochar (BC) was derived from wood chips gasification (150 kW/h gas and 300 kW/h heat production) at the temperature range 700–900 °C. The chemical characteristics of amendments are shown in Table 1.

Various amendments, both alone and in mixtures, were used to adjust soil pH values to the desired levels. The soil pH is widely reported as the strongest factor which influences the mobility of certain TE (Adriano, 2001; Tlustoš et al., 2006). Therefore, this approach was considered as providing better comparability of the tested amendments. We defined three increasing pH values (6.5, 7.0, and 7.5) of the final soil plus amendment mixture from the experiment carried out prior to this study to find out proper amendment doses and their combinations (data not shown). Final treatments were chosen based on reasonable application doses of the amendments. In the case of BC amendment, we failed to reach pH 7.5 when a 10% dose was applied. Similarly, we considered the dose of HS above 1% as economically inviable. The final experimental design is shown in Fig. 1.

Each portion of a 200 g air-dried soil sample was mixed with a dried amendment and placed into 0.5 l plastic pot (10 cm diameter and 15 cm height). The pots were irrigated in order to reach a moisture content of 60% of the water holding capacity. The

Table 1
Chemical characteristic of amendments and calculated atomic ratios (C/N, O/C, H/C).

Amendment	N	C	H	S	O	C/N	O/C	H/C	Ash	pH
	%	%	%	%	%				%	
Biochar (BC)	0.44	88.20	0.82	0.19	6.49	233.86	0.05	0.11	3.86	8.9
Humic substances (HS)	0.25	33.47	3.72	4.84	17.72	156.19	0.39	1.33	40.0	9.0
Wood ash (WA)	0.00	8.50	0.16	0.33	4.79	0.00	0.42	0.23	86.22	11.2

All values are given on moist-free basis. Atomic ratios C/N, H/C and O/C are calculated on ash-free basis.

Table 2
Correlation coefficients (Pearson) for the pH, ΔTOC, trace elements (Cu, Cd, Zn), and biological properties of the soil samples.

Variables	pH	ΔTOC	Cd	Cu	Zn	*D. magna* (IT,%)	*S.alba (root, mm)*	qCO_2	Cmic
pH	**1**								
ΔTOC	0,44	**1**							
Cd	**−0,66****	−0,19	**1**						
Cu	−0,20	−0,30	0,20	**1**					
Zn	**−0,73****	−0,29	**0,92****	0,15	**1**				
D. magna (IT)	**−0,94****	−0,38	**0,57***	0,01	**0,69****	**1**			
S. alba (root, mm)	0,37	0,26	−0,36	−0,38	−0,37	−0,24	**1**		
qCO_2	−0,11	0,16	0,26	0,03	0,18	−0,07	−0,24	**1**	
Cmic	−0,32	−0,32	0,13	−0,04	0,29	0,47	0,12	−0,51	**1**

Values in bold correspond to significant correlation at the $P < 0.05$ level,**$P < 0.01$ level. ΔTOC was calculated according to the added amount of amendments.

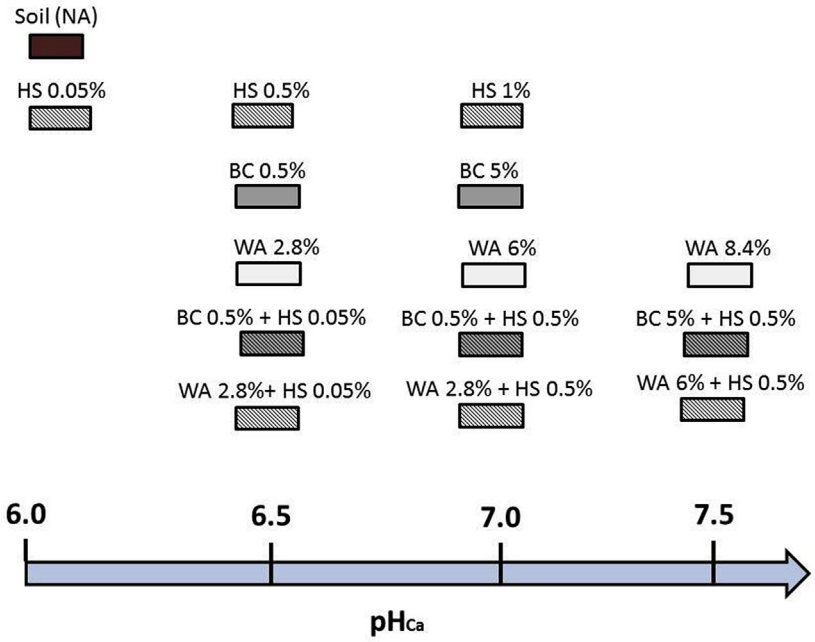

Fig. 1. Experimental design showing amendment types, their concentration and resulting pH_{Ca} values in soil. NA—non-amended soil, HS—humic substances, BC—biochar, WA—wood ash.

moisture content was maintained by weighing the pots every third day and replenishing the evaporated water. The pots were exposed to the greenhouse conditions at 21/18 °C (day/night). The non-amended (NA) and amended soils were collected after 3, 30, and 60 days of incubation.

At the collection time, each soil sample was divided into two parts. The first part was kept refrigerated (4 °C) and was used to determine soil respiration activities and bioassays. The second part of the sample was dried at 120 °C and used for the analysis of pH and plant-available fractions of TEs.

2.2. Chemical properties

The pH values of the non-amended and amended soils were measured in 0.01 M $CaCl_2$ at a ratio of 1:2 (w/v) using WTW pH 340i meter with glass, ion-selective electrode (WTW, Weilheim, Germany).

Plant-available fractions of Cd, Cu, Zn were determined by $CaCl_2$ extraction which is regarded to be a reliable indicator of TE's bioavailability in a range of contaminated soils (Novozamsky et al., 1993). Briefly, 5.0 g of a soil sample were shaken in 12 ml 0.01 M $CaCl_2$ solution for 6 h at 120 rpm. The suspensions were then centrifuged at 9000 rpm for 12 min. The trace element concentrations in the supernatants were then determined by inductively coupled plasma — optical emission spectrometer (ICP-OES, Agilent 720, Agilent Technologies Inc. USA).

2.3. Soil respiration parameters

The samples were preincubated for 3 days with the constant moisture of 60% of water holding capacity at room temperature. Two grams of the soil were placed in 13 ml glass vials and closed with rubber caps. Substrate induced respiration (SIR) was assessed after the addition of glucose (32 mg per g soil) over 4 h, and was converted to microbial biomass C (Cmic) as Cmic ($\mu gC\ g^{-1}$ soil) = SIR($\mu l\ CO_2\ g^{-1}$ dry soil h^{-1}) × 40.04 + 0.37 (Anderson and Domsch, 1978). Microbial basal respiration was measured in the same manner as the substrate-induced except for the fact that glucose had not been added and exposure time was 24 h. The microbial quotient qCO_2 ($\mu g\ CO_2$—C g^{-1} dry soil h^{-1}) was calculated as a ratio of basal respiration (BR, $\mu gCO_2\ g^{-1}\ ml^{-1}\ h^{-1}$) and microbial Cmic (μgC g-1 soil), where qCO_2 = BR/Cmic. For CO_2 quantification, M3700-400 gas analyzer (Kristall, Granat Co. Russia) was used.

2.4. Bioassay

The bioassays were selected to represent different taxonomic groups and directly performed the assess toxicity of the soils and the soil water extracts. The direct soil phytotoxicity test was run according to the modified protocol of Martignon (2009) using seeds of mustard (*Sinapis alba* L.). The seeds were pre-sterilized in 10% sodium hypochlorite solution for 10 min to prevent fungal growth and washed twice in distilled water; the seed germination potential was also examined. Germination rates higher than 95% demonstrated the viability of the seeds. For each sample, 10 g of the soil were placed in a Petri dish (d 80 mm), completely hydrated with deionized water and, next, spread over the entire surface of the test plate to obtain a homogeneous flat layer. Then, the wet soil samples were covered with a wet paper filter (Whatman® #1) and ten seeds were placed on the top. All dishes were closed with lids and wrapped with Parafilm™. The Petri dishes prepared in this way were incubated in a horizontal position at 24.5 ± 0.5 °C in the darkness for 72 h. Afterwards, the image was registered with a digital camera and the root length was measured using "Image Tools" program for image analyses. The test was replicated three times.

Determining the inhibition of *Daphnia magna* mobility is an acute toxicity assay. Its objective is to identify the initial concentration of a pollutant in solution or an aqueous mixture that may immobilize 50% of the *Daphnia* exposed to a polluted source within 48 h (ISO 6341, 2012). The soil water extraction was carried out at a liquid/solid ratio of 10/1 (50 g soil in 500 ml deionized water) at 20 °C in 1 L glass flasks for 2 h. After decantation for 15 min, the soil suspension phase was centrifuged at 5000 g during 10 min and

stored at 4 °C until tests. The sensitivity of the laboratory species was controlled by regular tests with potassium dichromate. Only young female *Daphnia* aged less than 24 h were used. The tests were conducted in quadruplicate in the darkness and at 20 ± 2 °C, using 5 daphnids in the final 50 mL volume. The control tests (the normal medium, without EDTA) were also run in parallel series. The test parameter considered was the number of died individuals in each replica after 48 h of exposure, compared to the number of individuals initially exposed. Each assay was regarded valid only if the *Daphnia* immobilization in the control solution was less than 10% or equal to it. The toxicity effect of the water extracts was compared with a toxicant-free control (the normal medium, without EDTA) to obtain percent of toxicity index (IT,%); thus

$$IT(\%) = \left(1 - \frac{N_{sample}}{N_{control}}\right) * 100\%$$

where N_{sample} – number of living daphnids in samples, $N_{control}$ – number of living daphnids in toxicant-free control (the normal medium, without EDTA). When evaluating the results of the tests, the following toxicity criteria were accepted: non-toxic samples IT < 10%; low-toxic samples 10% < IT < 50%; toxic samples 50% < IT < 100%; highly-toxic samples IT = 100%.

2.5. Statistics and data analysis

Each treatment for each time of collection was set up in four replicates. One-way analysis of variance (ANOVA) was carried out to determine statistical differences among treatments for different factors, and means were compared by *post-hoc* Fisher's least significance test (LSD) (Supplementary Table A). Differences in mean value were tested with the help of two-way ANOVA with the presence/absence of time and the type of amendment as factors. Principal Component Analysis (PCA) was performed, and component extraction was made by means of the Pearson's correlation matrix using the STATISTICA software package version 8 (Statsoft Inc.). All figures were prepared in SigmaPlot 12.5 (Systat).

The amount of amendment, which is needed to immobilize a certain amount of TE, plays a crucial role when *in situ* stabilization takes place in field scale. In order to compare the relative effectiveness of amendments and their application rates to reduce plant-available contents of TE in soil, immobilization efficiency (IE) [μg TE g^{-1} amendment] of tested amendments was calculated, as follows:

$$IE = \frac{TE_{NA(60\ day)} - TE_{treatment(60\ day)}}{m_{treatment}}$$

where $TE_{NA(60\ day)}$ is the amount of plant-available TE in control (NA) treatment, $TE_{treatment(60\ day)}$ is the amount of plant-available TE in amended treatment, and $m_{treatment}$ is the mass of amendment applied.

3. Results

3.1. Soil pH and plant-availability of trace elements (Cu, Pb and Zn) in the presence of amendments

The pH was monitored at the beginning and at each time-point of the experiment; the initial soil pH of NA was 6.0 ± 0.1. The levels of pH significantly increased by the amendments (except for HS 0.05) in comparison with the control, and the values were relatively constant over the experimental time (Fig. 2; Table SA). Wood ash showed higher alkalinity compared to BC and HS, the amount of WA needed to increase pH value of the amended mixture by 0.5 was more than 3-fold higher than the amount of BC (Table 1; Fig. 1).

Plant available fractions of both Cd and Zn were strongly influenced by the applied dose of amendment ($p < 0.001$) and the incubation time ($p < 0.001$) in all treatments (Table SB). The available Cd content in the soil was reduced predominantly by WA to about 83% (Fig. 3A–C). Similar results were obtained for WA + HS treatments; reduction by 60–84% was found after 3 days of incubation (Fig. 3A, Table SA). Other treatments were much less effective in contrast to WA and WA + HS in decreasing the Cd plant-availability: reduction did not exceed 40% after 3 days and reached 43–60% at the 60th day. Combining WA with HS improved the Cd immobilization efficiency at pH 7 and 7.5 (Table 3). With regard to BC, strong Cd immobilization improvement by the combination with HS was found at pH 7 while there was no change at pH 6.5 (Table 3).

A similar tendency was found for Zn mobility (Fig. 3D–F, Table SA). The humic substances treatments were less effective in decreasing the Zn mobility compared to WA and BC treatments. Yet, combination of WA or BC with HS increased immobilization efficiency of the amendments at pH levels 7 and 7.5.

All amendments led to the steep decline of plant-available Cu concentrations in all collection periods (Fig. 3 H–M, Table SA) as it had been expected according to the dependency of Cu compounds solubility on pH (Lindsay, 1979). The concentration of Cu extracted by $CaCl_2$ most noticeably decreased by 50–75% in the presence of BC against the control treatment. Wood ash, as well as HS, also exerted a positive effect of 40–60% and 40–72% reduction, respectively. According to the ANOVA factor analysis (Table SB), the plant-available Cu contents were highly ($p < 0.001$) influenced by the applied dose of BC and WA while the effect of the time factor was not significant. This indicates fast establishment of equilibrium after the application of these materials. On the contrary, in HS, BC + HS, and WA + HS treatments, the considerable effect of the dose as well as the incubation time, were detected. It suggests time-dependent reaction between HS and Cu in the soil. The trend of available Cu concentration was identical in all HS-containing treatments and decreased over the time (Fig. 3 H–M, Table SA).

Calculated values of the immobilization efficiency (IE) coefficient were generally ordered in the following line: HS > BC » WA at pH ~6.5 and HS » BC > WA at pH ~7 when these materials were applied separately (Table 3). Generally, IEs of all amendments showed indirect proportion to the amount of the applied

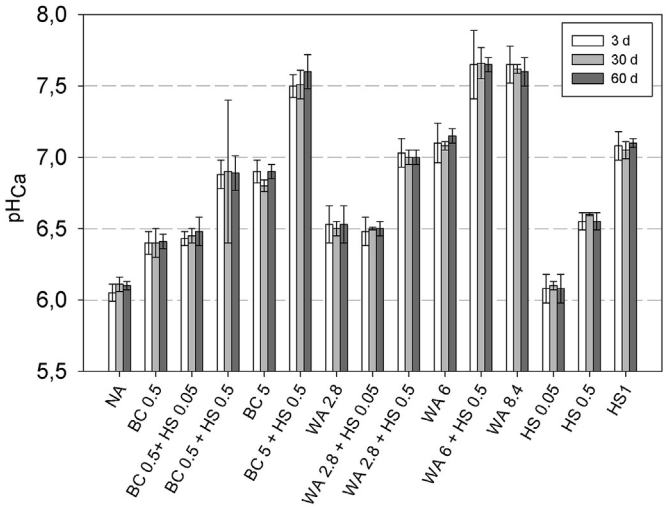

Fig. 2. Dynamics of the pHCa in 0.01 M CaCl2 extracts as a function of treatment concentrations and sample collection periods–3d, 30d, and 60d after treatment. The error bars represent the standard deviation of the mean (n = 4).

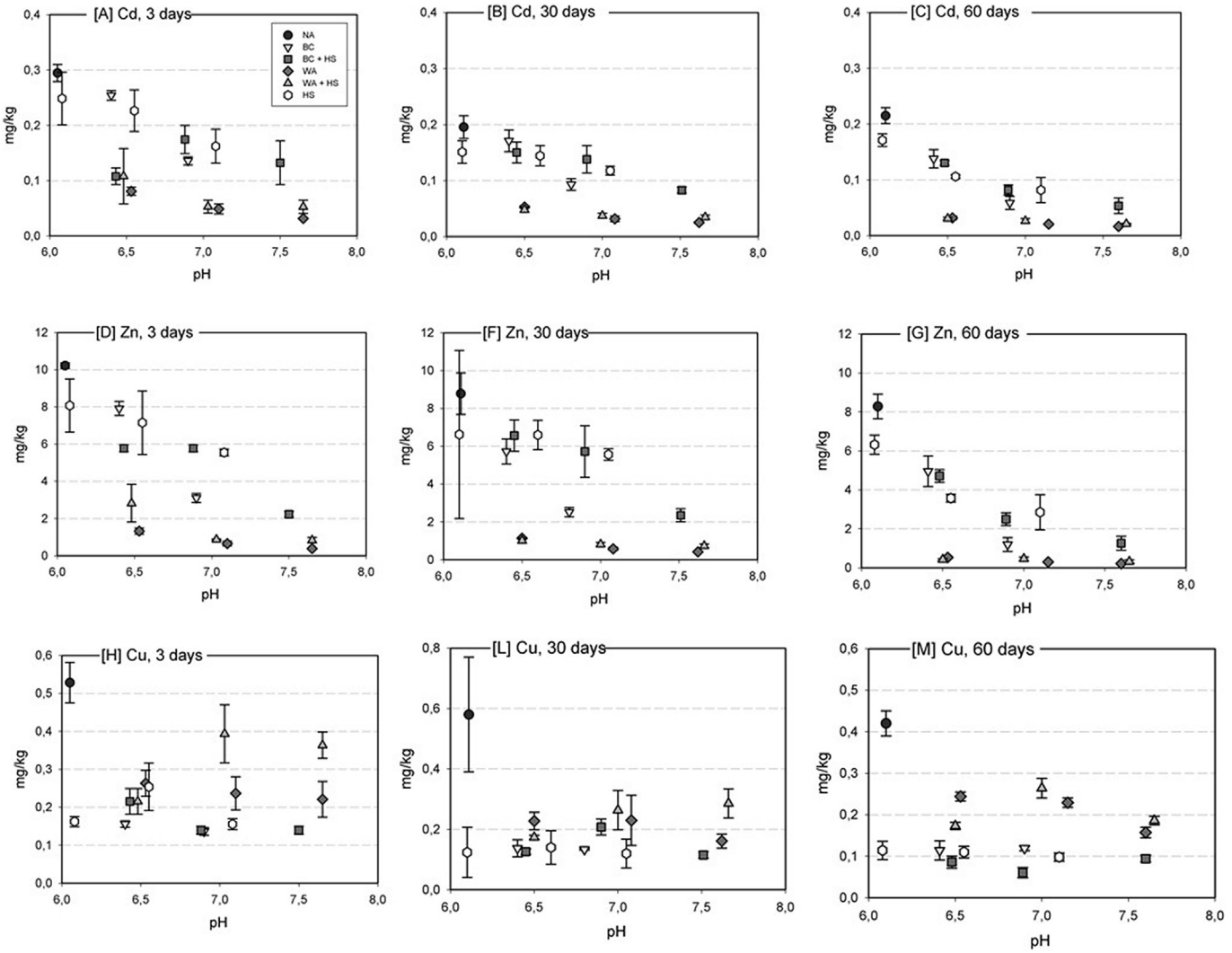

Fig. 3. Concentrations of Cd (A–C), Zn (D–G), Cu (H–M) in the $CaCl_2$ extract from samples in soil with different amendments in comparison with the non-amended soil (NA); the samples were collected after 3d, 30d, and 60d. The error bars represent the standard deviation of the mean (n = 4).

amendment. Cd, Cu, and Zn immobilization efficiency of BC treatments was found to be highly improved by HS addition at pH ~7, e.g.: 6.02 μg Cu g^{-1} and 36.0 μg Cu g^{-1} for BC 5.0% and BC 0.5% + HS 0.5%, respectively (Table 3). Similarly, adding HS to WA resulted in a strong improvement of Zn IE at pH ~7; but only slight improvement of IE at pH ~7.5 was found. Generally, the IE-improving effect of HS addition to WA was significant particularly for Zn. The content of C in the amendments also had a strong impact on the IE: at the same pH level amendments containing higher dose of C proved to be less effective in reducing Zn, Cu, and Cd bioavailability in the soils.

3.2. Effects on soil respiration activities

Table 4 showed the cumulative CO_2 evolution from the amended and non-amended soils throughout 60 days of incubation. Carbon dioxide production in the samples without the glucose additive (BR) greatly varied across the treatments and had significant differences over the control NA and all amended samples ($p < 0.05$). Generally, WA diminished CO_2 production by 12–21%, as well as WA + HS. Contrarily, a lower dose of BC (BC 0.5) increased BR during the first 30 days of incubation, but a higher dose (BC 5.0) significantly diminished BR even after 60d. The combination of BC + HS (BC 5.0 + HS 0.5) mitigated the negative impact of BC at 30 and 60 d of incubation. Higher doses of humic substances alone (HS 0.5; HS 1) considerably increased BR during the first 30d.

The glucose induced respiration data (recalculated to Cmic) testified to the stimulatory effects of HS and the inhibitory effect of

Table 3
Immobilization efficiency of individual amendments at 60 d (μg TE g^{-1} amendment).

Variables	pH (values)	ΔTOC(g/kg)	Cd	Cu	Zn
BC 0.5	6.41	4.41	15.4	61.2	667
BC 0.5 + HS 0.05	6.48	4.58	15.4	60.8	650
BC 0.5 + HS 0.5	6.89	6.08	13.3	36.0	579
BC 5	6.90	44.1	3.13	6.02	142
BC 5 + HS 0.5	7.60	45.77	2.94	5.93	128
WA 2.8	6.53	2.38	6.54	6.29	277
WA 2.8 + HS 0.05	6.50	2.55	6.47	8.67	276
WA 2.8 + HS 0.5	7.00	4.05	5.72	4.73	237
WA 6	7.15	5.10	3.24	3.18	133
WA 6 + HS 0.5	7.65	6.77	2.99	3.60	123
WA 8.4	7.60	7.14	2.36	3.13	96
HS 0.05	6.08	0.17	87.8	612	3931
HS 0.5	6.55	1.67	21.8	62.0	942
HS 1	7.10	3.35	13.3	32.2	543

ΔTOC was calculated according to the added amount of amendments.

Table 4
Effects of the amendments on the soil respiration activity. The data is represented as Mean ± SD (n = 4).

Samples	BR ($\mu gCO_2\ g^{-1}\ ml^{-1}\ h^{-1}$)			Cmic ($\mu gC\ g^{-1}$ soil)			qCO_2 (μgCO_2-C $mgCmic^{-1}\ h^{-1}$)		
	3d	30d	60d	3d	30d	60d	3d	30d	60d
NA	258 ± 2 d	217 ± 2 def	247 ± 9 def	58.6 ± 5.4 fg	51.2 ± 1.4 cd	55.5 ± 0.9 defg	4.39 ± 0.22 bcd	4.25 ± 0.21 cd	4.45 ± 0.22 cd
BC 0.5	277 ± 2 e	239 ± 4 gk	247 ± 26 def	47.4 ± 0.6 cd	48.4 ± 0.4 bc	47.0 ± 3.3 bc	5.85 ± 0.29 g	4.94 ± 0.25 fg	5.24 ± 0.26 f
BC 0.5 + HS 0.05	276 ± 2 e	223 ± 2 efg	194 ± 7 ab	56.7 ± 0.6 f	58.0 ± 1.5 e	49.2 ± 1.6 bcd	4.87 ± 0.24 def	3.85 ± 0.19 b	3.95 ± 0.20 ab
BC 0.5 + HS 0.5	263 ± 2 d	238 ± 14 gk	236 ± 16 cdef	53.8 ± 0.4 e	55.0 ± 3.1 de	58.2 ± 2.1 efg	4.52 ± 0.23 bcde	4.32 ± 0.22 cd	4.06 ± 0.20 b
BC 5.0	240 ± 5 c	190 ± 3 bc	200 ± 18 b	38.5 ± 1.3 a	44.7 ± 2.5 b	44.9 ± 5.7 b	6.25 ± 0.31 g	4.26 ± 0.21 cd	4.44 ± 0.22 cd
BC 5.0 + HS 0.5	242 ± 11 c	238 ± 14 gk	234 ± 23 cde	53.8 ± 0.4 e	55.0 ± 3.1 de	47.3 ± 2.3 bc	4.35 ± 0.22 bc	4.32 ± 0.22 cd	4.95 ± 0.25 f
WA 2.8	228 ± 2 b	208 ± 5 cde	213 ± 3 bc	45.5 ± 0.7 bc	45.0 ± 2.2 b	46.8 ± 4.1 b	5.01 ± 0.25 f	4.63 ± 0.23 def	4.56 ± 0.23 d
WA 2.8 + HS 0.05	238 ± 7 c	204 ± 3 bcd	228 ± 11 cd	49.5 ± 0.5 d	56.1 ± 7.4 e	58.4 ± 6.7 efg	4.80 ± 0.24 ef	3.64 ± 0.18 ab	3.90 ± 0.19 ab
WA 2.8 + HS 0.5	278 ± 2 e	209 ± 14 def	262 ± 12 f	60.5 ± 0.4 g	58.9 ± 1.7 e	51.5 ± 4.0 bcde	4.58 ± 0.23 bcde	4.46 ± 0.22 cde	5.08 ± 0.25 f
WA 6.0	205 ± 4 a	201 ± 2 bcd	189 ± 17 ab	43.3 ± 0.6 b	39.4 ± 0.8 a	45.0 ± 4.0 b	4.73 ± 0.24 cdef	5.11 ± 0.26 g	4.21 ± 0.21 bcd
WA 6.0 + HS 0.5	276 ± 2 e	166 ± 7 a	193 ± 18 ab	53.8 ± 0.5 e	50.1 ± 1.7 c	54.0 ± 0.8 cdef	4.36 ± 0.23 bc	3.31 ± 0.17 a	3.58 ± 0.18 a
WA 8.4	202 ± 2 a	186 ± 18 b	167 ± 16 a	40.1 ± 0.7 a	38.9 ± 1.8 a	33.6 ± 0.9 a	5.03 ± 0.25 f	4.77 ± 0.24 efg	4.99 ± 0.25 f
HS 0.05	261 ± 2 d	227 ± 14 fg	262 ± 20 f	70.4 ± 0.6 k	51.0 ± 1.1 cd	50.9 ± 9.4 bcd	3.71 ± 0.19 a	4.46 ± 0.22 cde	5.15 ± 0.26 f
HS 0.5	310 ± 1 f	248 ± 2 k	251 ± 23 def	72.2 ± 0.6 k	58.4 ± 1.6 e	60.7 ± 2.5 fg	4.30 ± 0.22 b	4.24 ± 0.21 c	4.13 ± 0.21 bc
HS 1	368 ± 5 g	289 ± 23 l	260 ± 12 ef	76.0 ± 0.7 l	56.9 ± 1.8 e	62.3 ± 5.7 g	4.84 ± 0.24 ef	4.85 ± 0.39 fg	4.18 ± 0.21 bc

Different letters denote significant differences at the 0.05 confidence level between treatments at the same exposure time.
BR — basal respiration, Cmic — microbial biomass C, qCO_2 — microbial quotient.

WA and BC. As a result, qCO_2 in BC treatments was increased by 16–42% over control, similarly to the WA treatments, where increase in qCO_2 ranged 8–12% after 60 days (Table 4). At the same time, HS and mixtures with HS (WA 2.8 + HS 0.05, WA 6 + HS 0.5, BC 0.5 + HS 0.05) exhibited a pronounced positive effect. These samples were characterized by the lowest metabolic quotient in comparison with the NA soil and with the samples amended by WA and BC alone.

3.3. Effects of treatments on soil ecotoxicological properties

A tendency to the root elongation in mustard was pointed out in the all amended treatments (Fig. 4A, Table SA). Treatment with HS and combined amendments (BC + HS, WA + HS) stimulated the plant growth compared with the NA soil. The shortest roots were always found in the NA soil throughout the whole testing period, but only four amendments (BC5; HS 0.5; HS 1; BC 5 + HS 0.5) were considerably different from the control. Therefore, it is interesting to note, that we observed a pronounced positive effect of HS on root elongation for all mixtures in comparison with HS or WA added alone.

The bioassay with *Daphnia magna* demonstrated that the toxicity index was higher than 10% in the majority of treatments and also in the NA control (Fig. 4B, Table SA). Only water extracts prepared from WA 8.4% and two mixtures BC 5 + HS 0.5 and WA 6 + HS 0.5 were non-toxic (IT < 10%). Most samples were low-toxic (10% < IT < 50%), and the extracts prepared from the NA soils were regarded as toxic (50% < IT < 100%).

3.4. Data analysis

For a more comprehensive assessment we compared all data blocks across the 60's days using PCA which indicated that Factor 1 and Factor 2 provided a reasonable summary of the data accounting for about 63% of the total variance (Fig. 5). Trace elements concentration (Cd, Cu, Zn), pH, ΔTOC, and toxicity for *D. magna* were best described by F1, while F2 was dominated by microbial respiration (Cmic, qCO_2) and *S. alba* root length.

4. Discussion

4.1. Amendments influence on the plant-availability of Cd, Cu, and Zn

The present experimental design focused specifically on the effects of biochar, wood ash, and commercial humic product alone

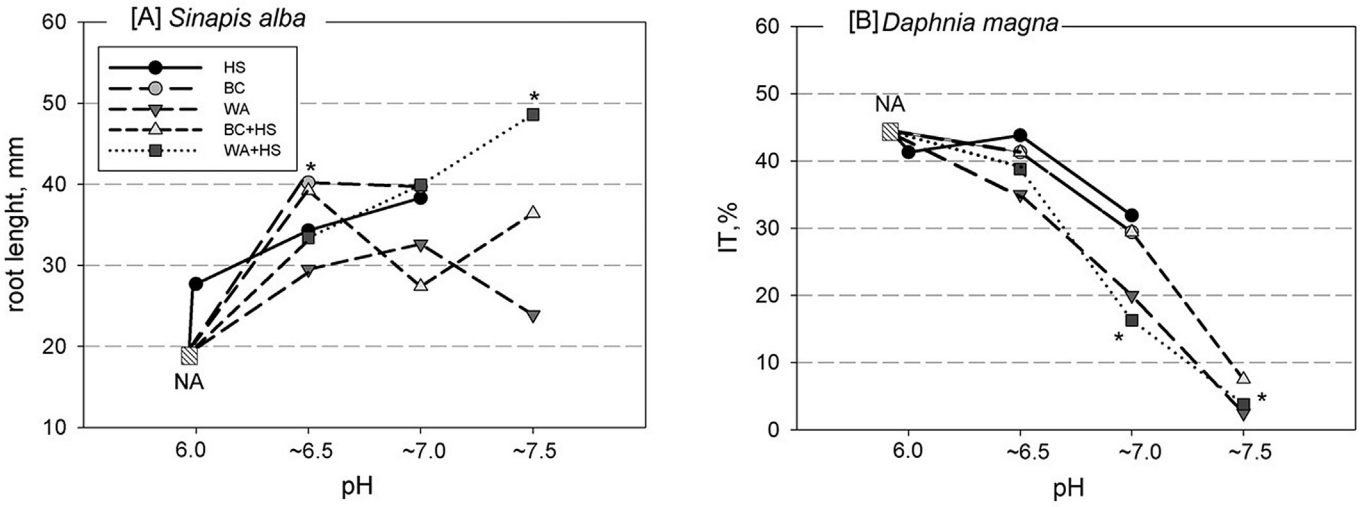

Fig. 4. Effects of the amendments on *Sinapis alba* root length (A) and *Daphnia magna* toxicity index (B); the samples were collected after 60d. * denote a significant difference of treatment in compare with control (NA) with $p < 0.05$.

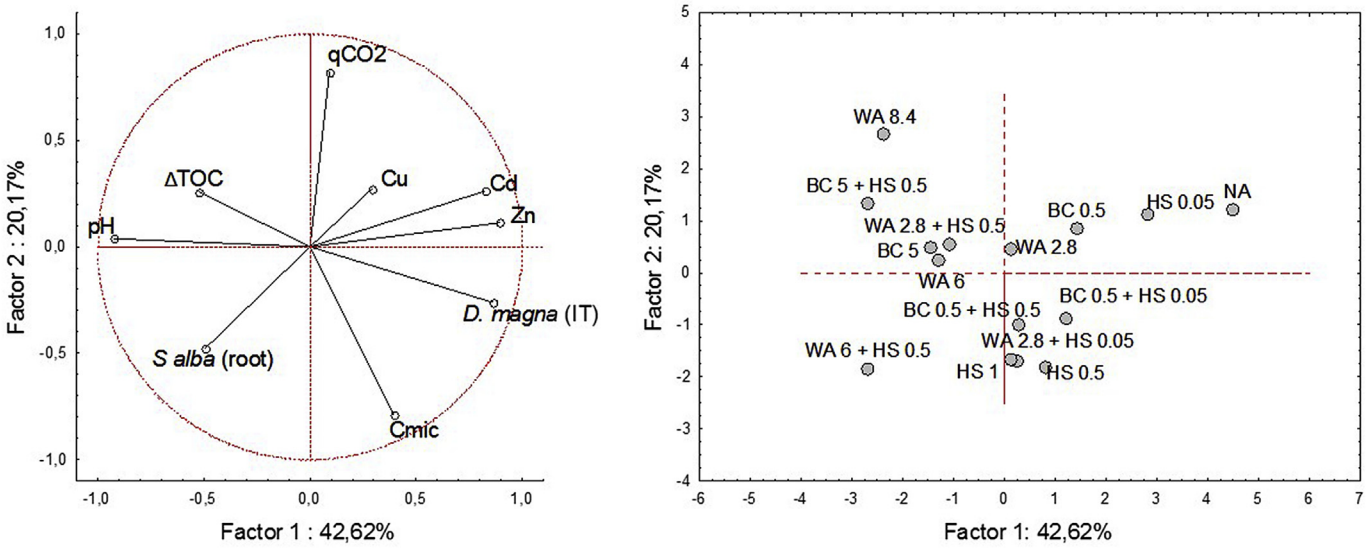

Fig. 5. PCA score plots based on soil parameters in 14 treatments and NA after 60 incubation days. Each treatment was replicated four times generating one mean point on the graphs.

and in mixtures to the plant-available fractions of Cu, Zn, and Cd. The results showed variable potential of the selected amendments to a short-term reduction of trace metals availability in the soil. The soils that were treated with wood ash evoked profound decrease in Zn and Cd plant-availability, while biochar and humic substances-treatments better reduced Cu plant-available content in the polluted soil. The minor amount of HS added as supplement to BC markedly increased immobilization efficiency to Cu, probably due to cumulative effects of the amendments.

There are several mechanisms responsible for soil TE concentrations and amendments selectivity after addition. Firstly, numerous studies have provided the evidence that inorganic and organic amendments are distinct in their TE immobilization mechanisms. There is a number of ways how inorganic materials such as wood ash, limestone or lime can provide the long-term retentive capacity for some heavy metals, including: i) enhanced adsorption onto soil particle surfaces due to increased pH, ii) co-precipitation with major hydroxide or carbonate phases (Lee et al., 2004), or iii) binding in lattice positions in aluminosilicates (Patterson and Passino, 1987). Carbon-based materials like biochar and humic substances provide remediation properties mainly according to their surface reactions potential (Sposito and Page, 1984), or by high content of oxygen-containing functional groups. However, due to alkaline character of carbon-based materials, pH-induced changes in chemical speciation of TE have to be also considered. Inyang et al. (2015) reviewed the biochar mechanisms responsible for TE removal from aqueous solution as: i) surface complexation, ii) surface precipitation, iii) electrostatic interaction, iv) physical sorption, and v) ionic exchange.

Secondly, the changes in soil pH can significantly affect metal species distribution and, as a result, modify metal sorption. Below pH 6.0–6.1 (NA soil), TEs such as Cd, Cu, and Zn present in soil solution at different forms including not only Cd^{2+}, Cu^{2+} and Zn^{2+}, but also Cd(s), Cu[+], Cu(s), $Cu(OH)_2$(s), and $Zn(OH)_2$aq forms (Takeno, 2005). The changes in soil pH may also enhance the overall negative charge of the soil clay minerals that provoke heavy metals absorption onto soil particles surface (Filep, 1999). Many previous studies have indicated that biochar and wood ash have a potential to increase soil pH due to their high alkalinity (Demeyer et al., 2001; Xu et al., 2016). Our research has also confirmed these results.

In the case of wood ash, the pH was influenced largerly by the dissolution of several oxides, hydroxides, carbonates, and bicarbonates contained in WA (Etiegni et al., 1991; Vassilev et al., 2013). As for biochar and humic substances, the general alkalinity is related to several forms of alkalinities: i) the low-pKa structural, ii) other organic, iii) carbonate, and iv) other inorganic alkalinities (Fidel et al., 2017). Chen et al. (2015) demonstrated that the acid/base group dissociation, mainly BC carboxyl groups, is responsible for proton uptake/release or hydroxyl uptake at different pH levels. Moreover, the authors determined the pKa value of the carboxyl groups for high temperature produced biochar at 6.47. According to McBride and Blasiak (1979), clay surfaces are responsible for strongly pH-dependent adsorption of Zn hydroxide. The reason for the lowest Zn concentrations in case of WA treatments is likely bought about by adding Fe- and Ca-carbonates by wood ash. Sorption of Zn on carbonates, Fe-oxides or precipitation as Zn-carbonates or Ca-zincate could occur in these treatments (Adriano, 2001).

Thirdly, applying the amendments provided the considerable increase of TOC in the soil (Table 3), yet no significant correlation between TE and ΔTOC value were found (Table 2). This fact suggests that the quality, not the quantity, of the applied C is the most important factor affecting the availability of TEs. Differences in Cu plant-availability reduction between wood ash, biochar, and humic product can probably be attributed to their chemical properties, in particular O/C and H/C atomic ratios (Table 1) determining their complexation and sorption potential. Atomic H/C ratio of tested BC (Table 1) clearly indicates a high pyrolysis temperature (>750 °C) and the preferable content of aromatic clusters (Xiao et al., 2016). On the contrary, tested HS contain relatively balanced amounts of aliphatic, carbohydrate, aromatic, and phenolic carbon (Novák et al., 2015). This fact is in line with the observed effect of HS on the Cu immobilization (Fig. 3 H, L,M). The mechanisms involved in decreasing the Cu content at pH 6.0–7.0 could be the surface-active properties of HS (Kulikowska et al., 2015). For example, Cu forms complexes with low molecular weight organic components from humic and fulvic acids of HS. Town et al. (2012) showed that the rate of complex formation between HS and Cu (II) is governed by both the diffusive supply of hydrated Cu ion to the HS particle and the formation of the inner-sphere complex. The authors stated that with decreasing ionic strength, the rate of inner-sphere complex formation is greatly accelerated and the reaction is limited by a

diffusive supply of metal ions. Almost all BC + HS combinations were found to exert the same effect on Cu concentrations as BC alone. The significant difference was found (Fig. 3H–M, Supplementary table A) at pH 7 treatments (BC 5.0% versus BC 0.5% + HS 0.5%) only after 60 days.

4.2. The effect of the amendments on soil microbial biomass and metabolic quotient

The benefits of carbon-rich amendments applications to the soil for the microbial biomass in short term studies are well documented in the literature and are usually associated with several points: (i) the direct immobilization of trace metals content with the presence of amendments induced microbial growth; (ii) labile C in biochar or humic substances could be an important driver (factor) for microbial growth as a microbial substrate for short periods of time (Ameloot et al., 2013); (iii) the porous structure of added materials may potentially provide a suitable habitat for microbes to grow (Warnock et al., 2007). In this study, higher microbial biomass, significant variations in soil respiration and consequently reductions in metabolic quotient were found under humic substances treatments or with minor addition of HS into BC or WA. The significant increase of Cmic in the HS amended soils may have been triggered by the amplified availability of substrate-C, which stimulates microbial growth, or the stimulation of microbial activity through additions of labile nutrients. Yet, a direct effect from microorganisms contained in HS is also possible (Ros et al., 2006). Notably, the application of that biochar (0.5 and 5%) markedly decreased the Cmic in the studied soil. This may be an example of "negative priming effect", possibly due to either the physical protection of soil particles from microbial degradation (Cely et al., 2014) or the ability of biochar to chemisorb CO_2, leading to underestimated microbial C mineralization rates (Ameloot et al., 2013). The data obtained in our study also suggests that the HS-containing amendments profoundly stimulated the heterotrophic microorganisms in the soil and consequently improved the Cmic value for WA and BC applications even after 60 d (WA and BC 0.5).

The metabolic quotient, i.e. the ratio of BR and Cmic, is inversely related to the efficiency, with which the microbial biomass consumes indigenous substrates (Anderson and Domsch, 1990). It has been widely used as a sensitive indicator for revealing heavy metal toxicity under natural conditions (Wardle and Ghani, 1995). Anderson (2003) identified the qCO_2 critical range between 0.5 and 2.0 μg CO_2–C mg Cmic $^{-1}$ h^{-1} for neutral soils, and Leita et al. (1995) reported higher qCO_2 in metal-contaminated soil than in the uncontaminated one. As we expected, the NA soil in our study is characterized by high value of qCO_2 4.25–4.45 μg CO_2–C mg Cmic $^{-1}$ h^{-1}, which could account for multi-pollution. Lower metabolic quotient found in humic substances treated soils suggested that the microorganisms in the HS-amended soils likely produced more cell mass per unit of C degraded than those in non-amended soil. It also indicated that even a small addition of HS into biochar or wood ash act as a better protection of microorganisms from disturbance or stress.

4.3. Changes in ecotoxicity

A battery of simple, rapid, and cost-effective soil and aquatic bioassays was performed in order to evaluate possible short-term soil remediation effects of the applied amendments. Our results indicated that a small addition of humic substances into biochar and wood ash successfully mitigated soil toxicity. The *Daphnia magna* toxicity index was mainly controlled by pH and Zn content (Table 2). The PCA analyses proved this fact: treatments with WA (WA 6.0, and WA 8.4, WA 2.8 + HS 0.5) and treatments with BC (BC 5, BC 0.5 + HS 0.5) were found more shifted towards the left along with F1 in comparison with all the other treatments. It also indicated that these variants had the most prominent impact on chemical properties: they reduced the mobility of trace elements (especially Zn and Cd), increased the soil pH, and decreased the soil ecotoxicity for *D. magna*. Detrimental effects of those treatments on *D. magna* could be explained through account for the changes in trace metals content (Teodorovic et al., 2009); significant correlations (Pearson test, $p < 0.05$) between pH and Zn, Cd in the soils were observed in this work (Table 2).

Results of phytotoxicity tests exhibited an opposite trend and minor addition of humic substances into WA or BC treatments came to the fore. According to the PCA results the mixture treatments (WA 6 + HS 0.5, WA 2.8 + HS 0.05, BC 0.5 + HS 0.5, BC 0.5 + HS 0.05) led to the shifts along F2 and were preferable in comparison to independent treatments in the point of reducing soil phytotoxicity (Fig. 5). This could be partly explained by the protective role of humic substances against the stress state of trace metals (Piccolo et al., 1992). These effects are usually associated with several factors: (i) HS can stimulate H + -ATPase of the root plasma membrane (Nardi et al., 1991); (ii) HS promote the nutrient uptake (Nardi et al., 2002); or (iii) they change carbon and nitrogen metabolism of a plant. However, the concentration of Zn, Cd, and Cu in the soils did not provide a clear pattern on mustard root length in the present work. Yet, other works reported a greater sensitivity of phytotest than the other bioassays like earthworm acute toxicity test (Rodriguez-Ruiz et al., 2014).

5. Conclusion

In light of the results obtained we conclude that adding biochar, wood ash, and humic substances resulted in significant changes in the soil chemical properties and altered microbial community and ecotoxicity after incorporation in a multi-contaminated sandy loam soil. That was especially notable at higher application rates of wood ash, and biochar. Wood ash turned out to be more effective in decreasing Cd and Zn mobility in soil, while biochar was more suitable as a soil amendment for Cu-pollution. However, both materials induced a detrimental effect on biological functions of the soil. These effects were successfully mitigated by humic substances addition. Addition of humic substances restored soil fertility functions, including soil respiration and ecotoxicity parameters. Moreover, the mixtures of humic substances with wood ash or biochar substantially increased the immobilization efficiency (Cu, Cd, Zn) of amendments compared to wood ash or biochar applied alone. Toxicity towards the organisms used in the bioassays significantly correlated to the metal concentration in the soil and, partly, to pH values in soil. The most promising amendments in terms of favorable chemical, biological and ecotoxicological conditions are the mixtures of biochar plus humic substances and wood ash plus humic substances. Combination of these materials may represent a promising strategy to achieve high quality *in situ* stabilization for multi-contaminated soils with added economic value.

Conflict of interests

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/j.envpol.2017.06.021.

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4.2) Biochar, wood ash and humic substances mitigating trace elements stress in contaminated sandy loam soil: Evidence from an integrative approach

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Biochar, wood ash and humic substances mitigating trace elements stress in contaminated sandy loam soil: Evidence from an integrative approach

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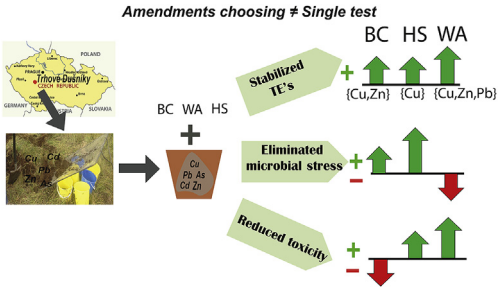
HIGHLIGHTS

- Wood ash is reliable to decrease the water- and weak acid-extractable Cu, Zn, and Pb soil content.
- Carbonaceous amendments decrease weak acid-extractable Cu, and Zn soil content.
- Wood ash depressed soil enzymes activity.
- Biochar addition may pose a risk to *Eisenia foetida* survival.
- Minor addition of humic substances creates favourable soil condition for enzymes and earthworms.

GRAPHICAL ABSTRACT



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ABSTRACT

We conducted a pot experiment with biochar (BC), wood ash (WA), and humic substances (HS) to investigate their effect on As, Zn, Cu, Cd and Pb mobility in soil, as well as enzyme activities involved in C-, N-, and P-cycles, and *Eisenia foetida* toxicity in multi-contaminated soils. Amendments were dosed to increase **epy** soil pH from initial 6.0 to ~6.5 and ~7.0. Applying amendments has revealed, that WA significantly immobilized Cu, Zn and Pb, BC – Cu and Zn, and HS decreased solely Cu mobility in soil. The partition indices of Zn, Cu, and Pb, quantitatively describing the bioavailable species of elements in soil, were the lowest for WA. Changes in the water-soluble species of metals were more pronounced than in the exchangeable ones for all amendments. An opposite effect was observed on enzyme activity and earthworm toxicity for the WA and carbonaceous amendments. The BC and HS provided favourable soil conditions to dehydrogenase, β-glucosidase, urease activity and fluorescein diacetate hydrolysis, while WA significantly decreased the activity of all the mentioned enzymes in soil. The results are supported by an enzymes-based weighted mean index, being the highest for BC and HS and the lowest for WA (lower than in the control sample). At the same time, WA was suitable to eliminate the trace elements' stress to earthworms (biomass endpoints and cocoons production). Our data revealed that each amendment has its own advantages and disadvantages. The choice of the most suitable amendment therefore should always be made within an integral approach and based on the purpose of remediation.

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1. Introduction

Trace element pollution of soils is the major concern threatening ecosystems, water bodies, food safety and human health. Therefore, favourable soil conditions are crucial, and the search for the most beneficial amendments is of great importance (Rao et al., 2017). Considerable attention has been paid to waste recycling materials, and several materials have been proposed for soil reclamation, such as wood ash (Demeyer et al., 2001), biochar (Kuppusamy et al., 2016), or humic substances (Perminova and Hatfield, 2005). The primary mechanisms of immobilizing by biochar (BC) in soils include alkalization, enhancement of ion exchange capacity and increment of physical sorption and precipitation (Beesley et al., 2015; Li et al., 2017). The beneficial properties of wood ash (WA) have been linked to its high alkalinity and nutrient concentration (Ca, Mg, P and K) (Mercl et al., 2016). The humic substances (HS) protective effect has been generally attributed to the formation of metal-humic complexes and presence of carboxyl, hydroxyl, and amino groups (Tan, 2014). Overall, the effect of carbonaceous soil amendments was recently reviewed by Ren et al. (2018). BC, WA, and HS having potential to restore the degraded or contaminated soils due to the abovementioned characteristics.

Environmental risk assessment of trace elements (TE) pollution is usually based on the analysis of their available concentrations in soil. However, the chemical analyses fail to reveal the complex interactions between the contaminants and soil environment; the formation of toxic intermediate metabolites or changes in TE mobility may increase soil toxicity during the remediation processes (Manzano et al., 2014). Consequently, the combined use of chemical analyses and biological assays is advantageous as it integrates the biological effects of all compounds present, taking into account the following factors: bioavailability, synergism, or antagonism (Stephenson et al., 2002; Fernández et al., 2005). Thus, the using the soil enzymes activities as screening tools to characterize contaminants in a variety of environmental matrices has become a popular, powerful and reliable tool in the environmental toxicology (Alkorta et al., 2003; Luo et al., 2017). Moreover, earthworms are generally used for toxicological tests as they are in direct contact with soil and are important in terrestrial food webs, soil productivity and fertility (Hirano and Tamae, 2011). In this study, we chose the *Eisenia foetida* earthworm species that is widely used as a model soil organism in research and governmental guidelines (Reinecke, 1992; Environment Canada, 2004).

Several studies reported negative or zero effects of BC, WA and HS on soil biological properties (Björk et al., 2010; Zhang et al., 2014). The reasons for this are diverse. Firstly, in many cases, these products are applied as recommended by manufacturers, sometimes with little or even no knowledge of the optimal rates, timing and methods of application. Secondly, the incorrect choice of product concentrations or disregarding the environmental aspects may contribute to a lack of response to amendments. Therefore, elucidating their specific selectivity to different types of pollution for remediation purposes is in high demand for soil studies of the new millennium. Our previous study (Pukalchik et al., 2017) focused on the effect of BC, WA, and HS amendments on the plant-available TE concentrations, microbial respiration and ecotoxicity (acute toxicity test with *Daphnia magna* and *Sinapis alba*) but the effect of the aforementioned amendments on soil enzymatic activities and soil habitat functions has not been previously evaluated.

The present study focusing to the relationships between water-soluble and exchangeable concentrations of Cu, Zn, Pb, Cd and As, soil enzymatic activities and earthworm's response in highly contaminated sandy loam soil amended with BC, WA, and HS. Hence, our study may establish the knowledge of soil-interlinked interactions required to manage and improve the waste material amendments used in soil reclamations.

2. Material and methods

2.1. Soils

The soil was sampled from the topsoil layer at the alluvium of the Litavka River in vicinity of the village of Trhové Dušníky (Czech Republic; 49°43′08.0″N 14°00′46.4″E). The history of excessive concentrations of TEs in the soil is associated with mining and smelting activities in this region (Vysloužilová et al., 2003; Vaněk et al., 2005). The physicochemical properties of experimental soil were: Clay 6%, Silt 49%, Sand 45%, pH 6.0 ± 0.1, TOC 3.60 ± 0.00%, CEC 149 ± 5.90 mmol kg^{-1}, P_{tot} 0.02 ± 0.00%, K_{tot} 0.25 ± 0.00%, Mg_{tot} 0.12 ± 0.00%, Ca_{tot} 0.14 ± 0.01%, S_{tot} 0.03 ± 0.00%, Fe_{tot} 11.76 ± 0.62%, Mn_{tot} 0.14 ± 0.00%, Zn_{tot} 7595.65 ± 194.76 ppm, Cd_{tot} 80.82 ± 4.58 ppm, Cu_{tot} 62.51 ± 6.45 ppm, Pb_{tot} 4346.13 ± 140.08 ppm, Ni_{tot} 5.61 ± 0.25 ppm, As_{tot} 177.54 ± 5.21 ppm.

