Degradation of polycyclic aromatic hydrocarbons in a soil co-contaminated with lead

Can red clover and soil amendments help?

Master Thesis

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Abstract

Plants and microbes can be used for sustainable soil remediation. Knowledge about the interactions between plants and microorganisms during degradation of organic pollutants in soils co-contaminated with metals is still limited. In this study the microbial degradation of polycyclic aromatic hydrocarbons (PAHs) in a shooting range soil was expected to be enhanced by the addition of soil amendments that should reduce toxicity of lead (Pb). Furthermore, degradation may be stimulated in the rhizosphere of clover due to root exudates and nitrogen (N) provided by its rhizobia symbionts.

A rhizotest was conducted comparing PAH degradation within 10 days in control and amended soils, as well as planted and unplanted treatments. To reveal microbial processes, stable isotope probing using a ^{13}C-labelled PAH (phenanthrene, PHE) was combined with analysis of microbial phospholipid fatty acids (PLFAs). The addition of 3% amendments (biochar + gravel sludge + iron oxides) could increase pH, but Pb bioavailability assessed by DGT and $CaCl_2$ extraction was not effectively reduced. However, plants grown on soils with amendments accumulated less Pb in roots, probably due to more pronounced exudation of organic compounds that could complex Pb. Despite the presence of nodules it remained unclear if rhizobia could fix N and support microbial degradation. Nevertheless, incorporation of ^{13}C into microbial PLFAs was higher in rhizosphere soils, due to the higher fungal biomass in the rhizosphere and because root exudates likely served as a source for carbon, energy and co-metabolism. Fungi and actinomycetes were primary PHE degraders, whereas Gram-negative bacteria started ^{13}C incorporation later indicating cross-feeding on metabolites.

This experiment revealed insights in interactions within the PAHs degrading microbial consortium at short time scale, demonstrated the rhizosphere effect and hence suggests phytoremediation as a promising approach for remediation of mixed-contaminated sites.

Kurzfassung

Phytoremediation ist eine nachhaltige, kostengünstige Variante zur Bodensanierung. Das Wissen über die Wechselwirkungen von Pflanzen und Mikroorganismen beim Abbau von organischen Schadstoffen in Böden kontaminiert mit Schwermetallen ist jedoch begrenzt. In dieser Arbeit wurde in einem Rhizotest ermittelt, ob der mikrobielle Abbau von polyzyklischen aromatischen Kohlenwasserstoffen (PAKs) in einem mit Blei (Pb) kontaminierten Boden durch Bodenzuschlagsstoffe (A) stimuliert werden kann, die die Toxizität von Blei reduzieren. Außerdem könnte der Abbau in der Rhizosphäre von Rotklee durch Wurzelexudate und Stickstoff aus der Symbiose mit Knöllchenbakterien weiter angeregt werden. Effekte von A und Bepflanzung auf den PAK-Abbau wurden durch die Zugabe eines markierten PAKs (^{13}C-Phenanthren) und Phospholipid-Analysen der Mikroorganismen (PLFAs) untersucht.

Die Zugabe von 3% A (Biokohle, Kiesschlamm, Eisenerz) bewirkte einen Anstieg des pH-Wertes, aber keine dauerhafte Blei-Immobilisierung ($CaCl_2$-Extrakt, DGT). Dennoch akkumulierten Kleewurzeln auf Böden mit A weniger Pb. Möglicherweise war die Ausscheidung von Wurzelexudaten, die mit Blei weniger verfügbare Komplexe bilden, ausgeprägter. Zwar hatten sich Wurzelknöllchen gebildet, aber es blieb unklar, ob die Stickstofffixierung effektiv war oder durch die Toxizität gehemmt wurde. Jedenfalls war der Einbau von ^{13}C des markierten Phenanthren in PLFAs höher in bepflanzten Behandlungen. Gründe dafür waren die höhere Biomasse von Pilzen in der Rhizosphäre, Wurzelausscheidungen, die als Kohlenstoff- und Energiequelle für den PAK-Abbau sowie für Ko-metabolismus dienten. Zu Beginn des Experiments dominierten Pilze und Actinomyceten den Abbau, später folgten Gram-negative Bakterien, die möglicherweise Phenanthren-Metabolite nutzten.

Das Experiment ermöglichte Einblicke in den komplexen PAK-Abbau und demonstrierte den Rhizosphären-Effekt in einem gemischt-kontaminierten Boden.

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Abbreviations

BNF	Biological nitrogen fixation
C, +A	Soil treatments: control, soil with amendments
$CaCl_2$	0.0125 M calcium chloride-extractable element concentration
CEC	Cation exchange capacity
DGT	Diffusive gradients in thin films, an assembly to assess bioavailable element concentrations in soil based on diffusion
DOC	Dissolved organic carbon
GC/c/IRMS	Gas chromatography-combustion-isotopic ratio mass spectrometry
GC-FID	Gas chromatography with flame ionisation detector
HM	Heavy metals
ICP-MS	Inductively coupled plasma mass spectrometry
K, P	Potassium, phosphorous
N	Nitrogen
NH_4NO_3	0.1 M ammonium nitrate-extractable element concentration
NO_3^-	Nitrate
PAHs	Polycyclic aromatic hydrocarbons
Pb	Lead
PHE	Phenanthrene
PLFA	Phospholipid fatty acids that form microbial membranes and are specific for certain taxonomic groups (biomarkers)
T0, T3, T6, T10	Harvest