2.2. Amendments and greenhouse experiment

Three materials, namely BC derived from wood chips gasification (150 kW/h gas and 300 kW/h heat production) at the temperature range 700–900 °C, WA which was collected from a fluidized bed reactor (15 MWt) for wood chips burning at a commercial biomass power plant, and commercial potassium HS Lignohumate (Amagro, Czech Republic) produced by alkaline extraction from lignin were used as soil amendments in this study. The elemental composition of BC, WA, and HS is shown in Table 1.

Amendments were applied to soil at different doses in order to achieve the equal pH values (from initial pH 6.0 in control treatment):

- pH~6.5 with the addition of the BC 0.5%, or HS 0.5%, or WA 2.8%;
- pH~7.0 with the addition of the BC 5%, or HS 1%, or WA 6%.

It should be noted, that each treatment induced changes in the initial total organic carbon concentration (TOC) in soil. The addition values for TOC with each treatment were: 4.4 g kg^{-1} with BC 0.5%, 1.7 g kg^{-1} with HS 0.5%, 2.4 g kg^{-1} with WA 2.8%, 44.1 g kg^{-1} with BC 5%, 3.3 g kg^{-1} with HS 1%, 5.1 g kg^{-1} with WA 6%.

Each portion of a 200 g air-dried soil sample was mixed with a dried amendment and placed into 0.5 L plastic pots (10 cm diameter and 15 cm height). The moisture content was maintained at 60% of the respective maximum water holding capacity (WHC) and kept constant by watering up to original weight every third day, which resulted in a maximum water loss of 5%. Pots were incubated uncovered at a temperature of 21/18 °C (day/night) in the natural light regime for 60 days. The non-amended (NA) and amended soils were collected after 30 and 60 days of incubation. At the end-point, each sample was properly mixed and divided into two parts: one part was stored at 4 °C for enzymes, and another part was dried at 105 °C for chemical analyses.

2.3. Chemical properties

The pH values of the non-amended and amended soils were measured in 0.01M $CaCl_2$ at a ratio of 1:2 (w/v) using WTW pH 340i meter with glass, ion-selective electrode (WTW, Weilheim, Germany).

The mobility of TEs (Cu, Zn, As, Cd, and Pb) was determined by means of sequential extraction procedure according to Száková et al. (1999):

Table 1
Chemical characteristic of amendments.

Characteristics	Amendment		
	BC	WA	HS
pH	8.9	11.2	9.0
N,%	0.44	0.00	0.25
C,%	88.20	8.50	33.47
H,%	0.82	0.16	3.73
S,%	0.19	0.33	4.84
O,%	6.49	4.79	17.72
Ash, total (%):	3.86	86.22	40.0
Na, %	<0.001	0.04	22.5
K,%	0.59	2.93	3.61
Mg, %	0.02	1.73	0.04
Al, %	0.04	2.84	0.01
Si, %	1.41	20.58	0.08
P, %	0.05	1.01	0.06
S, %	0.03	1.97	3.86
Ca, %	0.98	11.84	0.93
As, %	0.002	<0.001	<0.001
Zn,%	0.01	0.12	0.04
Cd, %	0.02	<0.001	0.01
Pb, %	<0.001	0.16	0.06
Cu, %	0.06	0.01	0.01
Other, %	0.64	42.99	8.79

All values are given on moist-free basis.

i) water-soluble fraction - 1 g of soil samples was mixed with 10 ml of demi-water and was shaken for 2 h at room temperature, the extract was separated from the solid by centrifugation (8000 g × 10 min), decanted into a polyethylene bottle and stored at 4 °C;
ii) exchangeable fraction - 40 ml of 0.11 M acetic acid (pH 2.0) was added to the residue and shaken for 16 h at room temperature, followed by the same procedure described for step *i.*

The TE's concentrations in the supernatants were determined by an inductively coupled plasma — optical emission spectrometer (ICP-OES, Agilent 720, Agilent Technologies Inc., USA). All plastic and glassware were acid-soaked overnight (5% HNO_3) and rinsed with distilled water before use.

The changes in the mobile species of trace elements in soil were evaluated using a modified partition index (PI) calculated according to Han et al. (2003):

$$PI_i = \frac{C_{i\ (water-soluble)} + C_{i\ (exchangeable)}}{C_{i\ (total\ )}} * 100\%$$

where *i* is the selected element (Cu, Zn, As, Cd, or Pb). C*i* (total) – the initial pdeuso-total concentration of TE in soil.

2.4. Soil enzyme activity

Dehydrogenase (EC 1.1) activity was evaluated according to Thalmann (1968) and (was) expressed as μg TPF g^{-1} soil 16 h^{-1}. The activities of acid phosphatase (EC 3.1.3.2) in the soil samples were assayed as outlined by Eivazi and Tabatabai (1977) and were expressed as μg pNP released g^{-1} dry soil h^{-1}. β-glucosidase activity (EC 3.2.1.21) was determined according to the procedure described by Dick et al. (1996). The Results are expressed as μg pNP released g^{-1} h^{-1} dry soil. The Urease activity (EC 3.5.1.5) was evaluated according to the method described by Klose and Tabatabai (2000) and (was) expressed as μg NH_4^+ g^{-1} soil 24 h^{-1}. The hydrolysis of fluorescein diacetate (EC 3.2.1.21) was determined by a modified procedure of Inbar et al. (1991) and expressed as μg FDA g^{-1} h^{-1} of soil. For all enzyme activities, the assays were performed in triplicate and were corrected for a blank.

Soil microbial activities were combined in to the weighted mean index (WMean) described by Lessard et al. (2014):

$$WMean = \sum_{i=1}^{n} w_i * y_i$$

where y_i - is the activity of i-enzyme, n - is the total number of soil enzymes, and w_i - is the 'weight' of each soil enzyme that was calculated as:

$$w_i = \frac{v_i}{\sum_{i=1}^{n} v_i}$$

being v_i the eigenvector for each soil enzyme activity associated with the first or second (depends on the data) principal component obtained from a PCA.

This index is regarded to be a reliable tool to integrate information from variables that possess different units that are featured by a range of variation indicator in a diversity of contaminated soils (Lessard et al., 2014; Sanchez-Hernandez et al., 2017). Compared with many other enzyme-based indices, WMean index is calculated according to the PCA-results and summarizes the 'weighted' values, so the relative importance for each response is evaluated objectively.

2.5. Earthworms bioassay

The *Eisenia foetida* mortality bioassay was carried out according to the Organisation for Economic Co-operation and Development (OECD) procedure (OECD 207/222). A homogeneous group of earthworms was acclimated for 2 weeks in 1 box with artificial soil (10% peat, 20% clay and 70% quartz sand, pH 6–7 adjusted with calcium carbonate) at 18 ± 1 °C, and 16:8 h light/dark regime. The earthworms were cleaned and kept in darkness for 24 h before use. After this acclimation every ten *E. foetida* earthworms (each organism weighing 0.2–0.5 g) were placed in an aluminium box containing 200 g of dry soil + amendment (70% WHC moisture) in four replicates. The container was covered by polyethylene material with dots in order to prevent evaporation. Approximately 2.5 g of food (oatmeal) with water war spread on the soil surface of each container every week. After 30 days of laboratory experiment, the earthworms were removed from the soil. The earthworms were cleaned from soil particles and the following variables were determined: survival rate (SR), individual biomass changes (IB). The number of cocoons was also counted after 60 days.

The survival rate (SR) was calculated as follows:

$$SR = \frac{Nadi}{Nad_{oecd}} * 100\%$$

where i – data for the samples (NA, or treatment), OECD – data for an artificial soil control, Nad – number of living adult earthworms after 30 days of exposure.

Individual biomass changes (%) were calculated as follows:

$$IB = \frac{IBi}{IB_{oecd}} * 100\%$$

where IBi – individual earthworms biomass in samples (NA, or treatment), IB_{OECD} – individual earthworms biomass in artificial soil control.

2.6. Statistical analyses

All treatments were conducted in 4 independent replicates. The analyses were iterated in 3 technical replicates for each sample and the mean value of these 3 was further used as a result of the measurement for each sample in further interpretation and statistical evaluation.

To test the effects of the experimental factors (type of amendment, dose of amendment, sampling time) in the variables analysed, a tree-way analysis of variance (ANOVA) with interactions was performed. In the case of significant F-tests, differences between group means were assessed by the Fisher's *post hoc* least significant difference test (LSD) with the significance level at $p < 0.05$. The variance homogeneity was verified by a graphical analysis of the residuals and no transformation was necessary. The correlation between characteristics was calculated using Pearson's rank correlation with the level of significance established at $p < 0.05$ by using Statistica 10.0 (StatSoft, Tulsa, OK). All graphs were prepared using SigmaPlot 12.5 (Systat, San Jose, CA).

Principal components analysis (PCA) was used for WMean index calculation and the component extraction was made by the covariance (n) matrix using XLSTAT-Ecology software.

3. Results

3.1. Amendments effect on soil chemical properties

In this study, the efficiency of the TE's immobilization was estimated on the basis of water-soluble and exchangeable species, and significant effects of BC, WA, and HS treatments on soil characteristics were revealed (Table 2). All treatments induced alkaline effect and changed As, Cu, Zn, Cd and Pb mobility (predominately in water-extractable species than in exchangeable ones). Moreover, the effects strongly depended on the type of amendment and the studied element (the results of the Factorial ANOVA test are presented in Supplementary Table A, ST A).

The pH_{CaCl2} values for all samples tested ranged from 6.47 to 7.18 in comparison with the non-amended control (pH 6.04–6.12) (Table 2). The BC 5%-treated soil exhibited a significant water-soluble Cu-immobilization effect in contrast with other amendments (Table 2; ST A), and in general, higher doses of amendments had more influence compared to lower doses at 30- days. The significant reduction in water-soluble and acid-extractable Cu concentration was found after exposure period of 30-days for higher doses of amendments.

Overall, BC demonstrated a weak influence on Zn-concentration (only slightly or insignificantly influenced). The only difference from NA was found in Zn water-soluble species after 60 days of exposure. HS seemed to have a prolonged effect on Zn immobilization as the significant reduction was found only after 60 days. The most intensive immobilization was determined in the case of WA. Generally, Zn concentration significantly and negatively correlated with the initial TOC and pH $_{CaCl2}$ values (Table 3).

Arsenic available concentration in soil was influenced by several factors: the type of amendment, treatment dose, time and pH-factor. The BC-treated soil was characterized by the lowest concentration of As (0.26–0.30 mg kg^{-1}), while HS and WA had a weaker impact on As compare to BC (Table 2; ST A). The immobilization effect also increased with the increasing doses of amendments and time of exposure, as well as the increased pH_{CaCl2} values which correlated with a decreased available As concentration in soil (Table 3).

All treatments significantly affected mobile Pb concentration in soil. The concentration of water-soluble Pb was the lowest after BC

Table 2
Leachability of trace elements from the polluted soil in the presence of amendments. The data represent Mean (mg kg^{-1}) ± SD (n = 4). Different letters denote significant differences at the 0.05 confidence level (Fisher's LSD) between treatments at the same exposure time.

Amendment	pH($CaCl_2$)		As			
	30d	60d	30 d		60 d	
			H_2O	CH_3COOH	H_2O	CH_3COOH
NA (control)	6.11 ± 0.05 a	6.10 ± 0.03 a	0.45 ± 0.06 a	1.61 ± 0.16a	0.34 ± 0.04 a	1.46 ± 0.06 a
BC 0.5	6.40 ± 0.10 b	6.41 ± 0.05 b	0.29 ± 0.01 b	1.30 ± 0.19 b	0.27 ± 0.03 b	1.21 ± 0.19 b
BC 5	6.80 ± 0.04 c	6.90 ± 0.05 c	0.30 ± 0.03 b	1.41 ± 0.15 ab	0.26 ± 0.04 b	1.25 ± 0.11 b
WA 2.8	6.50 ± 0.05 b	6.53 ± 0.13 b	0.45 ± 0.06 a	1.44 ± 0.22 ab	0.35 ± 0.06 a	1.31 ± 0.34 ab
WA 6.0	7.08 ± 0.03 c	7.15 ± 0.05 c	0.29 ± 0.01 b	1.34 ± 0.69 b	0.29 ± 0.01 ab	1.16 ± 0.13 b
HS 0.5	6.60 ± 0.01 b	6.55 ± 0.06 b	0.37 ± 0.06 ab	1.45 ± 0.23 ab	0.28 ± 0.02b	1.11 ± 0.23 b
HS 1	7.05 ± 0.06 c	7.10 ± 0.03 c	0.29 ± 0.01 b	1.53 ± 0.06 ab	0.28 ± 0.00b	1.21 ± 0.13 b

Amendment	30 d		60 d		30 d		60 d	
	H_2O	CH_3COOH	H_2O	CH_3COOH	H_2O	CH_3COOH	H_2O	CH_3COOH
	Zn				Pb			
NA (control)	8.04 ± 2.16 ab	1785.7 ± 161.6 a	7.01 ± 0.80 d	1511.0 ± 65.4 b	0.90 ± 0.50 c	95.1 ± 9.1 a	0.78 ± 0.11 d	86.0 ± 4.7 a
BC 0.5	5.72 ± 0.82 ab	1852.6 ± 223.6 a	4.58 ± 0.38 a	1422.9 ± 160.9 ab	0.31 ± 0.11 ab	92.4 ± 19.3 a	0.38 ± 0.02 c	82.3 ± 4.4 a
BC 5	4.40 ± 3.09 acd	1628.5 ± 80.5 a	3.01 ± 1.32 c	1379.5 ± 158.1 ab	0.20 ± 0.01 a	80.3 ± 9.1 a	0.18 ± 0.03 a	82.3 ± 2.4 a
WA 2.8	2.90 ± 1.44 cd	1418.1 ± 238.7 a	1.78 ± 0.06 bc	984.7 ± 78.4 d	0.49 ± 0.00 bc	67.8 ± 18.4 a	0.40 ± 0.01 c	55.4 ± 6.0 b
WA 6.0	1.62 ± 0.21 c	1576.9 ± 242.2 a	1.49 ± 0.10 b	838.1 ± 56.2 c	0.33 ± 0.15 ab	71.8 ± 24.8 a	0.29 ± 0.02 b	58.3 ± 1.6 b
HS 0.5	6.32 ± 1.47 ab	1551.6 ± 495.4 a	5.81 ± 0.60 ad	1380.8 ± 129.1 ab	0.25 ± 0.03 a	71.6 ± 32.2 a	0.27 ± 0.02 b	64.8 ± 2.8 c
HS 1	7.97 ± 2.96 b	1623.0 ± 108.8 a	5.03 ± 1.04 a	1277.1 ± 50.6 a	0.22 ± 0.05 a	80.9 ± 17.1 a	0.19 ± 0.00 a	65.1 ± 3.1 c
	Cd				Cu			
NA (control)	0.09 ± 0.02 a	14.8 ± 2.9 a	0.05 ± 0.00 a	15.2 ± 0.3 a	3.11 ± 0.34 a	1.59 ± 0.24 a	2.36 ± 0.26 a	
BC 0.5	0.07 ± 0.01 a	16.0 ± 1.4 a	0.05 ± 0.01 a	7.6 ± 1.1 b	2.41 ± 0.32 ab	0.21 ± 0.12 b	1.97 ± 0.21 a	
BC 5	0.06 ± 0.03 ab	13.5 ± 0.8 a	0.04 ± 0.01 a	7.6 ± 1.2 b	2.01 ± 0.18 b	0.25 ± 0.20 b	1.97 ± 0.33 a	
WA 2.8	0.04 ± 0.00 b	14.8 ± 1.8 a	0.05 ± 0.00 a	8.0 ± 0.6 b	1.97 ± 0.44 ab	0.15 ± 0.01 b	1.80 ± 0.13 a	
WA 6.0	0.06 ± 0.03 ab	12.1 ± 2.0 a	0.05 ± 0.01 a	7.4 ± 0.4 b	1.86 ± 0.43 b	0.11 ± 0.02 b	1.89 ± 0.08 a	
HS 0.5	0.08 ± 0.02 a	14.3 ± 2.7 a	0.05 ± 0.01 a	7.2 ± 0.2 b	2.23 ± 0.68 ab	0.29 ± 0.16 b	2.16 ± 0.16 a	
HS 1	0.08 ± 0.01 a	13.3 ± 4.8 a	0.04 ± 0.00 a	8.4 ± 1.1 b	2.15 ± 0.37 b	0.15 ± 0.01 b	2.04 ± 0.07 a	

AbbreviationH2O – the water-soluble species; CH3COOH – the exchangeable species of the elements.

Table 3
Pearson correlations matrix for soil variables, soil enzymes activity, and earthworms bioassay endpoints.

Variables		TOC	pH($CaCl_2$)	Soil enzyme activity					Earthworms		
				Dehydrogenase	Urease	Acid phosphatase	FDA	β-glucosidase	Survival rate	Individual biomass	Cocoons
TOC		**1**	**0.75**	**0.42**	0.11	**−0.55**	**0.50**	−0.15	−0.37	−0.23	−0,21
pH($CaCl_2$)		**0.75**	**1**	0.36	0.09	−0.17	0.36	−0.17	0.03	0.19	0,27
H2O	As	**−0.47**	−0.35	−0.49	−0.44	0.04	**−0.48**	−0.33	0.28	0.29	0,00
	Pb	**−0.66**	**−0.68**	−0.25	−0.06	0.26	**−0.61**	−0.08	0.03	−0.09	−0,29
	Zn	**−0.56**	**−0.67**	0.15	0.32	**0.55**	−0.17	0.33	−0.18	−0.37	−0,30
	Cu	**−0.61**	**−0.68**	−0.23	−0.02	0.28	**−0.56**	−0.11	−0.08	−0.20	−0,36
	Cd	**−0.57**	**−0.62**	0.00	0.21	0.38	−0.20	0.13	−0.14	−0.34	−0,21
CH3COOH	As	0.10	−0.07	0.01	−0.08	−0.13	−0.16	−0.03	−0.23	−0.13	−0,39
	Pb	**−0.40**	**−0.40**	0.14	0.17	0.36	0.02	0.32	−0.17	−0.16	−0,22
	Zn	**−0.42**	**−0.47**	−0.01	0.15	0.26	−0.13	0.18	−0.07	−0.15	−0,22
	Cu	**−0.52**	**−0.54**	0.02	0.26	0.34	−0.29	0.11	−0.03	−0.32	−0,22
	Cd	**−0.39**	−0.24	0.08	0.18	0.34	−0.02	0.21	0.08	−0.06	0,05

The values in bold mark significant correlation at the 0.05 level.

and HS amendments, while WA predominantly affected the exchangeable Pb species (Table 2). Increasing the doses of amendments had marked effects on Pb-concentration (ST A), and the pH_{CaCl2} and TOC concentration showed a negative correlation with Pb concentration in soil (Table 3).

Cd available concentration in soil had a slight trend to decrease water-soluble and exchangeable species with a presence of BC 5%, WA 6%, and HS 1% (Table 2). Overall, Cd immobilization was stronger with increasing the doses of amendments and time of exposure (ST A). WA had more impact than BC and HS.

A partition index indicates the percentage of trace element presented in the water-soluble and exchangeable species versus the pseudo-total concentration. The decrease in the partition index demonstrates that the chemical species of the element are changed to less available form (Fig. 1). All the amendments were demonstrated to be able to immobilize trace elements but with different extent. BC treatments influenced preferably Zn and Cu mobility (Fig. 1A, E). WA proved to be more effective in reducing Zn and Cu in both treatment doses, and decreased Pb mobility with higher application dose (WA 6) (−25.7%) (Fig. 1 A, B, E). HS decreased Cu and Zn concentration and a slight decrease was found also for Pb (Fig. 1 A, B, E). It should be noted that we observed a trend to decrease As mobility only with 0.5% HS treatment (Fig. 1 C).

3.2. Effects in the enzymes activity

Dehydrogenase activities tend to increase with the elevated doses of all amendments (Fig. 2A; ST B) and according to the elevated level of initial TOC values (Table 3), but the absolute values of DHA activity were decreased over time (60 days). BC and HS-treated soils supported dehydrogenase activity, while WA had slightly negative or no effect on dehydrogenase. Generally, the dehydrogenase activity increased by 45–80% after 30 days, and 57–240% after 60-days in BC and HS treatments and no effect was

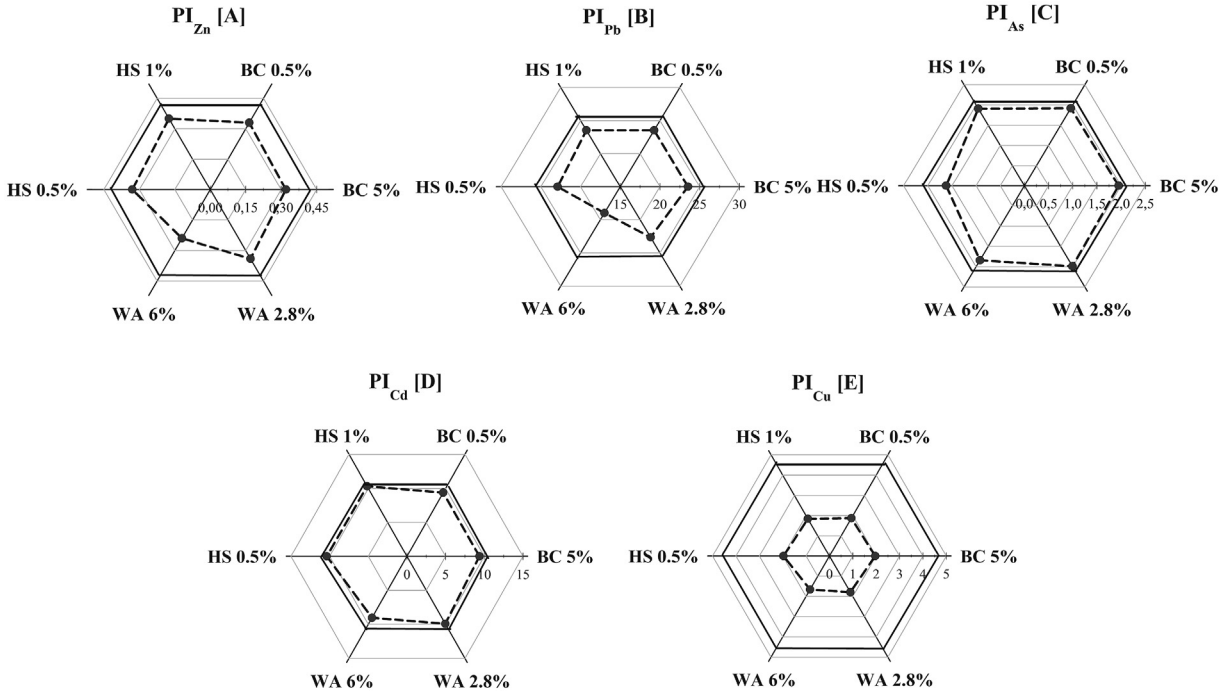

Fig. 1. Changes in the mobility of trace elements in soil according to the Zn (A), Pb (B), As (C), Cd(D), Cu (E) Partition index with a presence of different amendments at the end of experiment. The partition index for NA shows in black solid line, for each element – in medium dash line.

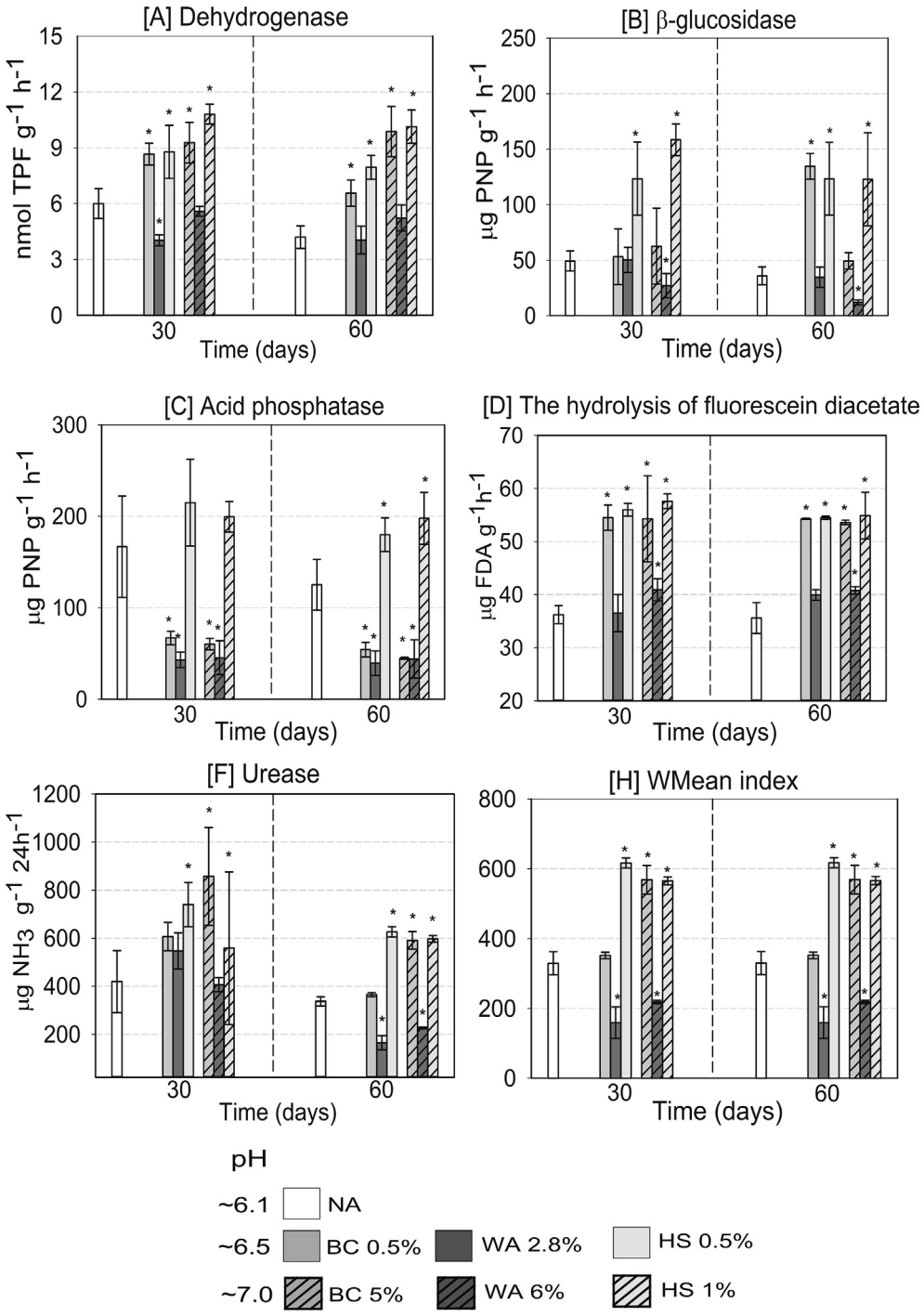

Fig. 2. Enzyme activities (A–F) and summarized Weighted Mean index (H) in soil with varying doses of amendments (Mean ± SD, n = 4) after 30, and 60-days of exposure. The * highlights significant differences in treatments compared to control (NA) (Fisher's LSD; $p < 0.05$).

found in case of WA after 60 days.

BC and HS additions have also a positive impact on the β-glucosidase activity (Fig. 2B; ST B). The effect of HS was well expressed in both doses at both incubation periods while BC showed significant improvement of β-glucosidase only in the lower dose after 60 days. Generally, the influence of amendments was more expressed in 0.5% BC and 0.5% HS treatments than in higher doses. The activity of β-glucosidase also decreased over time through during the experiment. WA demonstrated a trend to decrease the β-glucosidase activity. Moreover, a higher application dose of WA led to a significantly inhibition of β-glucosidase (lowest values 4.21 ± 0.21 μg pNP g^{-1} dry soil h^{-1} after 60 days).

The quantification of acid phosphatases activity revealed a significant promotion by HS-treatments at 60 days (Fig. 2C; ST B), while the other types of amendments had significant inhibitory effects when compared with non-amended soil. The acid phosphatases activity slightly decreased with increasing the exposure time, at HS treatments (only at 60 days).

The hydrolysis of fluorescein diacetate in soil treated with carbonaceous amendments (BC and HS) markedly improved by

both application doses at both sampling periods, while the positive effect of WA was determined only in case of higher application dose (Fig. 2D; ST B).

The urease soil activity dramatically increased with BC and HS additions (Fig. 2F; ST B), and the effect was intensified with an elevated application dose. WA-treated soils were characterized by no effect (30 days) or significant decrease (60 days) in activity of these enzymes. During the experiment, the values of urease activities significantly decreased in all treatments over time.

Finally, according to the calculated Weighted Mean index (WMean), BC and HS amendments triggered a marked improvement of soil microbial conditions, while WA treatments had a trend to depress microbial activity despite the highest trace element immobilization efficiency (Fig. 2E).

3.3. Earthworms responses

The earthworm mass decreased by 40% in NA treatment after 30-days of exposure in comparison with OECD artificial soil. The average number of cocoons per 10 earthworms in NA soil was 21 as compared with 53 in the OECD soil, indicating a considerable effect of adverse soil conditions on cocoon production (Fig. 3C). Similarly, a lower dose of BC and HS resulted in 40–50% body mass decrease (no significant difference from NA) (Fig. 3A). Surprisingly, applying a higher dose of BC 5% led to annihilated of 100% earthworms with no cocoons detectable after 60 days (Fig. 3C). Contrarily, the application of both WA doses resulted in a significant increase of earthworm's body mass (Fig. 3A), 100% survival rate (Fig. 3B) and the higher dose of WA also led to slightly higher number of cocoons compared to OECD control (Fig. 3C).

4. Discussion

4.1. Mobility of trace elements

Many types of amendments have been reported as effective supplements to remediate the contaminated sites. The multi-contaminated soil near the Trhové Dušníky was also previously studied with regards to the use of different materials for soil recovery. For example, lime was proved to reduce Cd and Zn mobility, yet turned to be ineffective to reduce Pb and As mobility (Vondráčková et al., 2013). Digestate and fly ash were observed as successful amendments for Cd, Zn and Pb immobilization (Garcia-Sanchez et al., 2015). The WA, BC, and HS also have been effective to reduce Cu, Zn and Cd plant-available concentration in soil (Pukalchik et al., 2017). In this study, we focused on the effects of BC, WA, and HS to the bind forms of Cu, Zn, As, Cd and Pb which may equilibrate with the aqueous phase and acetic acid, as those are considered to be rapidly bioavailable (Seguin et al., 2004; Kabata-Pendias, 2011). The soil water extraction is suitable for evaluating metal concentrations at a pseudo equilibrium in the soil solution (Meers et al., 2007).

The results demonstrated different potential of BC, WA, and HS to short-term reduction of water-soluble and exchangeable forms of TEs in soil. It was observed that BC (0.5 and 5%) evoked marked decrease in Cu, and had a tendency to decrease Zn available concentrations; WA-treatments (2.8 and 6%) had strong influence on the reduction of Zn, Pb and Cu mobile soil concentration, while HS (0.5 and 1%) primary changed Cu soluble concentration in polluted soils.

The selectivity of these amendments to reduce mobile fractions of TEs were largely expected. More important findings demonstrate different mechanisms of remediation efficiency of three tested amendments. The BC addition affected Cu and Zn concentration, while a decrease in other elements was negligible. It may be linked with formation of metal hydroxides, oxides, carbonates, and phosphate precipitates and with the activation of surfaces caused by the increase in pH (Uchimiya et al., 2011). Han et al. (2017) investigated the competitive adsorption of Pb and Cd by biochars produced from 12 sources, indicating that the Pb adsorption process was inhibited in the mixed solutions with Cd. This competition also was obtained in other binary systems such as Cu and Zn (Chen

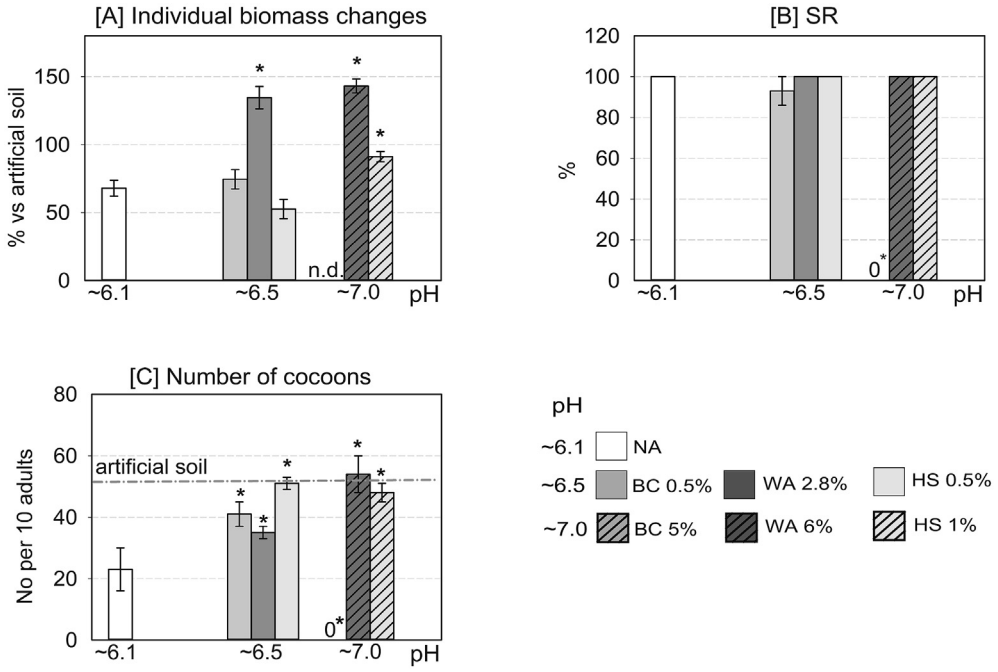

Fig. 3. Individual biomass changes (A), survival rate (B), and number of cocoons production (C) of *Eisenia foetida* after exposure in soils with different amendments (Mean ± SE, n = 4). The * highlights significant differences in treatments compared to control (NA) (Fisher's LSD; $p < 0.05$); n. d. – data not detected.

et al., 2011), and multi-metals system (Park et al., 2015). Those results may imply that metals could be adsorbed by the same sites and functional groups, while competing with other metals to produce reciprocal inhibition, and there are different mechanisms and bindings for various metals (Chen et al., 2011).

HS primary changed Cu concentration in contaminated soil. As all heterogeneous macromolecules, HS contains multifunctional groups, among them the carboxylic and phenolic−OH, which are known to be the most important in forming the negative charges of humic substances for metal complexation (Wang et al., 2010; Shan et al., 2015). The dissociation constant (pKa) for COOH are 3.0 and 10.0 for phenolic−OH (Tan, 2014), implying that the phenolic−OH would be mainly protonated under this liming condition (pH 6.5–7.0), whereas COOH would be deprotonated to an effective complex with positively charged metals. It also has been reported that the sorption of Cu ions on HS is mainly through the formation of five-membered chelate rings via ion exchange and surface complexation, respectively (Karlsson et al., 2006).

Finally, adding WA was more effective than BC and HS treatments in removing Cu, Pb and Zn from the available pool of contaminated soils,. This effect may be attributed to high soluble concentrations of carbonates and phosphates in wood ash, and the main mechanism for metals sorption was suggested as the formation of precipitates, such as $ZnCO_3$, $Zn_5(CO_3)_2(OH)_6$ (Voegelin et al., 2005), $Pb_5(PO_4)_3OH$ (Cao et al., 2011), and $Cu(OH)^+$ (Chirenje et al., 2006).

According to Masscheleyn et al. (1991), the arsenic is normally enhancing solubility in soil with alkaline pH. In particular, the soil liming above 6.5 induced changes in As mineral saturation from As^0, H_3AsO_3 to $HAsO^{I-}$. In line with this, the addition of alkaline amendments should increase the As water-soluble and exchangeable species in soil. However, our data revealed quite the opposite effect: partition index for As did not significantly change, or slightly decreased at the background of amendments (Fig. 1). Thus, we can conclude that all tested amendments successfully neutralised the As mobility in soil. Similar effect was previously reported by Vandecasteele et al. (2002), and immobilization of As in contaminated soils with a presence of fly ash was linked with the formation of $Ca_3(AsO_4)_2$ and $CaHAsO_3$.

The decrease of mobile TEs concentration may be also attributed to the increasing soil pH_{CaCl2} value, significant negative correlation at $p < 0.05$ between water-soluble As, Pb, Zn, Cu, Cd, exchangeable Pb, Zn, Cu, Cd were observed (Table 3). At the same time, the applied amendments showed a considerable increase of TOC in the soil, so this effect may be linked with changes in carbon concentration.

4.2. Soil enzyme activities

The hydrolytic enzymes determined in our study provide an index of the potential to mineralize C (β-glucosidase, dehydrogenase), and the nutrients N (urease), P (phosphatase), which can be used as early indication of changes in soil quality (Ndiaye et al., 2000; Tejada et al., 2006). Fluorescein diacetate hydrolysis activities are related to the overall microbial activity, and have been investigated to determine the amounts of active fungi and bacteria, and hence may considerably affect the soil organic matter dynamics (Lundgren, 1981; Aseri and Tarafdar, 2006).

This study has shown that short-term WA exposure caused a significant decrease in the activity of dehydrogenase, acid phosphatase and β-glucosidase, as well as in soil microbial activity as indicated by the reduced FDA activities. This fact is in line with the observed effect of WA on the WMean index (Fig. 2E). Despite the data of WA impact on soil enzymatic activity are scarce, our results are consistent with those of other studies (Perucci et al., 2006; Björk et al., 2010). Perucci et al. (2008) showed that the inhibitory effect of wood ash on soil enzymatic activities lasts shorter (up to 12 months) and that its application does not result in long-term changes of enzymatic activities in field conditions. A recent study also demonstrated that the WA can decrease the ratio of fungi to bacteria in the soil microbial community (Noyce et al., 2016).

Application of BC has been reported to influence C and N mineralization in soil, and could increase the activity of specific enzymes related to C, N utilization (Bailey et al., 2011; Yang et al., 2016), and our data supported this fact. BC positively affected urease, β-glucosidase activities compared to non-amended soil. The β-glucosidase catalyses the last step of cellulose hydrolysis and release of glucose as energy source for the microorganisms. Its potential activity is associated with carbon substrate availability, so our data suggested that BC and HS might affect the soil community probably through increasing initial TOC rather than reducing the TE's mobility. The acid phosphatase activity decreased significantly with the application of BC; similar effects were previously observed with 5% bamboo BC (Yang et al., 2016), and BC from *Parthenium hysterophorus* (Kumar et al., 2013). Dehydrogenase and fluorescein diacetate hydrolysis activities both were significantly higher in BC soil than in NA soil. Possibly, it can be explained by the stimulation of a specialized subset of the microbial community by the biochar or growth of biomass in response to initially labile C (Bailey et al., 2011).

The results of the experiments revealed a stimulating effect of HS on the enzymes activity, which is not surprising if we consider its positive influence on microbial populations. It is noteworthy, that HS treatments differed from WA and BC treatments in promoting the acid phosphatase activities. A higher acid phosphatase activity in samples with HS are opposite to the fact that phosphomonoesterase is an enzyme which is very sensitive to changes in soil pH (Dick et al., 2000), and the optimum pH for the activity of acid phosphatase ranges from 4.0 to 6.5 (Wittmann et al., 2004). It has to be noted that in our experiment, we found no significant differences between treatments in activity of alkaline phosphatase and therefore, these data are not shown. According to Nannipieri et al. (2011), a linear relationship is commonly observed between the activity of acid phosphatases and the amount of phosphorus in soil solution, which could be provided from humic products. Tikhonov et al. (2010) observed that some fractions of HS are labile and can provide a ready-to-use substrate for microbial populations. A similar trend with fluorescein diacetate hydrolysis activities stimulation was previously pointed with HS from industrially mined raised bog peat in sandy loam soil spiked with a complex contamination (Muter et al., 2015).

Generally, the enzyme activity correlated with the TOC in soil (Ekenler and Tabatabai, 2003; Darby et al., 2006). We found positive and significant correlations of the initial added amount of TOC with dehydrogenase activity, and fluorescein diacetate hydrolysis activities (Table 3), however, there was no relationship with β-glucosidase, urease and acid phosphatase. This fact may indicate that only extracellular enzymes are fixed in the soil matrix by interacting with organic carbon. Moreover, the fluorescein diacetate hydrolysis activities was the only enzyme activity, which negatively correlated with all the measured water-soluble As, Cu and Pb concentration, while dehydrogenase and urease was negatively affected only by water-soluble As (Table 3). These results are opposite to Pan and Yu's conclusions (2011), who reported that the metals such as Cu, Cd and Pb induced marked inhibition of dehydrogenase activity. Our results suggested that fluorescein diacetate hydrolysis activities were the best representatives to be used as a biological indicator for soil recovery as compared to all other enzymes tested. This data also confirms a direct inhibitory effect of the most labile (water-soluble) species of metals on the overall microbial activity.

Applying the integrated enzymes-based index provides comprehensive information about the biological effects of contamination and may therefore serve as a useful tool for environmental managers (Paz-Ferreiro and Fu, 2016). Among them, the Wmean index has been satisfactorily validated in the assessment of metal-contaminated soils (Lessard et al., 2014) and pesticide-contaminated soils (Sanchez-Hernandez et al., 2017). To the best of our knowledge, this study is the first to use WMean index to assess soil health status based on soil microbial activities during the remediation. The highest improvements were obtained both in BC and HS treatments, while WA depressed the soil microbial community (Fig. 2E).

4.3. Earthworms survival

Earthworms are known for their sensitivity to toxic chemicals present in contaminated soils and hence have been widely used as indicator organisms for ecotoxicity studies. They can pose a risk to higher trophic levels that feed upon them (Suthar and Singh, 2009). However, the earthworm survival in such stress conditions - like contaminated soils - depends upon several physicochemical factors such as soil texture, pH_{CaCl2} of the medium, organic matter, nature and extent of the clay minerals (Maity et al., 2008). In our study, the TE's stress was probably to cause a significant body weight reduction in non-amended soil whereas WA (2.8% and 6%) and HS 1% showed the opposite as compared to NA soil. The decrease in body weight observed in control (NA) earthworms may account for the lower availability of food resources as the TOC was much lower than in amended soil. We also observed the 100% mortality of adult earthworms in the BC 5% treatment during the first 5 days of the experiment, whereas no acute mortality occurred in the control soil and other treatments. Toxicity of BC for earthworms was previously detected by Li et al. (2011), soil amended with 10% and 20% apple wood BC induced significant reduction in worm's body-mass during 28d test due to changes in soil water holding capacity. Liesch et al. (2010) observed higher earthworm death rate and weight loss in 67.5 t/ha poultry litter BC amended soil, due to the rapid increment in soil pH or excessive salinization. Earthworms in arable soil treated with a pruce chip BC (30 t/ha) tended to avoidans biochar after 2-week of exposure due to a decline in soil water potential (Tammeorg et al., 2014). Similarly, Malev et al. (2016) observed that BC application at the rate of 100 t/ha could cause damage to earthworm, with survival rates decreasing to 78% in clay soil and 64% in sandy soil.

4.4. Integrative approach

In our study, the mixtures of TE's in multi-contaminated soils interacted with contrasting amendments and induced the magnitude of effects in chemical, biological and ecotoxicity properties of soil. The generalized data for biological and ecotoxicity responses in presence of WA, HS, and BC from this study and the previous one (Pukalchik et al., 2017) are shown at Table 4.

We could suggest that each type and dose of amendment has unique advantages and disadvantage, making it very hard to choose the 'master key'- the universal variant that would miraculously solve a plethora of problems facing the soil protection today. We detected the adverse effect of WA, BC and HS on soil biological and ecotoxicity properties. BC 5% were harmful for earthworms, while WA application supported earthworms reproduction and biomass but depressed enzymes activity. Overall, the WA 6% and BC 5% had the greatest number of negative effects among all treatments, while WA 2.8%, BC 0.5%, HS 0.5% and HS 1% promoted good (or normal) microbial performance and created suitable conditions to the organisms assayed (at least has no harmful effect). Our data proved an idea that integral approach for soil assessment coupled with chemical measuring, biological and biological tests is crucial for monitoring the contaminated sited, and choosing of the best management practice option for soil remediation (Lors et al., 2010; Gonzalez et al., 2011).

Table 4
Summary table of effects observed in each biological parameter in soils with a presence of amendments.

Parameter	BC		WA		HS	
	0.5%	5%	2.8%	6%	0.5%	1%
Sinapis alba, root length[a]	▲	▲	●	▲	▲	▲
Daphnia magna, toxicity[a]	●	▼	●	▼	●	●
Basal respiration[a]	●	▼	▼	▼	●	●
Microbial biomass carbon, C_{mic}[a]	▼	▼	▼	▼	▲	▲
Microbial quotient, qCO_2[a]	▲	●	●	▼	▼	▼
Dehydrogenase activity	▲	▲	●	●	▲	▲
β-glucosidase activity	▲	●	●	▼	▲	▲
Acid phosphatase activity	▼	▼	▼	▼	▲	▲
Urease activity	●	▲	▼	▼	▲	▲
FDA activity	▲	▲	●	▲	▲	▲
Eisenia foetida, body weight	●	n.d.	▲	▲	●	▲
Eisenia foetida, No living earthworms	●	▼	●	●	●	●
Eisenia foetida, No cocoons	▲	n.d	▲	▲	▲	▲

Symbols ▲and ▼ stand for a corresponding decrease or increase of the parameter relatively to NA soil. Symbol ● is corresponding to zero-effect. Underlined symbols (_) represent changes in parameters that are considered deleterious. n.d. – not detected.

[a] For the data see Pukalchik et al. (2017).

5. Conclusions

The results of amendments' application to affect TE's mobility, along the changes in soil enzymes activities and ecotoxicity to earthworms, demonstrate that adding BC, WA or HS at relatively low doses can restore the quality of multi-contaminated soils. Moreover, high earthworm mortality occurred in BC 5% treatments, and significant depression in enzyme activity was detected in amended WA 6% soil compared to the non-amended (contaminated) samples.

This case study provides evidence, that an incorrect choice of amendment dose can exacerbate soil toxicity despite of immobilizing the trace metals. However, our results should be very carefully generalized as all the amendment materials tested, particularly BC and HS, may drastically vary in their structure and composition between the particular sources and therefore, their impact on soil properties may vary as well. The major advantage of the proposed approach is in drawing a multidimensional picture with most important variables taken into account. Thus, it should be neither overlooked nor dismissed as it brings the added value for sustainability and ecologically friendly remediation management.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at

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4.3) Influence of Rhizon MOM suction cup and *Triticum aestivum* L. on the concentration of organic and inorganic anions in soil solution

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SOILS, SEC 3 • REMEDIATION AND MANAGEMENT OF CONTAMINATED OR DEGRADED LANDS • SHORT ORIGINAL COMMUNICATION

Influence of Rhizon MOM suction cup and *Triticum aestivum* L. on the concentration of organic and inorganic anions in soil solution

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Abstract
Purpose Rhizon suction cups are widely used for soil water sampling. However, available literature about the variability and stability of organic and inorganic anions in solution extracted by Rhizon suction cup is very limited. The main purpose of this study was to examine the Rhizon MOM sampler's direct influence on soil solution composition.
Materials and methods In this study, two experimental approaches were used. In recovery test, reference solution containing organic and inorganic anions (NO_3^-, SO_4^{2-}, PO_4^{3-}, lactate, acetate, formate, propionate, pyruvate, malate, oxalate and citrate) was sampled using the Rhizon MOM samplers. In pot experiment, model plants (*Triticum aestivum* L.) were grown in outdoor precipitation-controlled vegetation hall, soil solution was sampled at major growth stages and at distinct day time. The influence of plants and different time scales on soil solution composition was assessed. The relative microbial degradability (stability test) of collected solution was examined by the addition of microbial inhibitor at the end of vegetation period. Ion-exchange chromatography was used for the analysis of solution samples.
Results and discussion Recovery test revealed strong influence of Rhizon on Cl^- and lactate anions. Increased concentrations of these anions in reference solution were found probably due to their initial leaching from the sampler. Significant decrease caused by the Rhizon sampler was found for acetate, propionate and formate. However, recovery rates of these mono-carboxylates ranged from 98 to 93 %. The variability in soil solution composition was found between weeks, not within 1 day (no significant differences between morning and evening sampling). However, organic anions detectable in soil solution on regular basis were acetate, lactate, formate, oxalate and pyruvate, implying further need for sample concentration increase. Stability test of collected solution revealed an accumulation of NO_2^- and formate. On the contrary, lactate, acetate and Cl^- concentrations were partly decreased after 24 h of incubation. After 1 h of incubation, no significant differences were found.
Conclusions Rhizon MOM samplers seem to show good performance for the sampling of di- and tri-carboxylates (average recovery rates 100 ± 2 %) as well as NO_3^-, SO_4^{2-} and PO_4^{3-} in solution. Collected soil solution should be treated to prevent microbial degradation within 1 h after collection. The composition of soil solution, in heavily rooted soil-plant system of *T. aestivum* L., did not vary in 24 h, but over longer period. Sampling of soil solution by described procedure may provide useful insight into temporal variation of soil processes.

Keywords Inorganic anions · Low-molecular-weight organic acids · Soil solution · Spring wheat · Suction cup

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1 Introduction

Knowledge of soil solution composition is of great importance for many studies in, e.g. plant nutrition and prediction of nutrient fluxes from soil to plants (Wang et al. 2013), biogeochemical cycles of elements (Dold et al. 2005) or geochemical modelling of element speciation (Appelo and Postma 2005).

Low molecular mass organic acids (LMMOAs) represent labile sources of C in soils, and once released into the soil solution, they may be quickly taken up and broken down by the soil microbial community. The occurrence of many LMMOAs in soil is related to the citric acid cycle (the Krebs cycle), e.g. oxalic acid is a by-product of the hydrolysis of oxaloacetate (Plassard and Fransson 2009). Some other LMMOAs, such as lactate or acetate, may represent rather stable fermentation end-products of the soil microbe metabolism. LMMOAs can play important role in many processes. Castaldelli et al. (2013) showed acetate as best predictor for denitrification in soils. Ma et al. (2008) showed crucial role of soil formate in the regulation of nitrous oxide emission from soil fungi. Dold et al. (2005) described a crucial role of di- and mono-carboxylates (e.g. oxalate, formate) in chemistry of acid mine drainage from sulfidic mine waste. However, LMMOAs half-life in soils varies largely depending on types of acid and soil (Oburger et al. 2009; Jones and Darrah 1994). Because of LMMOAs relative lability and spatial variability in soil, appropriate sampling techniques are needed.

Three different approaches are in use for soil solution sampling, according to Strobel (2001): (i) centrifugation, (ii) extraction and (iii) lysimeter/suction cup application. Due to the destructive sampling, a centrifuged sample can be conducted only once at a specific spot. This prevents the centrifugation method from being used for precise determination of long-term changes of soil solution composition. In the case of the extraction method, damage of root cells and soil microorganisms occurs. Consequently, cell contents are extracted along with the soil solution and thus results do not correspond to the real conditions (Jones 1998). Using the suction cup method, one is able to repeatedly collect samples from one specific spot for a long period without damaging roots and microorganisms there. It is especially suitable when heavily rooted soil systems are analysed. Several limitations of this method exist. First, due to various designs, suction cups differ in their final suction pressure, thus collected samples differ in their amount, and distinct soil solution fractions may be collected. Second, the material of suction cup can degrade or can be damaged in soil conditions. Hence, results can vary over longer period. Third, spatial resolution of suction cup is inversely proportional to the amount of sample collected. According to Puschenreiter et al. (2005), sampling of 1 mm soil layer using 6 mm disc-shaped micro-suction cup may result in 10 μL of the solution collected. Moreover, suction cup spatial resolution varies largely depending on soil texture and soil water content. Therefore, the interpretation of results might be difficult.

Rhizon soil moisture samplers represent widely used type of suction cup nowadays (Enell et al. 2016; Ettler et al. 2016; Qasim et al. 2016). The Rhizon is consisted of porous, chemically-inert, hydrophilic polymer plastic part and double walled tubing. Inner part of tubing is made of polyethylene (PE) whereas outer part of polyvinylchloride (PVC). The polymer material is reported as polysulfone (Reynolds et al. 2004), namely polyethersulfone (PES) (Di Bonito et al. 2008). The Rhizons usually have several advantages compared to other techniques. Seeberg-Elverfeld et al. (2005) state, among other, low mechanical disturbance of studied soil or sediment by Rhizon, and low dead volume (0.5 mL) including standard tubing. Rhizon pore size should ensure the extraction of microbial- and colloidal-free, ready-to-analyse solution (Knight et al. 1998). However, Rhizon PES polymer may, at fibre interface, retain colloidal Fe (Jones and Edwards 1993) and high molecular weight humic substances (Reynolds et al. 2004). The aims of this paper are (i) to specify the influence of Rhizon suction cup on LMMOA and inorganic anion composition of soil solution, (ii) to determine the temporal variability of soil solution during the vegetation period of model crop *T. aestivum* L. and (iii) to determine the stability of obtained solution.

2 Material and methods

2.1 Experimental design and soil solution sampling

Over whole experiment, Rhizon MOM samplers (distributed by Rhizosphere Research Products B.V, The Netherlands) with 10-cm porous part (0.12–0.18 μm pore size), 2.5 mm outer diameter, female luer lock and 12 cm PVC/PE tubing were used. For schematic diagram, see Seeberg-Elverfeld et al. (2005). Vacuum was generated by 20-mL syringes (B. Braun, Germany) covered by aluminium foil. During the collection of soil solution, syringes were wedged by wooden sticks of same dimension in order to satisfy equal vacuum among syringes.

The influence of the Rhizon MOM suction cup on anion composition of collected sample was studied in adsorption recovery experiment. Glass test tubes covered by aluminium foil were filled by reference solution (0.5 mg L^{-1} of organic and inorganic anions, 2 % methanol). Reference solution contained inorganic anions (NO_3^-, SO_4^{2-} and PO_4^{3-}), mono-carboxylates (lactate, acetate, formate, propionate and pyruvate), di-carboxylates (malate and oxalate) and tri-carboxylate (citrate). Rhizons were installed into test tubes, and after 4 h of incubation at room temperature, solution was collected by using Rhizons. Five millilitres syringes were used to create vacuum. As a control, treatment without Rhizons was set up and solution was collected only using syringes.

The pot experiment was performed in an outdoor precipitation-controlled vegetation hall using 6-L plastic pots. Each pot contained 5 kg (d.w.) of non-sterilized soil. Used soil was arable one common to the Czech Republic; loam (Cambisol) with the following characteristics: 30 % (*w*/*w*)

sand, 48 % (*w*/*w*) silt, 22 % (*w*/*w*) clay, pH_{CaCl2} 4.8 and oxidisable carbon (C_{ox}) 1.6 % (*w*/*w*). Twenty plants of spring wheat (*Triticum aestivum* L. variety Aranka) were grown in each pot from seed (VEG+) and were fertilized by 0.5 g N (NH_4NO_3) in the time of sowing. Control treatments without plants (VEG−) were also set up.

Soil solution was sampled at 27, 41, 55, 97 and 111 days after sowing (DAS). These terms corresponded to beginning of tillering, stem elongation, head emergence, dough development and ripeness, respectively. Before collecting of soil solution samples, pots were irrigated with an exact amount of demineralized water to reach 60 % of maximum water holding capacity (MWHC) (controlled gravimetrically) at 2 p.m.

Soil solution samples were collected in pairs (47 and 49 DAS) from each pot evening (EV) and morning (MO). Sampling of EV samples started 4 h after irrigation (6 p.m.), subsequently MO samples were collected 19 h (9 a.m.) after irrigation. Right after collection, samples of soil solution were transferred from the syringe to a 0.5-mL vial, and then 10 μL of 99.9 % (*v*/*v*) methanol (Lach-ner, Czech Republic) was added (final methanol concentration 2 % *v*/*v*) to prevent microbial degradation (Oburger et al. 2013). Right after collection, samples were frozen at −83 °C (Akhter et al. 2015; Gardolinski et al. 2001) and kept for further analysis. For the characterization of variability over the vegetation period, EV samples were used.

For the determination of solution stability, two series of 18 samples (nine individual pairs in each series, 36 samples in total) were collected and one sample from each pair was treated with methanol (M+ samples). No additive substances were added into M− samples. Subsequently, samples were incubated in darkness at room temperature. First series was analysed after 1 h of incubation, second series was analysed after 24 h of incubation.

2.2 Analytical procedures

Major LMMOA (lactate, acetate, propionate, formate, butyrate, malate, tartrate, maleate, oxalate and citrate) and inorganic (F^-, Cl^-, NO_2^-, NO_3^-, PO_4^{3-} and SO_4^{2-}) anions were determined by means of ion-exchange chromatography with suppressed conductivity; ion chromatograph ICS 1600 (Dionex, USA) equipped with IonPac AS11-HC (Dionex, USA) guard and analytical columns. The eluent composition was 1–37.5 mM KOH with a 1–50 min gradient, flow rate was set to 1 mL min^{-1}. To suppress eluent conductivity, an ASRS 300–4 mm suppressor (Dionex, USA) and Carbonate Removal Device 200 (Dionex, USA) were used. Chromatograms were processed and evaluated using the software Chromeleon 6.80 (Dionex, USA). Standards were prepared from 1 g L^{-1} anion concentrates (Analytika, CZ and Inorganic Ventures, USA) and deionized water (conductivity <0.055 μS cm^{-1}; Millipore, USA) in the range of 0.1–40 or 50 mg L^{-1}. Detection limits were calculated from a 3:1 signal-to-noise ratio (Shabir 2003). Quality control and assurance of ion chromatography analysis are described by Tejnecký et al. (2013). LMMOA and inorganic anion limits of detection (DL) are shown in Table 1.

2.3 Statistical analysis

Statgraphics XVI. I Centurion was used for statistical analyses such as simple regression and correlation (*r*—correlation coefficient), *t* test, paired *t* test and analysis of variance (ANOVA). Statistically significant differences (post hoc Fisher's LSD) were shown at the 95.0 % confidence level ($p < 0.05$). All figures were prepared in SigmaPlot 12. If any determined value was below DL of IC, then it was replaced (for statistical tests) by one-half of DL. This approach had been previously shown as acceptable method providing unbiased statistical estimation for incomplete environmental data sets (Antweiler and Taylor 2008).

3 Results and discussion

3.1 Rhizon adsorption recovery test

Rhizons had no significant effect (*t* test) on determined concentrations of NO_3^-, SO_4^{2-} and PO_4^{3-} (Table 2). Significant increase was found for Cl^- ($t = 12$; $p < 0.001$) which was, however, not spiked into reference solution. For organic anions, no significant differences were found in citrate, oxalate, malate and pyruvate concentrations (recovery 100 ± 2 %).

Table 1 Limit of detection for analysed anions (μmol L^{-1})

	Lactate	Acetate	Propionate	Formate	Butyrate	Malate	Tartrate	Maleate	Oxalate	Citrate
Detection limit (μmol L^{-1})	0.25	0.46	0.53	0.27	0.55	0.28	0.23	0.43	0.24	0.34
	Pyruvate	Valerate	F^-	Cl^-	NO_2^-	Br^-	NO_3^-	SO_4^{2-}	PO_4^{3-}	
Detection limit (μmol L^{-1})	0.42	0.49	0.23	0.28	0.39	0.53	0.49	0.21	0.87	

Table 2 Rhizon MOM adsorption recovery test of a reference solution (0.5 mg L^{-1} of organic and inorganic anions) ($n = 8$)

Analyte	Recovery	*t* test	
	%	*t*	*p*
Lactate	146	4.5	<0.01
Acetate	98.1	−3.1	<0.05
Formate	93.4	−4.0	<0.01
Propionate	94.0	−4.4	<0.01
Pyruvate	98.8	−0.5	n.s.
Malate	101	0.4	n.s.
Oxalate	102	1.8	n.s.
Citrate	102	1.1	n.s.
Cl^-	623	12	<0.001
NO_3^-	110	0.8	n.s.
SO_4^{2-}	122	1.8	n.s.
PO_4^{3-}	96.3	−0.5	n.s.

n.s. indicates not significant effect ($p > 0.05$)

Significantly lower concentrations in Rhizon samples were found for acetate, propionate and formate. However, recovery rates were relatively high: acetate (98 %), propionate (94 %) and formate (93 %). Significant increase was found for lactate ($t = 4.5$; $p < 0.01$), where recovery rate was in average 146 % (Table 2). This high recovery rate, along with the detection of Cl^-, suggested that these anions (Cl^-, lactate) were leached directly from the Rhizon sampler.

Leaching of Cl^- and lactate from Rhizon sampler may influence the final composition of collected aliquot as well as Rhizon close surroundings in soil. For example, it has been shown that increased concentrations of Cl^- may mobilize Cd in soil solution (Weggler et al. 2004).

The origin of impurities detected in recovery test is not clear. We suppose that both Cl^- and lactate represent Rhizon manufacturing residue rather than leached-out products from Rhizon materials. Jones and Edwards (1993) reported leaching of low amount of S and organic carbon from polysulfone fibres. Authors consequently proposed rigorous washing procedure with water. Chloride salts are generally highly soluble in water therefore washing by deionized water should be appropriate. However, lactate salts may differ substantially in their water solubility (Cao et al. 2001). In the light of these facts, washing of Rhizons prior to their installation should be recommended. In order to clarify this, we additionally performed simple washing experiment (data not shown). Deionized water (conductivity <0.055 μS cm^{-1}) was stepwise sampled from glass beaker using Rhizons (three replications) and 10-mL syringes. As a control, same procedure was done without Rhizons. After 50 mL of water collected by Rhizon, concentrations of lactate were not significantly (*t* test) different ($t = 0.80$; $p = 0.47$). This amount of water may be sufficient to wash out all lactate impurities from Rhizon. Concentrations of Cl^- were, however, still significantly higher even after 100 mL (289 %; $t = 2.78$; $p = 0.04$). The cumulative amount of Cl^-, released from Rhizon into 100 mL of water during the washing experiment, was 0.23 ± 0.05 μmol. Larger amounts of water is, therefore, needed to wash out Cl^- impurities from Rhizon. It should be kept in mind that Rhizon MOM used in this experiment, are, according to the distributor, not designed for direct pore water sampling.

3.2 Influence of sampling period and temporal variation

Concentrations of detected LMMOA did not statistically differ for EV and MO samplings (paired *t* test). However, the trend of lactate (EV 1.67 ± 0.80 and MO 3.23 ± 0.80 μmol L^{-1}) and acetate (EV 3.09 ± 0.36 and MO 3.35 ± 0.36 μmol L^{-1}) concentration slightly increased, and oxalate (EV 0.91 ± 0.15 and MO 0.66 ± 0.15 μmol L^{-1}) slightly decreased in MO compared to EV. A moderately strong relationship between MO and EV was found only in the case of oxalate ($r = 0.816$; $p = 0.014$). Diurnal rhythms in the exudation processes of higher plants with maximal exudation rates during the photoperiod were reported (Watt and Evans 1999; Oburger et al. 2014). No significant changes in acetate, formate, lactate or oxalate concentrations in soil solution between EV and MO samples were found.

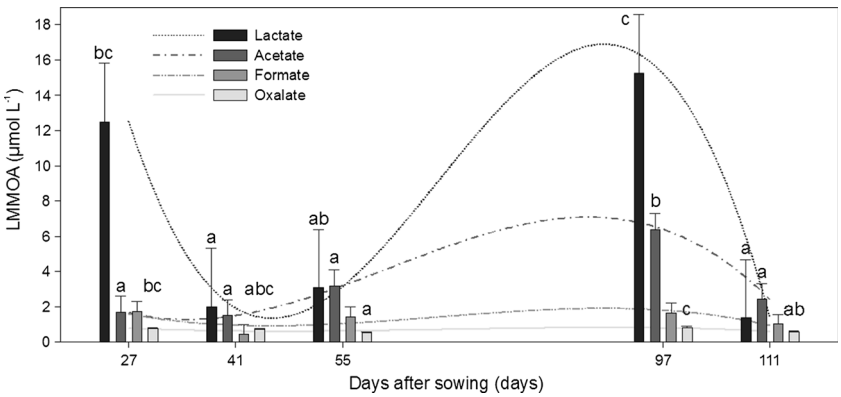

Fig. 1 Temporal changes in soil solution concentrations of LMMOAs (mean and std. deviation, μmol L^{-1}) in VEG+ treatment. Small letters (*a–c*) represent significant differences between DAS for each LMMOA species separately based on LSD value ($p < 0.05$). *Lines* represent nonlinear regression—dynamic fitting ($y = y_0 + ax + bx^2 + cx^3$)

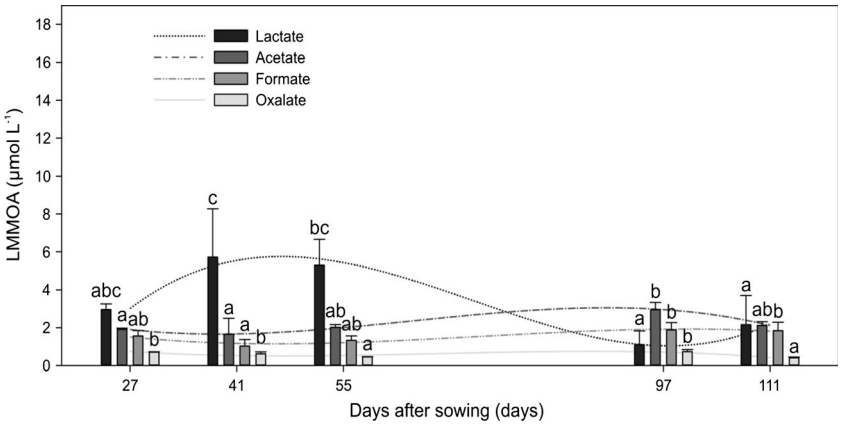

Fig. 2 Temporal changes in soil solution concentrations of LMMOAs (mean and std. deviation, µmol L^{-1}) in VEG− treatment. Small letters (*a–c*) represent significant differences between DAS for each LMMOA separately based on LSD value ($p < 0.05$). *Lines* represent nonlinear regression—dynamic fitting ($y = y_0 + ax + bx^2 + cx^3$)

Contents of F^-, Cl^-, SO_4^{2-} and PO_4^{3-} were not statistically different between MO and EV. Concentrations of these anions showed strong correlation between MO and EV (r from 0.993 to 1; $p < 0.001$). Statistically non-different concentrations of these anions indicate that the time of 4 h after irrigation was sufficient to obtain chemical equilibrium in soil solution for these anions. Increasing trend of NO_3^- (but not significant) concentrations was observed between MO and EV with higher concentrations in the case of EV. The correlation coefficient ($r = 0.864$; $p = 0.006$) indicates a moderately strong relationship in NO_3^- content between MO and EV.

Contents of detected LMMOA fluctuated during the growing season (Fig. 1 and Fig. 2). Significant differences ($p < 0.05$) between sampling days were observed for lactate, acetate and oxalate. No significant differences were observed for formate in VEG+ contrary to the VEG−, where formate slightly decreased at 41 DAS. The highest concentrations of all detected LMMOAs were determined at 97 DAS, while lactate, acetate and oxalate were higher in VEG+ contrary to formate which was slightly higher in VEG−. *T. aestivum* L. plants had significant influence on the concentrations of lactate, acetate and oxalate in soil solution (Table 3).

Although rhizodeposition of carbon is reported to decrease with increasing plant age (Nguyen 2003), the highest LMMOA concentrations in our study were determined in the later plant age (97 DAS) (Fig. 1). We assume that the observed increase of acetate is caused by changes in metabolism of soil microbial communities. However, lactate is rather produced in root cells during limited oxygen supply and can be released into the root environment in substantial amounts (Neumann and Römheld 2001). As wheat plants are finishing their life cycles, water consumption is decreasing, thus anoxic conditions in soil solution may occur due to longer retention time of water in pot. This can result in the accumulation of these carboxylates in soil solution. These increased carboxylate concentrations may, however, also indicate first stage of the fine root decomposition.

Table 3 Two-way ANOVA of time and plant effects on the detected LMMOA concentrations in soil solution during the vegetation period of *T. aestivum* L. ($n = 30$)

LMMOA	Source of variation					
	Time		Plant		Time*Plant	
	F	*p*	*F*	*p*	*F*	*p*
Lactate	2.5	n.s.	4.8	<0.05	5.2	<0.01
Acetate	6.6	<0.01	4.4	<0.05	2.5	n.s.
Formate	1.7	n.s.	1.1	n.s.	0.5	n.s.
Oxalate	11.2	<0.0001	6.1	<0.05	0.4	n.s.

n.s. indicates not significant effect ($p > 0.05$)

3.3 Stability test of collected soil solution

After 1 h of incubation, no significant effect (paired *t* test) on anion (F^-, Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, PO_4^{3-}, lactate, acetate, formate and oxalate) concentrations was observed for nine pairs of samples with (M+) and without (M−) methanol. Under the lower confidence level ($p = 0.0608$), a difference was found for NO_2^-. After 24 h of incubation, no significant effect on F^-, NO_3^-, SO_4^{2-}, PO_4^{3-} and oxalate concentrations was found. Under the lower confidence level ($p = 0.064$), a difference was determined for SO_4^{2-}. However, significant differences between M+ and M− samples were found for Cl^- ($p = 0.037$), NO_2^- ($p < 0.001$), lactate ($p = 0.024$), acetate ($p = 0.002$) and formate ($p = 0.014$). The averaged differences between M+ and M− samples after 24 h of incubation for individual anions were in order: NO_2^- (+756 %), formate (+48 %), lactate (−46 %), acetate (−30 %) and Cl^- (−5 %). Cl^-, lactate and acetate concentrations were significantly lower in M− samples, indicating their microbial degradation or incorporation into organic chlorine compounds, respectively. Contrarily, formate and NO_2^- concentrations were increased in M− samples. Formate was accumulated probably due to degradation of longer chain C compounds (Leonhartsberger et al. 2002), while NO_2^- possibly due to nitrification processes (Appelo and Postma 2005).

3.4 Abundance and variability of LMMOA and main inorganic anion concentrations over the whole vegetation period

Lactate (mean 5.07 μmol L^{-1}) was the predominantly occurring LMMOA followed by acetate (2.93 μmol L^{-1}), formate (1.68 μmol L^{-1}) and oxalate (0.68 μmol L^{-1}) (Table 4). Medians of pyruvate and propionate were determined using one-half of the DL but a few samples represented higher contents of pyruvate and propionate (maximum 2.02 and 0.74 μmol L^{-1}, respectively). In the case of VEG+, pyruvate was detectable only at 27 DAS contrary to VEG−, where pyruvate was detectable during whole vegetation period. The main inorganic anions analysed were in order NO_3^- (mean 1475 μmol L^{-1}), followed by SO_4^{2-} (257 μmol L^{-1}), Cl^- (90.0 μmol L^{-1}), F^- (18.8 μmol L^{-1}) and NO_2^- (1.35 μmol L^{-1}) (Table 4). Concentrations of malate, butyrate, citrate, valerate, tartrate, maleate and Br^- were below or close to the DL of IC (data not shown).

Soil solution concentrations of lactate, acetate and oxalate were comparable with previously reports for bulk soil solution (Dessureault-Rompré et al. 2007). However, when using Rhizon suction cup method, malate and citrate were not detected, contrary to what was expected according to reports for *T. aestivum* L. rhizosphere (Pearse et al. 2007; Andrade et al. 2011). Adsorption recovery test revealed negligible/no influence of Rhizon on these di- and tri-carboxylates. Therefore, the lack of citrate and malate in our samples is probably due to fast sorption and microbial degradation of these anions (Jones et al. 2003); thus, their concentrations in our sampled solution were below DL. The use of analytical equipment with lower DL or sample lyophilization and subsequent concentration increase of these anions could solve this phenomenon.

4 Conclusions

Rhizon suction cups can be used for the sampling of LMMOA in soil solution. However, sampler should be cleaned prior to installation to avoid chemical interference in Rhizon surroundings. Using the procedure described here, only acetate, lactate, formate, oxalate and pyruvate were consistently detected in soil solution. No significant differences in organic or inorganic anion concentrations were observed within 1 day. Variations in the composition of collected soil solution were observed over longer periods (weeks), probably due to water residence period in soil and changes in soil microbial metabolism over life cycle of *T. aestivum* L. Concentrations of lactate, acetate and oxalate were influenced by *T. aestivum* L. Collected soil solution remained relatively stable within 1 h after collection. After 24 h, however, significant changes were found especially in the concentrations of NO_2^-, formate, lactate and acetate.

It should be kept in mind that only one plant species and one soil have been tested in this study. Additionally, soil solution was sampled using one tension pressure, which results in sampling of one soil water type. More effort is therefore required to assess Rhizon potential in different soil-plant systems as well as with different tensions. For this, comparison with other methods is crucial. Future work may also be focused on the sampling of other fractions of dissolved organic carbon.

Table 4 LMMOA and main inorganic anions in soil solution during vegetation period (μmol L^{-1}; $n = 45$)

Analyte	Average μmol L^{-1}	Median μmol L^{-1}	Standard deviation μmol L^{-1}	Coeff. of variation %	Minimum μmol L^{-1}	Maximum μmol L^{-1}
Lactate	5.07	3.37	5.59	110	0.13*	28.8
Acetate	2.93	2.39	2.15	73.5	0.23*	11.2
Formate	1.68	1.37	1.37	81.4	0.14*	8.74
Oxalate	0.68	0.62	0.36	53.2	0.36	2.81
Pyruvate	0.64	0.21	0.61	95.5	0.21*	2.55
Propionate	0.29	0.26	0.11	38.5	0.26*	0.74
F^-	18.8	17.4	5.58	29.7	11.2	31.5
Cl^-	90.0	44.6	103	114	4.23	403
NO_2^-	1.35	0.50	2.33	172	0.20*	13.0
NO_3^-	1475	238	1958	133	3.55	7933
SO_4^{2-}	257	175	210	81.7	4.27	631
PO_4^{3-}	2.65	2.45	1.43	54.0	0.43*	6.41

*represents one-half of DL

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4.4) Nutrient dynamics in soil solution and wheat response after biomass ash amendments

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Nutrient Dynamics in Soil Solution and Wheat Response after Biomass Ash Amendments

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ABSTRACT

Among the possible methods for biomass ash (BA) utilization, land application represents an important nutrient-saving approach of BA management. The land application of BA results in an increase of soil pH, but in contrast to conventional liming, ash application on agricultural land can supply additional nutrients to soil, such as K, Mg, or P. However, due to the complex mineral phase composition of ashes, release of nutrients from the ash matrix into soil solution is not well understood. In the presented pot experiment, two agricultural soils were amended using two common types of BA (wood and straw ash) at rate 1% (w/w). During the vegetation period of spring wheat (*Triticum aestivum* L.), soil solution was sampled and monitored for the concentrations of Ca, K, Mg, and P. Subsequently, yield and nutrient uptake of wheat were determined. The effect of ash application on the investigated parameters differed substantially between the tested soils. Positive yield responses were found in soil with higher N content. Straw ash application increased concentrations of all monitored nutrients in soil solution but simultaneously increased plant uptake of K and P only. Wood ash increased concentrations of Ca and Mg in solution, while its effect on nutrient uptake strongly differed between soils. Generally, higher relative increases of nutrients in soil solution were surprisingly found in soil with higher pH and higher cation exchange capacity (CEC). Factors influencing dynamics of ash-contained nutrients in soil solution are discussed.

Core Ideas

- Nutrient leaching from straw and wood ash matrix in soil-plant conditions is investigated.
- The influence of ashes on yield and nutrient uptake differed substantially depending on types of ash and soil.
- Highly soluble K-compounds in straw ash revealed by X-ray powder diffraction.
- Straw ash is a much more efficient P source than wood ash.

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Biomass ash, a by-product of biomass incineration, is produced in relatively high amounts. Vassilev et al. (2013a) estimated the global production of BA at 476 Tg annually. These ashes contain considerable amounts of mineral-nutrients, especially Ca, K, Mg, and P (Biedermann and Obernberger, 2005); therefore, land application of biomass ash is widely considered as a promising value-added utilization of this material. Of course, only ashes meeting regulatory requirements of contaminants should be applied to land. Currently, the majority of produced BA is landfilled, which is considered unsustainable, and due to increasing disposal costs, indirectly increasing the price of electricity and heat produced from renewable resources. Land application of BA should supply nutrients to agricultural ecosystems and save nutrient resources (James et al., 2012; Demeyer et al., 2001). Thanks to the fertilizing and liming potential of BA (Vassilev et al., 2013b; Biedermann and Obernberger, 2005; Tan and Lagerkvist, 2011), improvement of the soils nutrient status and soil pH level usually occurs, especially when the ash is applied on nutrient-poor acidic soils in reasonable amounts (Gómez-Rey et al., 2013; Moilanen et al., 2012; Park et al., 2012). Ochecová et al. (2016) reported significant increases in plant-available nutrients in loam and sandy clay loam soils after 5% wood ash (WA) addition, while 1% ash addition had negligible effect. Due to alkaline character of WA, it can also be used as an amendment to reduce the transport of some trace metals from contaminated soils to crops (Ochecová et al., 2014). A number of studies have investigated the use of WA in agriculture, but only a limited number have focused on straw ash (SA). Thanks to the rising demand for renewable energy resources, SA is produced in substantial amounts, but its chemical properties differ significantly from WA. This is due to two main reasons: (i) differences in chemical composition of wood and straw biomass, and, (ii) cereal straw is commonly burned at lower temperatures compared to wood biomass, which results in a different mineral phase composition and solubility of formed ashes. Dissolution of ashes in soil is a complex process,

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Abbreviations: BA, biomass ash; CEC, cation exchange capacity; CON, control treatment; DAS, days after sowing; ICP–OES, inductive coupled plasma–optical emission spectrometer; MWHC, maximum water holding capacity; PUE, phosphorus use efficiency; SA, straw ash; WA, wood ash; XRD, X-ray powder diffraction; XRF, X-ray fluorescence.

as is the rate at which nutrients become plant available. This is due to the fact that each cation present in BA usually occurs in several forms such as oxides, hydroxides, carbonates, and bicarbonates (Vassilev et al., 2013a; Demeyer et al., 2001). Published research has focused on the utilization of BAs on forest soils. Several studies have examined the influence of WA on soil solution chemistry of forest soils (Park et al., 2004, Gómez-Rey et al., 2012), but there is a lack of studies on the influence of BA on agricultural soils and crops. There is evidence that the soil solution chemistry of BA-amended soils is a good indicator for solubility, mobility, and availability of nutrients and in general of the nutrient status of the soil (Nkana et al., 2002).

The objective of this study was to compare two BAs from different feedstock (wood chips and cereal straw) in terms of nutrient release, their influence on soil solution composition, subsequent yield response and nutrient uptake of spring wheat on two contrasting arable soils.

MATERIALS AND METHODS

Experimental Design

The experiment was conducted in an outdoor precipitation-controlled vegetation hall in 6 L plastic pots (h = 20.5 cm, d_{top} = 21 cm, d_{bottom} = 18 cm). Each pot contained 5 kg (dry wt.) of soil collected from the plow horizon (0–20 cm). A Cambisol soil was sampled from a long-term trial close to the city of Humpolec, Czech Republic, where no fertilizer was applied for 20 yr. A Fluvisol soil was sampled from a field near to the city of Poděbrady, Czech Republic, which is managed by a conventional farming system with low P inputs. Soil was air dried and passed through a 10 mm stainless steel sieve. Soil was thoroughly mixed with ash in the dose of 1% (50 g of ash/pot) prior to filling the pots. No ash was added to the control treatment (CON). Two types of BA from two industrial heating plants were used. Straw ash originated from the combustion of cereal straw in a grate-fired boiler (15 MWt) and was a mixture of fly and bottom ash. Wood ash originated from the combustion of wood chips in a fluidized bed reactor (15 MWt) (chemical characteristics of both soils and ashes are shown in Table 1). All pots were filled with soil or soil-ash mixture 1 d before sowing. Twenty-five wheat seeds (variety Aranka) were sown in each pot and plants were thinned to 20 at the age of the third leaf emergence. All pots were fertilized with N in the dose of 100 mg N kg^{-1} soil (NH_4NO_3 water solution) immediately after sowing. The soil in pots was maintained at 60 to 70% of maximum water holding capacity (MWHC) by watering with demineralized water daily (controlled gravimetrically once per week). Maximum water holding capacity of soils was determined as follows: Mitscherlich

Table 1. Chemical characteristics of soils and ashes.

Soil type soil texture	Cambisol loam	Fluvisol sandy loam	Straw ash	Wood ash
pH_{CaCl2}	4.5 ± 0.1†	6.0 ± 0.1	10.2 ± 0	11.2 ± 0
pH_{H2O}	5.2 ± 0.1	6.8 ± 0.0	10.3 ± 0	11.2 ± 0
Cation exchange capacity, mmol kg^{-1}	121 ± 2	168 ± 4	261 ± 0.1	125 ± 1.2
Total C, %	1.6 ± 0	1.9 ± 0	4.8 ± 0.3	8.0 ± 0.7
Total N, %	0.16 ± 0	0.19 ± 0	0.07 ± 0.01	0.02 ± 0.01
NO_3^-, mg L^{-1}‡	379 ± 84	980 ± 90	–	–
Total CO_3^{2-}, %	0.15 ± 0	0.14 ± 0.01	3.13 ± 0.02	4.23 ± 0.18
Pseudototal/Total Ca§	2 739 ± 91	3 303 ± 45	56 460 ± 160	117 789 ± 200
Pseudototal/Total K§	9 629 ± 72	4 461 ± 62	159 900 ± 200	58 938 ± 170
Pseudototal/Total Mg§	8 473 ± 266	3 516 ± 54	9 030 ± 160	17 478 ± 280
Pseudototal/Total P§	587 ± 11	384 ± 3	13 610 ± 20	10 195 ± 50
Available $Ca_{Mehlich\ 3}$	1 542 ± 10	2 854 ± 43	5 931 ± 438	23 360 ± 1 071
Available $K_{Mehlich\ 3}$	173 ± 3	225 ± 7	37 464 ± 2 889	5 096 ± 224
Available $Mg_{Mehlich\ 3}$	168 ± 3	202 ± 7	838 ± 75	2 607 ± 65
Available $P_{Mehlich\ 3}$	99 ± 2	66 ± 0	1 977 ± 45	249 ± 10
Available $Ca_{CH3COOH}$	1 416 ± 24	2 037 ± 19	15 035 ± 215	40 824 ± 174
Available $K_{CH3COOH}$	105 ± 3	107 ± 1	83 403 ± 657	6 607 ± 571
Available $Mg_{CH3COOH}$	107 ± 1	104 ± 0	4 220 ± 2	3 990 ± 97
Available $P_{CH3COOH}$	14 ± 1	18 ± 1	1 819 ± 116	315 ± 1
Available Ca_{CaCl2}	–	–	–	–
Available K_{CaCl2}	83 ± 1	93 ± 0	34 018 ± 1 160	4 934 ± 443
Available Mg_{CaCl2}	96 ± 1	82 ± 0	102 ± 2	27 ± 0
Available P_{CaCl2}	2 ± 0	3 ± 0	10 ± 0	<5
Available Ca_{H20}	278 ± 2	247 ± 4	354 ± 4	8 208 ± 314
Available K_{H20}	61 ± 1	116 ± 2	56 037 ± 2 058	3 944 ± 121
Available Mg_{H20}	52 ± 1	69 ± 1	64 ± 4	23 ± 2
Available P_{H20}	9 ± 1	12 ± 0	868 ± 20	<5

† Shown values represent arithmetic means ± standard deviation (n = 3). Pseudototal, total and available portions of Ca, K, Mg, and P are given in mg kg^{-1}.

‡ Content of NO_3^- in soil solution of CON VEG+ treatments at 27 DAS; for the method, see section Soil Solution Analysis within the Materials and Methods section.

§ Pseudototal contents are shown for soils (aqua regia digestion), total contents for ashes (XRF); for the method, see section Plant, Soil, and Ash Analysis within the Materials and Methods section.

columns were filled by air- dried soil (approximately 150 g) of known moisture and weight. Columns were soaked in water for 2 h and subsequently were left to drain at room temperature. After 12 h, MWHC was determined gravimetrically as the amount of water retained by known amount (dry wt.) of soil. The experiment was made in a randomized design (three replications per treatment) with four randomization procedures during the vegetation period.

Soil Solution Sampling

Soil solution samples were collected at 27, 41, 55, 69, 83, and 97 days after sowing (DAS) using Soil Moisture Samplers- Rhizon MOM (Rhizosphere Research Products, Wageningen, the Netherlands). Twenty-milliliter syringes (B. Braun, Melsungen, Germany) were used to create the vacuum. Rhizons with 10 cm porous part (0.12–0.18 μm pore size) were installed vertically in the middle of each pot. Pots were irrigated with an exact amount of demineralized water to reach 60% of MWHC. Collection of soil solution samples started 4 h after irrigation to maintain the solution chemical equilibrium. Collected solution was transferred to test tubes, pH was measured using an Argus pH meter (Sentron, Roden, the Netherlands) with transistor CupFET probe (Sentron, the Netherlands), and samples were kept at 7°C for further analysis (Ca, Mg, K, P). Part of the sample was transferred to 0.5 mL vial and frozen at −83°C for further NO_3^- analysis.

Soil Solution Analysis

Concentrations of Ca, Mg, and K in soil solution were determined by inductive coupled plasma–optical emission spectrometer (ICP–OES, Agilent 720, Agilent Technologies Inc., Santa Clara, CA). Concentrations of P in soil solution were, in some treatments, below or close to the detection limit (DL) of ICP–OES (DL = 0.05 mg L^{-1}). Therefore, P concentrations in all soil solution samples were determined by inductively coupled plasma– mass spectrometer (ICP–MS, 7700x, Agilent Technologies Inc., USA). Nitrate concentrations in soil solutions were determined by means of ion-exchange chromatography with suppressed conductivity; ion chromatograph ICS 1600 (Dionex, Sunnyvale, CA) equipped with IonPac AS11-HC (Dionex) guard and analytical columns. The eluent composition was 1 to 37.5 mM KOH with a 1 to 50 min gradient, flow rate was set to 1 mL min^{-1}. To suppress eluent conductivity, an ASRS 300 4 mm suppressor (Dionex) and Carbonate Removal Device 200 (Dionex) were used.

Plant, Soil, and Ash Analysis

Aboveground mature biomass was harvested at 98 DAS, separated to grain, leaves and stems and dried at 65°C to constant weight. The yield of dry biomass was determined. Samples were then milled and digested with concentrated (65% v/v) HNO_3 (Analytika) and (30% v/v) H_2O_2 (Analytika) in an Ethos 1 (MLS, Leutkirch, Germany) microwave-assisted wet-digestion system. Nutrient concentrations were then determined by ICP–OES (Száková et al., 2013); only K concentrations were determined using flame atomic absorption spectrometer F-AAS (Varian AA285S, Varian Australia, Mulgrave) (Száková et al., 2013).

The soil and ash pH values were determined after extraction with 0.01 M $CaCl_2$ in the ratio 1:2.5 (w/v), or with demineralized water in the ratio 1:2 (w/v) using an Argus pH meter (Sentron) with transistor CupFET probe. For the determination of total C and N, a CHNS Vario MACRO cube (Elementar Analysensysteme GmbH, Hanau, Germany) analyzer was applied. Carbonate content was determined using the volumetric calcimeter method (Loeppert and Suarez 1996). Cation-exchange capacity (CEC) was determined using a three-step saturation of the sample with $BaCl_2$ and subsequent Ba^{2+} release using $MgSO_4$ according to the method of Gillman (1979). Total contents of Ca, K, Mg, and P in ashes were measured using X-ray fluorescence (XRF) spectrometry (Spectro IQ, Kleve, Germany). The tested samples were pressed into pellets prior to XRF analysis. Four grams of ash (particle size 15– 20 μm) was mixed with 0.9 g of the binding additive (HWC Hoechst way, Frankfurt am Main, Germany). The pressing power was 80 kN. Pseudototal contents of Ca, K, Mg, and P in soils were determined by ICP–OES after microwave-assisted aqua regia extraction, as described by Száková et al. (2013). Soil silicates are not totally dissolved during the aqua regia extraction, therefore, these contents are referred here as "pseudototal". Available portions of nutrients in soils and ashes were determined by extraction of samples in Mehlich 3 solution (Mehlich, 1984); 0.11 M CH_3COOH 16 h extraction in the ratio of 1:20 (w/v) for soils and 1:40 (w/v) for ashes, 0.01 M $CaCl_2$ and deionized water 2 h extraction 1:10 (w/v) for soils and 1:100 (w/v) for ashes. The X-ray powder diffraction (XRD) analysis of ashes was performed at room temperature with X'Pert PRO diffractometer (PANalytical, Almelo, the Netherlands) using CuKa radiation (l = 1.5418 Å, U = 40 kV, I = 30mA). Data were scanned with an ultrafast detector X`Celerator over the angular range 5 to 60° with a step size of 0.017° and a counting time of 19.69 s.

Statistics and Data Analyses

The effects of ash application on individual plant component yield, nutrient uptake, and phosphorus use efficiency (PUE) were examined by one-way ANOVA followed by Fisher's least significant difference test (LSD) separately for each soil (not in the case of PUE). Factors influencing soil solution pH and concentrations of individual nutrients in soil solution were examined by repeated measures analysis of variance (rANOVA) with three between-subjects factors (ash amendment, soil type, plant), their interactions, and one within-subject effect (time). STATISTICA 12.0 (Statsoft, Tulsa, OK) was used for the statistical analysis. All figures were prepared in SigmaPlot 11.0 (Systat, San Jose, CA). Phosphorus use efficiency was calculated according to Brod et al. (2015a) as:

$$\mathrm{PUE}(\%) = 100 \times \frac{P_{\mathrm{uptake}}(\mathrm{BA}) - P_{\mathrm{uptake}}(\mathrm{CON})}{P_{\mathrm{total}}(\mathrm{BA})}$$

where P_{uptake} (BA) = total P uptake (mg pot^{-1}) from individual biomass ash treatment, P_{uptake}(CON) = total P uptake (mg pot^{-1}) from control treatment, P_{total} (BA) = total amount of P supplied by ash in individual biomass ash treatment (mg pot^{-1}).

Table 2. Mineralogy of ashes according to X-ray diffraction analysis.

Material†	Relative quantity	Formula	Name
SA	Major	SiO_2	Quartz
		SiO_2	Cristobalite
		KCl	Sylvine
	Minor	K_2SO_4	Arcanite
		$KCaPO_4$	K-Ca phosphate
		$NaAlSi_3O_8$	Albite
		$MgCa(Si_2O_6)$	Diopside
WA	Major	SiO_2	Quartz
	Minor	$CaAl_2Si_2O_8$	Anorthite
		KSi_3AlO_8	Orthoclase
		$Ca(CO_3)$	Calcite
		TiO_2	Rutile

† SA, straw ash; WA, wood ash.

RESULTS AND DISCUSSION

Element and Mineral Phase Composition of Ashes

Wood ash and SA differed in their total contents (determined by XRF) of investigated nutrients (Table 1). Wood ash contained almost double the amount of total Ca and Mg compared to SA, while almost three times more K was present in SA. Total contents of P were similar in both ashes, but slightly more was present in SA. The XRD revealed differences between WA and SA in mineral phase composition. The major mineral in both ashes was quartz (SiO_2) but remaining minerals differed substantially (Table 2). This is in a good agreement with an overview from Vassilev et al. (2013a) and it reflects common differences between woody and straw biomass ashes. Presence of sylvine in SA points out to the combustion temperature lower than 700°C (Wang et al., 2016; Zevenhoven et al., 2012), while thanks to the presence of albite, one can estimate the combustion temperature higher than 575°C (Vassilev et al., 2013c). On the contrary, anorthite, which was found in WA, is usually formed at temperatures 800 to 1100°C (Vassilev et al., 2013c).

Vassilev et al. (2013a) showed the contents of water-soluble fractions of major nutrients from biomass ashes in the following order: K > > > Mg > Ca > > P. Ohno and Erich (1990) found 48% of total Mg, 40% of total K, and 5.7% of total P extractable in ammonium acetate at pH 3 from wood ash. Meiwes (1995) showed 81% of total Ca, 57% of total Mg, 34% of total K and 20% of total P extractable in the same extractant from wood ash at pH 4.2. In our study (Table 1), we found 34% of total Ca, 11% of total K, 22% of total Mg, and 3% of total P extractable

Table 5. Phosphorus use efficiency (PUE, %) for individual ashes.

Soil	Ash†	PUE
Cambisol	SA	1.66AB‡§
	WA	0.02A
Fluvisol	SA	5.50C
	WA	3.20B
F test		13.5
p value		<0.01

† SA, straw ash; WA, wood ash.

‡ Shown values represent arithmetic means (n = 3).

§ Different letters indicate significant difference (Fisher`s LSD test).

Table 3. The yield of dry biomass (g pot^{-1}).

Soil	Material	Stem	Leaf	Grain	∑ aboveground biomass
Cambisol	SA	17.9B† (0.90)	7.8B (0.33)	20.7A (1.03)	46.4AB (2.09)
	WA	15.9A (0.16)	6.5A (0.22)	21.1A (0.22)	43.5A (0.59)
	CON	17.7B (0.26)	7.0A (0.08)	24.4B (1.17)	49.0B (0.83)
F test		7.8	16.8	9.9	8.5
p value		<0.05	<0.01	<0.05	<0.05
Fluvisol	SA	23.5C (0.25)	11.1B (0.75)	32.5B (1.67)	67.1B (2.16)
	WA	19.2B (0.57)	11.4B (0.37)	33.9B (0.68)	64.5B (1.34)
	CON	15.7A (0.36)	8.2A (1.62)	28.2A (1.30)	52.0A (2.41)
F test		179.2	5.6	10.9	31.6
p value		<0.001	<0.05	<0.05	<0.001

† Shown values represent arithmetic means and standard deviations in parentheses (n = 3). Different letters indicate significant differences (Fisher`s LSD test) among the treatments in individual soils.

Table 4. Nutrient uptake by aboveground biomass (mg pot^{-1}).

Soil	Material	Ca	K	Mg	P
Cambisol	SA	103.3 (11,6)†	564.2B‡ (50.5)	33.7 (1.8)	67.5B (4.3)
	WA	102.5 (3.6)	388.1A (22.6)	34.8 (0.26)	56.3A (0.22)
	CON	107.2 (15.7)	384.5A (20.5)	36.2 (2.3)	56.2A (0.7)
F test		0.1	18.2	1.1	12.4
p value		ns§	<0.01	ns	<0.01
Fluvisol	SA	160.7A (17.6)	1012.8C (43.3)	50.3A (3.7)	99.2C (9.5)
	WA	215.1B (7.1)	606.5B (21.9)	68.7B (2.7)	78.1B (4.6)
	CON	147.5A (32.2)	432.1A (31.4)	49.3A (4.6)	61.8A (0.48)
F test		5.5	159.4	16.8	18.8
p value		<0.05	<0.001	<0.01	<0.01

† Shown values represent arithmetic means and standard deviations in parentheses (n = 3).

‡ Different letters indicate significant differences (Fisher`s LSD test) among the treatments in individual soils.

§ ns = not significant.

Table 6. Summary of rANOVA of the effects of ash amendment, soil type, plant and time on soil solution properties (n = 216).

Soil solution properties	Source of variation													
	Amendment			Soil			Plant			Soil × Amendment	Soil × Plant	Amendment × Plant	Soil × Amendment × Plant	Time
df	2			1			1			2	1	2	2	5
Solution properties		Mean‡	F		Mean	F		Mean	F	F	F	F	F	F
pH	SA†	7.5B§	56***	Cam	7.0A	159***	PL	7.5B	166***	0.3ns¶	3.1ns	6.7**	1.0ns	11***
	WA	7.4B		Flu	7.5B		NoPL	6.9A						
	CON	6.9A												
Ca, mg L^{-1}	SA	432C	169***	Cam	90A	1041***	PL	214	3.4ns	67***	0ns	35***	24***	250***
	WA	313B		Flu	463B		NoPL	303						
	CON	158A												
Mg, mg L^{-1}	SA	43C	178***	Cam	13A	409***	PL	25 A	4.6*	34***	0.7ns	16***	11***	295***
	WA	27B		Flu	40B		NoPL	28 B						
	CON	13A												
K, mg L^{-1}	SA	152B	1882***	Cam	34A	270***	PL	44A	47***	229***	3.7***	9.1**	0.5ns	94***
	WA	8.5A		Flu	70B		NoPL	56B						
	CON	4.8A												
P, µg L^{-1}	SA	602B	261***	Cam	223A	9.5**	PL	144A	89***	15***	1.2ns	50***	4.3*	3.2*
	WA	99A		Flu	318B		NoPL	372B						
	CON	60A												

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

† WA: wood ash treatment; SA: straw ash treatment; CON: control; Cam: Cambisol; Flu: Fluvisol; PL: planted treatment; NoPL: no-planted treatment; df, degree of freedom.

‡ Mean values for each factor are averaged over 27 to 97 days after sowing (DAS).

§ Different letters indicate significant differences (Tukey`s HSD test) among the treatments for each property separately. Shown F values are given for the factors of repeated measures analysis of variance.

¶ ns, not significant.

in 0.11 M acetic acid (pH 2.5) from WA. Straw ash used in our experiment had higher (except Ca) relative portions of available nutrients (26% of total Ca, 52% of total K, 46% of total Mg and 13% of total P) extractable in acetic acid. Among the extraction agents used, acetic acid released the highest available amounts of Ca, K, and Mg from both ashes, whereas Mehlich 3 was the most effective agent in the case of soils. The trend in efficiency of Ca release from ash matrix could be related to the presence of calcite in WA and K–Ca phosphate in SA, as these minerals are reported as soluble in acid and in root exudates (Vaněk et al., 2013). Mehlich 3 and $CaCl_2$ were comparable in the extracted amount of K from both ashes, but strong differences were found in water soluble K between WA and SA. In the case of SA, more K was extracted by water compared to Mehlich 3 and $CaCl_2$, which can be explained by the presence of highly soluble phases (sylvine, arcanite). This is in contrast to WA, where the lowest amount of K was extracted in water compared to other extraction agents. Magnesium in both ashes seems to be highly dependent on extractant pH and time of extraction, since strong differences were found between acidic (acetic acid and Mehlich 3) and neutral ($CaCl_2$ and water) extractants. Concurrently, much more Mg was extracted in Mehlich 3 (5 min of extraction) compared to acetic acid (16 h of extraction). Calcium chloride seems to be unsuitable for determination of highly soluble fractions of P from BA because only 1% of water extractable P from SA was found in $CaCl_2$ extraction, probably due to precipitation of insoluble Ca-phosphates. The only P mineral found in SA was K–Ca phosphate. We suggest that this mineral is not soluble in water; therefore, the amount of P extracted by water could represent amorphous P compounds, which were not detected by XRD. The results listed above highlight difficulty in predicting the real dissolution behavior of BAs in soil.

Yield and Nutrient Uptake

The influence of ash application on the aboveground biomass yield of wheat differed between investigated soils (Table 3). In Cambisol, SA significantly decreased grain yield by 15% and increased leaf yield by 11% compared to the control, but the total aboveground biomass yield was not significantly affected. Wood ash in Cambisol had a negative influence on yield of stem, grain and consequently on total aboveground biomass (reduction of 11%). In Fluvisol, both types of ash amendments showed increases in yield. Both of ashes increased total yield (129% in SA and 124% in WA), grain (115% in SA and 120% in WA) and leaf yield of wheat. In Fluvisol, the only differences in the yield response of individual plant parts between ashes were found in stem biomass, where SA treatment had significantly higher stem yield (150%) compared to WA (122%). Etiegni et al. (1990) reported a 25 to 69% increase in biomass yield of winter wheat in wood ash treatments as compared to no ash control. Patterson et al. (2004) observed an 18 to 25% increase in grain yield of barley (*Hordeum vulgare* L.) in wood ash treatment. Ochecová et al. (2014) reported a 7 to 23% increase in grain yield of spring wheat after wood fly ash amendment. Schiemenz et al. (2011) reported an increase in grain yield of summer barley by 16% after SA treatment on loamy sand soil, but also reported a decrease in the yield of wheat grain grown on sandy loam soil by 3% after SA addition. Unfortunately, the studies listed above do not give information about the natural N level in soil. Patterson et al. (2004) describe only slight positive impact of WA application on the yield of barley when ash was applied without additional N. They also found a slight decrease in the yield of barley grain in the first year after WA amendment applied without additional N. According to several studies (Gómez-Rey et al., 2013; Demeyer et al., 2001; Clarholm, 1994), WA application in soil can cause N immobilization. This could explain such contrasting effects of ashes between soils in our experiment, where a positive impact of ash amendment was found only in Fluvisol; the soil with higher total N and NO_3^- content (Table 1) compared to Cambisol. According to Nkana et al. (1998), a positive effect of WA on the biomass yield is mainly caused by Ca and K supplementation. However, this was confirmed only in the case of Fluvisol in our experiment, where increased uptake of Ca and K as well as Mg and P was found after WA addition. Straw ash application increased uptake of K and P in both soils but not Ca and Mg uptake (Table 4). However, elevated K doses supplied by SA lead to a decrease of Ca and Mg concentrations in wheat biomass (data not shown), possibly as a result of competitive uptake between Mg^{2+}, Ca^{2+} and K^+. Calculated PUEs (Table 5) were much lower in Cambisol compared to Fluvisol for both ashes, while SA had higher PUE values in both soils compared to WA. From these results it can be concluded that SA was a more efficient P source compared to WA, which is in agreement with the availability of P from both BAs (Table 1). Similar conclusions were recently published by Brod et al. (2015b) for wide range of waste products. Authors showed that relative agronomic P efficiency of secondary waste products can be determined by simple laboratory extractions. Furthermore, the importance of soil N level for effective ash-contained nutrient use has been partly demonstrated in our study.

Soil Solution

pH

According to Demeyer et al. (2001), the alkalinity of ash decreases with increasing combustion temperature. However, WAs have generally higher pH values than SAs, due to the higher Ca and lower S concentrations (Vassilev et al., 2013a). This corresponds well with our data (Table 1), where WA had a higher pH_{H20} than SA by 0.9. However, there are only a few papers showing the influence of BA on soil solution pH. In our experiment, the addition of both ashes increased the pH of soil solution, but differences between WA and SA were negligible (Table 6). The soil solution pH was mainly influenced by soil type ($F = 159$; $p < 0.001$) and plants ($F = 166$, $p < 0.001$). In treatments with plants, there was a visible increase of pH up to 69 DAS (corresponding to flowering stage), and was followed by a sharp decrease between 69 and 83 DAS (Fig. 1). The increase in pH was probably due to prevalent nitrate uptake from soil solution. It has been demonstrated that plant uptake of nitrate occurs with the influx of two protons into the root (Brimecombe et al., 2007; Mistrik and Ullrich, 1996). This process had a presumably stronger impact on soil solution pH compared to nitrification, which is associated with the acidification of soil solution due to H^+ release during NH_4^+ oxidation. Interestingly, the differences in soil solution pH between CON and ash treatments almost disappeared in the case of treatments with plants on Fluvisol. Although only speculative,

a possible explanation could be in the higher natural nitrate content in Fluvisol (Table 1), and consequent intensive nitrate uptake by plants, which could increase pH to a similar level as with ash addition. Similarly, precipitation reactions could play a role in ash treatments. It is known, that an excess of Ca may cause $CaCO_3$ to precipitate and buffer the pH to a value near 8 (Troeh and Thompson, 2005; Kirk et al., 2015). However, further research will be necessary to elucidate these findings with clarity.

Calcium

Addition of both BAs increased Ca concentrations in soil solution of both investigated soils. The strongest factor influencing the Ca concentration in solution was soil type (F = 1041, $p < 0.001$) (Table 6). Higher contents of Ca in soil solution, as well as higher relative increase of solution Ca after ash addition, were found in Fluvisol (the soil with higher CEC [Table 1]), probably due to different Ca % saturation on soil exchange sites. This leads to the suggestion, that native content of exchangeable Ca in soil influences its release from ash to soil solution. The highest soil solution Ca concentrations were observed in SA treatments (Fig. 2). This is in contradiction with the results of extractable portions of Ca released with Mehlich 3, CH_3COOH, and water (Table 1), where the extractable portions of Ca were more than twofold lower in SA compared to WA. Thus, the increased Ca concentration in SA solution was probably caused by the competition for sorption sites between Ca^{2+} and K^+ ions. An excessive dose of K supplied by SA probably released Ca^{2+} from soil into soil solution due to mass action. Calcium ions, released from the exchange complex, may subsequently induce P precipitation from soil solution into insoluble Ca-phosphates (Tunesi et al., 1999). However, such a phenomenon was not observed probably due to different factors, such as ligand exchange reactions shifting the adsorption–desorption processes toward equilibrium (Hinsinger, 2001). On the other hand, taking into consideration higher content of CO_3^{2-} (Table 1) and the presence of calcite ($CaCO_3$) mineral (Table 2) in WA, the soluble portions of Ca from this ash could substantially differ in soil from those, obtained by the extraction procedures (Table 1). Due to a natural presence of Ca^{2+}, Mg^{2+}, and CO_3^{2-} ions in soil solution and the increase in pH, substantial part of ash-contained Ca could re-precipitate as a calcite ($CaCO_3$), aragonite ($CaCO_3$), and/or dolomite [$Mg,Ca(CO_3)_2$], if at all was solubilized after soil application. Troeh and Thompson (2005) describe the tendency of soil solutions to keep the optimum Ca/K ratio at 5:1. This can also be the reason why we did not find significant differences in soil solution pH between WA and SA.

Generally, plants did not have any significant effect on Ca concentrations in soil solution (Table 6). However, a nonsignificant decrease of Ca in soil solution due to plant uptake

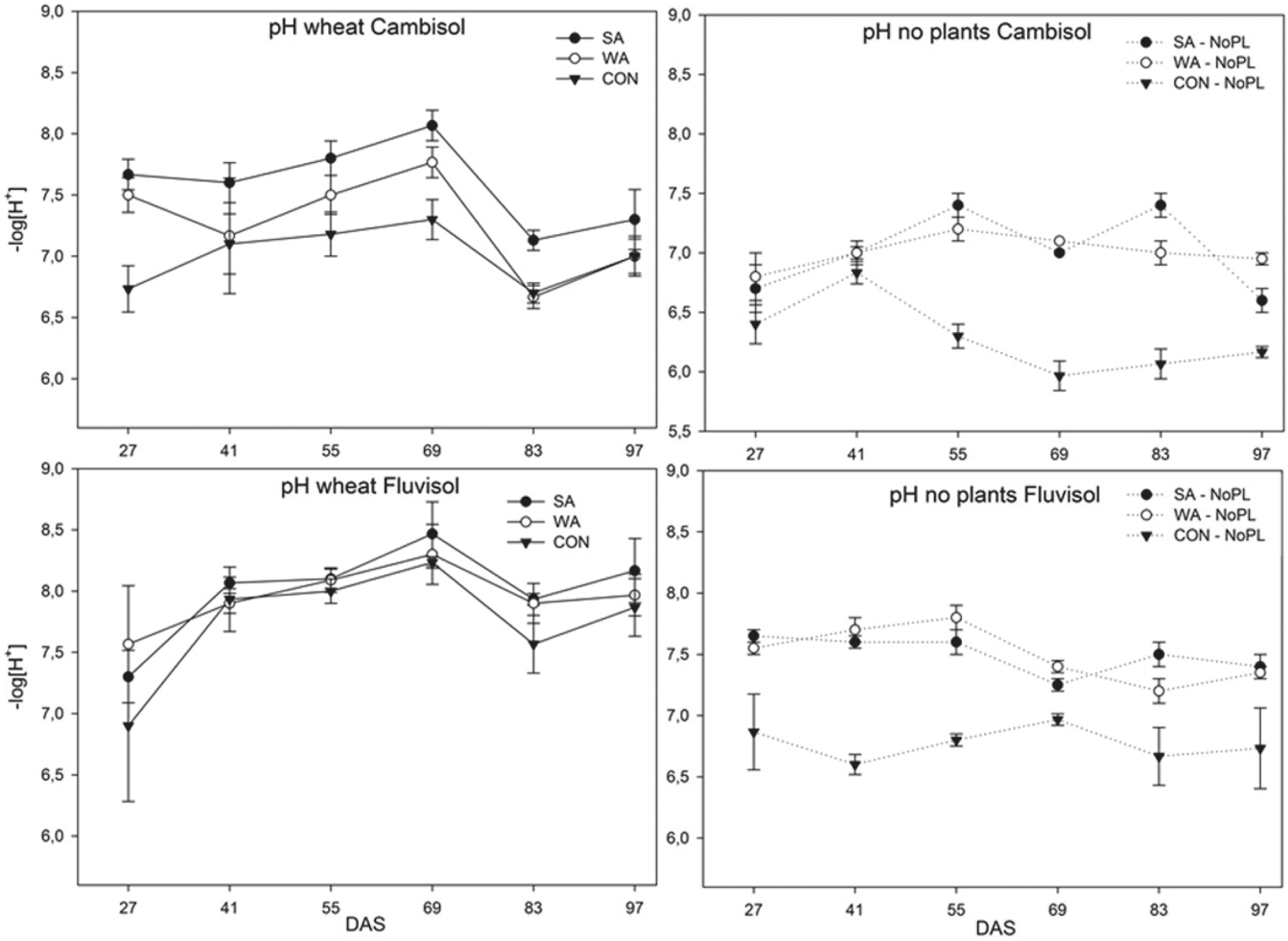

Fig. 1. Effect of biomass ash (BA) amendment on soil solution pH. Arithmetic means (error bars = standard deviation; n = 3) are shown; SA = straw ash; WA = wood ash; CON = not amended control; NoPL = treatment without plants; DAS = days after sowing.

was observed in CON variants, where relatively low Ca level occurred in both soils (Fig. 2). Calcium followed a similar decreasing trend over time in all treatments, possibly due to adsorption of Ca^{2+} on soil cation-exchange sites and/or precipitation reactions (the pH of soil solution in equilibrium with calcite ($CaCO_3$) at P_{CO2} = 0.5 kPa and [Ca^{2+}] = 10 mM is 7.1) (Kirk et al., 2015). It can be noticed that leaching of Ca could not occur because pots were irrigated to 60% MWHC, thus no percolate appeared. In no-plant treatments, we observed equilibrium between dissolved and exchangeable Ca at 55 DAS (Fig. 2). It is a slightly shorter time compared to planted treatments, where the same pattern was found mainly at 69 DAS.

Phosphorus

Ash amendment was the strongest factor influencing P concentration in soil solution ($F = 261$, $p < 0.001$) (Table 6). Phosphorus concentrations increased substantially in SA treatments, while the influence of WA on P concentrations only slightly differed between soils (Fig. 3). In Fluvisol, increased P concentrations after WA addition were observed during the whole growth period, contrary to Cambisol, where P contents of WA in planted treatments were almost similar to CON. It can be noticed that the soils, used in our experiment, varied in their pseudototal contents of Fe and Al (data not shown). This difference played a likely minor role in P soil solution dynamics in BA treatments as the pH of soil solution was generally higher than 6.5 (Fig. 1, Table 6). At neutral and alkaline pH, solution activities of Fe^{3+} or Al^{3+} ions are usually negligible and therefore, phosphate minerals like variscite ($AlPO_4 \cdot 2H_2O$) or strengite ($FePO_4 \cdot 2H_2O$) do not precipitate (Hinsinger, 2001; Lindsay 1979). In addition, wheat is reported to have negligible ability of rhizosphere acidification (Neumann and Römheld, 1999; Pearse et al., 2007; Liu et al., 2012) so markedly more acidic conditions in root close surroundings are not expected. Generally, P concentrations in WA were not significantly different from CON (Table 6). This is in good agreement with previously reported low P solubility from WA (Biedermann and Obernberger, 2005; Demeyer et al., 2001). Some discrepancies occur in the literature between P availability from WA in incubation experiments and the real P plant-uptake in soil–plant systems (Park et al., 2012; Demeyer et al., 2001; Vance, 1996; Erich, 1991). Etiegni et al. (1991) described a decrease in P uptake by wheat when WA was applied in doses up to 2%. In Cambisol with no-plant treatment, P contents in WA were even lower compared to CON, probably due to an increase of pH caused by WA addition. In this case, soil solution pH of CON was lower than 6.5, while WA had a pH of around 7; thus, solubility of Ca-phosphates decreased and their precipitation probably caused the decrease of P concentration in soil solution. However, Tunesi et al. (1999) showed that precipitation and adsorption phenomena can take place at the same

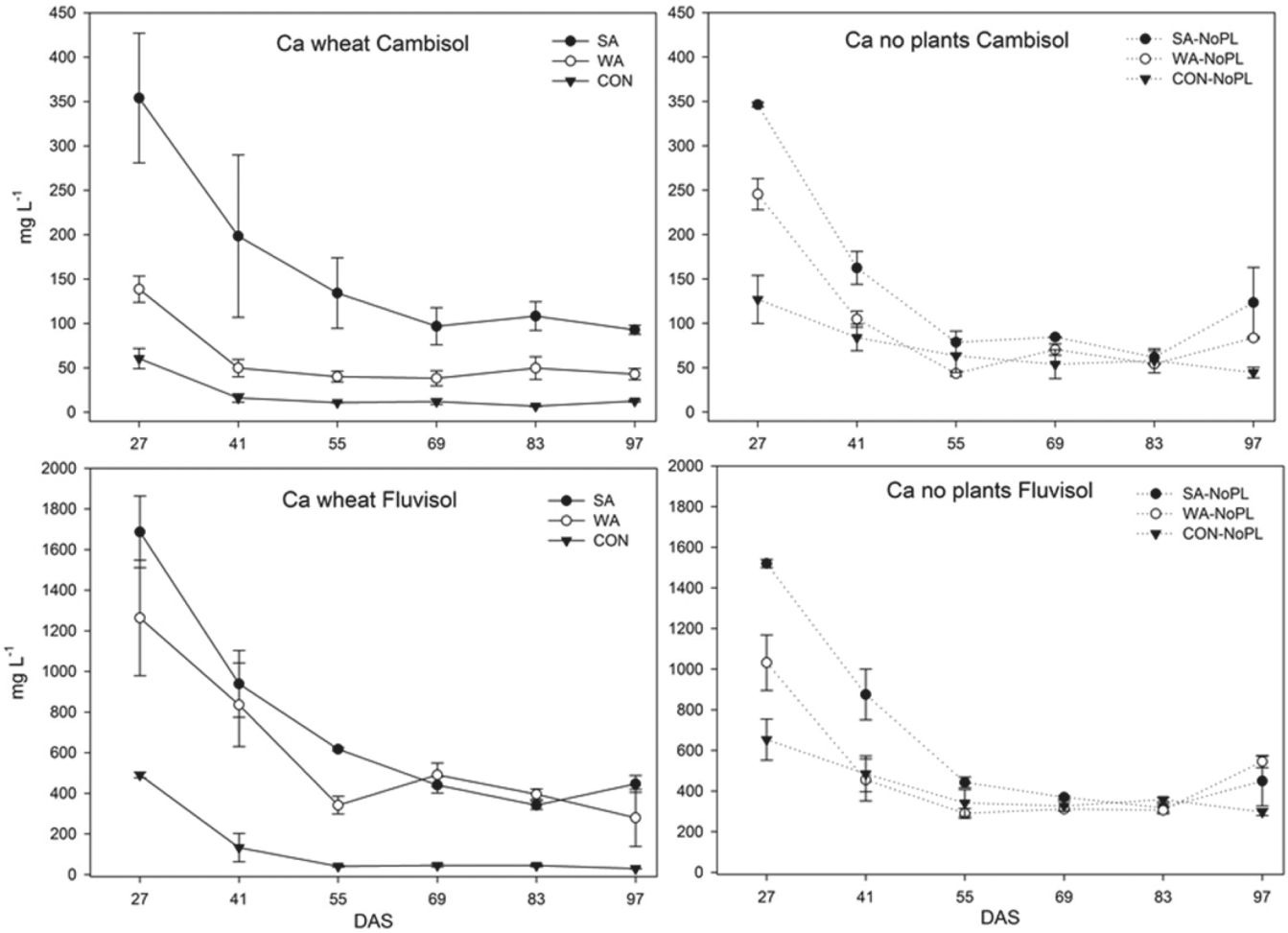

Fig. 2. Effect of biomass ash (BA) amendment on Ca concentrations in soil solution. Arithmetic means (error bars = standard deviation; n = 3) are shown; SA = straw ash; WA = wood ash; CON = not amended control; NoPL = treatment without plants; DAS = days after sowing.

time. The second factor influencing P in soil solution was plant factor ($F = 89, p < 0.001$) (Table 6). In planted treatments, we found significantly lower concentrations compared to no-plant. We suppose this is partly due to continuous P uptake by plants, and due to lower pH value of solutions in no-plant treatments. Erich and Ohno (1992) reported fast sorption of P released from ash onto soil surfaces. In our experiment, time had only a slightly significant effect ($F = 3.2, p < 0.05$) on P in soil solution. This indicates fast establishment of equilibrium between different P fractions in soil and soil solution after biomass ash addition. Soil type also had a significant effect on P concentration in soil solution (Table 6). We observed strong differences between soils in the percentage of P increase in soil solution after ash amendment. Although Fluvisol had a significantly lower content of available (Mehlich 3) P compared to Cambisol, the average increase of P in solution after ash addition was higher in Fluvisol in all cases. Phosphorus concentrations in soil solution of treatments with plants were, on average, increased (compared to CON) to 1230 and 208% after SA and WA addition in Cambisol, and to 3000 and 432% after SA and WA in Fluvisol, respectively. However, in no-plant treatments, average increases were 888 and 79% in Cambisol, and 2380 and 272% in Fluvisol after SA and WA additions, respectively. Higher increments in planted treatments likely reflect the influence of rhizosphere and root exudates (e.g., low molecular weight organic acids) on P release from the BA matrix. This trend was expected, as acidic extraction agents released more P from BAs (Table 1). However, this is in contradiction with the levels of soil solution pH, which were generally lower in no-plant treatments (Table 6).

Potassium

The strongest factor influencing K concentration in soil solution was the type of ash (Table 6), due to a significant increase of K concentration in solution after SA amendment. This phenomenon was likely a result of fast sylvite (KCl) and arcanite (K_2SO_4) dissolution (Table 2). Wood ash increased K in solution only slightly, although many studies reported high water solubility of K from WA (Vassilev et al., 2013a; Demeyer et al., 2001; Ulery et al., 1993). In Cambisol, K increase (over control) after WA addition was observed only at 27 DAS in no-plant treatments, while no increase was found in planted treatments (Fig. 4). In Fluvisol, we found K only increased in solution after WA addition in the planted treatment at 27 DAS, but in no-plant treatment, K increased during the whole growth period after WA addition (Fig. 4). Wheat plants seem to be able to take up substantial portions of easily soluble K from WA in the early stages of development. Generally, WA had no significant influence on K concentrations in soil solution (Table 6). In no-plant SA treatments of both soils, we found an almost linear decrease of K from 27 up to 69 DAS, and then concentrations equilibrated. This can be explained by K adsorption to cation-exchange sites or by fixation of K into

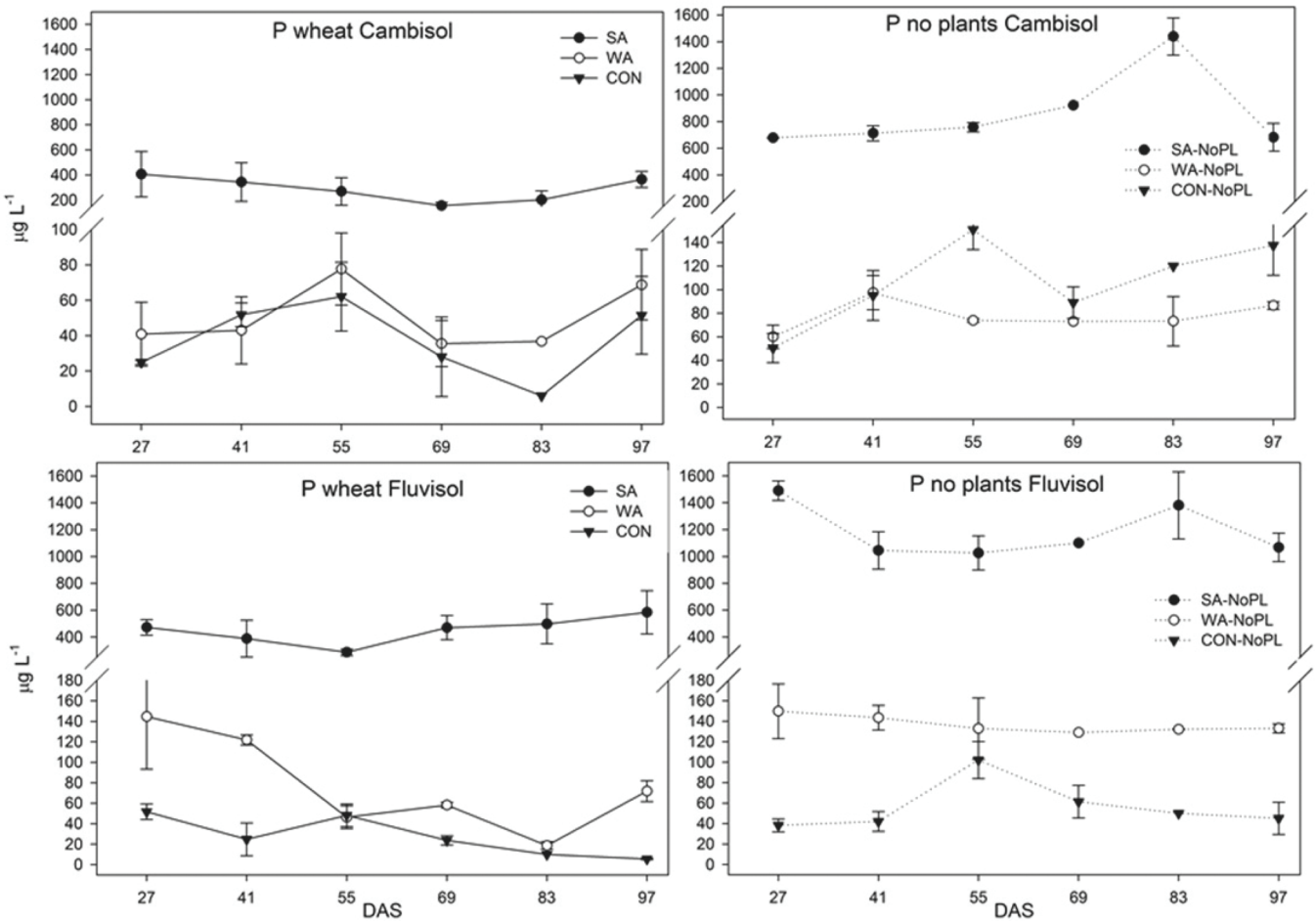

Fig. 3. Effect of biomass ash (BA) amendment on P concentrations in soil solution. Arithmetic means (error bars = standard deviation; $n = 3$) are shown; SA = straw ash; WA = wood ash; CON = not amended control; NoPL = treatment without plants; DAS = days after sowing.

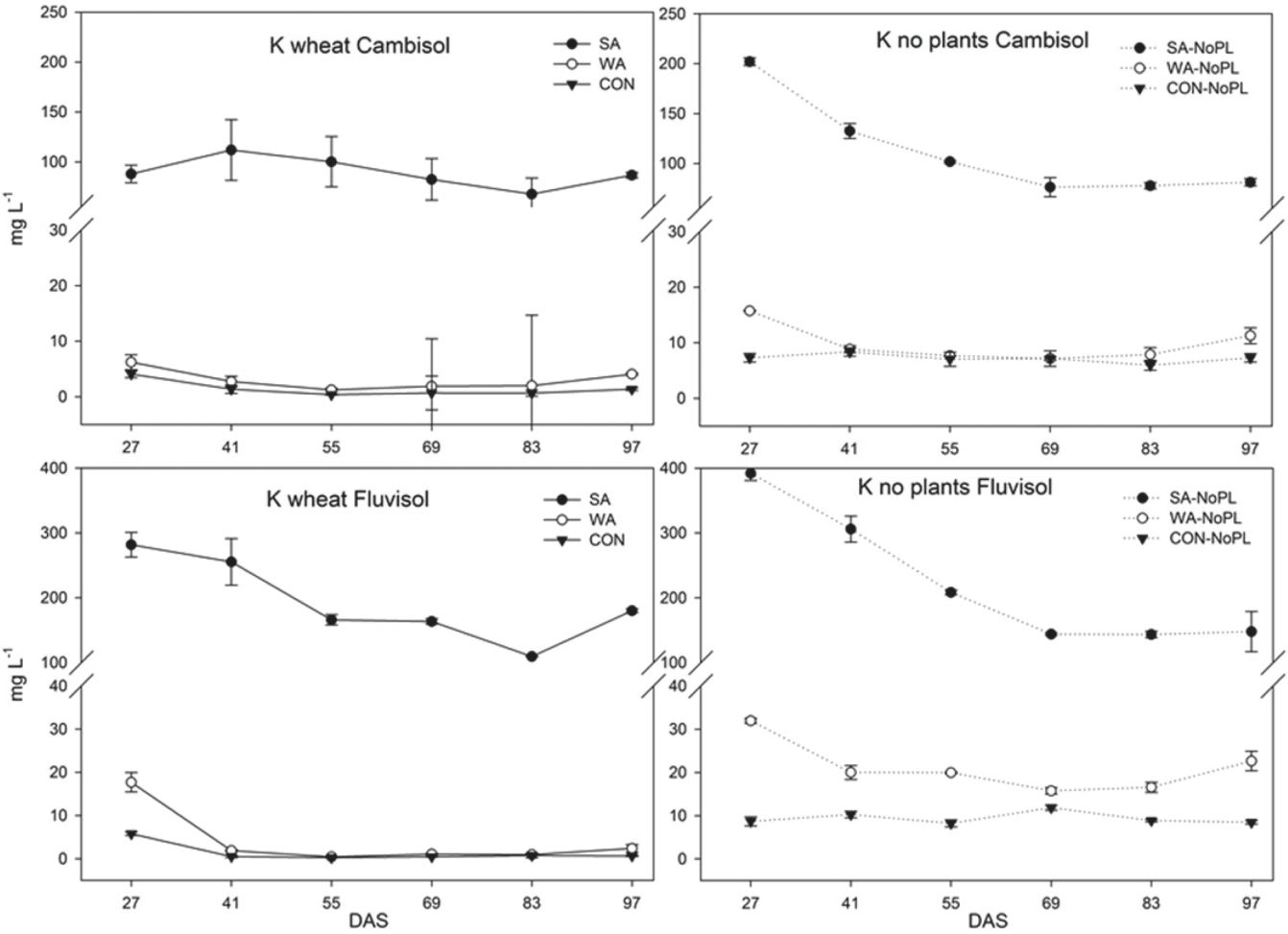

Fig. 4. Effect of biomass ash (BA) amendment on K concentrations in soil solution. Arithmetic means (error bars = standard deviation; n = 3) are shown; SA = straw ash; WA = wood ash; CON = not amended control; NoPL = treatment without plants; DAS = days after sowing.

mineral or organic structures. Such K becomes exchangeable, and is traditionally believed to form a reserve that can be available for plant uptake in the short term (Simonsson et al., 2007).

Magnesium

As was the case for Ca, application of both ashes increased Mg contents in soil solution of both soils. The higher concentrations were found in SA treatments (Table 6), although CH_3COOH extractable portions of Mg were comparable in both ashes and portions extractable by Mehlich 3 were even higher in WA compared to SA (Table 1). The reason may be similar to increased Ca concentrations. Namely, due to the high dose of K supplied by SA and subsequent mass action. Seggewiss and Jungk (1988) showed that K fertilization increases Mg concentration in soil solution. It is questionable whether there was competition for sorption-sites between Ca^{2+} or K^+ and Mg^{2+}. Gransee and Führs (2013) postulated that even though the ionic radius of Mg is smaller than that of Ca or K, its hydrated radius is substantially larger. Thus, Mg is less strongly bound to soil charges, leading to higher concentrations in the soil solution, and therefore higher mobility in soil compared to those listed above. However, higher Mg concentrations in soil solution in SA compared to WA could be expected according to $CaCl_2$ a H_2O extractable Mg portions from ashes (Table 1). Therefore, it is questionable if K mass action took place in the case of Mg. Similar to Ca, the strongest factor influencing Mg content in soil solution was soil factor (F = 409; p < 0.001) (Table 6); higher Mg concentrations were found in Fluvisol with higher CEC compared to Cambisol. Consequently, the proportion of initial Mg concentration increase in solution between ashes differed in the investigated soils. In Fluvisol (higher CEC and higher Mehlich 3 extractable Mg compared to Cambisol), the Mg concentrations at 27 DAS were much more similar in SA and WA (Fig. 5), reflecting the portions of Mg extractable by CH_3COOH from ashes (Table 1). However in Cambisol, Mg concentration at 27 DAS was more than twofold higher in SA compared to WA. This can be related to higher total Mg content in Cambisol. We assume that two phenomena could play a role in different behavior of ashes between soils. First, different portions of water- and acid-soluble Mg fractions present in ashes and second, mass action between ash-derived K and soil-derived Mg. However, taking into consideration that pH of soil solution was rather alkaline (Fig. 1, Table 6) and soils differed in their total Ca and Mg contents (Table 1), precipitation of magnesite ($MgCO_3$) and/or dolomite [$Mg,Ca(CO_3)_2$] cannot be excluded. Plants had a slightly significant (Table 6) influence on Mg concentrations in soil solution and similarly to Ca, differences between planted and no-plant treatments were found in relatively low Mg CON treatments.

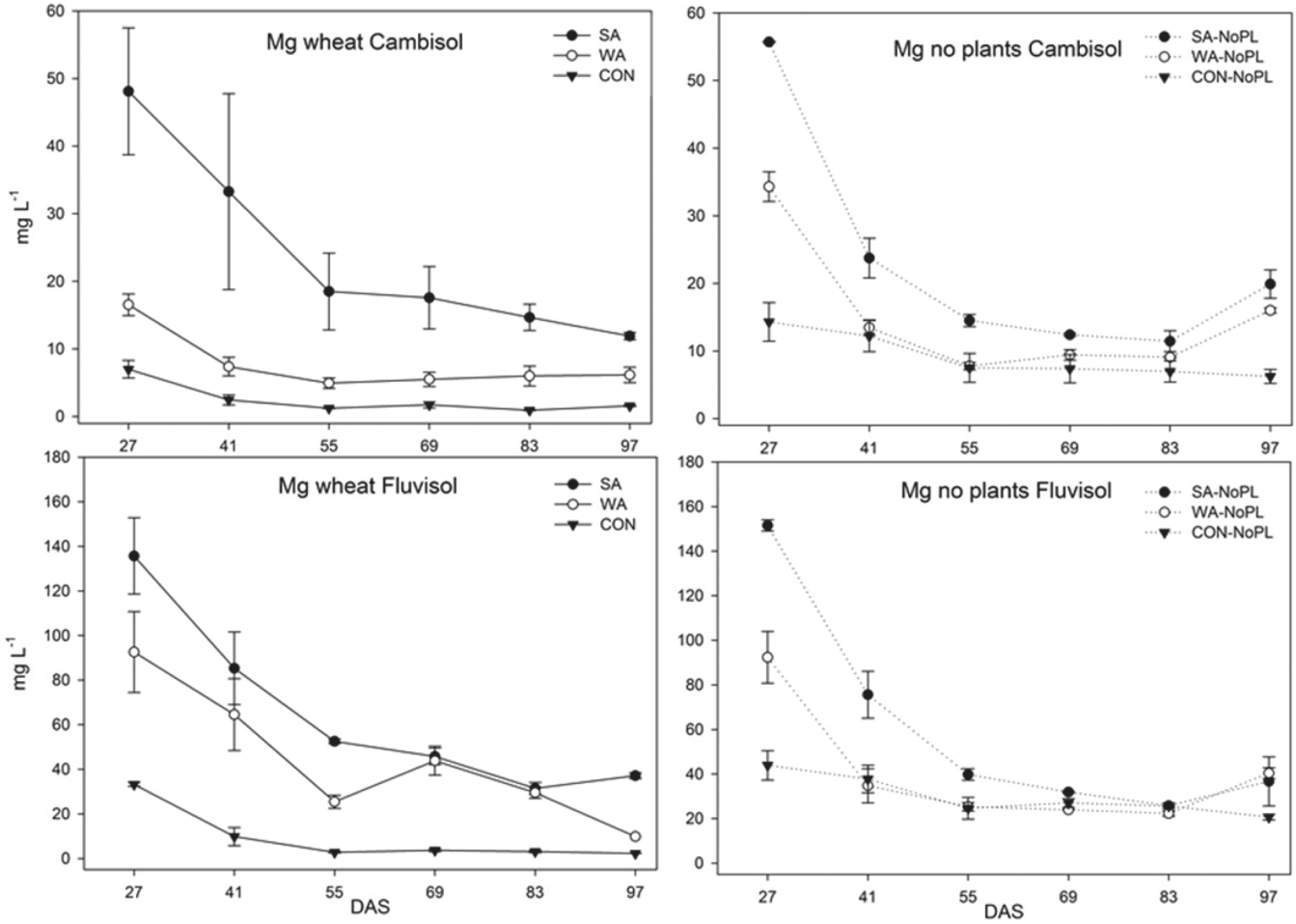

Fig. 5. Effect of biomass ash (BA) amendment on Mg concentrations in soil solution. Arithmetic means (error bars = standard deviation; *n* = 3) are shown; SA = straw ash; WA = wood ash; CON = not amended control; NoPL = treatment without plants; DAS = days after sowing.

CONCLUSIONS

This study evaluated spring wheat and soil solution dynamics after wood and straw ash amendments. Positive yield responses after biomass ash amendment were found in soils with higher N content. Application of WA even resulted in a wheat yield decrease in Cambisol. Both ashes increased soil solution pH, and no differences were found between SA and WA. Predictability of BA behavior in soil, using simple extraction procedures, has shown to be difficult. Straw ash amendment resulted in higher concentrations of Ca, K, Mg, and P in soil solution compared to WA. High K doses supplied by SA in the form of KCl and K_2SO_4 likely caused a release of soil-derived Ca and Mg from the sorption complex into soil solution. This phenomenon may pose a risk of leaching of these nutrients in field conditions, especially shortly after SA application. Subsequently, SA only increased plant uptake of K and P. Calcium and Mg uptake was not influenced by SA amendment possibly due to uptake antagonism with K. This effect was found in both investigated soils. Wood ash increased Ca and Mg concentrations in soil solution, while no changes in nutrient uptake were found in Cambisol. On the contrary, in Fluvisol, WA increased uptake of Ca, K, Mg, and P. Straw ash amendment resulted in higher PUE than WA in both soils, due to higher solubility of P compounds contained in SA. However, the majority of these soluble P compounds could not be determined by XRD, due to their amorphous character. Higher relative increases of Ca, K, Mg, and P in soil solution after ash addition were found in Fluvisol, which had higher CEC compared to Cambisol. This leads to the suggestion that soil CEC does not have any major impact on the release of nutrients from BA into soil solution. This is in accordance with recently published results of Komonweeraket et al. (2015), who showed same phenomenon for coal fly ashes.

This study demonstrated that the behavior of two BAs in soil may vary largely, depending on types of ash and soil. These differences are governed by the complex of reactions and simple extraction procedures of ash are unlikely to predict its real behavior in soil–plant systems. Prior to the soil application of BA, detailed analysis of soil is necessary to sufficiently predict ash behavior. More effort is therefore needed to develop effective practices in the utilization of these materials as alternative fertilizers and to identify crucial factors influencing the release of BA-contained nutrients into soil.

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4.5) Fertilization efficiency of wood ash pellets amended by gypsum and superphosphate in the ryegrass growth

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Fertilization efficiency of wood ash pellets amended by gypsum and superphosphate in the ryegrass growth

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ABSTRACT

Ochecová P., Mercl F., Košnář Z., Tlustoš P. (2017): Fertilization efficiency of wood ash pellets amended by gypsum and superphosphate in the ryegrass growth. Plant Soil Environ., 63: 47–54.

Application of biomass ash to soil can save mineral nutrients due to its relatively high contents of Ca, K, and P. The study assessed the effect of powdered ash and pellets made from wood fly ash (WFA), combined moreover with additives rich in S (flue gas desulfurization gypsum – FGDG) and P (single superphosphate – SP) on the yield and uptake of nutrients (Ca, K, P, and S) by ryegrass (*Lolium perenne* L.), the accumulation of nutrients in plant biomass at individual four cuttings, and the available nutrients amount in the acidic loamy soil after the last harvest. Plants grown in pots enriched by wood ash showed significantly higher yield and nutrient uptake than in the unamended treatments. The uptake of nutrients by plants, content of nutrients in plants and in soil was substantially positively influenced by both components added to the wood ash, especially by FGD gypsum. The combination of wood ash with additives proved to be effective. The soil enrichment by WFA + SP + FGDG increased the availability of SP-contained P and available P content in soil even after harvest.

Keywords: plant nutrition; soil amendments; sulfur; phosphorus; plant growth

Currently, there is a growing interest in the agricultural utilization of biomass combustion by-product – ash. Returning biomass ash to agricultural land is beneficial thanks to the fertilizing potential which is determined by the Ca, K, P, and Mg content (Pels et al. 2005) as well as micronutrient contents (Ochecová et al. 2014), and thanks to the highly alkaline pH (Mercl et al. 2016, Ochecová et al. 2016). The application of untreated wood ash creates severe dust problems and stabilization is necessary to make uniform spreading feasible. The use of a compaction technique can help to solve dust problems, decrease heterogeneity of ash application and costs of transport. The compaction process also permits to control particle size and composition of the ash products (Holmberg et al. 2000). Ash contains usually sufficient amounts of Ca and K but P is represented by smaller amounts of approximately 1%, and the most S is lost as gas during combustion process similarly to nitrogen. The biomass ash and materials rich in P or S implemented into ash pellets could be beneficial for plant nutrition. Superphosphate (SP) is a commonly used P fertilizer; flue gas desulfurization gypsum (FGDG) represents a new large source of S. FGDG ($CaSO_4 \cdot 2\ H_2O$) is produced when brown coal is burned (Chen and Dick 2011). Concentration of Ca and S in gypsum usually ranges between 20–24% for Ca and 17–19% for S. FGDG can provide a continual release of S to the soil and it has been

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verified for grass and also for many crops such as corn, soybean, alfalfa, wheat, sorghum, etc. (Chen et al. 2005, 2008, Lee et al. 2008, Chen and Dick 2011, Shi et al. 2011).

The main objective of the present study was to test the efficiency of dust ash application with comparison to the compacted ash pellets and the amendments of P and S in compacted ash on the growth and nutrient uptake by ryegrass (*Lolium perenne* L.) in the pot experiment.

MATERIAL AND METHODS

Soil. The experimental soil was Cambisol (loam – the textural class was classified according to the FAO Soil Classification) originated from the field near the town of Humpolec (49°33'N, 15°21'E) in the Czech Republic. Soil samples were collected from topsoil in the layer at 0–20 cm depth, air-dried at 20°C, ground in a mortar, and passed through a 15 mm sieve before establishment of the pot experiment. Soil characteristics are presented in Table 1.

Pot experiment. The pot experiment was set up in 6 treatments, each in 4 replicates. Air-dried soil (5 kg) was either mixed with powdered or pelleted ash alone or in combination with SP, FGDG or both (SP and FGDG were applied as a constituent of the pellet), and a treatment without ash addition was used as control (Table 2). The amount of the applied ash was 100 g in all treatments, representing 2% w/w per pot. However, since the pellets were partly formed by other additives, 100 g of ash corresponded to different amounts of ash mixture in the different treatments (Table 2). The amounts of nutrients added in each treatments in form of amendment are shown in Table 3.

Table 1. Physico-chemical characteristics of the experimental soil

Soil property		Cambisol
Altitude (m a.s.l.)		527
Mean annual temperature (°C)		7.1
Mean annual precipitation (mm)		603
pH_{CaCl_2}		5.0
Cation exchange capacity ($mmol_+/kg$)		68
Total organic carbon (%)		1.3
Pseudototal Ca (%)		0.2
Pseudototal K (%)		0.04
Pseudototal P (%)		0.09
Pseudototal S (%)		0.03
Soil texture		loam
Soil particle size (%)	sand	30.2
	silt	48.4
	clay	21.4

All values represent means (n = 3)

Wood fly ash (WFA) originated from a combustion plant with production of 27 000 tons of ash per year. The producer uses a specific combustion technology – fluid burning (820°C).

Soil-ash mixtures were mixed thoroughly, placed into 5 L plastic pots, and moistened by deionized water to keep 60% of water holding capacity (determined for each treatment separately).

As the experimental plant, ryegrass (*Lolium perenne* L.) was chosen in the seed rate 20 g/m^2. Sowing took place on 9 May 2013. During the vegetation, weeds were removed to avoid interplant competition. The pots received N fertilization (0.5 g N in the form NH_4NO_3) before sowing, and after 1st and 2nd harvest.

Table 2. Experimental design

Treatment	Ash form	Ash portion in amendment (%)	Amount of applied amendment (g/pot)	Pellet enrichment (%)
I	no ash	0	0	–
II	powdered	100	100	–
III	pelleted	90	111.1	–
IV	pelleted	88	113.6	SP (2)
V	pelleted	78	128.2	SP (2) + FGDG (10)
VI	pelleted	70	142.9	FGDG (20)

SP – single superphosphate; FGDG – flue gas desulfurization gypsum. Pelleted amendments contained 10% (w/w) of inert binder

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Table 3. Total amount of nutrients applied as an amendment (g/pot)

Treatment	Ca	K	P	S
I	–	–	–	–
II	10.2	5.10	0.79	1.20
III	10.2	5.10	0.79	1.20
IV	10.8	5.11	0.95	1.58
V	13.6	5.13	0.95	4.02
VI	16.4	5.15	0.79	6.61

The experiment was conducted in the rain-controlled vegetation hall (50°13'N, 14°37'E). The pots were randomized side by side on the bench exposed to daylight and outdoor climate. Plants were irrigated regularly by deionized water to maintain optimal growth conditions.

Biomass was harvested manually with scissors at a height of 2 cm from soil surface on 27 June, 23 July, 26 August, and 8 October (four cuts) in order to distinguish possible differences in nutrient release dynamics among treatments. For each pot, plant material was dried at 40°C, dry matter was weighed, ground through 0.75 mm sieve and homogenized. The soil samples were taken from 25 cm deep profile using the soil sampler (five samples from different places of each pot with total weight of approximately 100 g) at the end of the experiment. Then, the soil samples were air-dried at ambient temperature, ground in a mortar, passed through a 2-mm plastic sieve, and homogenized.

Analytical procedures. Non-destructive X-ray fluorescence (XRF) spectrometry (Spectro IQ, Kleve, Germany) was used for the determination of total nutrient contents in the ash and amendments.

Harvested plant material was pressure-digested according to Száková et al. (2013). The Ca and K concentrations were determined by flame atomic absorption spectrometry (F-AAS, Varian 280FS, Varian, Mulgrave, Australia), while P and S were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian, VistaPro, Mulgrave, Australia) (Száková et al. 2013). RM IAEA V-10 Hay Powder (Analytika, Prague, Czech Republic) was used as the certified reference material. The soil pH was determined after extraction with 0.01 mol/L $CaCl_2$ (w/v =

Table 4. Nutrient concentration in dry matter biomass of plants (%) at individual harvests

Harvest/Treatment		I	II	III	IV	V	VI
Ca	1st	0.67^{aB}	0.69^{aB}	0.67^{aB}	0.65^{aB}	0.61^{aBC}	0.69^{aB}
	2nd	0.53^{aA}	0.52^{aA}	0.49^{aA}	0.51^{aA}	0.46^{aA}	0.50^{aA}
	3rd	0.62^{cAB}	0.59^{bcAB}	0.52^{abcA}	0.51^{abcA}	0.49^{abAB}	0.47^{aA}
	4th	0.67^{aB}	0.59^{aAB}	0.66^{aB}	0.66^{aB}	0.63^{aC}	0.71^{aB}
K	1st	3.61^{aBC}	4.20^{abC}	4.37^{abC}	4.21^{abB}	4.67^{bC}	4.69^{bC}
	2nd	3.21^{abB}	3.39^{abB}	3.64^{bB}	3.03^{aA}	3.50^{abA}	3.12^{abA}
	3rd	3.82^{aC}	4.65^{cD}	4.67^{cC}	4.40^{bcB}	4.04^{abB}	4.00^{abB}
	4th	2.47^{aA}	2.80^{abcA}	2.94^{bcA}	2.65^{abA}	3.08^{cA}	2.85^{abcA}
P	1st	0.32^{aB}	0.31^{aB}	0.31^{aB}	0.24^{aA}	0.28^{aA}	0.29^{aC}
	2nd	0.24^{aA}	0.25^{aA}	0.22^{aA}	0.24^{aA}	0.25^{aA}	0.18^{aA}
	3rd	0.25^{abcA}	0.29^{cAB}	0.24^{abA}	0.28^{bcA}	0.28^{bcA}	0.22^{aAB}
	4th	0.23^{aA}	0.26^{aAB}	0.26^{aA}	0.27^{aA}	0.25^{aA}	0.25^{aBC}
S	1st	0.22^{aA}	0.48^{bA}	0.68^{bBC}	0.48^{bA}	0.57^{bA}	0.93^{cC}
	2nd	0.31^{aAB}	0.44^{bA}	0.54^{cA}	0.48^{bcA}	0.50^{bcA}	0.53^{bcA}
	3rd	0.37^{aB}	0.80^{bB}	0.78^{bC}	0.77^{bB}	0.80^{bB}	0.79^{bB}
	4th	0.32^{aAB}	0.49^{bA}	0.62^{cAB}	0.60^{bcAB}	0.69^{cAB}	0.80^{dB}

All values represent means (n = 4). Different lower case letters indicate significant differences (P < 0.05) among the treatments in individual harvests. Different capital letters indicate significant differences (P < 0.05) among harvest times for each nutrient and treatment individually

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1/2.5) and measured by WTW pH 340i meter with ion-selective electrode (WTW, Weilheim, Germany). Cation exchange capacity (CEC) was calculated according to the ISO 11260 (1994). Total organic carbon (TOC) was determined by the method described by Sims and Haby (1971). For determination of potentially available elements in soils after harvest, Mehlich 3 extraction procedure was applied (Mehlich 1984).

Statistical analysis. The effects of WFA application were examined by one-way analysis of variance including all treatments and harvests. When this test revealed significant differences, the mean values were compared by Tukey's means test ($P < 0.05$). The software Statistica 12.0 (Statsoft, Tulsa, USA) was used.

RESULTS AND DISCUSSION

The highest Ca concentration in ryegrass was found out at the first and last cuttings (Table 4), which correspond with the lowest biomass yield, but no significant differences were observed among the treatments even in the treatments with FGDG. Bailey (1995) observed no response to gypsum only at first cut of ryegrass.

The highest K concentration in ryegrass was recorded at the first and third cuttings. Treatments V and VI were significantly different from the control treatment I in the first cut although almost no K was present in SP or FGDG. The explanation was provided in the experiment of Sárdi et al. (2012) who found out that the better levels of P supply had a beneficial influence on K uptake and K concentrations in plants. During the uptake of K^+, root may exchange another cation, such as H^+ for K^+, or it may absorb an anion as NO_3^- or $H_2PO_4^-$ in order to maintain the electrical balance in cells (Marschner 1995). If the external pH declines, cation-anion imbalance may occur in the root tissues and therefore, anion absorption may be preferred. In our case, treatment VI had 70% of wood ash and 20% of FGDG. The acidic reaction of FGDG could therefore increase solubility of ash-contained nutrients, like P or K. On the other hand, synergism between K and S could also take place.

Only minor significant differences were found for P concentrations in ryegrass biomass. Generally, no amendment treatment was different from the control. This was probably due to soil's ability to supply plant-available P and therefore the possible P fertilization effects of amendment treatments were masked. However, from the results of Sárdi et al. (2012) it was evident that P accumulation by ryegrass and amounts of P taken up by plants both responded to the level of applied P.

The highest S concentration in ryegrass was reported mostly in the third and the last cut. Among the treatments, the most significant differences were observed from all tested nutrients. In the treatment VI with FGDG, on average almost 60% higher S concentration was noted compared to

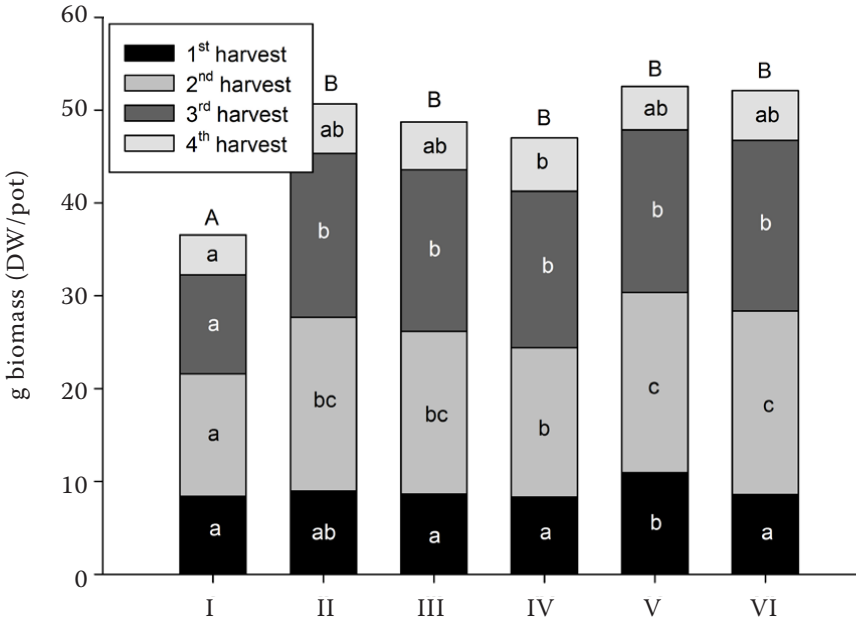

Figure 1. Total dry aboveground biomass yield of ryegrass (g biomass/pot). Different letters indicate significant differences ($P < 0.05$) among treatments. Differences among the individual harvests are represented by small letters, differences among the total biomass yield are represented by capital letters

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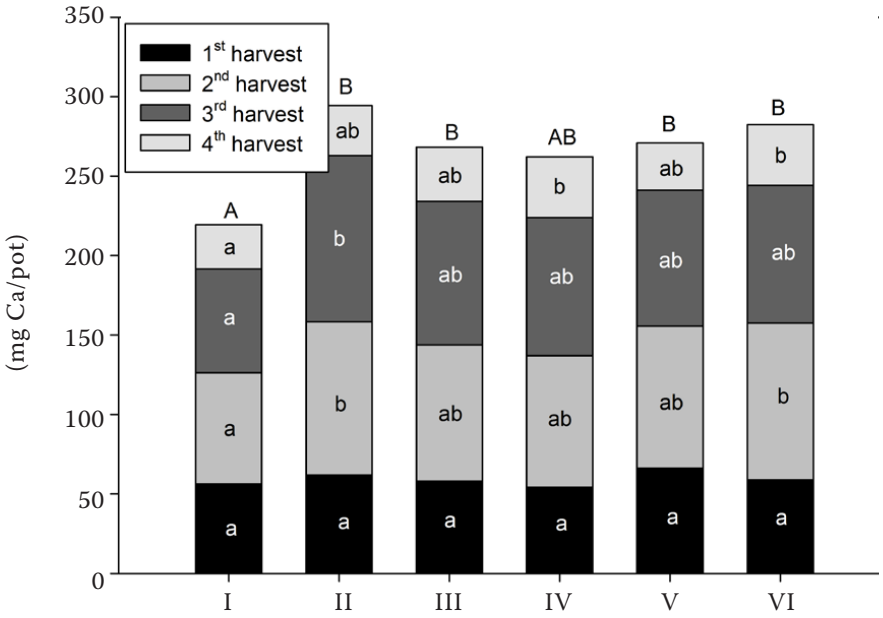

Figure 2. Calcium (Ca) uptake by ryegrass during four harvests. Different letters indicate significant differences ($P < 0.05$) among the treatments. Differences among the individual harvests are represented by small letters, differences among the total Ca uptake by plants are represented by capital letters

unamended treatment I. Warman and Sampson (1994) confirmed that gypsum was effective for supplying plant available S.

Effects of fertilizer treatments on biomass production. Application of wood ash in different forms and with different amendments resulted in significantly (α = 0.05) higher total biomass yield production compared to the control treatment (Figure 1). At the first harvest, yields were small, on average about 9 g of dry matter. The ash pelletized together with FGDG and SP resulted in the significantly (α = 0.05) largest yield. During the second harvest, plants grown in treatments enriched by FGDG resulted also in larger biomass. At third harvest, ryegrass that had been fertilized with ash developed well and resulted in the significantly (α = 0.05) larger yields compared to unamended treatment. At the last, fourth harvest, yields were small in general, probably because of lower temperature and light in autumn, and also due to the fact that the plants were not fertilized by nitrogen after the third harvest.

Overall, treatments with FGDG resulted generally in larger total yield than with SP alone, but

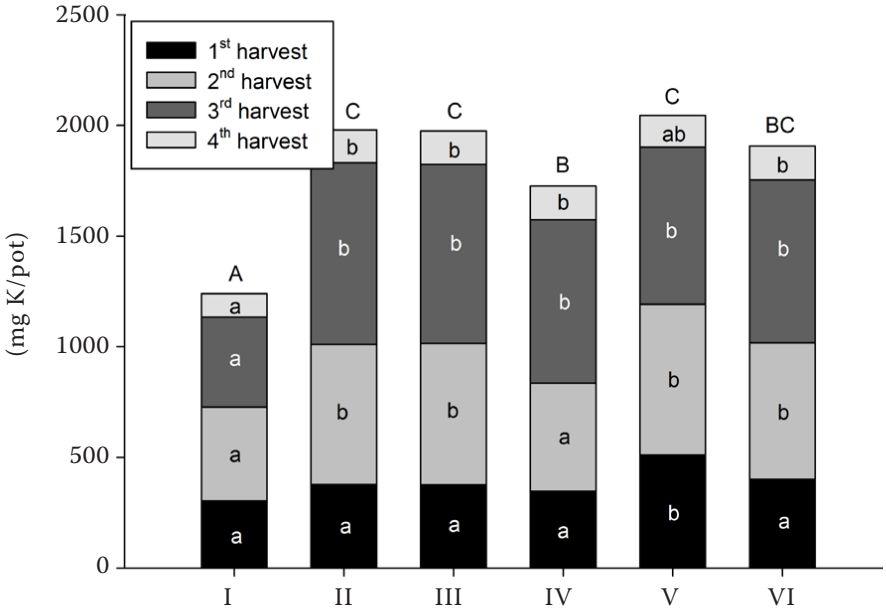

Figure 3. Potassium (K) uptake by ryegrass during four harvests. Different letters indicate significant differences ($P < 0.05$) among the treatments. Differences among the individual harvests are represented by small letters, differences among the total K uptake by plants are represented by capital letters

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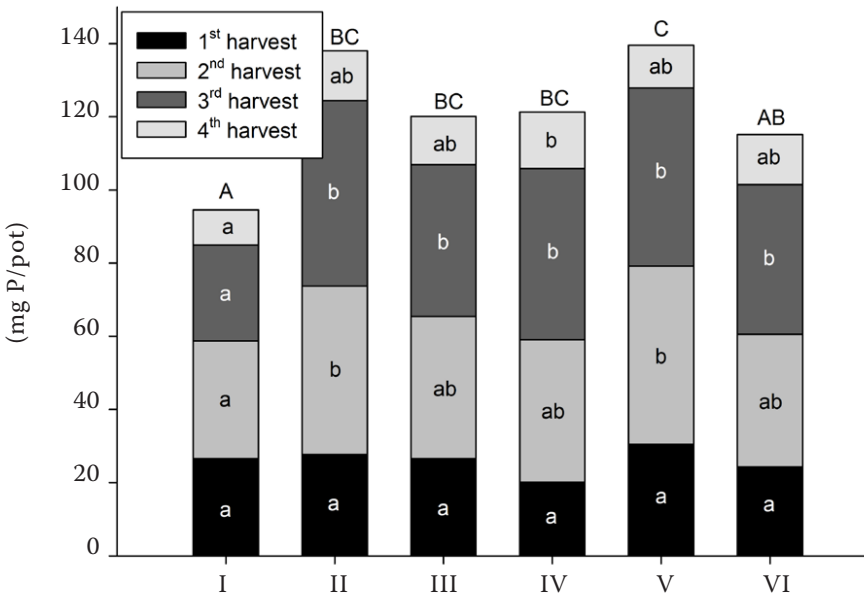

Figure 4. Phosphorus (P) uptake by ryegrass during four harvests. Different letters indicate significant differences ($P < 0.05$) among the treatments. Differences among the individual harvests are represented by small letters, differences among the total P uptake by plants are represented by capital letters

differences between these effects were not significant (α = 0.05), although the relative increase at the treatment V were 30% compared to untreated soil. The yield increase after FGDG application was observed also by Chen et al. (2005) in the experiment with alfalfa growing on silt loam soil.

Effects of fertilizer treatments on Ca uptake by plant biomass. Although plants utilized less than 1% of Ca applied with the powdered ash, Ca uptake in the treatment II was by 25% higher compared to the treatment I. The treatment II was the most successful in the case of Ca uptake by ryegrass but not significantly compared to the other enriched treatments (Figure 2). During the vegetation period, plants in the amended treatments, except the treatment IV, took up significantly more Ca than in the control treatment. It corresponds to the results of Mercl et al. (2016) that the addition of biomass ash increased Ca concentrations in soil. The higher Ca content in the FGDG treatment did not affect the Ca uptake by plants. It is contrary to the statements of Álvarez-Ayuso et al. (2011) that Ca in gypsum is well soluble but in agreement with the experiment of Buckley and

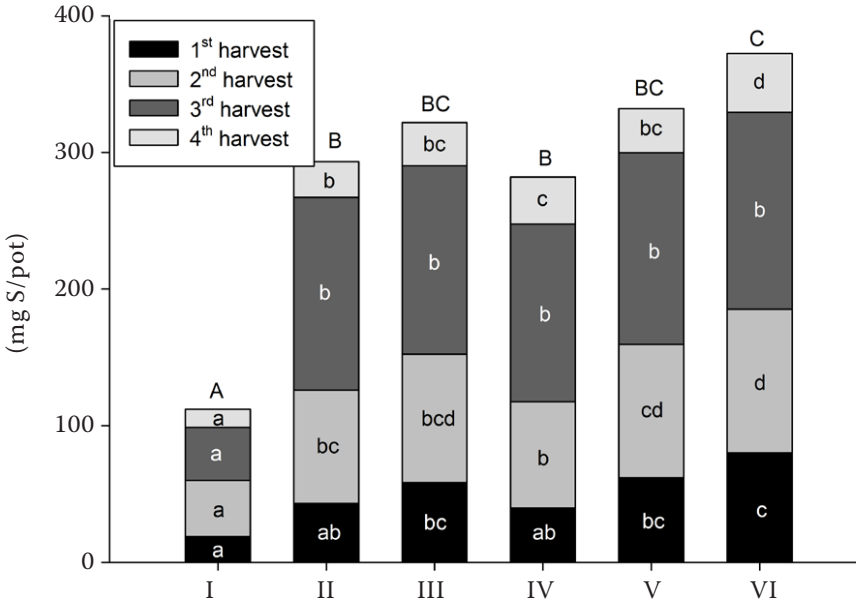

Figure 5. Sulfur (S) uptake by ryegrass during four harvests. Different letters indicate significant differences ($P < 0.05$) among the treatments. Differences among the individual harvests are represented by small letters, differences among the total S uptake by plants are represented by capital letters

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Table 5. Plant available nutrients in soil after the fourth harvest (mg/kg DW)

Treatment	I	II	III	IV	V	VI
Ca	1084[a]	1759[b]	1504[ab]	1398[ab]	1794[b]	1710[b]
K	55[a]	131[b]	130[b]	126[b]	111[ab]	105[ab]
P	36[a]	61[a]	58[a]	73[a]	111[b]	46[a]
S	12[a]	30[a]	27[a]	65[a]	251[ab]	420[b]

All values represent means (n = 4). Different letters indicate significant differences (P < 0.05) among the treatments in individual harvests. DW – dry weight

Wolkowski (2012) where the concentration of Ca in corn and soybean were largely unaffected by application of FGDG.

Effects of fertilizer treatments on K uptake by plant biomass. There were significant differences in K uptake after WFA fertilization (Figure 3). Overall, utilization of K supplied in WFA significantly increased the K uptake on average more than 40% compared to the treatment I. The highest total K uptake by ryegrass was in the treatment V although only 2% of K contained in these pellets was utilized by plants. K content in SP and FGDG was negligible (Table 3) but presence of these additives could improve the K uptake as also observed Blum et al. (2014).

The application of the WFA pellets with SP or FGDG alone was not as successful as their combination.

Effects of fertilizer treatments on P uptake by plant biomass. The total P amount taken up by ryegrass was significantly higher by 26% on average in the treatments II–VI compared to the treatment I (Figure 4). Although a significant increase of P uptake by plants after SP addition alone was expected, surprisingly, these pellets were not such efficient as the combination of SP with FGDG. It is in agreement with Philips et al. (2000) who also found out that applications of P and gypsum increased wheat grain and forage yields compared to P banded without gypsum. The latter authors suggested that P fertilizer bands with respect to Ca^{2+} could induce precipitation of applied P as dicalcium phosphate or dicalcium phosphate dihydrate which would slowly become plant available with time. Contrary to the findings stated above, results from the experiment of Silva et al. (2013) with strawberries or the studies of Murphy and Stevens (2010), and Clark et al. (2001) showed that the combination of P and gypsum were not beneficial for plants, and P solubility decreased with increased Ca concentration.

Effects of fertilizer treatments on S uptake by plant biomass. Among the analysed nutrients, S was found to be the most sensitive element to application of different additives to soil. In all harvests of amended treatments were found significantly higher uptakes of S compared to the treatment I (Figure 5). As expected, the highest uptake was observed at treatment VI – by 70% higher than in the treatment I. Our results are in agreement with Baligar et al. (2011) who stated that FGDG is an excellent source of S to plants.

As seen from Table 5, the amount of plant available Ca, P, and S in the soil was significantly influenced by the amendments added to the WFA. The most noticeable changes were observed in the case of S where the amount of plant available S in the treatment VI was 35 times higher than in the control treatment. Even though, WFA increased the uptake of Ca, K, P and S in our experiment, a significant increase in plant-available portions in soil after harvest was found for K only. This indicates a possible effect of long-term K fertilization with WFA. Ohno (1992) observed that the increase of soil available K levels resulted from the release of wood ash K as well as from the replacement of K on soil exchange sites by Ca and other exchangeable cations released directly from wood ash into the soil suspension.

The application of WFA in various forms to the soil resulted in the increase of yield, nutrient contents and nutrient uptake by ryegrass biomass and plant available portion of nutrients in soil after harvest. Compaction of ash did not significantly affect nutrient release and biomass growth.

The addition of FGDG into WFA pellets did not influence total biomass yield but resulted in a significant increase of S concentrations in ryegrass

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biomass. This may represent strong potential for further waste-recycling in agriculture. However, it was shown that nutritional effects are difficult to predict and due to complex character of waste materials may not necessarily lead to higher yields. More effort is therefore needed to optimize final composition of waste-based fertilizers.

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4.6) Effect of bioeffectors and recycled P-fertiliser products on the growth of spring wheat

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Chemical and Biological Technologies in Agriculture

RESEARCH Open Access

Effect of bioeffectors and recycled P-fertiliser products on the growth of spring wheat

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Abstract

Background: The recycling of waste products into P fertilisers in agriculture is advisable from the perspective of sustainability. Bioeffectors (BEs), which have the ability to increase the plant uptake of P from recycled fertiliser products, may increase the fertiliser value of these products. This paper investigated the effect of a range of different recycled fertilisers on the growth and P uptake of wheat in pot experiments conducted at three different locations in Europe. Furthermore, investigations were undertaken as to whether the addition of a range of bioeffectors could significantly enhance P availability, P uptake and plant growth.

Results: BE additions were found not to significantly increase the aboveground biomass of wheat plants or the uptake of P when plants were fertilised with recycled fertiliser products. This was shown across a range of pot experiments with soils of different P status. Only in the case of the positive control P fertiliser (TSP) was a positive effect of Proradix and RhizoVital on plant growth observed in one of the experiments, while in the same experiment RhizoVital and Biological fertiliser DC had a negative impact on plant biomass when the P fertiliser was Thomas phosphate. With regard to P uptake, there was only a slight positive effect of Proradix in plants not supplied with P fertiliser in this experiment. Clear differences were seen in the efficiency of P fertilisers. Generally, sewage sludge ash performed quite poorly (20–40 % of TSP), while sewage sludge, Thomas phosphate, P-enriched slag and the fibre fraction of pig manure all had a high availability of P (>74 % relative to TSP). Compost composed mainly of garden/park waste and sewage sludge was intermediate in availability (40–70 %). The elemental composition of the harvested wheat plants was significantly affected in all cases by the different P fertilisers added. The BE treatments significantly affected the elemental composition of the aboveground biomass in one of the experiments where the product Proradix had the greatest effect on elemental composition.

Conclusions: In conclusion, the experiments revealed a wide difference in the bioavailability of P in the different waste products, but the added microorganisms demonstrated a limited capacity to influence plant P uptake across a range of soils and waste products.

Background

Phosphorus (P) is a non-renewable resource [1], and currently the majority of P added as fertiliser in agriculture is in the form of inorganic fertilisers. From a sustainability point of view, it is sensible to make better use of P resources that are discarded as waste from urban areas; hence, there is a need to improve the recycling of P from agricultural and urban wastes [2]. Recycled fertiliser products are by no means homogenous and the availability of P for plant uptake from recycled fertiliser products may vary considerably, depending on the feedstock of the fertiliser and the subsequent type of processing [3]. Sewage sludge as a fertiliser may contain a range of different P forms depending

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on the specific process used to recover P from the sewage water, but a precipitation reaction using Al, Fe, Mg or Ca to precipitate P is often employed [3]. Sewage sludge may contain different types of organic contaminants [4]. Although it is still used as a fertiliser in many countries across Europe [5], the use of sewage sludge has declined in a number of European countries, while sewage sludge application on agricultural land was banned in Switzerland in 2008 [6]. A common practice that eliminates the organic contaminants in sludge is to incinerate the sewage sludge, thus producing sewage sludge ash. However, the availability of P in sewage sludge ash is quite low, but is also observed to be variable depending on the type of treatment in the sewage treatment plant [7]. Techniques such as acid leaching and thermal treatment have been investigated for their potential to recover P from sewage sludge ash while separating it from the detrimental heavy metals with varying success [5, 7], but there is also a possibility of combining the upgrading of sewage sludge ash with the recycling of metallurgical slags. Slags from the metallurgical industry had been recycled as fertiliser in Germany in the form of Thomas phosphate for more than 100 years, but this is no longer produced [8]. BOF (basic oxygen furnace) or LD (Linz–Donawitz) slag from steel production may be used as a liming agent in agriculture [8]. It is also possible, however, to use sewage sludge ash as a means to enrich the hot liquid BOF slag with P, resulting in a fertiliser with a markedly higher P availability compared to the feedstock sewage sludge ash, due to a conversion of $Ca_3(PO_4)_2$ (whitlockite) of low neutral ammonium citrate (nac) solubility into Ca-silico-phosphates with a higher nac solubility [9]. P contained in animal manures can be used more sustainably if it is up-concentrated, and thereby more easily transported from areas with a P surplus to areas where soils have a P deficit [3]. The majority of P (60–90 %) found in pig manure is inorganic [10] and only very little is in the form of phytate [11, 12]. Biomass ash (e.g. wood and straw ash) from bioenergy-plants could also potentially serve as a P fertiliser [13].

Different types of biostimulants or bioeffectors (BEs) have been investigated for their ability to increase plant productivity in agricultural systems [14]. The concept of BEs covers quite a diverse group of natural products [15]. In the present paper, the scope was limited to plant growth-promoting microorganisms (PGPM) focusing on plant growth-promoting rhizobacteria (PGPR) [16] and free-living fungi, such as species of the genus *Trichoderma* [17]. PGPM may have the potential to enhance plant uptake of P from soil [14, 18, 19]. Improved growth under P-limiting soil conditions as a result of microbial inoculations has been observed in many different plant species, such as mung bean [17], bean [20], maize [21] and wheat [22]. In soil, a large proportion of the total P pool is not directly available for plant uptake [23], and the ability to solubilise phosphates in the rhizosphere has been viewed as an important function of PGPM [24]. The plant growth-promoting effect may, however, be overestimated due to a publication bias, and an observed positive plant growth response may be due to mechanisms other than an increased availability of P in the rhizosphere brought about by PGPM solubilisation of P, e.g. changes in root architecture and total root length [24].

Further research is therefore required into the potential of BEs to facilitate the plant uptake of nutrients from soil. Fungi of the ascomycete genera *Trichoderma* and *Penicillium* have been extensively studied for their potential as PGPM [25], and fungi of the genus *Trichoderma* may have an ability to increase plant nutrient uptake from soil [26, 27]. The specific *T. harzianum* strain Rifai 1295-22 (T22) has been observed to increase the solubilisation of sparingly soluble calcium phosphates [28] and to have a plant growth-promoting effect in willow [29], chickpea [17] and maize [30]. Fungi of the genus *Penicillium* have been shown to have P-solubilising capabilities [31, 32], and members of this genus have been observed to have a positive effect on biomass and P uptake of wheat and bean [20]. Wakelin et al. [33] found that a strain of *P. bilaii* is both capable of increasing the yield of medic and lentil in the field and of significantly increasing the level of HCO_3-extractable P in soil microcosms, and more recently *P. bilaii* has been found to increase yield in maize in field trials [21]. On the other hand, the positive effect of *P. bilaii* under P-limited conditions has also been linked in pea to an increase in the root adsorptive capacity under P-limited conditions rather than through increased P solubilisation [34]. However, an investigation across a number of field studies involving wheat showed that *P. bilaii* does not significantly affect P uptake and yield [35]. Gram-negative gammaproteobacteria of the genus *Pseudomonas* are ubiquitous bacteria in soil, are known to proliferate greatly in the rhizosphere [36] and have been studied for their plant growth-promoting activities for many years [37]. Bacteria from this genus have been observed to increase plant productivity under P-limiting conditions [38, 39]. As a representative of *Pseudomonas* PGPR, the product *Proradix* was selected. This product has primarily been developed and investigated for its effects on plant resistance to pathogens [40–42], but there is also evidence that this product may improve plant growth under nutrient-limiting conditions [43]. Low G + C Gram-positive bacteria of the genus *Bacillus* have been shown to solubilise calcium phosphates and increase the dry matter yield of wheat in a pot trial in which no P fertiliser or calcium phosphates were applied [22]. A number of *B. amyloliquefaciens* strains have been investigated for their biocontrol capabilities [44] and the type strain (FZB42) for the subspecies *plantarum* of *B. amyloliquefaciens* [45] is reported to work as a biofertiliser and provide protection against various soil-borne diseases [46, 47].

The aim of the present paper was to investigate whether a variety of BE organisms could significantly enhance

the availability and uptake of P from a range of recycled P-fertiliser products with very different P availability. The paper encompasses a number of pot experiments carried out in Denmark, Germany and the Czech Republic. The experiments included a negative control as well as a positive control (in two out of three studies) in which highly available triple superphosphate was added.

Sewage sludge ash in particular is an example of a product with quite low P solubility, offering considerable potential for improvement by BEs. The microorganisms were expected to have a positive effect on the solubilisation of fertiliser-derived P in the soil, as well as a direct effect on the plants through hormonal effects. The latter effects might occur in all P-addition treatments, whereas the positive effects on soil P availability are expected to be of greater significance in treatments with lower P availability, such as sewage sludge ash. It was therefore hypothesised that: (i) inoculation with the selected BE strains would increase the availability of P from the recycled fertilisers and (ii) inoculation with the selected BE strains would increase the uptake of P by wheat from soil, leading to a larger production of aboveground biomass.

Due to differences in the conditions between the individual experiments, it is not possible to compare the concentrations of elements or biomass produced per kg soil across experiments. We therefore only analyse the relative changes in, for instance, biomass compared to the negative and positive controls (where included) across experiments. We analyse the effect of BEs in the individual experiments. The fact that some of the BEs are tested using different soils and slightly different growing conditions serves as a stronger test of their performance than a single pot experiment would have.

Methods

The paper deals with the results of three separate pot trials. The pot trials were carried out at Arbeitsgemeinschaft Hüttenkalk e.V., Germany (HK Kalke experiment), the University of Copenhagen, Denmark (UCPH experiment), and the Czech University of Life Sciences Prague, Czech Republic (CULS experiment).

Sampling of soil and soil characterisation

The three pot experiments included in this publication were performed with four different soils (Table 1). Soil for the HK Kalke experiment was sampled from the plough layer of a field with arable feed production that had not had any P fertilisation for over 30 years, located in Marienmünster-Vörden in East Westphalia, Germany. Soil for the UCPH experiment was sampled from the plough layer of the long-term nutrient depletion trial at the University of Copenhagen's experimental farm in Taastrup, Denmark, where cereals have been grown continuously for more than 50 years without the addition of P fertilisers. Finally, the soils used in the CULS pot experiment were sampled from the plough layer of either the long-term experimental farm in Humpolec, Czech Republic, where wheat, potato and barley were grown in rotation continuously without fertilisation for 20 years (Humpolec soil), or a field managed by a conventional farming system with low P inputs (Poděbrady soil). The air-dried soil was analysed in the laboratory of the *Landesanstalt für Landwirtschaftliche Chemie* at the University of Hohenheim, Germany, for the HK Kalke and UCPH experiments. Selected results of these analyses are presented in Table 1. Texture was analysed using a combination of wet sieving and pipetting according to the VDLUFA standard method C 2.2.1 [48]. Organic carbon content was measured according to the VDLUFA standard method A 4.1.3.1 [48]. pH was measured in 0.01 M $CaCl_2$ according to the VDLUFA standard method A 5.1.1 [48]. Finally, calcium-acetate lactate-extractable P (P_{CAL}) was measured according to the VDLUFA standard method A 6.2.1.1 [48]. The soils for the CULS experiments were analysed at the Czech University of Life Sciences following the same protocols.

Table 1 Soil data

Soil	Management	Texture (%)			OC (%)	pH[a]	P_{CAL} (mg kg^{-1})	P_{TOT} (mg kg^{-1})
		Sand	Silt	Clay				
Vörden[b]	Conventional farming system. Arable land food production. No P addition for more than 30 years	41.1	46.9	12.0	0.8	5.0	26	310
NDT-A[c]	Continuous cropping. No addition of P fertiliser for more than 30 years	55.4	31.2	13.4	1.1	5.8	35	397
Humpolec[c]	Continuous cropping (potato, wheat, barley). No addition of P fertilisers	30	49	21	1.6	4.5	59	587
Poděbrady[c]	Conventional farming system. Low P input	57	24	19	1.9	6	30	384

[a] pH measured in $CaCl_2$

[b] Data were recorded on the 2:1 soil:sand mixture used in the pot experiments

[c] Data were recorded on the pure soil

Bioeffector (BE) treatments

A range of different bioeffectors (BEs) was added to the growth medium at sowing. A control treatment without the addition of BEs was included (BE0). The BEs investigated were a *Trichoderma harzianum* isolate marketed as Trianum-P by Koppert (TrP), Proradix (Pro) from Sourcon Padena containing *Pseudomonas* sp., RhizoVital 42 (RhVi) produced by ABiTEP containing *Bacillus amyloliquefaciens* ssp. *plantarum*, strain FZB42, biological fertiliser DC (Bio-DC) produced by Bayer Crop Science Biologics GmbH containing *Penicillium* sp. and BactoProf (BaPr) produced by Terra Bioscience, Germany, which contains isolates of *T. harzianum* and five species of *Bacillus*. BE suspensions were prepared in 0.25 mM $CaSO_4$. The concentrations used for the inoculation are given in Table 2.

P fertilisers and P-fertilisation treatments

A number of recycled P fertilisers were applied in the experiment (Table 3). Thomas phosphate was obtained from the Luxengrais steel plant in Luxembourg. Sewage sludge and sewage sludge ash for the HK Kalke experiment were obtained from a municipal treatment plant in Bonn, processing wastewater mainly from households and from an attached sewage sludge incineration plant. The sewage sludge and sewage sludge ash used for the UCPH experiment originated from a public treatment plant receiving wastewater from households and industries (Spildevandscenter Avedøre). A P-enriched steelmaking slag (LDS/SSA in the HK Kalke experiment) was produced by blowing sewage sludge ash into a liquid 1500 °C basic oxygen furnace slag (prepared by the Linz–Donawitz process) from a steelwork in Salzgitter [9]. A fibre fraction of pig manure (FFPM) was obtained using the decanter centrifuge method. A compost (Comp) consisting of a mixture of mainly garden park waste and sewage sludge (42 % garden park waste, 36 % sewage sludge, 14 % straw and horse manure, 8 % wood mass) was obtained from the private company KomTek, Denmark. Straw ash (StA) was a mixture of fly and bottom ash from a grate-fired boiler (15 MWt) and originated from cereal straw combustion. Finally, wood ash (WoA) was obtained from a fluidised bed reactor (15 MWt) in which wood chips were combusted.

For the majority of the fertilisers, the equivalent of 50 mg P kg^{-1} soil was added. There were, however, some deviations from this in the CULS experiment (Table 3). For both the HK Kalke experiment and the UCPH experiment, sewage sludge (SS) and sewage sludge ashes (SSA) were included. Furthermore, both TSP and a low-grade type of TSP, termed superphosphate (SP) here, were included in both these experiments as positive controls. In all three experiments, a negative control without the addition of P fertiliser (P0) was included. An overview of the BE and P-fertilisation treatments included in the three pot experiments is presented in Table 4.

Pot trial setup, growing conditions and harvest

Soil preparation, growing conditions, harvest days and nutrient application are presented in Table 5.

HK Kalke experiment

The air-dried and sieved soil (mesh size 5 mm) was mixed with water-washed quartz sand in a proportion of 2:1. This substrate was mixed with 0.843 g kg^{-1} $Ca(NO_3)_2{\cdot}4H_2O$ and 0.719 g kg^{-1} Patentkali (27.8 % K_2O, 9.49 % MgO, 15.8 % S). Each pot was filled with 6 kg of the fertilised soil/sand mixture and watered to 70 % of WHC. Before watering, Bio-DC was mixed into the substrate of the Bio-DC treatment. Spring wheat (cultivar Aranka) was sown in 32 separate sowing holes (approximately, 2 cm deep). To each of the sowing holes, 2 ml of the Pro or RhVi suspensions were added in the corresponding BE treatments. After germination, plants were reduced to 24 wheat plants per pot. The pots were placed in a randomised design, with four replicates per treatment, in an outdoor roofed vegetation hall. Pots were irrigated with demineralised water to 60–70 % WHC (controlled gravimetrically once a week) during the whole vegetation

Table 2 Bioeffector (BE) products applied in the experiments

Product	Producer	Abbr.	Name of organism(s)	Type of organism	Application rate (cfu g^{-1} soil)
Control	n.a.	BE0	n.a.	n.a.	n.a.
Trianum-P, T22	Koppert, The Netherlands	TrP	*Trichoderma harzianum*, strain T-22	Fungi	$2.5{\cdot}10^4$
Proradix	Sourcon Padena, Germany	Pro	*Pseudomonas* sp., strain DSMZ 13134	Bacteria	$2{\cdot}10^6$
RhizoVital 42	ABiTEP, Germany	RhVi	*Bacillus amyloliquefaciens*	Bacteria	$2{\cdot}10^6$
Biological fertiliser DC	Beyer/Prophyta, Germany	Bio-DC	*Penicillium* sp.	Fungi	$1{\cdot}10^5$
Bacto prof	Terra Bioscience, Germany	BaPr	*T. harzianum* and five species of *Bacillus*	Bacteria + fungi	$2{\cdot}10^6$

Table 3 P-fertilisation treatments applied in the experiments

P fertiliser	Treatment abbreviation	Total P content in product (g kg^{-1})	Water-extractable P (% of total P)	App. rate (g dry product kg^{-1} soil)	P app. rate (mg P kg^{-1} soil)
Negative control	P0	n.a.	n.a.	n.a.	n.a.
Triple superphosphate	TSP	200	43.3	0.25	50
Superphosphate	SP	81	11.4	0.62	50
Thomas phosphate	Thph	68	0	0.73	50
Sewage sludge, HK Kalke	SS	36	n.d.	1.40	50
Sewage sludge, UCPH	SS	37	n.d.	1.36	50
Sewage sludge ash, HK Kalke	SSA	103	0.18	0.48	50
Sewage sludge ash, UCPH	SSA	89	n.d.	0.56	50
Fibre fraction of pig manure	FFPM	2.4	n.d.		50
SSA-enriched LD slag	LDS/SSA	17	0	2.92	50
Compost mainly consisting of sewage sludge and garden/park waste	Comp	3.6[a,b]	n.d.	13.8[b]	50
Ashes from cereal straw	StA	13.6	6.5	10	136
Ashes from wood chips	WoA	10.2	0.05	10	102
Dipotassium phosphate	DKP	178	100	0.18	32

[a] According to information from the producer

[b] These measurements are in g kg^{-1} fresh matter

period. The plants were supplemented with an additional 50 mg N kg^{-1} soil on day 40 in the form of $Ca(NO_3)_2$. The plants were harvested 8 weeks after sowing. The plants were at stage 59 (without P fertilisation) or stage 63 (with P fertilisation).

UCPH experiment

Soil was partially air dried and sieved (mesh size 5 mm). The soil was mixed with quartz sand in the proportion of 1:1. The water-holding capacity of the soil/sand mixture was determined. For each pot, 2.5 kg of the soil/sand mixture was mixed with 0.645 g kg^{-1} $Ca(NO_3)_2$ and 0.667 g kg^{-1} Patentkali (30 % K_2O, 10 % MgO, 42.5 % SO_3). Subsequently, this substrate was mixed with either 50 g sand (P0 treatment) or 50 g sand mixed with one of the P fertilisers being investigated (Table 5). The fertilisers were mixed with sand prior to being added to the soil to ensure thorough mixing throughout the whole soil volume. The soil was watered to 40 % of WHC. Fifteen wheat seeds (cultivar Scirocco, KWS) were sown in separate sowing holes (approximately, 2 cm deep). After the seeds were sown, 1 mL of BE suspension (or 0.25 mM $CaSO_4$ in the BE0 controls) was added to each of the sowing holes before these were closed. For each treatment, five replicate pots were set up, resulting in a total of 140 pots in the experiment. The pots were placed in a greenhouse in a randomised block design. After germination, the wheat plants were thinned out, leaving ten plants in each pot. During the experiment, the pots were watered to weight (initially, 60 % and subsequently 70 % of WHC) at regular intervals (every 1–3 days). The blocks were rotated and reshuffled once or twice a week during the experiment. At 25 days after sowing, the youngest fully developed leaf was removed from one plant in three replicates of the BE0 treatments (all P treatments), giving a total of 21 samples. After 32 days, five plants from each pot were harvested. After 42 days, extra N was added to each pot (33 mg N kg^{-1} soil). After 54 days, the remaining five plants were harvested from each pot. At harvest, the plants were at stage 55. A follow-up experiment partially replicating the UCPH experiment was carried out as well (see Additional file 1 for details).

CULS experiment

Soil was air dried and sieved (mesh size 10 mm). No sand was added to the soil. For each pot, 5 kg (d.w.) of soil was used, which was mixed with 1.67 g of NH_4NO_3. 50 g of WoA or StA was thoroughly mixed with the soil prior to filling the pots (final dose 10 g of ash per kg soil). K_2HPO_4 was applied as a water solution and was also thoroughly mixed into the whole soil volume. The soil was watered to 40 % of WHC, 25 wheat seeds (cultivar Aranka) were sown in separate sowing holes (approximately, 2 cm deep) and 2 ml of BE suspension (or 0.25 mM $CaSO_4$ in the BE0 controls) was applied to each hole prior to closing. After germination, the number of plants was reduced to 20 wheat plants per pot, and these were inoculated again by irrigation with 100 ml of BE suspension per pot. The pots were placed in an outdoor roofed vegetation hall. Pots were irrigated with demineralised water to 60–70 % WHC (controlled gravimetrically once a week)

Table 4 Overview of the treatments applied in the different experiments (soils)

Soil	1. HK Kalke (Germany)	2. UCPH (Denmark)	3. CULS (Czech Republic)	
	Vörden	NDT	Humpolec	Poděbrady
Bio-effectors				
Negative control (BE0)	X	X	X	X
TrP		X		
Pro	X	X		
RhVi	X	X	X[a]	X[a]
Bio-DC	X			
BaPr			X[a]	X[a]
P fertilisers				
Negative control (P0)	X	X	X[b]	X[b]
DKP			X[b]	X[b]
TSP	X	X		
SP	X	X		
Thph	X			
SS	X	X		
FFPM		X		
Comp		X		
SSa	X	X		
LDS/SSA	X			
StA			X	X
WoA			X	X

[a] Only in combination with StA and WoA

[b] Only in combination with BE0

during the entire vegetation period. The experiment was undertaken in a randomised design with four randomisation procedures during the experiment. The plants were harvested after 16 weeks. At harvest, the plants were at full maturity.

Soil data recorded during the HK Kalke experiment

Soil (40 g) was sampled 27 days after sowing. The soil was air dried and completely passed through a 2 mm mesh sieve. For pH measurement, 10 g of soil was suspended in 25 ml of a 0.01 molar $CaCl_2$ solution for 1 h, stirred twice and pH determined using a pH electrode (VDLUFA standard method A 5.1.1). For water extraction of soil phosphate according to Van der Paauw [49] and Murphy and Riley [50], 4.25 ml of soil was suspended in demineralised water for approximately 22 h. Thereafter 250 ml water was added; the mixture was mechanically shaken for 1 h and filtered. P determination was undertaken using a spectrophotometer and molybdenum blue method.

Plant analyses

HK Kalke experiment

After harvest, the aboveground wheat plant material was dried at 60 °C. 400 mg of plant material was digested with 8 ml 69 % HNO_3 supra and 1 ml 15 % H_2O_2 in high-pressure MARS express vessels in a MARS microwave digestion system. The element analyses of P, K, Mg, Ca, Mn and Na were performed by ICP-OES.

UCPH and CULS experiment

The plant material was dried at 65 °C and weighed to measure the dry aboveground biomass. For elemental analysis, the dry plant material was finely ground. Subsequently, 100 mg of dry plant material was mixed with 2.5 ml 70 % HNO_3 and 1 ml 15 % H_2O_2, followed by digestion in a pressurised single-chamber microwave oven (UltraWAVE, Milestone Srl, BG, Italy). Samples were then diluted to 50 ml using Milli-Q water and analysed for their elemental content (B, Ca, Cu, Fe, K, Mg, Mn, P, S, Zn in UCPH and Ca, K, Mg, Mn, Na, P in CULS) by ICP-OES. For the samples from the final harvest in the UCPH experiment, only P was measured using flow injection analysis.

Data analyses and statistics

The measurements of aboveground biomass were normalised relative to the control treatment (P0, BE0):

$$\text{Normalized biomass}_{\text{sample}} = \frac{\text{biomass}_{\text{sample}}}{\text{biomass}_{\text{control(P0, BE0)}}}. \quad (1)$$

In the CULS experiment, the normalisation was undertaken separately for the two soils. Significance testing of differences between treatment means was performed using one- and two-way ANOVAs and Dunnett's test (for comparisons versus the control only) or Tukey's test (for all possible comparisons) for post hoc multiple comparisons. These were performed using the statistics module in Sigma Plot 13.0. In the UCPH experiment in which two separate samplings had been performed, the difference in normalised biomass between sampling days was tested using a paired t test. For the CULS experiment, all the P-fertiliser treatments were combined with the BE0 treatment only. The effect of different P substrates was therefore analysed by a two-way ANOVA, excluding data for the RhVi and BaPr BE treatments. The effect of BE treatments in the CULS experiment was tested in two separate two-way ANOVAs for the two soils, where only data from the straw and wood ash treatments were included.

The efficiency of the fertilisers relative to TSP (positive control) was calculated as the mean efficiency measured

Table 5 Growing conditions in pot experiments

Exp.	Wheat cultivar	Soil:sand ratio (mass)	Size of pots (L)	Mass of substrate (kg)	No of plants	No. of harvests	Final harvest (weeks)	Rep	App. of macronutrients at setup (mg kg^{-1} substrate)				
									Ca[a]	K[b]	Mg[b]	N	S[b]
HK Kalke	*Aranka*	2:1	6	6	24	1	8	4	158	166	40	100[a]	114
UCPH	*Scirocco*	1:1	3	2.5	10	2	8	5	158	166	40	100[a]	114
CULS	*Aranka*	1:0	6	5	20	1	16	3	0	0	0	100[c]	0

[a] Supplied as $Ca(NO_3)_2$

[b] Supplied as Patentkali (30 % K_2O, 10 % MgO, 42.5 % SO_3)

[c] Supplied as NH_4NO_3

in n replicate pots. The efficiency in the individual pots was calculated as follows where data were available:

$$\mathrm{FE}(\%) = 100 \times \left(\frac{\mathrm{biomass}_{\mathrm{sample}} - \overline{\mathrm{biomass}_{\mathrm{P0,BE0}}}}{\overline{\mathrm{biomass}_{\mathrm{TSP}}} - \overline{\mathrm{biomass}_{\mathrm{P0,BE0}}}} \right). \quad (2)$$

Similarly, the P-uptake efficiency from the different fertilisers was calculated as follows where the necessary data were available:

$$\mathrm{PUE}(\%) = 100 \times \left(\frac{\mathrm{P\ content}_{\mathrm{sample}} - \overline{\mathrm{P\ content}_{\mathrm{P0,BE0}}}}{\overline{\mathrm{P\ content}_{\mathrm{TSP}}} - \overline{\mathrm{P\ content}_{\mathrm{P0,BE0}}}} \right). \quad (3)$$

The data for biomass in the HK Kalke experiment were expressed as a function of the available P level (P_{H2O}) in the pot experiment and the three-parameter exponential rise to maximum (Mitscherlich) curve was fitted to the data [51]:

$$y = y_0 + a(1 - e^{-\mathrm{bx}}). \quad (4)$$

The same model was used to express biomass as a function of the P concentration in the youngest fully developed leaf at day 25 in the UCPH experiment. These regressions and simple linear regressions were performed using the regression wizard in SigmaPlot 13.0 (Systat).

Principal component analysis (PCA) was performed on data for elemental concentrations. Beforehand, PCA data were standardised by subtracting the mean for each element and then dividing this by the standard deviation. PCA was performed in R version 3.1.1 [52] using the ade4 package [53] with a chosen number of principal components of 10.

Results

Aboveground biomass and P content

HK Kalke experiment

In the KALKE experiment (Fig. 1a), the normalised aboveground biomass was significantly different between the P-fertilisation treatments (two-way ANOVA, $P < 0.001$) and BE treatments (two-way ANOVA, $P < 0.05$), and there was a significant interaction between the two factors (two-way ANOVA, $P < 0.001$). For the TSP treatment, the inoculation with Proradix and RhizoVital resulted in a significantly higher normalised aboveground biomass (Dunnett's test, $P < 0.05$). When Thomas phosphate (Thph) was applied as a P fertiliser, inoculating with Proradix and biological fertiliser DC resulted in a significantly lower normalised aboveground biomass compared to the BE0 control (Dunnett's test, $P < 0.05$). For the remaining P fertilisers, the applied BEs (Pro, RhVi and Bio-DC) did not have a significant effect when compared with the control. The post hoc analysis of P-content data (Table 6a) showed that although there was a highly significant effect of both the P fertiliser, the BE application and the interaction between the two (two-way ANOVA, $P < 0.001$), there was not a significant positive effect of any of the BE treatments on P uptake from any of the P fertilisers compared to the BE0 control (Dunnett's test, $P > 0.05$). Only in the P0 treatment, there was a significant positive effect of Pro and RhVi on total aboveground P content (Table 6a).

UCPH experiment

In the UCPH experiment, plants were harvested after 32 (Fig. 1b) and 54 days (Fig. 1c). The normalised biomass across all treatments was significantly different between harvests (paired t test, $P < 0.001$); therefore, the normalised aboveground biomass data from the two harvests were analysed individually. The normalised biomass was significantly different between P-fertilisation treatments at both harvests (two-way ANOVA, $P < 0.001$). In contrast to this, the different BE inoculations did not affect the biomass obtained (two-way ANOVA, $P > 0.05$) and no interaction was observed between the two factors (two-way ANOVA, $P > 0.05$). At both harvests, all treatments in which a P fertiliser was applied resulted in a significantly higher aboveground biomass than the control without added P fertiliser (Dunnett's test, $P < 0.05$). The absence of a significantly higher aboveground biomass when inoculating with the three BEs (TrP, Pro, RhVi) compared to the uninoculated control (BE0) was confirmed for sewage sludge, sewage sludge ash and compost as the P fertilisers in a follow-up experiment at the University of Copenhagen in which only five wheat plants were grown in each pot (Additional file 1: Fig. S1). P uptake was only evaluated for the P fertilisers SSA and FFPM at the second harvest (Table 6b). No effect was produced by either of the two main factors (P fertiliser and BE addition) and there was no interaction between the two factors in relation to the aboveground P content (two-way ANOVA, $P > 0.05$).

CULS experiment

In the CULS experiment (Humpolec and Poděbrady soils), there was no significant effect of the soil type on the aboveground biomass in the control (P0/BE0) treatment (one-way ANOVA, $P > 0.05$, data not shown). For the Humpolec soil, the DKP treatment yielded a significantly lower normalised biomass compared to the control (two-way ANOVA on BE0 data with soil and P fertiliser as factors, Dunnett's test $P < 0.05$), while in the Poděbrady soil the normalised aboveground biomass was not significantly different from the control when adding DKP (Dunnett's test, $P > 0.05$). The addition of straw

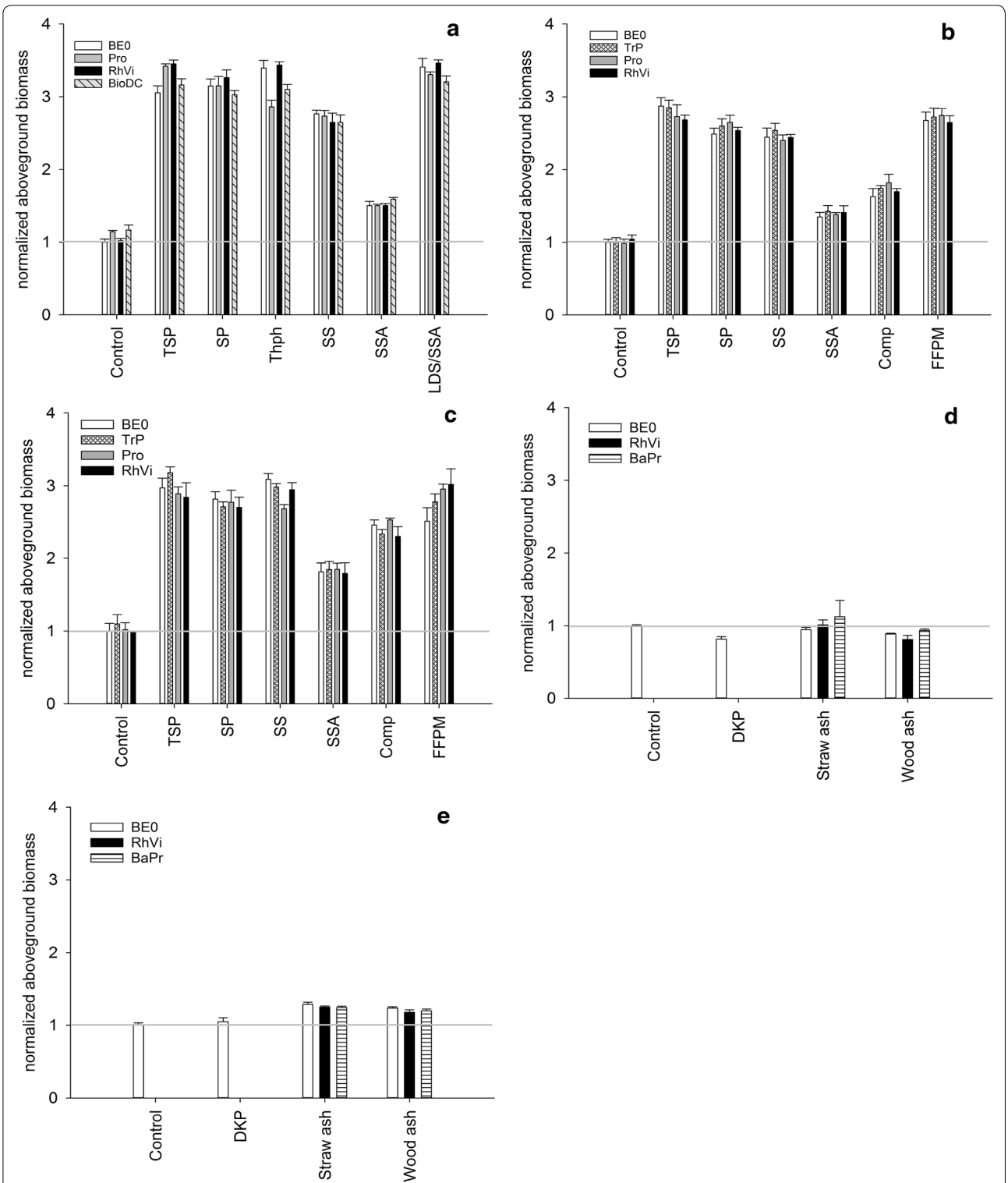

Fig. 1 Normalised aboveground biomass of wheat plants in the HK Kalke (**a**), UCPH (**b**, **c**) and CULS (**d**, **e**) experiments. The data are normalised by dividing by the mean of the control treatment (no P fertiliser and BE0) in each experiment and in each of the two soils in **d** and **e**. The following P fertilisers were added in the experiments: no P fertiliser (Control), triple superphosphate (TSP), superphosphate (SP), Thomas phosphate (Thph), sewage sludge (SS), sewage sludge ash (SSA), compost of sewage sludge and garden/park waste (Comp), P-enriched steelmaking slag (LDS/SSA), fibre fraction of pig manure (FFPM), K_2HPO_4 (DKP), straw ash and wood ash. The soil was either not inoculated (BE0) or inoculated with Proradix (Pro), RhizoVital (RhVi), biological fertiliser DC (Bio-DC), Trianum P (TrP) or BactoProf (BaPr). Data in **b** are for four plants harvested from each pot after 32 days, while data in **c** are for five plants harvested after 54 days. The Humpolec soil was used in **d** while the Poděbrady soil was used in **e**

Table 6 P content in aboveground biomass (mg P kg^{-1} soil) in the HK Kalke (a) , UCPH (b) and CULS (c) experiments

a

P fertilizer	Tukey's test (BE0 results)[a]	Control (BE0)	Pro	RhVi	Bio-DC
Control (P0)	a	3.8	***5.4***	***4.7***	4.3
TSP	c	8.9	10.5	10.2	9.5
Superphosphate	c	9.4	9.6	9.4	8.6
Sewage sludge	c	8.7	9.8	9.8	7.8
Sewage sludge ashes	b	5.3	6.1	6.1	*4.2*
Thomas phosphate	c	10.3	*7.5*	10.7	8.5
LDS/SSA	c	9.3	9.0	9.1	8.0

b

	Tukey's test (BE0 results)	Control (BE0)	TrP	Pro	RhVi
Sewage sludge ashes	n.s.	5.9	5.8	6.1	5.9
FFPM	n.s.	4.1	6.2	5.6	6.1

c

P fertilizer	Tukey's test (BE0 results)[a]	Control (BE0)	RhVi	BaPr
Humpolec soil				
Control (P0)	a	11.2	n.a.	n.a.
DKP	a	10.4	n.a.	n.a.
Straw ash	b	13.5	14.4	16.5
Wood ash	a	11.3	10.2	12.4
Poděbrady soil				
Control (P0)	A	12.4	n.a.	n.a.
DKP	A	14.4	n.a.	n.a.
Straw ash	B	19.8	20.2	19.1
Wood ash	AB	15.6	14.2	14.9

a Data were analysed by two-way ANOVA. Data were log-transformed prior to statistical analysis due to unequal variances (Brown–Forsythe test, $P < 0.05$). Tukey's post hoc test was used to test whether there was a significant difference in P uptake between the different P fertilisers within the BE0 treatment. For each P fertiliser, values in bold italics are significantly higher than the BE0 treatment and values in italics are significantly lower than the BE0 control according to Dunnett's test

b Data were log-transformed prior to statistical analysis due to unequal variances (Brown–Forsythe test, $P < 0.05$)

c For each of the two soils independently, Tukey's post hoc test was used to test if there was a significant difference in P uptake between the different P fertilisers within the BE0 treatment. Small letters indicate differences for the Humpolec soil, while capital letters indicate differences within the Poděbrady soil. For each row, values in bold italics are significantly higher than the BE0 treatment and values in italics are significantly lower than the BE0 control according to Dunnett's test (one-way ANOVA with data for straw ash and wood ash)

[a] Different letters indicate means that are significantly different

ash resulted in a significantly higher biomass at harvest in the Poděbrady soil (Dunnett's test $P < 0.05$), while the biomass after adding straw ash was not significantly different from the control without the addition of P fertiliser in the Humpolec soil (Dunnett's test $P > 0.05$). Finally, the addition of wood ash led to a significantly lower biomass compared to the control in the Humpolec soil (Dunnett's test $P < 0.05$), while in contrast the addition of wood ash led to an increase in biomass compared to the control for the Poděbrady soil (Dunnett's test $P < 0.05$). Overall, there was only a fairly limited difference in the harvested biomass in a comparison across P-fertilisation treatments (Fig. 1d, e). The maximum increase observed when looking across the BE0 treatments was 22 %. This increase was observed for both straw and wood ash in the Poděbrady soil. The effect of the addition of the two different ash types (straw and wood ash) in combination with the different BE inoculation treatments included here (BE0, RhVi, BaPr) was analysed in two separate two-way ANOVAs for the two soils (Humpolec & Poděbrady) included in this experiment. No significant effect was observed of ash type or BE addition or an interaction between the two factors for any of the two soils investigated ($P > 0.05$, two-way ANOVA). There was a significant effect of soil on P uptake in the P0/BE0 treatment (Table 6c, one-way ANOVA, $P < 0.001$). The data for P content (Table 6c) were subsequently analysed for the two soils independently, and a significantly higher P content was observed in the straw ash treatment compared to the control in both soils (Tukey's test, $P < 0.05$), but the

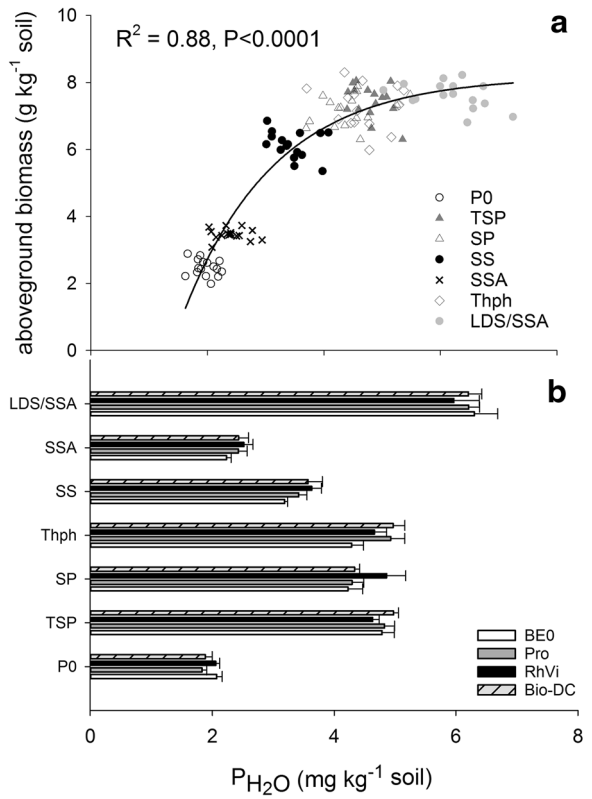

Fig. 2 **a** Total aboveground biomass of wheat plants in the KALKE experiment as a function of P_{H_2O} measured after 27 days and **b** the measured values of P_{H_2O} across P fertilisers and BE treatments. In **a,** data are fitted to three-parameter functions of the form $y = y_0 + a \cdot \left[1 - e^{(-b \cdot x)}\right]$. In **b,** each *bar* represents the mean of data from four replicate samples. *Error bars* represent SEM. The following P fertilisers were added in the experiment: no P fertiliser (P0), TSP, superphosphate (SP), sewage sludge (SS), sewage sludge ash (SSA), Thomas phosphate (Thph), P-enriched LD slag (LDS/SSA)

BE treatments did not result in a total P content that was significantly different from the control (Dunnett's test, $P > 0.05$).

Soil-available P in the KALKE experiment and relationship with biomass

Water-extractable P (P_{H2O}) was able to explain a large part of the variation in the aboveground biomass in the HK Kalke experiment across P-fertilisation treatments and BE treatments (Fig. 2a). Using the Mitscherlich equation, a model with P_{H2O} measured after 27 days explained 88 % of the variation in the aboveground biomass (Fig. 2a, $R^2 = 0.88$, $P < 0.0001$). The water-extractable P in soil was significantly different between the P-fertiliser treatments, and all P-fertiliser treatments were significantly different from the control (Fig. 2b, two-way ANOVA, $P < 0.001$). The sequence was as follows: P0 < SSA < SS < TSP = SP = Thph < LDS/SSA. There was no significant effect of BE inoculation on the level of water-extractable P in the pots (two-way ANOVA, $P > 0.05$) and there was no significant interaction between the two factors (two-way ANOVA, $P > 0.05$).

Correlation between plant P data and aboveground biomass

The variation in the aboveground biomass in the UCPH experiment in the BE0 treatments at both harvests (after 32 and 54 days) was explained by the P concentration in the youngest fully developed leaf harvested from one plant during early growth after 25 days. Using the three-parameter exponential rise to maximum (Mitscherlich) curve equation, highly significant relationships between the two variables were found (Fig. 3: first harvest, $R^2 = 0.73$, $P < 0.0001$; second harvest, $R^2 = 0.78$, $P < 0.0001$).

Fertiliser use efficiencies

Thomas phosphate and sewage sludge ash-enriched BOF slag both had a relative fertiliser efficiency and relative P-use efficiency comparable to or higher than TSP (Table 7). Sewage sludge tended towards slightly lower relative efficiencies compared to TSP (76–106 %), but these differences were only significant in the follow-up experiment at UCPH (Table 7). Sewage sludge ash, on the other hand, gave quite low efficiencies (24–31 %) and

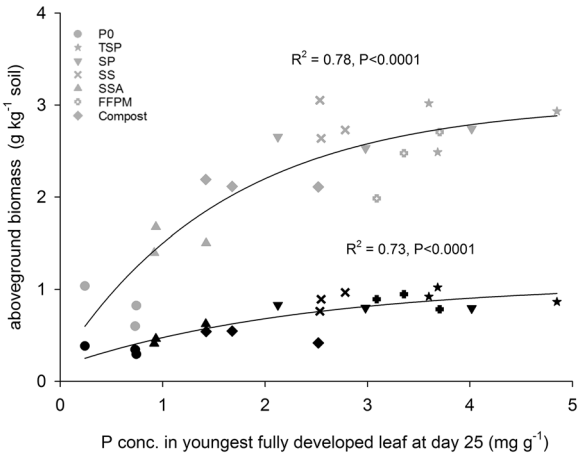

Fig. 3 Total aboveground biomass of wheat plants in the UCPH experiment from the first harvest (32 days after sowing, *black symbols*) or the second harvest (54 days after sowing, *grey symbols*) as a function of the P concentration in the youngest fully developed leaf from one plant measured at 25 days after sowing. Only data from three replicate pots were included for each treatment and only BE0 treatments were included. P treatments are: negative control (*P0*), *TSP*, low-grade TSP (*SP*), sewage sludge (*SS*), sewage sludge ashes (*SSA*), fibre fraction of pig manure (*FFPM*), compost of sewage sludge and garden/park waste (*Compost*). The data are fitted to three-parameter functions of the form $y = y_0 + a \cdot \left[1 - e^{(-b \cdot x)}\right]$

Table 7 Relative fertiliser efficiencies

P fertiliser	HK Kalke ($n = 4$)		UCPH ($n = 5$)		UPCH_2[a] ($n = 4$)
	FE (% of TSP)	PE (% of TSP)	FE (% of TSP), first harvest	FE (% of TSP), second harvest	FE (% of TSP)
Negative control	0 a	0 a	0 a	0 a	0
Triple superphosphate	100 cd	100 b	100 c	100 cd	100 c
Superphosphate	104 cd	110 b	79 c	92 cd	103 c
Thomas phosphate	117 d	127 b	n.a.	n.a.	n.a.
Sewage sludge	86 c	96 b	76 c	106 d	80 b
Sewage sludge ash	24 b	31 a	18 ab	41 b	36 a
Fibre fraction of pig manure	n.a	n.a.	93 c	77 c	n.a.
SSA-enriched LD slag	117 d	108 b	n.a.	n.a.	n.a.
Compost of sewage sludge and garden/park waste	n.a.	n.a.	33 b	74 c	54 a

The efficiencies are calculated for the BE0 treatment only. Efficiencies are calculated relative to TSP as the positive control (see "Methods"). For each column, different letters after the mean values represent significantly different means (Tukey's test, $P < 0.05$)

[a] This experiment was partly a replication of the UCPH experiment limited to five plants pot^{-1} (see Additional file 1 for details)

the relative P-use efficiency recorded in the HK Kalke experiment and the relative fertiliser efficiency recorded at the first harvest in the UCPH experiment were not significantly different from the negative control without the addition of a P fertiliser (Table 7). The compost included in the UCPH experiment had a relative fertiliser efficiency (33–74 %) between those of sewage sludge ash and sewage sludge (Table 7).

PCAs on plant compositional data

UCPH experiment

For the UCPH experiment, the elemental composition (B, Ca, Cu, Fe, K, Mg, Mn, P, S, Zn) was analysed in the youngest fully developed leaf sampled after 25 days from the different P-fertilisation treatments (all BE0). The composition was found to be very similar in treatments P1 (TSP), P4 (sewage sludge) and P6 (fibre fraction of pig manure), whereas the treatments P5 (sewage sludge ash) and particularly P0 (no P fertiliser added) were clearly separated in a PCA plot showing the first two principal components (Fig. 4a). The two groups of treatments were partly separated along the first principal component, but were more clearly separated along the second principal component. The loadings of the second principal component (Fig. 4b) showed that the most negative loading seen was for the element P followed by Zn and K.

HK Kalke experiment

The samples were grouped according to the P fertiliser applied along the first principal component in a PCA on the elemental composition (Ca, K, Mg, Mn, Na, P) of the aboveground biomass from the final harvest in the HK Kalke experiment (Fig. 5a). There was also a tendency towards a grouping along the second principal component due to the different BE inoculations across P-fertiliser treatments (Fig. 5b). Thus Proradix treatment was separated from the uninoculated control (BE0) in this plot. When looking at the loadings of the second principal component (Fig. 5c), higher concentrations of Mn in the BE0 plants were observed to be important for the separation in elemental composition between BE0 and Proradix plants.

CULS experiment

There was a clear grouping of pots according to the P fertiliser applied when concentrations of Ca, K, Mg, Mn, Na and K in leaves, stems and grain were used in a PCA (Fig. 6a). The clearest separation was between plants that had received no P fertiliser (P0) and plants that had received straw ash (StA). The treatments were primarily separated along the second principal component, which explained 30.8 % of the variation in the dataset. The loading plot of PC2 (Fig. 6d) shows that higher concentrations of P, K and Mn were especially important for the grouping of samples along the second principal component and that plants that had received straw ash as a fertiliser generally contained higher concentrations of P and K in the three tissues investigated compared to the remaining treatments, while higher concentrations of Mn in plant tissues pulled the samples that had received P0, DKP and WoA in the opposite direction in the PCA plot (Fig. 6a, d). The samples were grouped along the first principal component according to soil (Humpolec or Poděbrady), where higher concentrations of P and Mn were generally observed in plants grown in the Humpolec soil, while higher concentrations of Ca and Mg were recorded in plants grown in the Poděbrady soil (Fig. 6a, c).

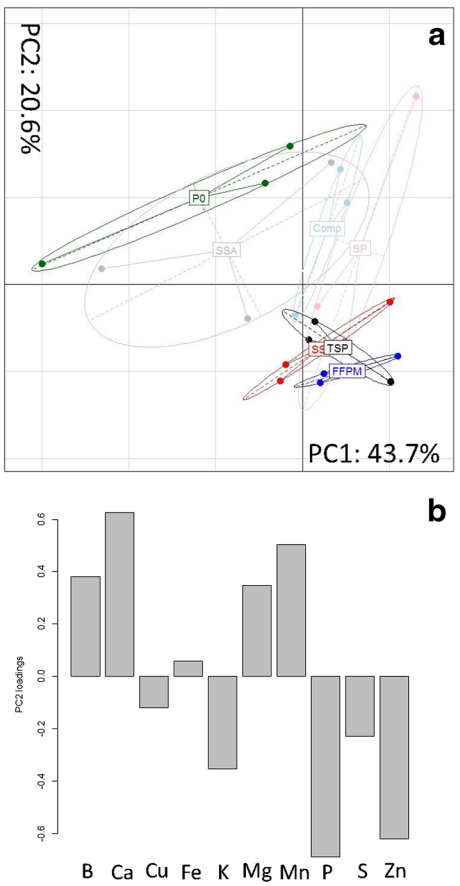

Fig. 4 Score plot (**a**) showing the result of a PCA on the elemental (B, Ca, Cu, Fe, K, Mg, Mn, P, S, Zn) composition of the youngest fully developed leaf measured at 25 days after sowing in the UCPH experiment. Loading plot (**b**) showing the loadings of the second principal component. Data are from three replicates of the P-fertilisation treatments P0 (control), TSP, SP (superphosphate), SS (sewage sludge), SSA (sewage sludge ash), FFPM (fibre fraction of pig manure) and Comp (compost of sewage sludge and garden/park waste). Pots were not amended with a BE (BE0)

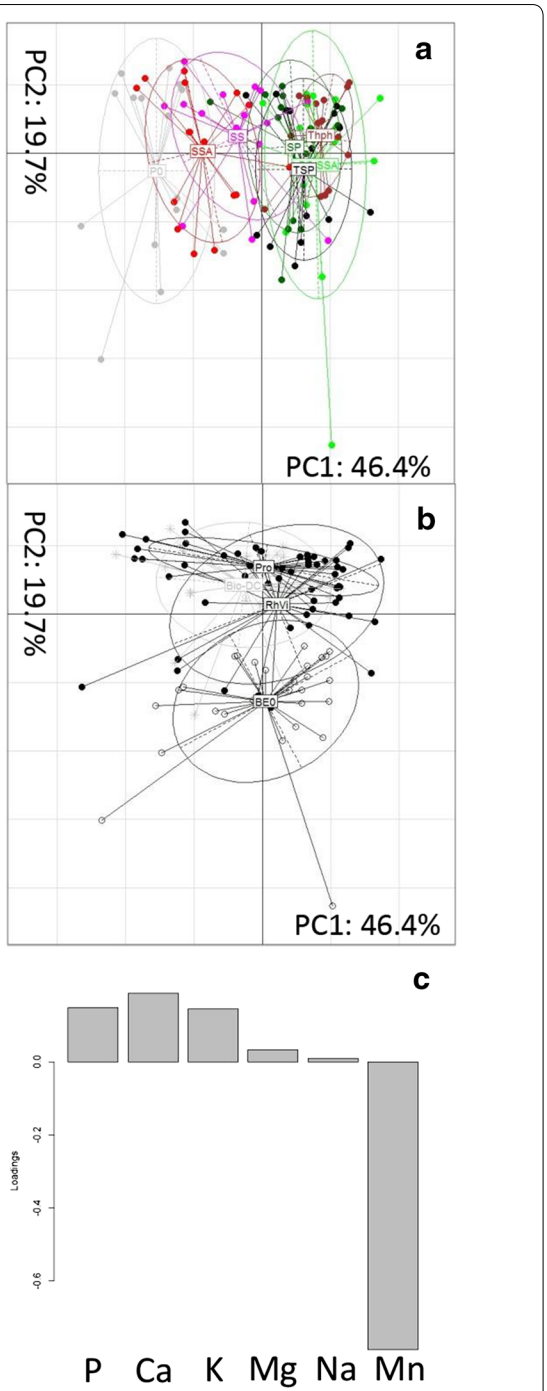

Fig. 5 Score plots (**a**, **b**) showing the result of a PCA on the elemental (Ca, K, Mg, Mn, Na, P) composition of the aboveground biomass grouped either according to P fertiliser applied (**a**) or BE inoculation (**b**). Loading plot (**c**) showing the loadings of the second principal component. Data are from the HK Kalke experiment. Codes for P fertilisers in **a** negative control (P0), TSP (TSP), superphosphate (SP), Thomas phosphate (Thph), sewage sludge (SS), sewage sludge ash (SSA), SSA-enriched LD slag (LDS/SSA, light green points)

Discussion

Was P the limiting factor in these experiments?

These pot experiments were undertaken on the assumption that P was the limiting factor in these trials. In the case of the UCPH experiment, the clear saturation-type relationship between P concentration in the youngest fully developed leaf during early growth and the subsequent biomass production (Fig. 3) served as validation that P limitation was in fact being studied in the UCPH experiment. Furthermore, the concentration of P recorded in leaves from the unfertilized treatment (Additional file 1: Table S3) was as low as 0.24 mg g^{-1} in one case and therefore probably within the deficiency

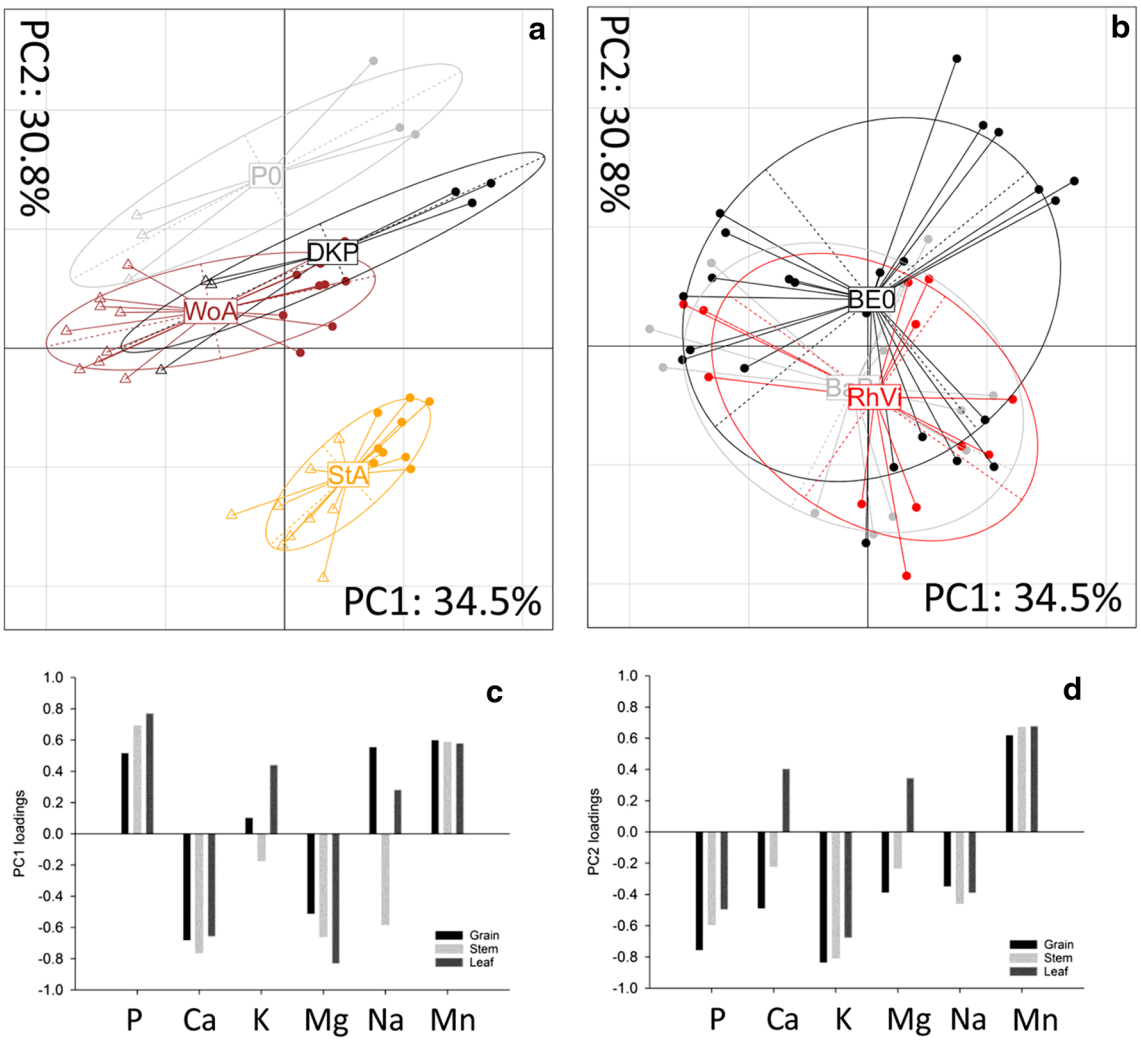

Fig. 6 Score plots (**a**, **b**) showing the result of a PCA on the elemental (Ca, K, Mg, Mn, Na, P) composition of the grain, stem and leaves. Samples are grouped either according to the P fertiliser applied (**a**) or BE inoculation (**b**). In **a** *filled circles* represent samples from the Humpolec soil, while *open triangles* represent samples from the Poděbrady soil. Loading plot (**c**) showing the loadings of the first principal component. Loading plot (**d**) showing the loadings of the second principal component. Data are from the CULS experiment

range at this stage [54]. Along the same lines, the clear relationships between soil P status and aboveground biomass in the HK Kalke experiment (Fig. 2) was validation that P was the limiting nutrient in the experiment. Here, we did not observe a positive response of P fertilisation on P concentration which shows that the P concentration of the whole shoot after 8 weeks of growth is not a robust measure of P deficiency. In contrast to the above, P could not be considered the sole limiting factor in the CULS experiment, since a positive growth response of adding readily soluble DKP (32 mg kg^{-1}) as the P fertiliser was not observed in this experiment. In general, the concentration of P was probably in the deficient range across all treatments (below 0.1 % in stem and leaves, Additional file 1: Table S4). This may partly be explained by a nitrogen limitation in the Humpolec soil, since soil solution nitrate levels in the Humpolec soil during the pot experiment were three times lower than those recorded in the Podêbrady soil (data not shown).

Were other nutrients limiting or present in toxic concentrations?

In the HK Kalke experiment, the concentrations of Ca, Mg, Ca, Mn and K (Additional file 1: Table S2) were in the adequate range for these elements in wheat shoots at the given growth stage [54].

In the UCPH experiment, the concentrations of Fe, K, S, Zn in the youngest fully developed leaf 25 days after sowing (Additional file 1: Table S3) were within the

adequate range at this stage [54]. For B, Ca, Cu, S and Zn this was also generally the case (Additional file 1: Table S3), but the leaf from one of the three control plants analysed showed concentrations (see minimum in Additional file 1: Table S3) in the deficiency range [54]. For B, the highest concentration recorded (156 μg g^{-1}) might be at the limit of toxicity at this stage [54]. However, no clear symptoms were observed.

In the CULS experiment, the grain concentrations of K (Additional file 1: Table S4) indicated deficiency in this element across all treatments, while Mn (Additional file 1: Table S4) was in the adequate range for grain at maturity [54].

Did the added BEs enhance the availability of P from recycled fertiliser products?

As stated in the introduction, one possible mechanism for improving plant growth by a BE would be to increase the availability of P in the soil. When used in combination with recycled fertilisers, it is of interest whether or not the introduced organisms directly affect the solubilisation of the introduced P. In the HK Kalke experiment, no significant effect was observed for any of the tested BEs (Pro, RhVi, Bio-DC) on the level of available P in the soil (P_{H2O}). Since we do not have soil data for the other experiments, we cannot make claims regarding the soil P availability in these experiments. This is in accordance with previous studies showing that although microbial inoculants may demonstrate potential for solubilisation of sparingly soluble P sources (such as Ca-phosphates) in vitro, this does not necessarily translate into increased plant availability of P in the soil [55]. In the present study, there was no support for an increase of plant-available P in the soil as a result of inoculation with two bacterial products (Proradix and RhizoVital 42) and one fungal product (Biological fertiliser DC). There may be several possible explanations for the lack of a significant positive effect on P availability: (i) a limited proliferation of the introduced microorganisms in soil due to competition with native microorganisms, for example, (ii) the soil P level may not have been sufficiently low to promote the up-regulation of enzymes involved in P solubilisation, (iii) released P may have been taken up by the introduced microorganisms without subsequent release to the soil within the time frame of the experiments and finally (iv) the native microbial community of the soil and/or organic waste materials may have been optimal already in making P available from the introduced fertilizers.

Did the added bioeffectors affect the growth of plants and plant P uptake?

Despite previous reports that the tested organisms may enhance plant growth [30, 43, 46], only a small positive effect on aboveground biomass of Pro and RhVi in combination with TSP was found (Fig. 1a). The fact that there was only a positive effect in combination with TSP as a fertiliser may point towards a direct effect of the BEs on the plants rather than an effect on P availability in the soil. This interpretation was also supported by the fact that the uptake of P from TSP-fertilised soil was not significantly different between BE treatments (Table 6a). The direct effects of these microbial inoculants on the plants are in line with earlier work showing that Pro and RhVi may elicit defence responses in plants [41, 56], thus directly affecting the plant's metabolism. In the P0 treatment, a positive effect of Pro and RhVi was observed on the total P content of the aboveground biomass, which seemed to indicate that under these P-limited conditions the two BEs did improve plant P uptake, even though a BE-mediated increase in P_{H2O} was not observed.

As a prerequisite for an effect of BEs on the growth of wheat plants, the successful establishment of organisms in the rhizosphere may be required, and it has been stated that rhizosphere competence may be a key factor in the effectiveness of PGPM [57, 58]. On the other hand, there is also an example of a study where the supernatant of the culture medium in which *T. harzianum* T22 was grown resulted in a stronger effect on the growth of maize plants compared to inoculating with spores [30]. This indicates that active growth in the rhizosphere may not always be a prerequisite for an effect of a PGPM and that a direct hormonal effect on the plants is a possible mode of action of these organisms. The present study did not measure whether the microorganisms established themselves in the rhizosphere of the wheat plants, meaning that it cannot be ruled out that the lack of a plant growth-promoting effect of the added BEs was due to an unsuccessful colonisation of the wheat rhizosphere. On the other hand, the fact that a significant BE effect was seen on the elemental composition of the aboveground biomass in the HK Kalke experiment may be an indication that the added microorganisms were in fact able to establish in the wheat rhizosphere in these pot experiments. In the CULS experiment, the plant elemental composition of the aboveground biomass did not give any indication of a BE effect.

Do the different recycled fertiliser products tested have potential as P fertilisers?

A low availability of P in the soil after fertilisation with sewage sludge ash was observed, which translated into a relative fertiliser efficiency based on biomass production of 24–41 % and P uptake of 31 %. This result was in line with earlier work, showing that phosphorus in sewage sludge ash is generally not readily taken up by plants [9]. On the other hand, there may be considerable variations

between different sewage sludge ashes, depending on the processing of sewage sludge in the water treatment plant [7]. Sewage sludge, Thomas phosphate and sewage sludge-enriched BOF slag (LDS/SSA) all resulted in levels of available P similar to or higher than TSP. In fact, fertilisation with LDS/SSA resulted in a significantly higher level of P_{H2O} compared to TSP. This was probably related to an increase in soil pH from ~5.6 in the TSP treatment to ~6.5 in the LDS/SSA treatment (Additional file 1: Table S1), since the availability of phosphates in soil is generally highest close to neutrality [59]. Severin et al. [9] found that the LDS/SSA product had high efficiency as a P fertiliser [9] in accordance with this study's results, yielding a P-fertilisation effect comparable to TSP. This shows the potential of this technology to produce a highly effective P fertiliser, partly based on sewage sludge devoid of any organic contaminants. However, the content of heavy metals could potentially be problematic. The content of Cr (1712 mg kg^{-1}, data not shown) for instance is above the current Danish limits [60], while in Germany contents above 300 mg kg^{-1} have to be declared [61]. An alternative to using sewage sludge ash could be to use sewage sludge as a fertiliser instead. Concerns may be raised regarding organic contaminants and problematic microorganisms, which are not relevant in the case of sewage sludge ash. However, organic contaminants probably do not pose a great threat here when the quality of present-day sewage sludge is taken into account [4]. In the present study, sewage sludge was observed to possess high potential as a P fertiliser, resulting in responses that are 76–106 % of those observed when using TSP. This was in relatively good agreement with a pot trial using English ryegrass in which the efficiency of different sludges was 62–86 % of monocalcium phosphate [62]. In the case of wood and straw ash, it was not possible to clearly evaluate their potential as P fertilisers based on the results presented here. This was due to the fact that (i) the CULS experiment lacked a positive control with the addition of a comparable level of total P and (ii) the input of P with the two different ash types was different. These problems aside, from the results presented here, it would not appear that wood ash and straw ash have great potential as P fertilisers, since the relative increase in biomass yield was not above 25 % in comparison to the HK Kalke and UCPH experiments showing yield increases of 50 % or more, even for sewage sludge ash. This result contradicted an earlier study in which a high P-fertilisation effect was found for rape meal, straw and cereal ashes [63]. However, as observed from the PCA plot, a small effect was observed on the plant elemental composition due to the wood ash and DKP treatments and greater effect of the straw ash treatment, but these differences were not clearly associated with differences in the aboveground biomass. These effects were observed to be independent of soil type. The fibre fraction of pig manure (FFPM) prepared using a decanter centrifuge was shown to have a high fertiliser efficiency that was not significantly lower than the positive TSP control. This was in accordance with previous results showing a high P availability after application of this solid manure fraction to soil [64].

Conclusions

Based on the results from the HK Kalke experiment, we did not find evidence to support the hypothesis that BE products increase the availability of P in the soil. Furthermore, the BE products only had a very limited effect on the growth of wheat plants across all experiments. Further work is therefore needed to elucidate whether inoculation with BEs has agronomic potential in wheat production. A number of the tested recycled P-fertiliser products (sewage sludge, P-enriched BOF slag and fibre fraction of pig manure) were shown in the HK Kalke and UCPH experiments to have a high potential as P fertilisers without a requirement for further processing.

Additional file

Additional file 1. Fig. S1. Biomass in the UCPH follow-up experiment. **Table S1.** Data on soil pH from the HK Kalke experiment and **Tables S2–S4**. Data on plant elemental composition from the HK Kalke (**Table S2**), UCPH (**Table S3**) and CULS (**Table S4**) experiments.

Authors' contributions

JDSL carried out the UCPH experiments, performed the majority of the data analysis in the paper and wrote the paper. MR carried out the HK Kalke experiment, contributed to data analysis and discussions of data. FM carried out the CULS experiment and performed the plant analyses for this experiment. MK supervised the analyses in the CULS experiment. PT supervised the experimental design in the CULS experiment. JM contributed to discussions regarding data interpretation. AN contributed to experimental design, data interpretation and the writing of the paper. All authors contributed to initial discussions of data. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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4.7) Co-application of wood ash and *Paenibacillus mucilaginosus* to soil: the effect on maize nutritional status, root exudation and composition of soil solution

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REGULAR ARTICLE

Co-application of wood ash and *Paenibacillus mucilaginosus* to soil: the effect on maize nutritional status, root exudation and composition of soil solution

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Abstract
Aims Improvement in nutrient efficiency of recycled fertiliser products represents a crucial step for sustainable agriculture. In this context, ash from biomass combustion belongs to the materials of interest.
Methods Novel strain of potential plant growth-promoting bacterium (*Paenibacillus mucilaginosus* ABi13) was tested for its ability to increase the plant availability of nutrients from wood ash (WA) in P-deficient soil-plant systems. Maize plants were grown in soil microcosms in semi-natural conditions, enabling rhizospheric- and bulk-soil solution analysis with special emphasis on low-molecular-mass organic acids (LMMOA).
Results Wood ash, as a sole fertiliser, increased biomass yield and improved nutritional status of maize plants. Concomitantly, application of WA led to lower root exudation rates of malate and isocitrate likely due to improved P status of plants. *P. mucilaginosus* ABi13 was inefficient in mobilising P from plain, acidic soil, but increased P solubility in ash-amended soil. However, *P. mucilaginosus* ABi13 consequently decreased NO_3^- concentrations in soil solution and induced N deficiency in maize, which led to decreased biomass yield and LMMOA exudation rates.
Conclusions This study demonstrated the importance of plant nutritional status on the final outcome of PGPR inoculation and contributes to our understanding of interactions between introduced PGPR, soil microbiome and plants.

Keywords *Paenibacillus mucilaginosus* · Low molecular mass organic acids · Recycled fertiliser products · Soil solution · Wood ash · Maize · Rhizosphere

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Introduction

The majority of biomass ash produced is represented by wood ash, since wood is the single most important biomass fuel worldwide (FAO 2017). Major mineral nutrients contained in wood ash are Ca, K, Mg and P, which are present in various forms of minerals. Wood ash exhibits several other valuable properties, which may significantly improve soil quality. In addition to possible improvement in hydraulic conductivity or water holding capacity, high alkalinity of wood ash reduces soil acidity, which, in turn, reduces Mn and Al toxicity and nutrient leaching into groundwater (Demeyer et al. 2001). Transfer of some risk elements, e.g. Cd, from soils to crop production can be also reduced by ash application (Ochecová et al. 2014; Li et al. 2016). However, the plant-availability of ash-bearing macronutrients is generally very low and is inversely proportional to combustion temperature due to mineral-phase transformation into various forms of insoluble silicates and phosphates (Vassilev et al. 2013). This is especially so with industrial wood ash where combustion temperatures are rarely lower than 800 °C (Vos 2005). Very low plant-availability of ash-bearing nutrients associated with low nutrient use efficiency discourages from wide use of wood ash as a recycled fertiliser product. Nonetheless, effective recycling of biomass ash as fertilisers is an important step in agricultural sustainability as wood ash recycling on agricultural land achieves a closing of the loop for phosphorus and several other nutrients. Consumption of P and K fertilisers in particular, which are associated with high costs and limited supply of these materials, is reduced and, therefore, overall energy input into agriculture may be lowered.

Paenibacillus mucilaginosus (syn. *Bacillus mucilaginosus*) is a typical silicate-weathering bacterium characterised as a gram-positive and facultatively anaerobic bacterium with extracellular polysaccharide production (Xiao et al. 2016). Hu et al. (2006) described phosphate and potassium solubilisation ability of *P. mucilaginosus*. According to Xiao et al. (2016), *P. mucilaginosus* can secrete carbonic anhydrase (CA), which catalyses the conversion of CO_2 to carbonate and protons accelerating the acidification by dissolved CO_2. Such effect consequently promotes the microbial conversion of silicate minerals and releases a number of nutritional ions, especially Ca and Mg, from minerals and soil. However, the most well-known mechanism of inorganic nutrient solubilisation, for Ca-associated P in particular, is due to organic acid (OA) production. This was the case in studies by Hu et al. (2006) and Lu et al. (2014), which reported OA production for all tested strains of *P. mucilaginosus*. Moreover, Liu et al. (2006), proposed significant participation of both exopolysacharides and OA produced by *P. mucilaginosus* in the dissolution of silicates. Authors further suggested formation of bacterial-mineral complexes where polysaccharides strongly adsorb the organic acids and formation of an area of organic acids at a high concentration near the minerals (Liu et al. 2006). Several studies reported plant growth-promoting ability of *P. muclaginosus* due to improved K and P nutrition when combined with sudan grass (Basak and Biswas 2009), maize, wheat (Singh et al. 2010), tobacco (Li et al. 2007), cucumber and pepper (Han et al. 2006). Thus, the use of *P. mucilaginosus* may represent a cost-effective and ecological solution, improving the bioavailability of nutrients contained in wood ash. To our knowledge, there is no study which has focused on the combination of *P. mucilaginosus* and ash.

The main objective of this study was to test novel strain *P. mucilaginosus* ABi13 in terms of its influence on maize and on soil solution composition, with particular emphasis on its potential to increase nutrient availability (P in particular) from wood ash in the soil-plant system. We tested the effect of *P. mucilaginosus* ABi13 on maize plant parameters and soil solution composition using a microcosm approach with natural soil reflecting in vivo P-limiting conditions. In order to understand and sufficiently predict the possible influence of the tested strain, a rhizospheric microcosm was developed with emphasis on low-cost and easy-to-use methods. Nutrient-poor, acidic soil with low cation-exchange capacity (CEC) was chosen in order to simulate highly limiting conditions. In such conditions, the influence of PGPR is expected to be maximized as: i) soil buffering capacity is low and, therefore, potentially solubilised nutrients are likely to be taken up by the plant and not to be adsorbed on soil particles; ii) plant growth is limited by nutrient deficiencies and even a small increase in soil-nutrient availability is likely to result in better growth performance of the plant.

Material and methods

Experimental design

The experiment was conducted using soil microcosms, each containing 5 kg dry wt of soil and placed in an outdoor precipitation-controlled vegetation hall. Each microcosm was made from a 6 L polypropylene pot (h = 20.5 cm, d_{top} = 22 cm, d_{bottom} = 18.5 cm). Inner space of each microcosm was vertically divided into two compartments by nylon (PA 6.6) membrane with a 30-μm pore size (SEFAR, Thal, Switzerland). The root zone compartment (70% of inner volume, 3.5 kg of soil) was sown with maize and the bulk zone compartment (30% of inner volume, 1.5 kg of soil) was without plants. The membrane was used to provide comparable conditions between both compartments, mainly in terms of water saturation level, water residence time and consequently redox potential, factors which are known to have a strong influence on soil solution properties. Simple segregation of planted and non-planted pots may lead to bias and incomparable results for carboxylate concentrations in soil solution (Mercl et al. 2017). One Rhizon MOM suction cup (Rhizosphere Research Products, Wageningen, Netherlands) was installed vertically in the centre of each zone. The Rhizons had a 10-cm porous section with a 0.12–0.18 μm pore size. Schematic representation of the microcosm is shown in Fig. 1.

Experimental soil (Haplic Cambisol) was collected one week prior to sowing from a field (plow depth, 0–20 cm) near the city of Žamberk, Czech Republic (50°8′ 38.102 N, 16°30′51.351 E). Soil was air dried, passed through a 10-mm stainless steel sieve and manually homogenised prior to the experiment. Wood ash originated from the combustion of wood chips in a fluidised bed reactor (15 MWt) and is described in more detail elsewhere (Mercl et al. 2016). In this study, wood ash treatment is denoted W, whereas control treatment (plain soil with no wood ash added) is denoted C. In the case of W treatment, 24 mg P was supplied in form of wood ash by thorough mixing of corresponding amount of ash with soil. This dose of P resulted in theoretical application rate of 7 tons of wood ash per hectare. The basic characteristics of experimental soil and ash are given in Table 1. Results of P fractionation are shown in Table S1.

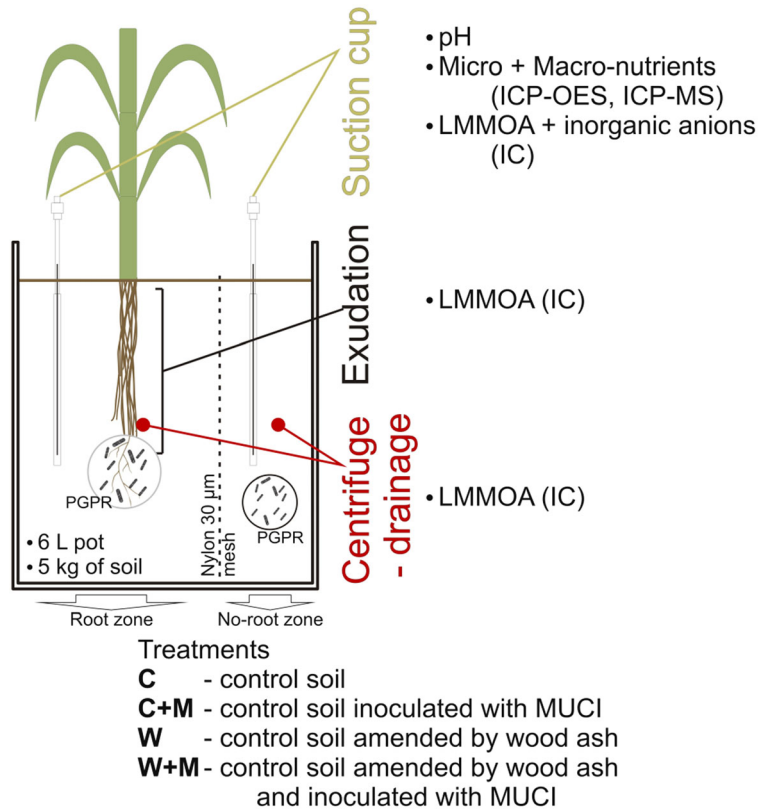


Fig. 1 Schematic representation of the microcosm and description of individual treatments. ICP-OES inductively coupled plasma optical emission spectrometry; ICP-MS inductively coupled plasma mass spectrometer; IC ion-exchange chromatography; PGPR plant growth-promoting rhizobacterium

Table 1 Physicochemical properties of the experimental soil and ash

Characteristics	Experimental soil		Wood ash[a]	
Textural class	Silt loam		–	
Clay (%)	13		–	
Silt (%)	56		–	
Sand (%)	31		–	
CEC (mmol kg^{-1})	74.9 ± 3.7		125 ± 1.20	
pH_{CaCl2}	5.20 ± 0.01		11.2 ± 0	
TC (%)	1.90 ± 0.01		8.00 ± 0.7	
DOC (mg kg^{-1})	63.6 ± 2.0		–	
CO_3^{2-} (%)	0.10 ± 0.05		4.23 ± 0.18	
	Pseudototal (aqua regia)	Available (Mehlich 3)	Total (XRF)	Available (CH_3COOH)
N (%)	0.19 ± 0.01[†]	55.9 ± 3.82[††]	0.02 ± 0.01	–
P (mg kg^{-1})	551 ± 26.7	22.0 ± 0.02	10,195 ± 50.0	315 ± 1.00
K (mg kg^{-1})	3802 ± 169	59.6 ± 0.36	58,938 ± 170	6607 ± 571
Mg (mg kg^{-1})	2838 ± 50.5	27.7 ± 0.28	17,478 ± 280	3990 ± 97.0
Ca (mg kg^{-1})	1722 ± 114	1418 ± 8.31	117,789 ± 200	40,824 ± 174
S (mg kg^{-1})	193 ± 16.4	10.3 ± 0.13	15,816 ± 16.0	5313 ± 129
Fe (mg kg^{-1})	20,682 ± 84.6	178 ± 0.63	38,339 ± 84.0	10.8 ± 1.77
Mn (mg kg^{-1})	939 ± 50.1	70.4 ± 0.51	15,292 ± 61.6	1119 ± 13.2
Zn (mg kg^{-1})	53.5 ± 0.69	1.84 ± 0.02	1154 ± 98.0	47.0 ± 1.50
Cu (mg kg^{-1})	7.24 ± 0.13	1.12 ± 0.01	98.8 ± 6.48	0.13 ± 0.04
Al (mg kg^{-1})	21,835 ± 122	1098 ± 0.26	57,028 ± 200	14.4 ± 1.42

Shown values represent arithmetic mean ± standard deviation (SD)

[a] Previously described in more detail by Mercl et al. (2016)

[†] Total contents of N are given in % and were determined using CNS analyser

[††] Available N contents are given in mg kg^{-1} as a sum of NH_4^+ and NO_3^- extractable in 0.01 M $CaCl_2$ (determined by means of colorimetry)

Seven untreated maize seeds (organic variety Colisee; KWS Saat, Germany) were sown in the root zone compartment and plant numbers were thinned to five after germination. Immediately after sowing, both compartments were inoculated with *P. mucilaginosus* ABi13 (MUCI). Tap water (200 mL), instead of inoculum, was applied in the case of control (non-inoculated) treatments (C and W). Treatments involving MUCI inoculation are denoted C + M and W + M in this study.

Each treatment was replicated four times. During the course of the experiment, microcosms were maintained at 60 ± 5% of maximum water-holding capacity (MWHC) by irrigation with demineralized water. MWHC was controlled gravimetrically once every two days.

Bacterial strain *Paenibacillus mucilaginosus* ABi13

Tested strain *Paenibacillus mucilaginosus* Abi13 was isolated from the rhizosphere of wheat growing in Alpine foothills area in Germany and was cultivated in liquid Aleksandrov medium (Hu et al. 2006) at 30 °C. Strain was identified as *Paenibacillus mucilaginosus* by 16S rDNA gene sequencing using universal primers 27F (AGAGTTTGATCMTGGCTCAG), 1492R (TACGGYTACCTTGTTACGACTT) and the resulting PCR fragment was sequenced by Sanger method (Sanger et al. 1977). The sequence was compared with NCBI-Database and matched 100% with *Paenibacillus mucilaginosus*. The strain was grown in a bioreactor at 30 °C in a complex media until complete sporulation. A pure spore product (MUCI) was formulated by

membrane-separation of spores from broth culture and subsequent washing out of possible residues of broth and metabolites and was provided by ABiTEP (Berlin, Germany) as a liquid suspension containing living spores of *P. mucilaginosus* ABi13 at a concentration of 1.0×10^9 CFU/mL. MUCI suspension was diluted with a tap water and 200 mL of inoculum was applied directly on seeds in the planting holes resulting in a concentration of 1×10^{10} CFU/kg of soil.

Soil solution sampling

Two methods were used to collect soil solution, namely suction cup and centrifuge drainage method. Using suction cups, soil solution was sampled twice during the experiment (at 14 and 28 days after emergence; DAE) and was used for analysis of available nutrients. Four hours prior to sampling, microcosms were irrigated with a precise amount of demineralized water to reach 60% MWHC and soil solution was collected using 20-mL syringes (B. Braun, Germany) as described elsewhere (Mercl et al. 2017). Immediately after collection, pH of soil solution was measured using a Sentron SI400 pH meter with ISFET electrode (Sentron Europe BV, Leek, Netherlands). Aliquots of collected solution (5 mL) were separately frozen (−42 °C) and kept for subsequent NO_3^- and NH_4^+ analysis. Solutions collected by suction cup method are denoted as root zone solution and no-root zone soil solution.

LMMOA concentrations in rhizospheric and bulk soil solutions were determined at the end of the experiment (28 DAE). These solutions were obtained using a centrifuge drainage method. Immediately after collection of suction cup solutions, individual compartments of microcosms were carefully dismantled. Rhizospheric soil was collected by careful shaking of the whole root system to remove non-adhering soil (this was discarded) and subsequent gentle brushing of roots to remove root-adhering soil which was analysed. Special emphasis was taken to minimize any damage of the roots. Collected soil was thoroughly checked for tiny roots which were then removed with tweezers. Bulk soil was collected from bulk zone compartment by taking representative samples, avoiding both the upper (3-cm) layer and the bottom (3-cm) layer of soil. Approximately 20 g of fresh collected soil was placed in Falcon Maxi-Spin Filter Tubes (Ciro Manufacturing Corp., Deerfield Beach, USA) with a 0.45-μm pore size nylon membrane and was immediately centrifuged at 4 °C for 30 min. No storage of freshly collected soil took place since it is known that LMMOA concentrations are strongly influenced by sample storage (Mimmo et al. 2008). As rhizospheric soil samples inevitably contained root hairs, the relative centrifugal force (RCF) was set to 2500 *g* to avoid symplastic sap leaching and therefore root cell rupture, as reported by Yu et al. (1999). After centrifuging, supernatant was transferred to a 0.5-mL vial; 10 μL of 99.9% (*v*/v) methanol (Lach-ner, Czech Republic) was added (final methanol concentration 2% v/v) in order to prevent any microbial degradation. Samples were then frozen at −42 °C and kept for subsequent analysis.

Collection of exuded LMMOAs

Immediately after collection of the rhizospheric soil, the plant root system was briefly rinsed with a gentle stream of demineralized water and carefully placed for 30 min in a 0.5 L glass beaker covered with aluminium foil and filled with 1 mM $CaCl_2$ solution. During this period, stabilization of root cell membranes by Ca^{2+} is provided and protoplast of mechanically-damaged root cells is washed out into the solution (Neumann and Römheld 2001). After 30 min of stabilization, roots were removed from the solution, gently rinsed with demineralized water and transferred into a new 250 mL beaker filled with a fresh 1 mM $CaCl_2$ solution. Plants were left to exude for 1 h. Plants were then removed, divided into shoots and roots and dried at 60 °C to a constant weight. The exudate solution was stirred briefly and 10-mL aliquots were immediately filtrated through a nylon syringe filter (0.2 μm pore size), frozen at −42 °C after addition of 200 μL of 99.9% (*v*/v) methanol and kept for further analysis.

Analytical procedures

Dried plant biomass was milled and digested with concentrated HNO_3 (65% v/v; Analytika) and H_2O_2 (30% v/v; Analytika) in an Ethos 1 microwave-assisted wet-digestion system (MLS, Leutkirch, Germany). Nutrient concentrations (P, S, Mg, Ca, Fe, Mn, Zn, B, Cu) were then determined by inductive coupled plasma-optical emission spectrometry (ICP-OES; Agilent 720, Agilent Technologies Inc., Santa Clara, CA). Concentrations of K only were determined using flame atomic absorption spectrometry (F-AAS; Varian AA285S, Varian Australia, Mulgrave) (Száková et al. 2013). The

standard reference material used was 1515 Apple Leaves (NIST, Gaithersburg, USA).

For the determination of total C and N, a CHNS Vario MACRO cube analyser was used (Elementar Analysensysteme GmbH, Hanau, Germany). Carbonate content was determined using the volumetric calcimeter method (Loeppert and Suarez 1996). Cation-exchange capacity was determined according to Gillman (1979). Pseudototal contents of nutrients in soil were determined by ICP–OES after microwave-assisted aqua regia extraction, as described by Száková et al. (2013). Available fractions of nutrients in soil were determined by extraction of samples in Mehlich 3 solution (Mehlich 1984). Content of available N (NH_4^+ and NO_3^-) in soil was determined using a continuous flow colorimetric analyser (SAN plus System, Skalar Analytical, Breda, Netherlands) after 2 h extraction with 0.01 M $CaCl_2$ (1:10 *w/v*). Concentrations of available nutrients in ash were measured using ICP-OES after extraction for 16 h in CH_3COOH (1:40 w/v). Soil and ash pH values were determined after extraction with 0.01 M $CaCl_2$ at a ratio of 1:2.5 (*w/v*) (VDULFA 1991), using a Sentron SI400 pH meter with ISFET electrode (Sentron Europe BV, Leek, Netherlands).

Concentrations of nutrients (P, K, S, Mg and Ca) in soil solution (root zone and no-root zone solutions sampled with Rhizons) were determined by ICP–OES. Concentrations of P in these solutions were usually below or close to the detection limit (DL) of ICP–OES (0.05 mg L^{-1}). Therefore, P concentrations in all soil solution samples were determined by inductively coupled plasma mass spectrometer (ICP–MS, 7700×; Agilent Technologies Inc., USA). Nitrate, nitrite and LMMOA concentrations in soil solutions and/or exudate samples were determined by means of ion-exchange chromatography with suppressed conductivity, using an ion chromatograph ICS 1600 (Dionex, Sunnyvale, CA) equipped with IonPac AS11-HC (Dionex) guard and analytical columns. The eluent composition was 1 to 35.2 mM KOH with a 1 to 65 min gradient; flow rate was set to 1 mL min^{-1}. Quality control and assurance of ion chromatography analysis are described by Mercl et al. (2017). Concentrations of NH_4^+ in soil solution were determined by means of ion-exchange chromatography with suppressed conductivity. The ion chromatograph ICS 90 (Dionex) equipped with IonPac CS16 (Dionex) guard and analytical columns was used. The eluent composition was 38.0 mM methanesulfonic acid and flow rate was set to 1 mL min^{-1}. To suppress eluent conductivity, the CMMS 300–4 mm suppressor (Dionex) and 0.103 M tetrabutylammonium hydroxide reagent was used. The limit of detection was 0.046 mg L^{-1}.

Soil DNA extraction and Illumina MiSeq sequencing

DNA was extracted from the soil samples collected at the end of the experiment. Prior to DNA extraction, water content of air-dried soil (0.4 g) was firstly adjusted to 20% by adding sterile PCR-grade water and soil was incubated at 4 °C for 30 min (Clark and Hirsch 2008). The method of DNA extraction is described in Sagova-Mareckova et al. (2008). The method is based on bead-beating and phenol/chloroform extraction. The samples are purified by incubation with cetyl trimethylammonium bromide followed by chloroform extraction and incubation with $CaCl_2$, and finally cleaned with GeneClean Turbo kit (MP Biomedicals, Santa Ana, CA, USA).

From the DNA samples, a fragment of the bacterial 16S rRNA gene including the variable region V4 was amplified by PCR using universal primers with overhang adapters CS1-515F (5'-ACACTGACGACATG GTTCTACAGTGCCAGCMGCCGCGGTAA-3′) and CS2-806R 5'-TACGGTAGCAGAGACTTGGT CTGGACTACHVGGGTWTCTAAT-3′) (Caporaso et al. 2011). Construction of amplicon libraries and sequencing using MiSeq sequencer (Illumina, San Diego, USA) were done at the DNA Services Facility, Research Resources Center, University of Illinois (Chicago, USA). The resulting paired sequence reads were merged, filtered, aligned using reference alignment from the Silva database (Quast et al. 2013), and chimera checked using integrated Vsearch tool (Rognes et al. 2016) according to the MiSeq standard operation procedure (Miseq SOP, February 2018; Kozich et al. 2013) in Mothur v. 1.39.5 software (Schloss et al. 2009). A taxonomical assignment of sequence libraries was performed in Mothur using the recreated SEED database subset of Silva Small Subunit rRNA Database, release 132 (Yilmaz et al. 2014) adapted for use in Mothur (https://mothur.org/w/images/7/71/Silva.seed_v132.tgz) as the reference database. Sequences of plastids, mitochondria, and those not classified in the domain Bacteria were discarded. The sequence library was clustered into OTUs using the Uparse pipeline in Usearch v10.0.240 software (Edgar 2013), and the OTU table was further processed using tools

implemented in the Mothur software. Distance matrices describing the differences in community composition between individual samples were calculated using the Yue-Clayton theta calculator (Yue and Clayton 2005). Maximum-likelihood phylogram of the OTU representative sequences was constructed using FastTree 2 (Price et al. 2010). The Illumina MiSeq 16S rRNA gene amplicon sequences have been deposited in the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) as BioProject ID PRJNA448787.

Statistics and data analyses

The normality of the data was checked using the Shapiro-Wilk test. Data for individual LMMOAs concentrations in rhizospheric and bulk soil solution did not follow normal distribution when comparing mean values ($n = 16$) and the comparison of means was conducted using the non-parametric Mann-Whitney Rank Sum test. STATISTICA 12 (StatSoft, Inc., Tulsa, OK) and SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA) were used for statistical analyses, such as correlation and analysis of variance (ANOVA), and for preparation of figures. Statistically significant differences (post-hoc Tukey's honest significant difference test – HSD) are shown at the 95.0% confidence level.

For the determination of the most limiting nutrient, index values for individual nutrients were calculated using nutrient concentrations in shoot biomass based on the Diagnosis and Recommendation Integrated System (DRIS) (Beaufils 1973). Data published by Elwali et al. (1985) were used as reference parameters. Concentrations of N, P, K, S, Mg and Ca were used for the calculations. Shoot tissue contents of Fe, Mn, Zn, B and Cu were within sufficiency ranges in all treatments and are therefore not shown in this study. No significant differences between treatments were found in soil solution concentrations of NH_4^+ and NO_2^- and, therefore, these results are not shown.

Results

Nutritional status and uptake of nutrients

The application of wood ash (WA) significantly increased the yield of both shoot and root biomass (Table 2). Shoots with wood ash treatment (W) were increased by 33% compared to control (C) and the roots increased by 17%. The influence of MUCI on the biomass yield was inconsistent and varied between treatments. No significant difference in yield from C was found in C + M while a significant decrease in both

Table 2 Biomass yield and nutrient concentrations in individual plant parts at 28 DAE

Plant part		Yield (g pot^{-1})	N (mg g^{-1})	P (mg g^{-1})	K (mg g^{-1})	S (mg g^{-1})	Mg (mg g^{-1})	Ca (mg g^{-1})
Shoots	C	8.18 ± 0.30a	16.6 ± 0.38b	0.99 ± 0.03ab	16.3 ± 0.41b	1.26 ± 0.02b	2.53 ± 0.09b	7.66 ± 0.29bc
	C + M	7.79 ± 0.41a	11.3 ± 0.77a	0.91 ± 0.04a	13.8 ± 0.78a	0.93 ± 0.02a	2.42 ± 0.12b	7.79 ± 0.46c
	W	10.9 ± 0.88b	14.8 ± 1.21b	1.00 ± 0.05b	17.7 ± 1.28b	1.19 ± 0.13b	2.35 ± 0.15b	6.62 ± 0.40a
	W + M	7.05 ± 0.45a	9.73 ± 0.27a	1.25 ± 0.02c	23.0 ± 0.41c	0.98 ± 0.04a	2.00 ± 0.01a	6.91 ± 0.14ab
F-value		36.6***	51.4***	46.6***	70.4***	15.7***	13.0***	8.20**
Nutrient sufficiency ranges[†]			27.6–35.0	2.50 – 4.00	17.1–25.0	1.50–4.00[††]	2.10 – 6.00	2.10–10.0
Roots	C	7.51 ± 0.67a	8.93 ± 0.31c	0.54 ± 0.05ab	3.83 ± 0.24a	0.85 ± 0.03a	1.14 ± 0.10a	5.50 ± 0.30b
	C + M	7.66 ± 0.38ab	6.83 ± 0.32ab	0.54 ± 0.07ab	4.80 ± 0.54a	0.86 ± 0.03a	1.09 ± 0.11a	3.86 ± 0.21a
	W	8.77 ± 0.74b	7.72 ± 1.23bc	0.52 ± 0.03a	4.92 ± 0.40a	1.45 ± 0.12b	1.25 ± 0.13ab	5.48 ± 0.61b
	W + M	6.81 ± 0.26a	5.78 ± 0.28a	0.68 ± 0.08b	7.44 ± 0.99b	1.90 ± 0.22c	1.51 ± 0.14b	4.71 ± 0.46ab
F-value		8.71**	11.9***	4.32*	19.2***	44.4***	7.29**	10.1**

Values shown represent arithmetic mean ± SD ($n = 4$); different letters indicate significant difference (Tukey's HSD test; $p < 0.05$) between treatments in individual plant parts; all results are given on a dry basis

C control; C + M control inoculated with MUCI; W wood ash treatment; W + M wood ash treatment inoculated with MUCI; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

[†] Nutrient sufficiency ranges for maize ear leaf taken at silk according to Jones et al. (1990)

[††] Sufficiency range for maize biomass content in early growth according to Campbell and Plank (2000)

shoot and root yields after MUCI inoculation was found in the case of W + M compared to W (Table 2).

Phosphorus concentration in biomass was not influenced by WA application, but WA significantly increased the total P content. No significant effect of MUCI on biomass P concentration and subsequently on P content was found in plain soil treatment (C + M) (Table 2; Table S2). However, application of MUCI on ash-treated soil (W + M) significantly increased P concentration in both shoots and roots, but the P content in shoots was significantly decreased. Phosphorus concentrations in shoots were generally lower than the sufficiency range with all treatments (Table 2). Highest P concentration in shoots was found with W + M treatment (1.25 mg g^{-1}), while the lowest value was with C + M (0.91 mg g^{-1}). Phosphorus was identified by DRIS to be the most deficient nutrient in C, C + M and W treatments in contrast to W + M, whereas N was determined as the most limiting one (Table 3).

Wood ash had no significant influence on N concentration in shoots or roots, but significantly increased N content in shoots (Table 2; Table S2). Application of MUCI significantly decreased N concentration in both shoots and roots with both treatments and this reduction led to a decreased shoot and root N contents with C + M and W + M treatments. In general, N concentration in shoot biomass in this experiment ranged between 9.73 mg g^{-1} and 16.6 mg g^{-1} (Table 2). All treatments therefore suffered from N deficiency.

Biomass concentration of K remained the same in W compared to C, but increased K content in shoots and roots was evident after WA addition. Application of MUCI did not affect K content in roots, but had a significant effect on shoots where it resulted in a decreased content with both treatments. Concentration of K in shoot biomass was generally close to the lower level of sufficiency. Only in the case of C + M did K concentration fall slightly more below the sufficiency limit and could be considered as deficient with a value 13.8 mg g^{-1}.

Sulphur nutritional status of maize plants was significantly improved by WA; WA application resulted in increased S concentration in shoots and roots, as well as S content in these parts. The effect of MUCI on biomass S concentration differed between treatments and plant parts. A significant decrease in S concentration in shoot biomass after MUCI application was found with both C + M and W + M treatments. Conversely, the S concentration in roots was increased by MUCI in the case of W + M treatment but no effect was found in C + M treatment. Sulphur concentrations in shoot biomass ranged between 0.93 mg g^{-1} and 1.26 mg g^{-1} in this experiment. These values are lower than the sufficiency range (Table 2).

Finally, Mg and Ca content in shoots and roots were increased by WA application. Application of MUCI resulted in lower shoot Mg concentration in W + M treatment, and subsequent decrease in Mg content and lower shoot Ca content in C + M without any effect on the content of Ca in shoots. Concentrations of Mg and Ca were within the sufficiency ranges (Table 2).

Soil solution properties and nutrient concentrations

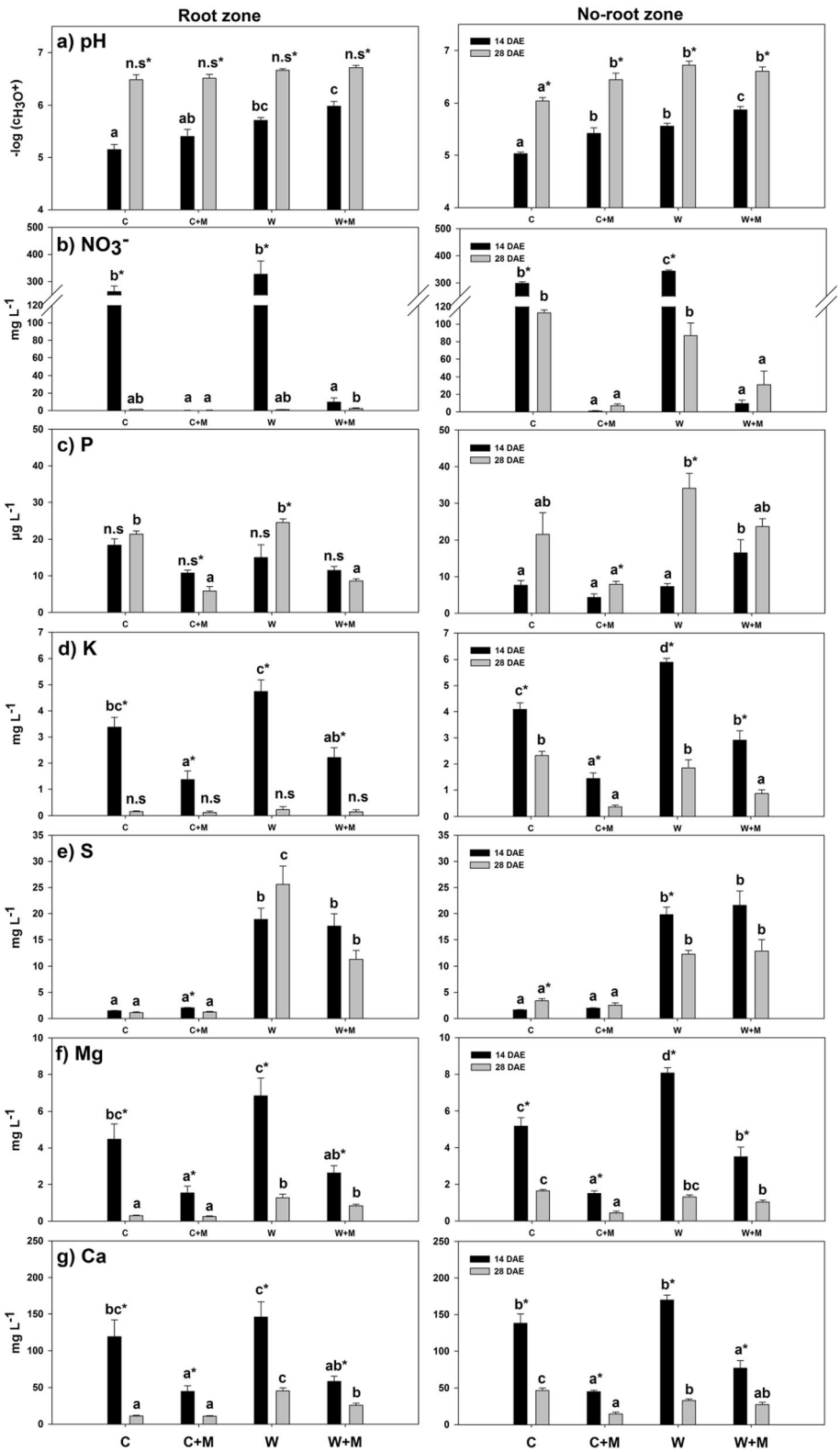
Soil solution pH was in the range 5.03–6.72 and increased approximately by 1 unit over time (14 DAE – 28 DAE) with all treatments (Fig. 2; Table S3). Solution pH was significantly increased by wood ash application in both zones at 14 DAE (by 0.56 and 0.53 in root and no-root zones, respectively). The effect of wood ash on solution pH disappeared in the root zone at 28 DAE but was still significant in the no-root zone. Concentrations of NO_3^- showed a decreasing trend over time in both zones. Wood ash slightly affected NO_3^- concentrations in soil solution, but a significant increase was found only in the no-root zone during the first sampling

Table 3 Nutrient DRIS index values for individual treatments

Treatment	N_{index}	P_{index}	K_{index}	S_{index}	Mg_{index}	Ca_{index}
C	-11	−56	12	−17	25	46
C + M	−31	−51	13	−30	35	64
W	−16	−49	20	−18	24	39
W + M	−49	−32	43	−31	20	48

C control; C + M control inoculated with MUCI; W wood ash treatment; W + M wood ash treatment inoculated with MUCI

Fig. 2 Soil solution properties. Data shown represent arithmetic ▶ mean ($n = 4$); error bars indicate standard error of the mean; different letters above bars indicate significant differences (Tukey's HSD; $p < 0.05$) between treatments for individual zone and sampling time separately; asterisks above bars indicate significant differences (Tukey's HSD; $p < 0.05$) between sampling times for individual treatments separately; F-values of ANOVA are shown in Table S3 n.s not significant; DAE days after emergency; C control; C + M control inoculated with MUCI; W wood ash treatment; W + M wood ash treatment inoculated with MUCI


Root zone
No-root zone
a) pH
-log (cH3O+)
b) NO3-
mg L-1
c) P
µg L-1
d) K
e) S
f) Mg
g) Ca
14 DAE
28 DAE
C
C+M
W
W+M

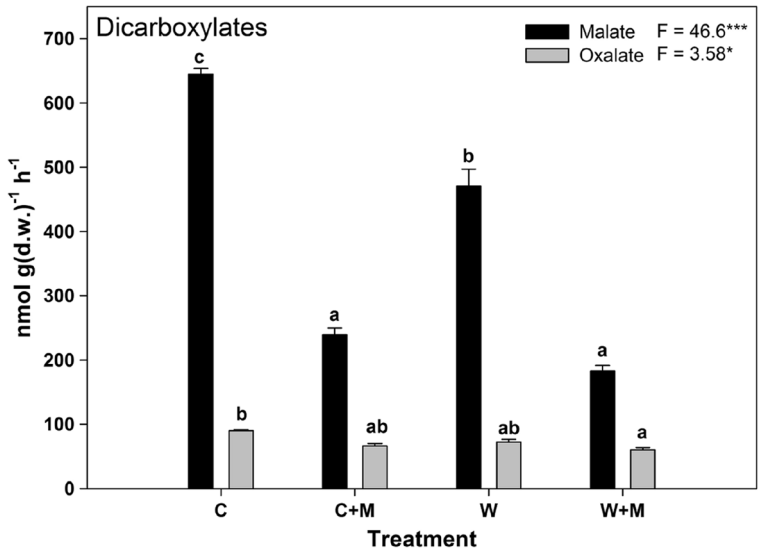


Fig. 3 Exudation rates of individual dicarboxylates at 28 DAE. Data shown represent arithmetic mean ($n = 4$); error bars indicate standard error of the mean; different letters above bars indicate significant differences (Tukey's HSD; $p < 0.05$) between treatments for individual LMMOA separately; C control; C + M control inoculated with MUCI; W wood ash treatment; W + M wood ash treatment inoculated with MUCI; *** $p < 0.001$; * $p < 0.05$

period. Application of wood ash did not affect P concentrations in soil solution within this experiment. Wood ash did increase K concentrations in solution but this effect was significant only in the no-root zone at 14 DAE. Concentrations of S were also increased by WA both at the first and at the second sampling period in both zones. At 14 DAE, concentrations of Mg were increased by wood ash application only in the no-root zone, while the increase became significant in the root zone over time (28 DAE). Finally, wood ash did not influence Ca concentrations in soil solution at 14 DAE, but changes appeared over time.

Application of MUCI significantly increased pH in the no-root zone with both treatments at 14 DAE. In the latter stage of the experiment, the significant increase in pH was recorded only for C + M treatment (Fig. 2). Effects of MUCI on P concentrations in soil solution were relatively inconsistent. At 14 DAE, MUCI affected only soil solution of W + M treatment in no-root zone where a significant increase of 9.24 μg P L^{-1} was found compared to W. No effect of MUCI was obtained for the same treatment at 28 DAE, while P concentrations decreased in root zone after MUCI application at 28 DAE. Application of MUCI resulted in a significant decrease in NO_3^- in both zones of soil solution at 14 DAE, but its effect at 28 DAE was significant only in the no-root zone. Potassium concentrations in solution were lowered with MUCI treatments in both solution types at 14 DAE. The effect of MUCI on S in soil solution was evident only at 28 DAE with W + M treatment when a decrease of 14.3 mg L^{-1} was recorded. The effect of MUCI on Mg concentrations in both zones was visible and significantly lower Mg concentrations were detected at 14 DAE. A significant decrease in Ca concentrations in solution at 14 DAE was found with all MUCI treatments, whereas this effect remained significant at 28 DAE in root zone of W + M.

Low molecular mass organic acids

Wood ash application had no significant influence on the exudation rates of oxalate, but those of malate were significantly decreased by WA application (Fig. 3). This led to the decreased total sum of exuded dicarboxylates (Table 4). Similar to malate, exudation rates of isocitrate

Table 4 Rate of total amount of carboxylates (nmol g(d.w.)$^{-1}$ h^{-1}) exuded at 28 DAE

Treatment	Dicarboxylates	Tricarboxylates
C	734 ± 37.1c	1978 ± 361
C + M	305 ± 38.9a	1258 ± 292
W	542 ± 111b	1585 ± 315
W + M	243 ± 39.3a	1738 ± 340
F-value	48.7***	$3.38^{n.s}$

Values shown represent arithmetic mean ($n = 4$) ± SD; different letters indicate significant differences (Tukey's HSD; $p < 0.05$) between soil treatments for each LMMOA group

C control; C + M control inoculated with MUCI; W wood ash treatment; W + M wood ash treatment inoculated with MUCI; *** $p < 0.001$; $^{n.s}$ not significant at $p < 0.05$

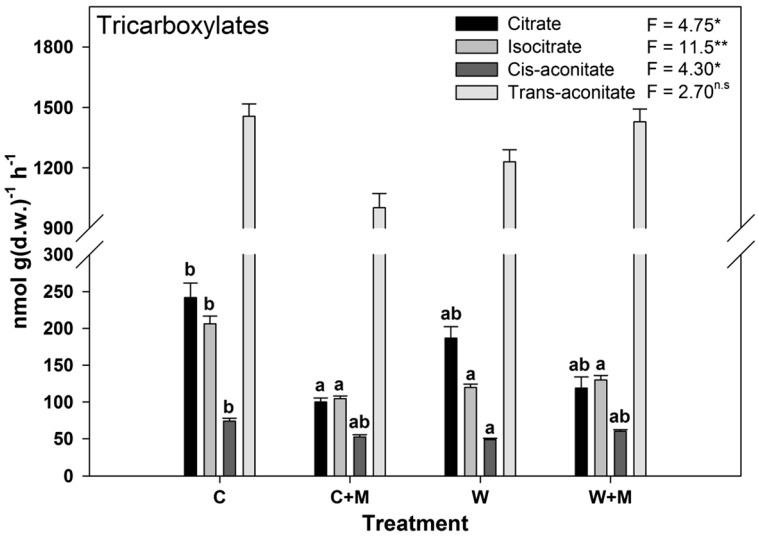


Fig. 4 Exudation rates of individual tricarboxylates at 28 DAE. Data shown represent arithmetic mean ($n = 4$); error bars indicate standard error of the mean; different letters above bars indicate significant differences (Tukey's HSD, $p < 0.05$) between treatments for individual LMMOA separately; C control; C + M control inoculated with MUCI; W wood ash treatment; W + M wood ash treatment inoculated with MUCI; ** $p < 0.01$; * $p < 0.05$; $^{n.s}$ not significant at $p < 0.05$

and cis-aconitate were also suppressed in W compared to C (Fig. 4). Two-way ANOVA showed significant and homogenous effect of wood ash on the exudation rates of malate and isocitrate and consequently on the lower total exuded rate of dicarboxylates (Table S4).

Application of MUCI resulted in a significant decrease of malate exudation rate with both C + M and W + M treatments and consequently in lower total amount of dicarboxylates exuded (Fig. 3; Table 4). The response in terms of exudation of tricarboxylates to MUCI application was different between C and W. In the case of ash-treated soil, total amount of tricarboxylates remained unchanged after MUCI application (Table 4) and no changes in the exuded amount of individual tricarboxylic LMMOAs were detected (Fig. 4). However, exudation rates of citrate and isocitrate were significantly lower when MUCI was applied to plain soil. According to two-way ANOVA, MUCI had significant and consistent lowering effect on the exudation rates of malate, oxalate and citrate (Table S4). In the case of isocitrate, cis- and trans-aconitate and, consequently, the rate of total amount of tricarboxylates, the interactions between the effects of MUCI and WA were observed (Table S4), suggesting a difference in MUCI effects between plain and ash-treated soil.

Concentrations of LMMOAs were determined in rhizospheric and bulk soil solutions at 28 DAE. Significantly higher concentrations (Mann-Whitney Rank Sum test) were generally found in rhizospheric than bulk soil solutions (Table S4; Table S5). Only acetate showed similar concentrations between both solution types. In addition, more types of LMMOA were found in the rhizospheric soil solution (Table S5). Isobutyrate, maleate and isocitrate were below the DL (DL = 0.005, 0.007 and 0.011 mg L^{-1}, respectively) in bulk soil solutions, but were found in rhizospheric ones. No differences in individual LMMOA concentrations between all treatments were found in bulk soil solutions (Table S4). In the case of rhizospheric soil solution, WA showed decreasing influence (two-way ANOVA) on the concentrations of acetate. In the same solution type, MUCI had a significant influence on the concentrations of acetate, formate, malate and, consequently, on the concentrations of total monocarboxylates (Table S4).

Survival of MUCI in soil

We obtained 803,196 high quality bacterial sequences divided to 3821 OTUs on the 3% similarity level. *Paenibacillaceae* were represented by 17,911 sequences divided to 126 OTUs. In the NMDS plot, *Paenibacillaceae* were separated by treatments of both, wood ash and MUCI additions (Fig. S1). The known sequence of MUCI belonged to OTU 37, which was higher by two orders of magnitude in the inoculated treatments (C + M; W + M) compared to the control. Above that bacterial communities were affected by the treatments. In particular, OTUs from *Actinobacteria* were upregulated with the 20 most affected OTUs (Table S6).

Discussion

Verification of the experimental and methodological approaches

Simplified soil microcosms were used in this experiment in order to study rhizospheric processes after PGPR inoculation of maize. The choice of proper growth system and exudates sampling technique still remains challenging since all known approaches have some advantages and drawbacks (Neumann and Römheld 2001; Fitz et al. 2006; Oburger et al. 2013). The soil microcosms were used for following reasons: i) experimental soil underwent only minimal disturbance since it was air dried and sieved through 10 mm sieve. Such procedure should keep soil structure, aggregates and natural microbial communities relatively unchanged; ii) root architecture is also expected to be similar to field conditions, at least in early growth stages since plants were soil-grown and microcosms were placed in outdoor conditions with natural sunlight and temperature. These parameters, when different from natural ones, may strongly influence the rate and composition of root exudates, which, in turn may have strong impact on the structure of rhizoplane and rhizosphere microbiome as well as PGPR colonization and vice versa (Nguyen 2003; Schulze and Pöschel 2004; Lakshmanan et al. 2014; Giagnoni et al. 2016). However, when quantification of LMMOA exudation rates is needed, it is impossible to remove intact root system from soil. Damage of root hairs is inevitable even when maximum effort is taken during roots removal. Exudation rates determined in our study were generally in the same magnitude as reported in published literature (Gaume et al. 2001; Oburger et al. 2013) suggesting minimal damage of the roots.

Sampling for LMMOA determination in soil solution has been subject to other problems. Rapid biodegradation (Neumann and Römheld 2001), adsorption on the suction cup material and spatial resolution of suction cups (Puschenreiter et al. 2005), distinct LMMOA concentrations between methods of sampling (Shen and Hoffland 2007) or changes in LMMOA extractability caused by sample storage (Mimmo et al. 2008) need to be taken into account when interpreting the results. Even though Rhizons used in this study were previously tested for LMMOA sampling from soil solution (Mercl et al. 2017), their spatial resolution is low compared to micro-suction cups (Göttlein et al. 1996; Puschenreiter et al. 2005) and sample collected by suction cup represents an average soil solution concentration over the whole sampling time (Shen and Hoffland 2007). It is notable that in this study LMMOA concentrations in soil solution sampled by suction cups were much lower than in solution obtained by centrifuge drainage and the maximum correlation coefficient (r) between these methods was $r = 0.5$; $p = 0.03$ (for oxalate in bulk soil; data not shown). Despite high root density in root-zone compartment, individual LMMOA concentrations in suction cup solutions were not statistically different between root zone and no-root zone (data not shown). Lack of differences for suction cup solution between rhizospheric and bulk soil solution was previously reported by Oburger et al. (2013) who sampled soil solution using micro-suction cups in mm scale distance from roots. However, these results are not in agreement with the generally accepted concept of rhizosphere where higher carbon concentrations are expected close to the roots due to rhizodeposition (Nguyen 2003; Sauer et al. 2006; Hinsinger et al. 2009; Jones et al. 2009). The centrifugation-drainage method showed better distinction between rhizosphere and bulk soil solution since the concentrations of almost all determined carboxylates were higher in rhizospheric soil solution (Table S5). We hypothesise that the superior performance of the centrifugation-drainage method is likely to be a result of collection of different fractions of pore water (e.g. micropore water), which are not collected by suction cups because of lower suction tensions (Di Bonito et al. 2008). However, one cannot rule out the possibility that higher concentrations of LMMOAs in soil solution obtained by centrifugation-drainage method were caused by disruption of microbial cells even though such phenomenon is generally reported for RCFs higher than 5000 *g* (Peterson et al. 2012).

Suction cup solution was used in our study for the interpretation of plant-available nutrients and pH, as was previously suggested and tested by Argo et al. (1997) and Rais et al. (2006). In general, initial pH of soil solution corresponded well with soil pH determined by $CaCl_2$ extraction (Table 1; Fig. 2) and it increased over time most probably because of predominant NO_3^- uptake by plants (Mistrik and Ullrich 1996; Brimecombe et al. 2007). The shift in pH led to increased P concentrations in soil solution over time (Fig. 2); however, this phenomenon was masked in root zone and was detectable only in no-root zone solution. We assume that the decrease in concentrations of NO_3^-, and subsequent

increase in pH in no-root solution was caused by diffusive transport of soil solution from no-root to root zone compartment.

The effect of wood ash

The positive impact of wood ash on crop yield has been widely reported (Erich 1991; Erich and Ohno 1992; Insam and Knap 2011; Ochecová et al. 2017), its agronomic efficiency was shown to be relatively high (Brod et al. 2015) but its influence on crops may vary among soils (Mercl et al. 2016). As we found significant increase in maize yield (Table 2), the combination between soil and rate of wood ash application used in this experiment can be concluded as suitable approach for the soil fertility improvement. According to Nkana et al. (1998), a positive effect of WA on the biomass yield is mainly caused by Ca and K supplementation. In our experiment, the application of WA resulted in increased biomass uptake of all studied nutrients (N, P, K, S, Mg and Ca). The higher uptakes of K, S, Mg and Ca can be easily explained by increased concentrations of these nutrients in soil solution after WA addition (Fig. 2). This may be also partly true for N as significantly higher NO_3^- concentrations in no-root zone soil solution were found at the early stage of the experiment (Fig. 2). Since WA contained a negligible amount of N (Table 1), increased NO_3^- concentrations in soil solution should be related rather to N mineralisation and/or enhanced rate of nitrification as reported by several studies (Park et al. 2004; Patterson et al. 2004; Odlare and Pell 2009). However, maize plants in our experiment were limited mainly by P as determined by both, nutrient sufficiency ranges (Table 2) and calculated DRIS index values (Table 3). One would expect that higher biomass yield in W compared to C as well as higher P uptake was therefore caused by supplementation of P-rich ash. However, results of soil solution P (Fig. 2) cannot confirm this since there was no significant increase of solution P in W compared to C treatment. The lack of difference in P concentrations in soil solution may be caused by sorption and precipitation reactions in soil or may be masked by intensive and continuous uptake by plants since P was still the most limiting nutrient in W treatment (Table 3). A similar trend was reported by Li et al. (2007) who found higher P and K uptake by plants but no increase in soil available P or K content. Such phenomenon may also lead to the suggestion that these nutrients were taken up by plants not directly from soil solution but likely from "biofilm cover" as proposed by several authors (Seneviratne and Jayasinghearachchi 2005; Liu et al. 2006; Bogino et al. 2013). Our suggestions are partly confirmed by the results of root exudation (Figs. 3 and 4). Increased citrate and malate exudation rate from roots in maize has been reported by Gaume et al. (2001) to be the adaptation to P-limiting conditions. Our results showed a significantly lower rate of malate and isocitrate exudation in W compared to C which may, therefore, indicate higher supply of P to plants. However, Lyu et al. (2016) reported rather root morphological than physiological response of maize to P-limiting conditions. In order to study the influence of individual treatments on LMMOA concentrations in soil solution, rhizospheric and bulk soil solutions were analysed. It is known that once carboxylates are released from root to the rhizosphere, they may be quickly utilised and/or transformed by the soil microbial community or may be adsorbed onto soil particles. Oburger et al. (2009) suggested that sorption is a major regulator of bioavailability of di- and tri-carboxylates. A recent study of Gunina et al. (2017) showed the carbon oxidation state to be a dominant factor governing half-life of low-molecular-weight organic substances in soil solution. Moreover, it has been suggested by Oburger et al. (2013) that the majority of LMMOA in soil solution sampled from the close vicinity of the root (1–3 mm) are not of plant origin. In our study, two-way ANOVA revealed an influence (decreasing trend) of WA application on the acetate concentrations in rhizospheric soil solution (Table S4). Since no effect was observed in bulk soil solution, the direct effect of WA on shifts in microbial metabolism is unlikely. We hypothesise that the lower concentration of acetate in rhizospheric soil solution was an indirect result of suppressed carboxylate rhizodeposition due to better P supply to maize plants. It is noteworthy that WA significantly decreased carboxylate C flux from maize plants to soil (Table 4), but only with a little effect on carboxylate concentration in rhizospheric soil solution (Table S4; Table S5).

The effect of *Paenibacillus mucilaginosus* ABi13

The inoculation was successful and MUCI was detectable in soil even after 35 days from the application as seen from the differences in counts of OTU 37 between the treatments. However, it seems that the inoculated strain as well as ash supplementation changed the

bacterial community so the observed effect on soil chemistry and plant performance might have been partly a result of more complex bacterial interactions (Panke-Buisse et al. 2015).

Bacterial inoculation has been reported to have better stimulatory effects on plant growth in nutrient deficient (Egamberdiyeva 2007), unfertile, stressed soils or in soils with poor microbial biomass and activity (Strigul and Kravchenko 2006; Fliessbach et al. 2009; Mäder et al. 2011). According to several studies (Krey et al. 2011; Krey et al. 2013; Mosimann et al. 2017), positive effects of P-solubilising bacteria on maize growth and nutrition are to be expected in P-deficient soils, but it cannot be taken for granted that inoculation with well-colonising and persistent PGPR will lead to a positive effect on the yield (Mosimann et al. 2017). The wood ash-amended soil (W) and control soil (C) and in our experiment can, therefore, be considered as suitable for PGPR use, since with both treatments maize plants exhibited P deficiency (Tables 2 and 3). However, application of MUCI had no beneficial effect on maize growth when applied in plain soil (C + M), possibly due to inefficient P solubilisation since P remained to be the most limiting nutrient, even after inoculation (Table 3). On the one hand, such an effect could be expected as the soil was strongly acidic (pH_{CaCl2} 5.2) and, therefore, a considerable amount of P was present as precipitated Fe- and Al-phosphates, or was sorbed on Fe- and Al-oxides (Hinsinger 2001). Potential localised simple acidification induced by MUCI, as reported by Hu et al. (2006), Liu et al. (2006) and Lu et al. (2014), would thus be expected to have lower efficiency as the solubility of these phosphates decreases with decreasing pH (Lindsay 1979; Bashan et al. 2013). On the other hand, organic acids formed may act through other mechanisms, such as ligand exchange and occupation of P sorption sites and/or ligand-promoted dissolution of Fe- or Al-oxides and subsequent desorption of P into soil solution (Jones et al. 2003; Oburger et al. 2011). However, to mediate significant desorption of P, relatively high carboxylate concentrations are required (Neumann and Römheld 2001). Such "hotspots" could be theoretically possible, as was hypothesised by Liu et al. (2006), for the combinatory carboxylate and polysaccharide excretion by MUCI. More favourable conditions for microbial-induced P dissolution were expected with W + M treatment, as the soil was supplied with WA containing a considerable amount of acid-soluble P (Table 1; Table S1). In this case, we found significantly higher P concentrations in soil solution, but only at 14 DAE in no-root zone (Fig. 2). We attribute the fact that no significantly different P concentrations were found in root zone due to the continuous P uptake by maize roots. However, part of P solubilized by MUCI could be hidden in "biofilm cover" and therefore, not sampled by suction cups. Nevertheless, inoculation of MUCI to W + M shifted the nutritional status of maize in favour of N deficiency (Table 3), which consequently led to lower biomass yield (Table 2) and N uptake (Table S2). Soil solution analysis (Fig. 2) revealed that the application of MUCI lowered NO_3^- concentrations in solution irrespective of the treatment. It is questionable whether N was utilized by MUCI into organic forms, denitrified and lost from the system or simply sorbed onto exopolysacharides produced by MUCI, since we found no differences in NO_2^- or NH_4^+ concentrations in soil solution between treatments. Interestingly, MUCI also decreased solution concentrations of K, Mg and Ca (Fig. 2). Tang et al. (2014) studied exopolysaccharides produced by *P. mucilaginosus* as a possible bioflocculant in waste water treatment. Authors showed that the production of polysaccharides was enhanced by the addition of Mg^{2+}, Ca^{2+} and Fe^{3+} ions into the culture medium and they hypothesised a metabolic reaction of *P. mucilaginosus* to these ions. Moreover, these polysaccharides contained hydroxyl, carboxyl and phosphate functional groups, making them a possible sorbent for mentioned ions since divalent cation bridging of polysaccharides is reported to be the main mechanism of bioflocculation (Sobeck and Higgins 2002). Nevertheless, as soil conditions were not K, Mg or Ca limiting, the decreased concentrations of these ions in soil solution induced by MUCI were unlikely to affect growth of maize. As mentioned above, inhibition of maize growth in W + M treatment was related to N deficiency. Lower N status of plants probably led to lower exudation rates of malate in C + M and W + M treatments (Fig. 3) since carboxylate exudation of maize is known to be affected by nitrate supply due to involvement of carboxylates in nitrate reduction in roots (Neumann and Römheld 2001). This is partly confirmed by Pearson correlation analysis between exudation rates of individual carboxylates and nutrient concentrations in shoot biomass. This analysis revealed strong correlation for malate ($r = 0.94$; $p < 0.001$), oxalate ($r = 0.70$; $p < 0.01$), citrate ($r = 0.72$; $p < 0.01$) and isocitrate ($r = 0.58$; $p < 0.05$), particularly with N concentration in shoots. As a consequence, the effect of MUCI on the concentrations of

acetate, formate and malate in rhizospheric soil solution was noticeable (Table S4). As mentioned above, lower concentrations of carboxylates in the rhizosphere may, in turn, decrease P acquisition by roots. This effect could be theoretically overcome by increased N supply but such hypothesis needs be tested in future research.

Conclusions

Wood ash, as a recycled fertiliser product, represents an important source of mineral nutrients which are, however, of low plant availability as demonstrated in this experiment. Application of *P. mucilaginosus* ABi13 led to changes in soil microbiome from which *Actinobacteria* were mostly affected. Inoculation of *P. mucilaginosus* in untreated, nutrient-poor, acidic soil had no significant influence on biomass yield of maize due to inefficient mobilisation of soil P. When soil was amended by addition of wood ash, increased P concentrations in soil solutions were detected after *P. mucilaginosus* inoculation. In this case, however, the growth of maize was inhibited due to significant decrease in NO_3^- concentrations in soil solution induced by *P. mucilaginosus*. As a consequence, the rate of LMMOA rhizodeposition in maize was lowered but decreased LMMOA exudation rates resulted in negligible concentration changes in rhizospheric soil solution sampled using centrifuge drainage method. To the best of our knowledge, this is the first report of increased P solubility from wood ash amended soil by PGPR. However, the mechanism by which *P. mucilaginosus* ABi13 immobilises N needs to be elucidated in future research in order to optimise N fertilisation strategies. Moreover, *P. mucilaginosus* ABi13 could be theoretically used for prevention of K, Ca, Mg and particularly NO_3^- leaching from soils after further tests. This study demonstrated the importance of plant nutritional status on the final outcome of PGPR inoculation and contributes to our understanding of interactions between introduced PGPR, soil microbiome and plants. Lower LMMOA rhizodeposition induced by PGPR may be clearly of ecological significance.

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4.8) Improved phosphorus fertilization efficiency of wood ash by fungal strains *Penicillium* sp. PK112 and *Trichoderma harzianum* OMG08 on acidic soil

Autoři: Mercl, F., García-Sánchez, M., Kulhánek, M., Košnář, Z., Száková, J., Tlustoš, P.

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Article type	Research Paper

Abstract

Effective recycling of phosphorus remains a critical issue in sustainable agriculture. Wood ash represents valuable soil amendment and potential source of phosphorus for agriculture, but its solubility and subsequent P-fertilisation efficiency is extremely low. This study tested fungal inoculants (Penicillium sp. PK112 and Trichoderma harzianum OMG08) applied alone and in combination with wood ash on P-deficient acidic soil to determine if they can improve P-nutrition in maize. Wood ash alone did not have any significant P-fertilising effect. Application of both inoculants, when combined with wood ash, led to significant increment of plant-available P content in soil, increased P uptake by maize plants and consequently to higher yield of maize biomass. Both inoculants suppressed overall microbial activity in soil as determined by the activity of dehydrogenase, alkaline phosphatase and microbial P content. Only T. harzianum led to higher activity of soil acid phosphatase. This study demonstrated that tested strains may be co-applied with wood ash and improve its P-fertilisation efficiency. The positive influence of inoculants on P availability was mainly due to stronger acidification of rhizosphere and decreased content of microbial P. However, both effects seemed to be hindered by the P sorption capacity of the soil in the case of inoculation without wood ash. Such findings may lead to development of novel formulations of recycled fertiliser products and boost nutrient recycling in agriculture.

Keywords	P-solubilizing microorganisms; wood ash; bioeffector; recycled fertilizer products; maize
Taxonomy	Microcosm Studies, Soil Enzyme Assays, Rhizosphere Biology, Agricultural Waste Management
Manuscript category	Microorganism-related Submissions

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Cover Letter

Dear Editorial Board,
I wish to submit a new manuscript entitled "**Improved phosphorus fertilisation efficiency of wood ash by fungal strains *Penicillium* sp. PK112 and *Trichoderma harzianum* OMG08 on acidic soil**" for consideration by Applied Soil Ecology journal.

I hereby confirm that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere. Professional proofreading of the manuscript was provided by Proof-Reading-Service.com.

In our study, wood ash is used as an alternative P fertiliser as it globally represents significant sink of P. However, it has been demonstrated that P fertilisation efficiency of wood ash is very low which discourages from its wide application to soil. In our study, we report two fungal strains which are able to significantly improve P nutrition of maize when wood ash is used as a fertiliser. To the best of our knowledge, this is the first report of inoculants which are successfully combined with wood ash in terms of P solubilisation. The modes of action responsible for boosted maize growth are thoroughly studied in our manuscript. We revealed that both tested inoculants not only caused acidification in the rhizosphere, but they also significantly inhibited overall microbial activity in soil likely because of competition for carbon sources and caused mobilization of microbial phosphorus. Only this joint action enabled higher uptake of P from wood ash by maize. In general, tested strains are able to significantly improve P fertilisation efficiency of wood ash, which may lead to formulations of novel recycled fertiliser products and thus increase recycling of nutrients and subsequently the sustainability of agriculture.

The paper should be of interest to a wide range of readers, especially in the areas of rhizosphere ecology, soil management, plant growth-promoting microorganism application and plant nutrition.

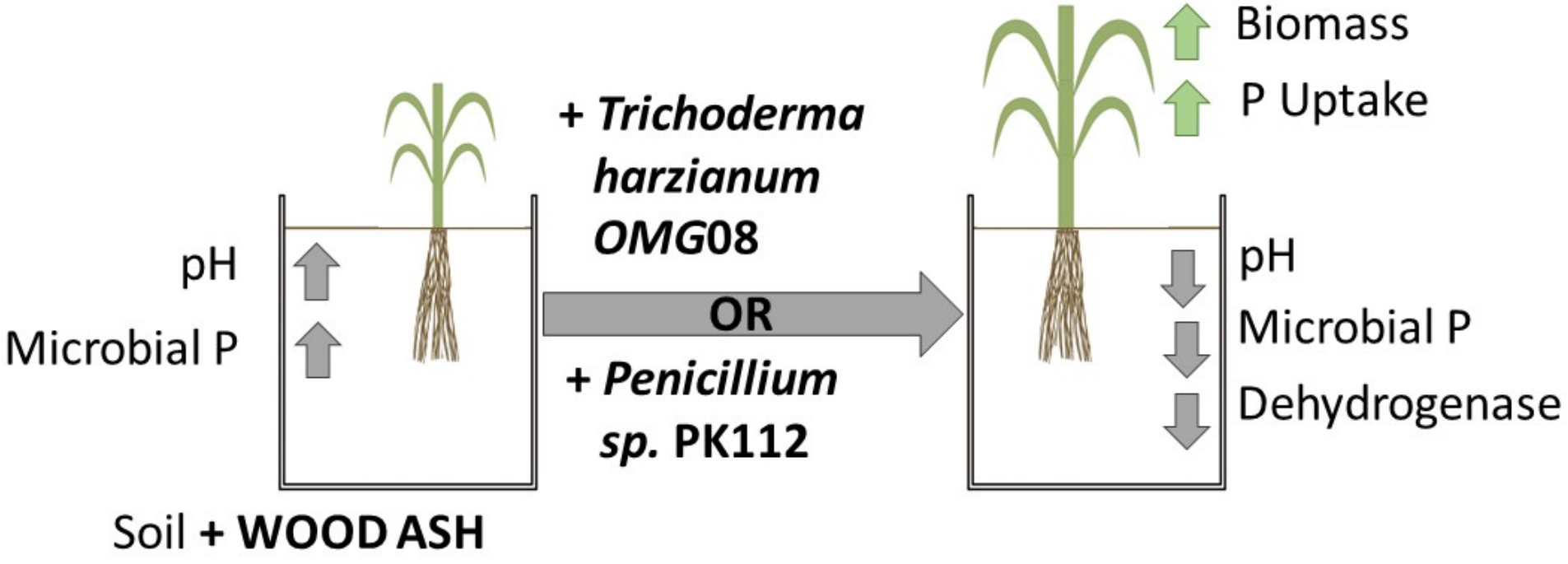
Please address all correspondence concerning this manuscript to myself at: mercl@af.czu.cz

Thank you for your consideration of this manuscript.

Sincerely,
Filip Mercl

Highlights

- Tested fungal strains enhance uptake of P from wood ash by maize
- Both strains inhibit dehydrogenase activity and mobilize microbial P
- P sorption capacity of soil inhibits the performance of P-solubilisers


Biomass
P Uptake
+ Trichoderma harzianum OMG08
pH
Microbial P
OR
+ Penicillium sp. PK112
pH
Microbial P
Dehydrogenase
Soil + WOOD ASH

Improved phosphorus fertilisation efficiency of wood ash by fungal strains *Penicillium* sp. PK112 and *Trichoderma harzianum* OMG08 on acidic soil

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Abstract

Effective recycling of phosphorus remains a critical issue in sustainable agriculture. Wood ash represents valuable soil amendment and potential source of phosphorus for agriculture, but its solubility and subsequent P-fertilisation efficiency is extremely low. This study tested fungal inoculants (*Penicillium* sp. PK112 and *Trichoderma harzianum* OMG08) applied alone and in combination with wood ash on P-deficient acidic soil to determine if they can improve P-nutrition in maize. Wood ash alone did not have any significant P-fertilising effect. Application of both inoculants, when combined with wood ash, led to significant increment of plant-available P content in soil, increased P uptake by maize plants and consequently to higher yield of maize biomass. Both inoculants suppressed overall microbial activity in soil as determined by the activity of dehydrogenase, alkaline phosphatase and microbial P content. Only *T. harzianum* led to higher activity of soil acid phosphatase. This study demonstrated that tested strains may be co-applied with wood ash and improve its P-fertilisation efficiency. The positive influence of inoculants on P availability was mainly due to stronger acidification of rhizosphere and decreased content of microbial P. However, both effects seemed to be hindered by the P sorption capacity of the soil in the case of inoculation without wood ash. Such findings may lead to development of novel formulations of recycled fertiliser products and boost nutrient recycling in agriculture.

Keywords

P-solubilizing microorganisms; wood ash; bioeffector; recycled fertilizer products; maize

1 Introduction

Effective recycling of P is one of the main challenges of sustainable agriculture. Wood ash represents an important source of P and valuable soil amendment. Wood fuel provides 40% of today's global renewable energy supply and thanks to its net carbon emissions being zero; its production is expected to grow in future. Wood ash is also one of the cheapest and most available soil amendments in developing countries (FAO 2018). Soil application of wood ash supplies mineral nutrients, especially Ca, K, Mg and P (Ochecová et al. 2017) and increases soil pH with concomitant benefits such as element leaching reduction, mitigation of possible Mn- and Al-toxicities (Demeyer et al. 2001) or reduction of the heavy metal uptake by crops (Ochecová et al. 2014). However, the solubility, and therefore the plant-availability of wood ash-bearing P is generally very low. The predominant P-minerals present in wood ash are apatite ($Ca_5(PO_4)_3(F,Cl,OH)$) and whitlockite ($Ca_9(MgFe)(PO_4)_6PO_3OH$) (Boström et al. 2011; Vassilev et al. 2013). Due to the very low water solubility of these minerals, the P fertilisation efficiency of wood ash is very low, incomparably lower than that of soluble commercial fertilisers (Demeyer et al. 2001; Nkana et al. 1998; Ohno & Erich 1990; Park et al. 2012). One strategy for improving the P availability from wood ash may be the application of P-solubilising microorganisms. Such approach represents environmentally friendly, easy-to-use and low-cost strategy, especially when compared with industrial leaching or thermochemical P-recovery methods used for ashes (Ohtake & Tsuneda 2019).

Some soil fungi species are well known for their strong P-solubilisation potential and/or mobilisation of organic P in soils. Their general involvement in P cycling in soils is therefore well recognised. Fungi belonging to genera *Penicillium* and *Trichoderma* have been studied extensively in recent decades. Many successful applications were demonstrated mostly in their use for

54 biological control of plant diseases (Howell 2003). However, their use in terms of P-solubilisation
55 still remains challenging. A perfect example is work of Karamanos et al. (2010) demonstrating the
56 positive effect of *Penicillium bilaiae* application only in 5 out of 47 field experiments. Although
57 some recent studies (Gómez-Muñoz et al. 2017; Lekfeldt et al. 2016; Raymond et al. 2019) reported
58 very limited capacity of the inoculants to influence P uptake of crops, many authors rather report
59 strong influence of soil type and P-source on the outcome of fungal inoculants (Mpanga et al. 2018;
60 Sánchez-Esteva et al. 2016; Thonar et al. 2017). However, soil property determining the
61 performance of inoculants is rarely identified due to complexity of soil-plant systems (Gómez-
62 Muñoz et al. 2018; Leggett et al. 2015).
63 The objectives of this study were (1) to test P-solubilising strains of *Penicillium* and
64 *Trichoderma* whether they can be co-applied with wood ash for improving its P-fertilisation
65 efficiency and (2) to identify key mechanisms responsible for their influence on P transformations
66 in soil-plant system.

2 Materials and Methods

2.1 Soil and wood ash characteristics

Experimental soil (Haplic Cambisol) was collected from a field (plow depth, 0-20 cm) near the city of Žamberk, Czech Republic. The soils textural class was silt loam and soil had following properties: cation exchange capacity (CEC) 74.9 $mmol_+ kg^{-1}$; pH 5.2 in 0.01M $CaCl_2$ 1:2.5 (w/v); C_{tot} 1.9%; P_{tot} 551 mg kg^{-1}; $P_{Mehlich\ III}$ 22 mg kg^{-1}. For more detailed characterisation and P fractionation of the soil we refer to Mercl et al. (2018). The soil was air dried and passed through a 2 mm mesh stainless sieve prior to using. Wood ash originated from industrial power plant from Czech Republic combusting exclusively wood chips in a fluidised bed reactor (15 MWt). Tested wood ash was of following properties: CEC 125 $mmol_+ kg^{-1}$; pH_{H2O} 11.2; C_{tot} 8%; P_{tot} 10 195 mg kg^{-1} and $P_{CH3COOH}$315 mg kg^{-1}. Detailed characterisation of the wood ash can be found in work by Mercl et al. (2016). Wood ash was also air dried and passed through 2 mm sieve prior to use. The ash was then thoroughly mixed with soil in the amount corresponding to addition of 24 mg P per kg of dry soil. This dose corresponded to theoretical ash application rate of 7 t/ha. Treatments without ash application (plain soil) are denoted as CON and treatments where ash was applied are denoted WA.

2.2 Tested inoculants

Two fungal inoculants were tested in this study, namely *Penicillium* sp. PK112,and *Trichoderma harzianum* OMG08. The *Penicillium* sp. PK112 was provided by Bayer Crop Science Biologics GmbH as a product Biological Fertilizer OD (BFOD) containing concentrated liquid spore culture. The *T. harzianum* (OMG) was provided as a dry spore powder by Institute of Bioanalytical Sciences at Anhalt University of Applied Sciences in Germany. Inoculants were dissolved (OMG) or diluted (BFOD) in tap water (free of chlorine) and applied on the soil surface

after sowing and immediately before first watering. The amount of applied solution (only tap water in the control treatments) was always 20 mL and the final concentration of inoculants was 1×10^9 CFU kg^{-1} soil (dw; dry weight). Treatments where *Penicillium* was inoculated are denoted as +BFOD and treatments inoculated with *T. harzianum* are denoted as +OMG.

2.3 Experimental setup

The experiment was established as a 2 × 3 full factorial design where each treatment was replicated four times. Plants of maize (*Zea mays* L. var. Colisee; KWS Saat, Germany) were grown in polyethylene pots placed in a greenhouse with natural light conditions under controlled temperature (22/18 ± 2 °C day/night). Each pot contained soil in an amount corresponding to 0.5 kg of dry soil. All pots received basal fertilisation of 100 mg N kg^{-1} in the form of NH_4NO_3 (aqueous solution) prior to sowing. Five untreated seeds were sown into each pot and plant numbers were thinned to two after germination. Immediately after sowing, pots were inoculated with tested microorganisms. During the experiment, pots were irrigated using demineralised water to reach 60% of maximum soil water holding capacity (gravimetrically monitored twice per week). Position of pots was fully randomised with three re-randomisations during the experiment. After 45 days from emergence, the above-ground biomass of maize plants was harvested, dried at 60 °C, milled to fine powder and analysed. Soil was passed through the 2 mm mesh stainless sieve in order to remove roots, and divided into two parts. One part was air dried and used for determination of pH, available P concentrations and P sorption capacity, whereas the second part was stored at 4 °C and used for determination of enzymatic activities and microbial P biomass (P_{mic}).

2.4 Analytical Procedures

2.4.1 Soil enzymatic activities

The activity of dehydrogenase (DHA) (EC 1.1) was assayed following the methodology described by García-Sánchez et al. (2018). Briefly, 1 g of fresh soil was incubated with triphenyltetrazolium chloride (TTC) dissolved in 0.1 M Tris–HCl buffer (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 (extract/acetone; v/v) and measured spectrophotometrically at 490 nm. The results are expressed as μg TPF g^{-1} dry soil day^{-1}. Activities of acid (EC 3.1.3.2) and alkaline (EC 3.1.3.1) phosphatases were determined according to Eivazi and Tabatabai (1977). In brief, the sample (1 g of fresh soil) was mixed with a modified universal buffer (pH 6.5 for acid and 11 for assays of alkaline phosphatase) and incubated for 1 h at 37 °C. The p-nitrophenyl-phosphate was used as a substrate and the concentration of produced p-nitrophenol was determined spectrophotometrically at 400 nm after addition of 0.5 M NaOH and 0.5 M $CaCl_2$.

2.4.2 Determination of nutrients

In dried plant biomass, total concentrations of P, K, Mg and Ca were determined by inductive coupled plasma-optical emission spectrometry (ICP-OES; Agilent 720, Agilent Technologies Inc., Santa Clara, CA, USA) after the dry-ashing procedure according to Mader et al. (1998). Nitrogen concentration in plant biomass was determined by the Kjeldahl method using the automatic distillation system Vapodest 50s (Gerhardt, Germany). The pH of soil samples was measured after extraction with 0.01 M $CaCl_2$ (VDLUFA 1991). The plant-available portion of inorganic P in the soil after harvesting (P_{AEM}) was assayed by a 16-h extraction of soil with demineralised water (1:60; w/v) with two anion-exchange membrane strips (AEM-PES membrane; FumaTech GmbH, Bietigheim-Bissingen, Germany). The P adsorbed by the membranes (P_{AEM}) was then extracted using 0.5 M HCl (Tiessen & Moir, 2007). This method provides more accurate information about the real amount of plant-available P in the soil compared to conventional soil-

liquid extractions because only inorganic phosphate ions (Pi) are continuously taken up by the membrane from solution. The adsorbed P is not desorbed back into the solution; the membrane therefore, mimics continuous uptake of P by the plant root. The amount of microbial biomass P (P_{mic}) in the soil was determined according to the method of Brookes et al. (1982) as a difference in Pi (determined according to Murphy and Riley (1962)) extractable by 0.5 M $NaHCO_3$ between fumigated ($CHCl_3$) and unfumigated samples. A conversion factor (K_p) of 0.4 was used for calculation. The P sorption capacity of the soil and soil + ash mixture was determined by shaking 1 g of air dried soil with 20 ml of 75 mg P L^{-1} solution (KH_2PO_4) for 24 h at 4 °C. The amount of sorbed P was determined by ICP-OES after centrifugation for 10 min at 4500×g. Water soluble organic carbon (WSOC) was determined in the supernatant by the Walkley-Black method (Walkley & Black, 1934) after extraction of soil with demineralised water (1:10; w/v).

2.5 Statistics and data analyses

STATISTICA 12 (StatSoft, Inc., Tulsa, OK) was used for statistical analyses. Figures were prepared using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). Statistically significant differences (post-hoc Fisher's Least Significant Difference - LSD) are shown at the 95.0% confidence level.

Diagnosis and Recommendation Integrated System (DRIS) indices (Beaufils 1973) were used to determine the most plant-growth limiting nutrient in individual treatments. For the calculation, biomass concentrations of N, P, K, Mg and Ca were used and data by Elwali et al. (1985) were used as a reference.

3 Results and Discussion

3.1 Nutritional status and biomass yield of maize

Phosphorus was the most growth-limiting macronutrient in all variants of the experiment. This is indicated by calculated DRIS index values (Table 1) and also by P concentrations in biomass ranging from 1.3 to 2 g kg^{-1} (data not shown) which are considered far below sufficiency range (Jones et al. 1990). The sole application of wood ash (WA) (~ 7 t ha^{-1}) did not result in higher yield of maize or P uptake as compared to CON (Fig. 1) indicating very low plant-availability of P in this material. Similar results were recently published by Cruz-Parades et al. (2017) who observed a P-fertilising effect of wood ash only at the application rate of 22 t ha^{-1}.

Inoculation of wood ash-treated soil with *Penicillium* (WA+BFOD) as well as *T. harzianum* (WA+OMG) led to significantly ($p < 0.05$) higher biomass yield (Fig. 1) and higher plant uptake of P (Fig. 2) as compared to WA treatment. This leads to the suggestion that the better growth performance was caused by higher availability of P in soil. The results of P_{AEM} in soil after harvest confirmed this hypothesis as both treatments (WA+BFOD and WA+OMG) showed significantly higher concentrations of this readily plant-available inorganic P in soil (Fig. 3). Contrarily, the inoculation of *Penicillium* or *T. harzianum* to plain, non-treated soil (CON+BFOD; CON+OMG) did not result in any significant difference in biomass yield, P uptake or the concentration of P_{AEM} as compared to corresponding non-inoculated treatments (CON).

(Table 1)

(Figure 1)

(Figure 2)

(Figure 3)

178 **3.2 Microbial activity in the rhizosphere of maize**

179 In order to elucidate the reason why maize plants exhibited positive and significant response
180 to tested inoculants only in the case of the wood ash-treated soil, a set of microbial analyses was
181 performed on soil samples after the harvest. Application of wood ash alone did not influence the
182 overall activity of DHA, acid phosphatase or the concentration of WSOC in the soil (Table 2). A
183 slight, but significant decrease in WA treatment was found in the activity of alkaline phosphatase.
184 Phosphomonoesterases are generally known to be very sensitive to pH. In the long term, alkaline
185 phosphatases are more present in alkaline soils (Ekenler & Tabatabai 2003), but application of ash
186 may result in a short-term disturbance of autochthonous microbial communities and lead to
187 decreased activity of these enzymes as demonstrated by several works (Noyce et al. 2017; Perucci
188 et al. 2006). Concomitantly, a significantly higher amount of P_{mic} was determined in WA compared
189 to CON treatment. This may point to the fact that potentially plant-available P from wood ash was
190 preferably immobilised into organic forms by the autochthonous soil microorganisms and
191 therefore, not directly available to the plants.

192 The effect of *Penicillium* and *T. harzianum* inoculation on soil enzymatic activities was
193 surprisingly the same in CON and WA treatments. In both cases, both inoculants significantly
194 decreased the activity of DHA (Table 2). Activity of DHA represents cumulative activities of many
195 microbial dehydrogenases involved in the multiple oxidation reactions during respiration
196 processes, and it is considered that all determined activity is intracellular (Prosser et al. 2011). The
197 lowered activity of these enzymes in inoculated treatments can be therefore considered as a
198 suppression of overall microbial activity in soil as induced by two tested fungi. Some strains of *P.*
199 *bilaiae* and *T. harzianum* had been reported to produce secondary metabolites with antibacterial as
200 well as antifungal activity (Cunningham & Kuiack 1992; Yang et al. 2011). However, it is difficult
201 to distinguish whether the inhibition of activity was caused by production of such secondary

metabolites or by competition for available carbon sources between inoculated fungi and autochthonous microbes. Results of WSOC in the soil (Table 2) likely confirms the latter hypothesis because significantly lower concentrations of WSOC were always found in inoculated compared to non-inoculated treatments. The inhibiting effect of tested inoculants on autochthonous microbes is further confirmed by significantly decreased activities of alkaline phosphatases (Table 2). As the soil alkaline phosphatases are not reported to be produced by higher plants but solely by soil microorganisms (Nannipieri et al. 2011; Spohn & Kuzyakov 2013), the activities of this enzyme confirm the overall inhibition of microbial activity in treatments inoculated by both, *Penicillium* and *T. harzianum*. Likewise, the amount of P_{mic} in inoculated variants was lower in all cases compared to non-inoculated treatments (Table 2). Although our data do not provide direct evidence of inoculant survival, taking into consideration that the effect of inoculation was the same in CON and WA treatments, we hypothesise that both fungi were able to survive in the control soil as well as in the ash-treated soil.

Inoculation by *T. harzianum* led to significantly higher activities of acid phosphatase in soil. The effect was the same for CON and WA treatments (Table 2). Acid phosphatases (namely phosphomonoesterases) are responsible for hydrolysis of phosphomonoesters in soil. Soil acid phosphatases may be produced by soil microorganisms as well as by plant roots (Spohn & Kuzyakov 2013).Therefore, it is impossible to distinguish whether the higher activity in +OMG treatments is caused by direct production of this enzyme by the inoculated fungi, other autochthonous microorganisms or by maize roots. Furthermore, all commonly used assays for determination of phosphatases in soil do not distinguish between extracellular and intracellular phosphatases, but likely measure their potential maximal activity at optimal conditions. Therefore, higher rates of phosphatase activity cannot be considered as a higher rate of organic P

225 transformations, but should be interpreted a higher potential to transform organic P (Nannipieri et
226 al. 2012).

227 (Table 2)

228 **3.3 Mobilisation of P by soil microbial activity**

229 The application of wood ash alone did not influence the concentration of plant-available P
230 in soil, but it increased the amount of soil P_{mic}. The amount of added ash-bearing P extractable in
231 acetic acid, usually considered as exchangeable or bioaccessible, was 0.7 mg P kg^{-1} of soil, but the
232 content of P_{mic} in the WA treatment increased by 24.2 mg P $kg^{-1}$of soil after application of wood
233 ash compared to the control (Table 2). Such significant disproportion indicates that autochthonous
234 microbes utilized, besides readily available ash-bearing P, also P of soil origin, which probably
235 became available due to chemical changes caused by wood ash. The soil used in this experiment
236 contained approximately 34% of total P in organic forms while more than 40% of total soil P was
237 associated with Fe/Al minerals (whether present as precipitated Fe- and Al-phosphates, or sorbed
238 on Fe- and Al- oxides) (Hinsinger 2001; Mercl et al. 2018). McLaren et al. (2015) reported that the
239 majority of organic P in soil is present as phosphomonoesters in supramolecular structures of humic
240 substances. As the solubility of Fe/Al-phosphates increases with increasing pH (Lindsay 1979); the
241 same goes for humic molecules (Piccolo 2001), only a slight increment in pH, as induced by wood
242 ash, could mobilise a certain amount of P. This P was probably rapidly utilised by soil microbes.

243 Contrarily, both applied inoculants inhibited the soil autochthonous microbial communities
244 and caused a significant decrease in amount of P_{mic}. This effect was same for plain soil as well as
245 ash-treated treatments. One would expect that such a decrease in P_{mic} will lead to a higher amount
246 of plant-available P; at least in treatments with a higher potential of phosphatase activity because
247 the phosphatases catalyse the hydrolysis of phosphomonoesters leading to the release of

orthophosphate. However, the decreased content of P_{mic} in plain soil treatments (CON+OMG; CON+BFOD) led neither to increased P_{AEM} nor to higher P uptake. Interestingly, even in CON+OMG treatment with significantly higher activity of acid phosphatase (Table 2), there was no detectable increment in plant-available P (Fig. 3). Such a result can be explained either that P released from microbial cells was rapidly sorbed and immobilised by soil particles or that the organic P (mainly nucleic acids and phospholipids) was not mineralised. However, these sources of P are usually mineralised within a few days (Spohn & Widdig 2017; Van Veen et al. 1987) so the preferential sorption of released orthophosphate is more likely.

In the case of wood ash treatments (WA+OMG; WA+BFOD), it is difficult to distinguish the origin of extra P taken up by maize plants compared to the WA variant because two main possible modes of action were detectable in these treatments, namely a decrease in P_{mic} and acidification (Table 2). Even though the wood ash-treated variants contained 0.7 mg kg^{-1} more of exchangeable P (extractable in 0.11M CH_3COOH supplied by wood ash) compared to plain soil treatments, sole acidification caused by inoculants cannot account for all of the excess available P and excess P taken up by plants (sum of differences over WA variant in P_{AEM} and P uptake were 3.46 and 3.12 mg kg^{-1} in WA+BFOD and WA+OMG, respectively). Similarly, the decrease in P_{mic} solely is insufficient to explain why the overall equilibrium of the soil-plant system shifted to more plant-favourable conditions because the same trend was observable in inoculated treatments without wood ash. The linear regression analysis revealed a significant effect ($r = -0.733$; $p < 0.001$) of P sorption capacity of soil on P uptake (Fig. 4). Wood ash-treated variants had clearly lower P sorption capacity and it is worthy to note that WA treatment (where no change in P uptake or P_{AEM} content was found) also showed lower P sorption capacity than other plain soil variants. The decrease in P sorption capacity of soil as induced by wood ash could be caused by many chemical factors. Surely pH and level of P played a role, but more importantly, the content of carbonates and

other anions competing with orthophosphate for sorption sites probably caused the decrease in sorption capacity. We therefore hypothesize that improved P uptake in WA+OMG and WA+BFOD treatments was caused by three mutual effects: acidification with subsequent release of acid-soluble P, and mineralisation of P_{mic}, both accompanied by lowered P sorption capacity of soil.

(Figure 4)

4 Conclusions

Wood ash is a valuable soil amendment, however, it has extremely low or no direct P-fertilisation effect when applied in agronomically relevant amounts. A combination of wood ash with *Penicillium* sp. PK112 and *Trichoderma harzianum* OMG08 significantly improved P nutrition, and subsequently, the yield of maize on acidic soil compared to application of wood ash alone. The tested inoculants therefore represent a promising and cost-effective way to improve the P-fertilisation efficiency of wood ash. Their modes of action were mainly soil acidification and reduction of microbial P content in soil. Our results further suggest that the P sorption capacity of soil may be an integral factor influencing the performance of P-solubilising inoculants. This property of soil and its management requires more attention as it may at least partly explain often reported soil-type dependent effects of applied phosphate-solubilising microorganisms.

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Table 1 Nutrient DRIS index values

Treatment	N_{index}	P_{index}	K_{index}	Mg_{index}	Ca_{index}
CON	-2,0	**-31**	-20	21	32
CON+BFOD	-10	**-27**	-13	19	31
CON+OMG	-7,9	**-23**	-9,4	16	24
WA	-6,9	**-27**	-15	24	24
WA+BFOD	-17	**-21**	-0,4	17	21
WA+OMG	-13	**-23**	-1,4	18	20

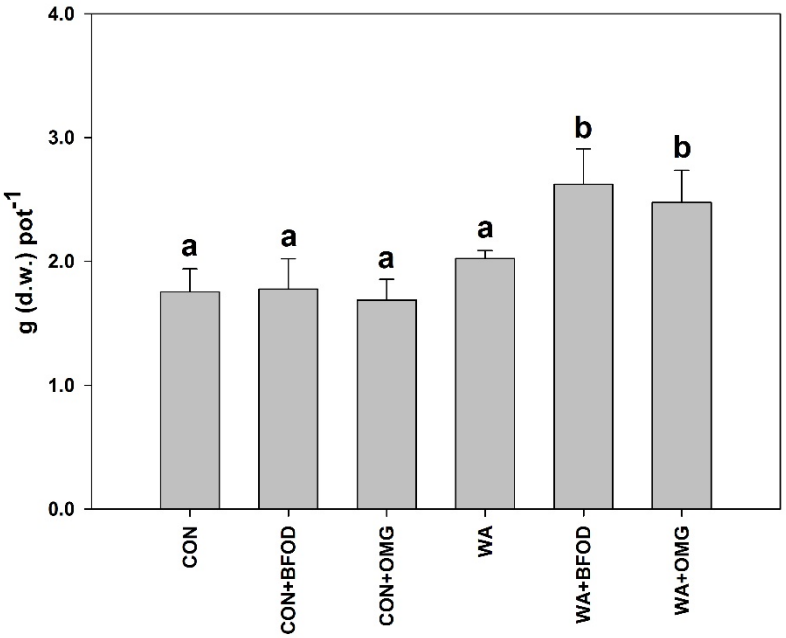
Values in bold represent the most limiting nutrient

Table 2 Soil physicochemical and microbial characteristics at harvest

Treatment	pH	WSOC	Dehydrogenase	Acid phosphatase	Alkaline phosphatase	Microbial P
	-log c(H^+)	mg C kg^{-1} (d.w.)	U_D	U_P	U_P	U_{PMIC}
CON	4.95 ± 0.07d	46.6 ± 8.54d	66.8 ± 6.22c	234 ± 21.4a	92.1 ± 1.45d	47.1 ± 4.64b
CON+BFOD	4.61 ± 0.13b	18.6 ± 4.33bc	39.1 ± 4.41b	223 ± 11.4a	89.6 ± 4.36cd	37.2 ± 0.76a
CON+OMG	4.41 ± 0.11a	21.1 ± 7.28c	25.7 ± 3.94a	331 ± 36.4b	76.5 ± 1.14b	43.9 ± 2.18ab
WA	5.16 ± 0.05e	48.7 ± 4.54d	77.5 ± 4.55c	221 ± 15.3a	85.4 ± 1.91c	71.3 ± 6.64d
WA+BFOD	4.74 ± 0.10c	8.66 ± 4.37a	45.9 ± 3.14b	251 ± 10.1a	74.2 ± 2.98b	47.3 ± 7.95b
WA+OMG	4.83 ± 0.03cd	9.92 ± 3.35ab	43.7 ± 7.09b	317 ± 22.7b	66.0 ± 0.75a	55.7 ± 5.86c

Data shown represent arithmetic mean (n = 4) ± standard deviation; different letters indicate significant differences (Fisher's LSD; $p < 0.05$) between treatments. Units: U_D µg TPF g^{-1} (dw) day^{-1}, U_P µg pNP g^{-1}(dw) h^{-1} and U_{PMIC} mg P_{mic} kg^{-1} (dw)

431 Figure 1 Yield of maize shoot biomass

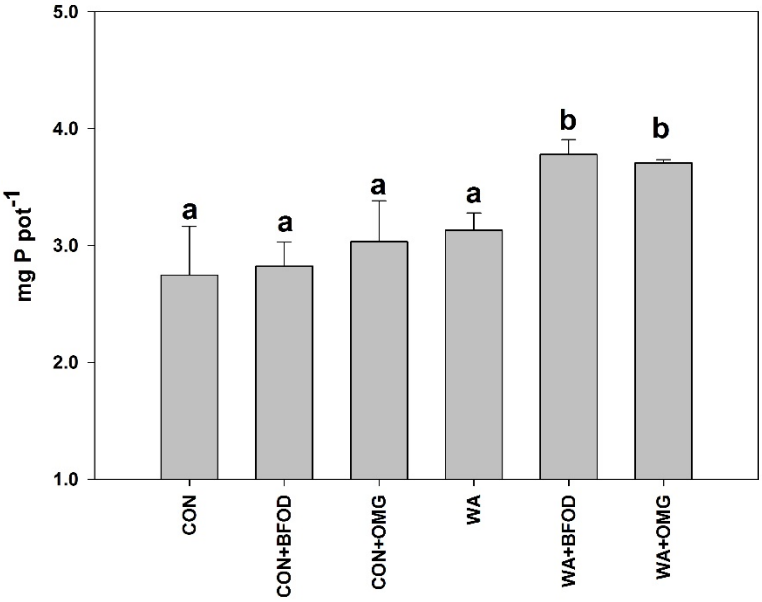


432

433 Data shown represent arithmetic mean (n = 4); error bars indicate standard deviation; different

434 letters above bars indicate significant differences (Fisher's LSD; $p < 0.05$) between treatments

435 Figure 2 Phosphorus content in maize shoot biomass

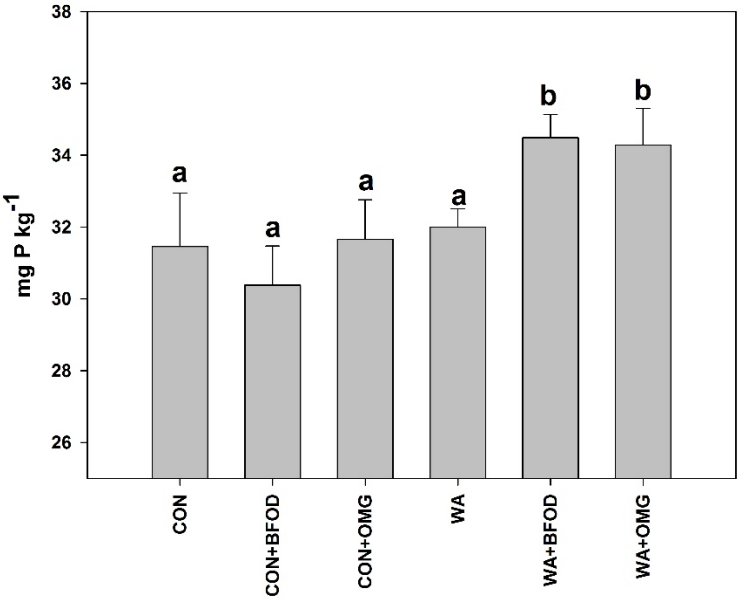


436

437 Data shown represent arithmetic mean (n = 4); error bars indicate standard deviation; different

438 letters above bars indicate significant differences (Fisher's LSD; $p < 0.05$) between treatments

439 Figure 3 Soil concentration of P_{AEM} after harvest

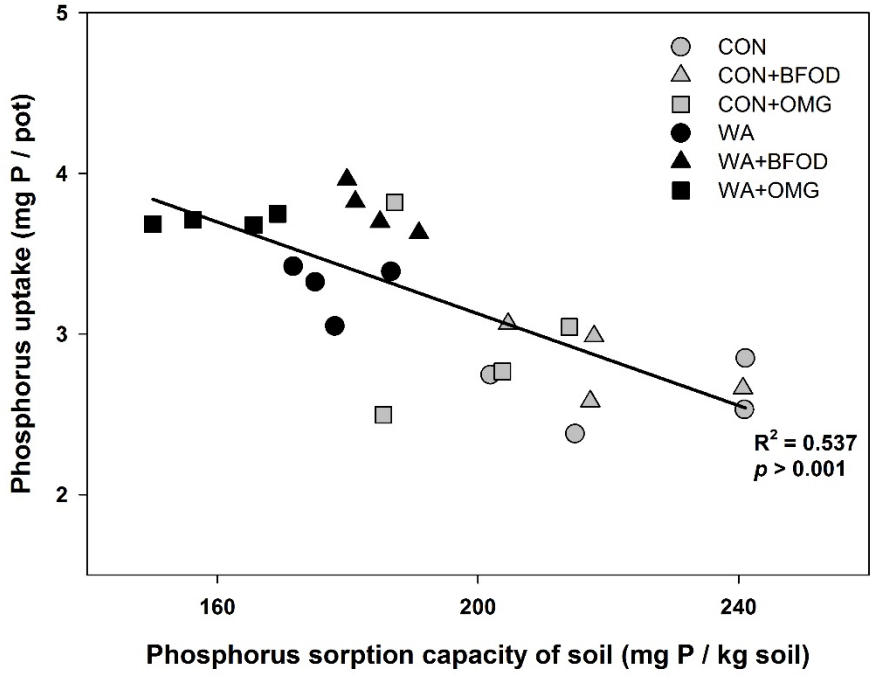


440

441 Data shown represent arithmetic mean (n = 4); error bars indicate standard deviation; different

442 letters above bars indicate significant differences (Fisher's LSD; $p < 0.05$) between treatments

443 Figure 4 Regression of P uptake by P sorption capacity of soil at the harvest



444

5) Sumární diskuze

Sumární diskuze této práce je rozdělena do tří hlavních podkapitol podle jednotlivých Cílů práce. V podkapitole 5.1 jsou shrnuty výsledky publikovaných prací 4.1 a 4.2 týkajících se významně odlišného vlivu dřevěného popela a dřevěného biocharu na chemické a biologické vlastnosti půdy. Podkapitola 5.2 pojednává o významu původu a zpracování odpadních materiálů na složení půdního roztoku a odběr živin rostlinami. Jako modelové materiály zde byly použity popel po spalování dřeva a popel po spalování slámy. V této kapitole jsou shrnuty poznatky z prací 4.3, 4.4 a 4.5. Poslední podkapitola 5.3 shrnuje výsledky dosažené v experimentech, kde byly pro zlepšení hnojivého potenciálu odpadních materiálů použity mikrobiální inokulanty. Výsledky této části jsou obsažené v pracích 4.6, 4.7 a 4.8.

5.1) Vliv aplikace dřevěného popela a biocharu na půdní vlastnosti

V rámci řešení Cíle 1 této práce byl realizován inkubační experiment s půdou velmi silně kontaminovanou rizikovými prvky, především Cd, Pb a Zn. Do této půdy byly jednotlivě aplikovány popel ze spalování dřeva a dřevěný biochar. Oba tyto materiály pocházely ze zpracování dřevěné štěpky a byly získány z průmyslových zdrojů. Oba materiály byly dávkovány tak, aby jejich dávka zvýšila hodnota pH směsi o 0,5, 1 a 1,5 jednotky. Nádoby se zeminou a směsmi byly inkubovány za řízených podmínek po dobu 60ti dnů.

5.1.1) Snížení mobility rizikových prvků aplikací dřevěného popela a biocharu

Ze získaných výsledků je patrné, že schopnost imobilizovat rizikové prvky se významně liší mezi popelem a biocharem. Aplikace obou materiálů sice vedla ke snížení rostlinám přístupných, popř. vyměnitelných forem rizikových prvků, avšak varianty, kde byl aplikován popel, vykázaly průkazně nižší koncentrace Cd, Pb a Zn oproti variantám s biocharem, a tudíž mnohem účinnější imobilizaci těchto prvků. Tato skutečnost je způsobena především rozdílnými mechanismy imobilizace u testovaných odpadních materiálů. Popel po spalování dřeva představuje směs anorganických sloučenin s nízkým obsahem organického uhlíku. Imobilizace se tudíž děje především dvěma způsoby, a to adsorpcí na povrch půdních částic a částic popela díky zvýšenému pH a potom srážením a tvorbou nerozpustných sraženin (Lee et al., 2004). Naproti tomu materiály s vysokým obsahem organického uhlíku, jako je biochar,

imobilizují rizikové prvky více způsoby. Tyto zahrnují komplexotvorné interakce s funkčními skupinami na povrchu biocharu, elektrostatické interakce, fyzikální sorpci, iontovou výměnu a srážení (Inyang et al., 2015). Uchimiya et al. (2010) uvádí, že za imobilizaci rizikových prvků v případě vysokoteplotního biocharu jsou více než iontová výměna zodpovědné děje jako koordinace π-elektrony dvojných vazeb mezi uhlíky biocharu a srážení. Vzhledem ke skutečnosti, že popel obsahuje mnohem více rozpustné minerální složky než biochar, lze předpokládat, že po jeho aplikaci se do půdního roztoku uvolní mnohem více solí, s kterými se rizikové prvky sráží. Mezi tyto soli patří především uhličitany, hydrogenuhličitany a fosforečnany (Voegelin et al., 2005; Cao et al., 2011). I v okamžiku, kdy bylo aplikováno přibližně dvakrát větší množství biocharu než popela po spalování dřeva, nedosahovala varianta s biocharem takové imobilizace, jako v případě popela. Dle našich výsledků lze tedy říci, že popel je efektivnější imobilizační agent než biochar.

5.1.2) Vliv dřevěného popela a biocharu na půdní respiraci a enzymatické aktivity

Účinky použitého dřevěného popela na půdní respirace byly průkazně negativní. Po aplikaci popela došlo ke snížení bazální i substrátem indukované půdní respirace, což může značit negativní vliv popela na půdní mikrobiální společenstva a celkovou mikrobiální biomasu v půdě. Aktivita dehydrogenázy v půdě po aplikaci popela značně poklesla. Vzhledem ke sníženým respiracím lze tedy usuzovat, že množství celkové mikrobiální biomasy v půdě se po aplikaci dřevěného popela snížilo. Podobně pak aplikace popela snížila aktivitu kyselé fosfatázy a vyšší aplikační dávka potom inhibovala i aktivitu ureázy a β-glukosidázy. Celkově tyto výsledky značí silný inhibiční efekt popela na půdní mikrobiální společenstva, jejich biomasu a narušení transformačních cyklů fosforu, dusíku a uhlíku. Podobně toxický vliv dřevěného popela ve své práci popisují autoři Noyce et al. (2017) a Perucci et al. (2006). Dle Perucci et al. (2006) je tento negativní vliv pouze krátkodobého charakteru a závisí na dávce popela a typu půdy. Krátkodobost negativního působení nelze z našich výsledků potvrdit, jelikož snížené aktivity kyselé fosfatázy, ureázy a bazální i substrátem indukované respirace byly prokazatelně nižší i po 60ti dnech od aplikace popela.

Podobně jako u aplikace popela, i v případě biocharu klesaly hodnoty půdních respirací, avšak aktivity dehydrogenázy, β-glukosidázy a hydrolýzy fluorescein diacetátu průkazně vzrostly. Enzymy dehydrogenázy jsou přítomné pouze v živých buňkách a zahrnují oxidační procesy během buněčné respirace, enzymy β-glukosidázy katalyzují poslední stupeň hydrolýzy

celulózy a hydrolýza fluorescein diacetátu pak značí celkovou mikrobiální aktivitu, jelikož je tato reakce katalyzovaná širokým spektrem enzymů. Celkově lze výsledky mikrobiálních aktivit interpretovat jako nárůst půdní mikrobiální biomasy. Skutečnost, že bazální i substrátem indukovaná respirace byly nižší lze tedy pravděpodobně připsat chemické sorpci CO_2 na částice biocharu. Díky tomu potom výsledky respirací mohou vykazovat nepravdivě nižší hodnoty, než reálně jsou (Ameloot et al., 2013; Thies et Rillig, 2009). Oba materiály se tedy odlišovaly v jejich účinku na půdní mikroorganismy. Popel měl jednoznačně negativní a inhibiční účinek, kdežto biochar naopak stimuloval jejich aktivitu.

5.1.3) Vliv popela po spalování dřeva a dřevěného biocharu na ekotoxicitu půdy

Pro stanovení vlivu testovaných materiálů na ekotoxicitu půdy bylo použito několik testů, konkrétně byl stanoven vliv na délku kořene hořčice seté (*Sinapis alba* L.), inhibici pohyblivosti hrotnatky velké (*Daphnia magna*) a změny v hmotnosti, míře přežití a množství kokonů žížaly hnojní (*Eisenia foetida*). Použitá půda byla písčito-hlinitá fluvizem silně kontaminovaná rizikovými prvky.

Aplikace obou materiálů v testech zvýšila průměrnou délku kořene hořčice, avšak statisticky průkazný rozdíl byl nalezen pouze v případě biocharu. Obdobně pak všechny varianty vykázaly nižší toxicitu v testech s hrotnatkou velkou oproti kontrole, avšak pouze nejvyšší dávka popela vedle ke statisticky průkaznému rozdílu a celkovému odbourání toxicity. Tuto skutečnost lze přisuzovat vysokému pH a velmi nízkým koncentracím Cd a Zn, na což je test s hrotnatkami velmi citlivý (Teodorovic et al., 2009). Aplikace popela vedla k průkazně vyššímu nárůstu hmotnosti žížal a statisticky vyššímu počtu kokonů. Podobný vliv měla i aplikace biocharu, kdy při nižší dávce byl pozitivně ovlivněn počet kokonů. Ovšem vyšší aplikační dávka vedla k úmrtí všech žížal v testu. Jelikož nižší dávka měla na žížaly pozitivní vliv, lze předpokládat, že důvodem úmrtí žížal nebyla přímá toxicita biocharu tak, jak popisují mnozí autoři (Liesch et al., 2010; Sanchez-Hernandez et al., 2019), ale spíše změny ve vodním režimu půdy (Li et al., 2011; Tammeorg et al., 2014). Z pohledu ekotoxicity půdy tedy popel vykazoval lepší vlastnosti oproti biocharu.

5.2) Vliv aplikace a úpravy popelů z biomasy na složení půdního roztoku a příjem živin rostlinami

V rámci řešení Cíle 2 této práce byly uskutečněny tři experimenty. Metodickou výzvu představovalo vzorkování a odběr půdního roztoku. Z předchozích zkušeností bylo známo, že vzorkovače vyráběné Gottfriedem Wieshammerem (Rakousko) nedosahují uspokojivých parametrů ve smyslu rychlosti a množství odebraného roztoku. Byly proto otestovány tzv. Rhizony dodávané firmou Rhizosphere Research Products (Nizozemsko). Tyto již byly schopné odebrat dostatečné množství půdního roztoku za relativně krátký časový úsek a byly tudíž použity v rámci celé této práce. Dále byly založeny dva nádobové pokusy, jeden s pšenicí jarní, kde byly použity popely po spalování dřeva a slámy, přičemž v druhé pokusu byl studován vliv peletizace dřevěného popela na růst a výživu jílku vytrvalého.

5.2.1) Odběr půdního roztoku

Je velmi dobře známo, že rostliny přijímají živiny téměř výhradně z půdního roztoku. Jeho složení má tedy přímý vliv na růst a výživu rostlin. V zásadě existují tři základní přístupy, jak získat půdní roztok. Jsou to centrifugace, mechanická extrakce a aplikace lyzimetrických zařízení (gravitační, vakuové) (Strobel, 2001). Nevýhodou centrifugace je skutečnost, že tato metoda je destrukční a vzorek je tedy během odběru zničen. Díky tomu také nelze odebírat půdní roztok z jednoho místa dlouhodobě. Výhodu naopak přináší centrifugační metoda v možnosti získat z půdy i silně vázanou vodu z mikropórů, jejíž doba zdržení v půdě je podstatně delší, než v případě gravitační nebo rostlinám dostupné vody. Extrakční metoda také neumožňuje nedestruktivní dlouhodobý odběr a během vzorkování jsou dále poškozeny buňky rostlin a mikroorganismů, jejich obsah je smíchán a extrahován s původním půdním roztokem. Výsledky tudíž mohou být velice odlišné od reálného stavu (Jones, 1998). Naproti tomu lyzimetrická zařízení umožňují dlouhodobý a nedestruktivní odběr z jednoho místa. Buňky půdních mikroorganismů ani rostlin nejsou při vzorkování porušeny a lze tudíž dosáhnout odběru vzorku nezatíženého chybou vzorkování. Nevýhodami u lyzimetrických zařízení jsou především množství odebraného vzorku, které bývá velmi často nedostatečné, dále pak velice proměnlivé prostorové rozlišení jednotlivých vzorkovačů, které se významně liší v závislosti na textuře půdy. Také platí, že prostorové rozlišení je nepřímo úměrné množství odebraného vzorku (Puschenreiter et al., 2005). V případě vakuových zařízení se pak jednotlivé konstrukce

vzorkovačů značně liší ve velikosti konečného podtlaku působícího na půdu v místě vzorkování a výsledky jsou tudíž obtížně srovnatelné mezi různými typy vzorkovačů.

Jak již bylo řečeno, námi testované Rhizony, jakožto typ vakuového lyzimetrického zařízení, byly schopné odebrat dostatečné množství (až 20 ml) roztoku během několika hodin při nasycení půdy na 60 % její maximální vodní retenční kapacity. Odebraný roztok je díky konstrukci Rhizonu již zfiltrován na velikost částic <0,16 μm, což dále díky dostatečnému množství vzorku umožňuje stanovení obsahu živin a rizikových prvků, ale také pH a organických i anorganických aniontů. V rámci testování bylo zjištěno, že pomocí Rhizonů lze s uspokojivou přesností stanovovat anorganické anionty (NO_3^-, SO_4^{2-}, a PO_4^{3-}), stejně jako di- a trikarboxylové anionty nízkomolekulárních organických kyselin (pyruvát, jablečnan, šťavelan, citrát). Další živiny a rizikové prvky byly testovány v předešlých pracích (Shotbolt, 2010; Seeberg-Elverfeldt et al., 2005; Knight et al., 1998; Argo et al., 1997). V našich testech nebyl nalezen statisticky průkazný rozdíl mezi ranním a večerním odběrem. Koncentrace testovaných aniontů se v půdním roztoku během vegetace pšenice měnily v horizontu týdnů, nikoliv dnů. I přes skutečnost, že rostliny měly statisticky průkazný rozdíl na koncentraci aniontů organických kyselin, při srovnání s centrifugační metodou nedokázaly Rhizony uspokojivě rozlišit mezi rhizosférní zeminou a zeminou bez rostlin. Test stability odebraných vzorků půdního roztoku dále prokázal, že vzorky jsou stabilní minimálně jednu hodinu po odebrání. Na základě těchto zjištění lze půdní roztok odebraný pomocí Rhizonů s výhodou použít pro sledování rostlinám přístupných koncentrací živin a rizikových prvků, pH a EC.

5.2.2) Rozdíl mezi dřevěným a slámovým popelem v jejich účinku na půdní roztok a růst pšenice jarní

V práci byly testovány dva popely získané z průmyslových provozů. Jednalo se o popel ze spalování dřevní štěpky a popel po spalování slámy a sena. Celkové obsahy živin se mezi těmito popely významně lišily. Dřevěný popel obsahoval dvakrát větší množství Ca a Mg oproti slámovému, který naopak vykázal více než dvakrát vyšší obsah K. Celkový obsah P se v popelech významně nelišil. Tyto rozdíly jsou ve velmi dobré shodě s literaturou a demonstrují standartní složení popelů z biomasy (Vassilev et al., 2013b; Tlustoš a kol., 2012). Při aplikaci 1 hm.% popelů do půdy se však jejich účinek značně lišil a absolutně nereflektoval celkové obsahy prvků v popelech. Koncentrace Ca a Mg byly výrazně vyšší ve variantách se slámovým popelem i přesto, že tento popel oproti dřevěnému obsahoval těchto živin poloviční množství.

Koncentrace K v půdním roztoku také nereflektovaly rozdíly v celkovém obsahu a koncentrace K v půdním roztoku byly více než desetinásobné ve variantách se slámovým popelem oproti dřevěnému. Vysoké koncentrace K v půdním roztoku pravděpodobně vedly k vytěsnění Ca a Mg z půdního sorpčního komplexu do půdního roztoku. I přes stejný obsah P v obou popelech dřevěný popel takřka nezvýšil koncentraci P v půdním roztoku, avšak slámový popel zvýšil koncentrace P desetinásobně. Účinek na odběr živin pšenicí se také lišil. Slámový popel i přes zvýšené koncentrace Ca a Mg v půdním roztoku zvyšoval odběr pouze K a P pravděpodobně kvůli antagonistickému vztahu mezi K a Ca a Mg. Dřevěný popel na jedné půdě neměl žádný vliv na odběr živin, avšak na půdě s vyšším množstvím minerálního N aplikace dřevěného popela zvýšila odběr Ca, K, Mg i P.

Rozdíly v prvkovém složení popelů jsou jasně způsobeny rozdílným složením vstupní biomasy. Na druhou stranu rozpustnost jednotlivých živin v popelech je přímo určena minerální formou, v kterých je daná živina v popelu přítomná. Je známo, že minerální formy jsou určeny jednak složením, ale především teplotou spalování. Tu lze jen obtížně u průmyslových provozů zjistit, jelikož v naprosté většině případů je teplota měřena až na výstupu spalin, ale ne přímo v kotli. Z výsledků rentgenové difrakční analýzy však lze teplotu spalování odhadnout. Ve slámovém popelu byly nalezen minerál albit ($NaAlSi_3O_8$), který vzniká při teplotách nad 575 °C (Vassilev et al., 2013a) a sylvín (KCl), který je stabilní pouze do teplot 700 °C (Wang et al., 2016; Zevenhoven et al., 2012). Dřevěný popel naproti tomu obsahoval minerál anortit ($CaAl_2Si_2O_8$), který se tvoří při teplotách 800 – 1100 °C (Vassilev et al., 2013a). Celkově lze říci, že slámový popel díky nižším teplotám spalování obsahoval mnohem více rozpustné minerály, díky čemuž byl jeho přímý hnojivý účinek významně vyšší oproti popelu dřevěnému. Díky obsahu sylvínu (KCl) a arkanitu (K_2SO_4) lze slámový popel považovat za dobře rozpustný zdroj draslíku.

5.2.3) Úprava popela peletizací a modifikace jeho složení pro lepší hnojivý potenciál

Polní aplikace popela v reálných podmínkách přináší několik omezení. Prvním je velmi jemná struktura popela, která způsobuje vysokou prašnost a nízkou homogenitu rozptylu. Dalším nedostatkem popela je jeho nevyrovnaný obsah hlavních živin. Během spalování dochází k oxidaci S, která pak z popela odchází se spalinami a popel tak obsahuje nedostatečné množství S ve srovnání s ostatními živinami. V případě dřevěného popela pak díky vysokým spalovacím teplotám dochází v popelu k transformaci sloučenin P do nerozpustných forem a

hnojivý účinek je tudíž značně omezen. Proto byl v rámci této práce otestován vliv peletizace dřevěného popela. Během peletizace pak byl do popela přidán jednoduchý superfosfát a elektrárenský sádrovec z odsíření spalin.

Samotná peletizace popela překvapivě neovlivnila při aplikaci 2 hm.% jeho hnojivý účinek a obě varianty dosáhly průkazně vyššího výnosu nadzemní biomasy jílku oproti kontrole bez popela. To by teoreticky mohlo být způsobeno vysokou dávkou popela, avšak obdobně Ingerslev et al. (2014) nenalezli rozdíl mezi peletovaným a nepeletovaným popelem v chemickém účinku na půdy plantáží smrku ani po 2,5 letech od aplikace. Z toho lze usuzovat, že peletizací popel v zásadě neztrácí svůj hnojivý potenciál. Nejvyššího výnosu biomasy dosáhla v pokusu varianta popela obohacená o jednoduchý superfosfát a sádrovec. Tato varianta dále dosáhla nejvyššího odběru P nadzemní biomasou a množství rostlinám přístupného fosforu v půdě po sklizni bylo statisticky průkazně nejvyšší v rámci celého pokusu. Při srovnání s variantou, kde byl aplikován pouze popel obohacený o superfosfát je patrné, že přídavek sádrovce podpořil jednak přístupnost P v půdě, ale i jeho příjem rostlinami. Mnozí autoři uvádí, že kombinace P se sádrovcem vede díky vysokému obsahu Ca v sádrovci ke srážení P a jeho omezené dostupnosti rostlinám (Clark et al., 2001; Murphy et Stevens, 2010; Silva et al., 2013). V našem pokusu byl však popel se sádrovcem aplikován do kyselé půdy (pH_{CaCl2} = 5) a jelikož rozpustnost Ca-P minerálů klesá se stoupajícím pH (Lindsay, 1979), lze předpokládat, že srážecí reakce nebyly tak intenzivní.

5.3) Možnosti zpřístupnění fosforu z odpadních materiálů pomocí aplikace mikroorganismů

Velkým nedostatkem většiny odpadních materiálů je nízká přístupnost jejich živin v porovnání s minerálními hnojivy. Díky tomu má aplikace těchto materiálů pouze minimální přímý vliv na výnos rostlinné produkce, což odrazuje od jejich použití ke hnojení. Jednou z možností, jak zvýšit přístupnost živin z odpadních materiálů je použití mikroorganismů. V rámci této práce byly experimenty zaměřeny především na zpřístupnění P jakožto strategické živiny pro evropské zemědělství. V rámci pokusů byly použity komerčně dostupné i nově izolované mikroorganismy se schopností rozpouštět $Ca_3(PO_4)_3$ in vitro (testováno producenty, nepublikováno). Mikroorganismy byly v experimentech testovány se širokým spektrem odpadních materiálů, avšak pozitivních výsledků bylo dosaženo pouze s popelem po spalování dřeva, a to ve zlomku testovaných případů.

5.3.1) Délka účinku aplikace mikrobiálních inokulantů

V prvním roce testování byly mikroorganismy aplikovány při setí a rostliny byly sklízeny po osmi týdnech růstu, popř. v období plné zralosti. Zkoušky proběhly s kukuřicí a pšenicí. Testované mikroorganismy zahrnovaly *Pseudomonas* sp. DSMZ 13134, *Bacillus amyloliquefaciens* FZB42, *Penicillium* sp. (Biological fertiliser DC, Bayer CropScience Biologics GmbH, Německo) a směsnou kulturu Bacto_prof obsahující *Trichodermu harzianum* a pět kmenů rodu *Bacillus* (Terra bioscience, Německo). Zkoušky proběhly se širokým spektrem, odpadních materiálů převážně anorganického charakteru. Konkrétně byly testovány čistírenské kaly, popely po spalování čistírenských kalů, Thomasova moučka, popel po spalování dřeva, popel ze spalování obilné slámy a kompost z čistírenského kalu a odpadu z údržby parků a zeleně. Na konci experimentů však nebyl zaznamenán ani jeden statisticky průkazný vliv mikrobiálního inokulantu na výnos biomasy nebo odběr P rostlinami. V části pokusu byl stanoven obsah vodorozpustného P v rhizosféře rostlin po 27mi dnech růstu, avšak ani v tomto případě se nepotvrdila schopnost testovaných mikroorganismů zvýšit uvolňování P do půdního roztoku. Obdobných výsledků dosáhli Thonar et al. (2017) v nádobovém pokusu s kukuřicí. V jejich pokusu nebyly nalezeny žádné průkazné rozdíly ve výnosu biomasy nebo odběru P rostlinami kukuřice sklizené po 15ti týdnech růstu. Autoři popisují pouze jeden statisticky průkazný vliv na výnos biomasy s použitím anorganického odpadního materiálu, a to ve variantě sklizené po 6,5 týdnech růstu. V tomto případě však navíc vyšší výnos biomasy pravděpodobně nebyl způsoben zlepšením příjmu P, avšak vyšším příjmem N. Naše nepublikované výsledky analýzy půdního roztoku ukázaly, že po aplikaci směsné kultury Bacto_prof bylo možné detekovat změny v obsahu nízkomolekulárních organických kyselin v půdním roztoku pouze do pátého týdne růstu. Dle Mosimann et al. (2017) se kmen *Pseudomonas* sp. DSMZ 13134 stal v rhizosféře nedetekovatelným mezi 4. a 8. týdnem růstu kukuřice. Gómez- Muñoz et al. (2017) dále popisují, že *Penicillium bilaiae* mělo pozitivní efekt na délku kořenů během prvních několika dní růstu kukuřice, avšak tento efekt zmizel a 27. den již nebyl detekován. Autoři dále popisují, že *P. bilaiae* nebylo na konci experimentu (27. den) nalezeno v rhizosféře, avšak zůstalo pouze v místě inokulace. Uvedená zjištění naznačují, že aplikované nepůvodní mikroorganismy mají v rhizosféře pouze krátkodobý vliv. To je způsobeno mnoha faktory, mezi které patří i krátká životnost inokulantů a jejich nízká mobilita v půdním potažmo rhizosférním prostředí.

5.3.2) *Paenibacillus mucilaginosus* ABi13 jako potenciální nástroj pro omezení vyplavování živin ze zemědělských půd

Bakterie *Paenibacillus mucilaginosus* ABi13 (MUCI) byla izolována z rhizosféry pšenice v podhůří Alp. Izolovaný kmen má schopnost rozpouštět $Ca_3(PO_4)_2$ in vitro, produkuje kyselinu indol-3-octovou a jelikož jsou bakterie *P. mucilaginosus* známy svou schopností narušovat stabilní matrice silikátů (Xiao et al., 2016; Hu et al., 2006), byl tento kmen testován na schopnost uvolnění živin z popela po spalování dřeva. Pro experiment byla použita kyselá půda s velmi nízkým obsahem přístupného P. Testovanou rostlinou byla v tomto případě kukuřice, která byla sklizena 28 dní po vyklíčení.

Aplikace MUCI na samostatnou půdu nevedla ke změnám v koncentraci P v půdním roztoku, ani k pozitivnímu ovlivnění odběru P biomasou kukuřice. Tento vliv byl očekáván, jelikož na kyselých půdách je P především vázán s ionty Fe a Al (Hinsinger, 2001). Když však byl MUCI aplikován do varianty s přídavkem popela, byla detekována zvýšená koncentrace rostlinám přístupného P 14 dní po vyklíčení, avšak pouze v části bez rostlin. Tento jev se při následujícím odběru (28. den) již neprojevil a koncentrace P v půdním roztoku bez rostlin se nelišila od varianty bez MUCI. To jasně potvrzuje již zmíněný krátkodobý vliv mikrobiálních inokulantů. Aplikace MUCI měla však zásadní vliv na koncentrace NO_3^-, K^+, Mg^{2+} a Ca^{2+} v půdním roztoku. Koncentrace všech těchto živin v půdním roztoku byly statisticky významně nižší po aplikaci MUCI ve srovnání s kontrolními variantami. Došlo tak k průkaznému snížení odběru těchto živin a díky nedostatku N MUCI způsobil nižší výnos biomasy. Horší zásobenost rostlin N dále vedla ke snížené kořenové exsudaci organických kyselin (Neumann et Römheld, 2001), díky čemuž následně poklesla i koncentrace P v půdním roztoku. Celkový odběr P rostlinami nebyl aplikací MUCI ovlivněn. V rámci této práce nebylo možné obsáhnout vysvětlení mechanismu imobilizace živin, však je nutné podotknout, že MUCI vykazoval stejný vliv na ionty v půdním roztoku ve čtyřech dalších experimentech a třech dalších různých půdách. Tang et al. (2014) popisuje zvýšenou produkci polysacharidů u *P. mucilaginosus* v kontaktu s ionty Ca^{2+}, Mg^{2+} a Fe^{3+} a Sobeck et Higgins (2002) uvádí tzv. „divalent cation bridging“ jako hlavní mechanismus bioflokulace polysacharidů. Imobilizace živin z půdního roztoku polysacharidy se tudíž zdá být pravděpodobná a po bližším objasnění mechanismu působení by *P. mucilaginosus* ABi13 mohl být teoreticky použit pro redukci vyplavování především NO_3^- do podzemních a povrchových vod.

5.3.3) Mechanismus zlepšení hnojivého účinku popela u houbových inokulantů

Ze všech realizovaných experimentů bylo pouze jedenkrát dosaženo úspěšné kombinace odpadního materiálu a mikrobiálních inokulantů ve smyslu zvýšení produkce biomasy rostlin. Pozitivní vliv měly houbové preparáty *Penicillium* sp. PK112 a *Trichoderma harzianum* OMG08 v kombinaci s dřevěným popelem aplikovaným na kyselou půdu s velmi nízkým obsahem rostlinám přístupného P ($P_{MehlichIII}$ = 22 mg kg^{-1}), který byl pro růst kukuřice limitující živinou. Rostliny byly sklizeny a analyzovány 45. den od vyklíčení.

Oba dva inokulanty, podobně jako *P. mucilaginosus* ABi13, nebyly schopné mobilizovat P ze samotné neošetřené zeminy, avšak když byl do zeminy aplikován popel, zvýšily oba inokulanty obsah rostlinám přístupného P v půdě. Díky tomu se zvýšil příjem P rostlinami a následně vzrostl výnos biomasy. Inokulanty shodně v půdě snížily obsah vodorozpustného organického C a aktivitu dehydrogenázy. To značí silnou inhibici autochtonních mikroorganismů v půdě, která byla dále potvrzena sníženou aktivitou alkalické fosfatázy jakožto půdního enzymu produkovaného půdními mikroorganismy, nikoliv však vyššími rostlinami (Spohn et Kuzyakov, 2013). Tato inhibice mohla být způsobena produkcí sekundárních metabolitů (Cunningham et Kuiack, 1992; Yang et al., 2011), nebo kompeticí o dostupné zdroje C, jak tomu napovídá snížení vodorozpustného organického C. Celkově inhibice autochtonních mikroorganismů vedla k průkazně nižšímu obsahu mikrobiálního P. Inokulanty dále snížily pH v rhizosféře. Tím lze vysvětlit jejich účinnost ve variantách s popelem, jelikož tento popel obsahoval většinu mobilizovatelného P ve formách rozpustných v kyselém prostředí. Celkovou bilancí P odebraného rostlinami však bylo zjištěno, že ani samotná acidifikace rhizosféry, ani samotná mobilizace mikrobiálního P nemohla pokrýt celkové navýšení odebraného P rostlinami. Pravděpodobně tedy byl vyšší odběr P a vyšší výnos kukuřice způsoben oběma mechanismy současně.

6) Závěr

Tématem této disertační práce je využití odpadních materiálů ve výživě rostlin, potažmo v zemědělství. Díky rostoucí lidské populaci stoupá tlak na produktivitu a udržitelnost zemědělské produkce. Udržitelnost zemědělství pak závisí na recyklaci živin, kterou lze realizovat právě aplikací odpadních materiálů na zemědělskou půdu. Tato práce je souborem osmi vědeckých článků, které jsou rozděleny do tří hlavních okruhů. V první části se práce zabývá vlivem průmyslově produkovaného popela a biocharu ze zpracování dřevní štěpky na chemické a biologické změny půdních vlastností. Druhá část práce se věnuje uvolnitelnosti živin z popelů po spalování dřeva a slámy a jejich hnojivým účinkům. Poslední okruh předkládané práce je pak zaměřen na snahu zlepšit hnojivý účinek popela ze spalování dřeva pomocí jeho společné aplikace s mikrobiálními inokulanty.

Ze srovnání chování a vlastností popela a biocharu produkovaných z dřevní biomasy je patrné, že efektivnější schopnost imobilizovat rizikové prvky má popel. Aplikace popela má, na rozdíl od biocharu, silně negativní vliv na půdní mikroorganismy, avšak tento negativní účinek může být utlumen přídavkem humínových kyselin. Popel ze spalování dřeva dále vykazoval mnohem nižší přístupnost živin, než popel ze spalování slámy a jeho hnojivý potenciál fosforem byl zanedbatelný. Jako klíčový faktor rozdílné přístupnosti živin byla identifikována teplota spalování. Slámový popel lze považovat za vynikající zdroj draslíku, přičemž dřevěný popel se jevil spíše jako výhodná náhrada vápenatých hnojiv. Aplikace širokého spektra mikrobiálních inokulantů nevedla k výraznějšímu zlepšení přístupnosti živin z odpadních materiálů. Aplikace *Paenibacillus mucilaginosus* ABi13 měla průkazně negativní vliv na růst rostlin díky imobilizaci půdního dusíku. Houbové preparáty *Penicillium* sp. PK112 a *Trichoderma harzianum* OMG08 byly úspěšně zkombinovány s aplikací popela a průkazně zvýšily výnos kukuřice díky okyselení rhizosféry a mobilizaci mikrobiálního P, ovšem pouze v ranných stádiích růstu a podmínkách nádobového pokusu.

Díky získaným výsledkům lze konstatovat, že aplikací odpadních materiálů lze zvýšit půdní úrodnost a zlepšit půdní vlastnosti. Ovšem vliv odpadních materiálů na půdu se významně liší v závislosti na původu odpadního materiálu, způsobu jeho zpracování a v neposlední řadě na aplikované dávce. Nevhodná aplikace odpadního materiálu může v krajním případě působit na půdní ekosystém značně negativně, jak bylo v rámci této práce ukázáno na příkladu úmrtí žížal po aplikaci vyšší dávky biocharu. Stejně tak se liší hnojivý

účinek jednotlivých odpadních materiálů a tento lze jen obtížně odhadnout z celkových obsahů živin v materiálech. Na příkladu popelů po spalování biomasy lze dobře demonstrovat např. zásadní vliv teploty spalování na přístupnost živin z těchto materiálů. To vede ke skutečnosti, že v zásadě velmi podobné materiály s velmi podobným celkovým složením se mohou v půdě chovat naprosto odlišně a stejně se tak může lišit jejich účinek na rostlinu. Možnosti zpřístupnění P z odpadních materiálů minerálního charakteru pomocí aplikace vybraných mikroorganismů se ukázaly jako značně omezené a z dostupných výsledků je zatím nelze doporučit pro praxi. Celková komplexnost a nevyváženost složení a vlastností odpadních materiálů v porovnání s běžnými minerálními hnojivy klade mnohem vyšší nároky na analýzu jednotlivých materiálů a nabízí rozsáhlé možnosti kombinací různých materiálů za účelem vývoje inovativních hnojiv na bázi odpadů. Toto však vyžaduje další výzkum a inovativní řešení v rámci budoucí zemědělské praxe.

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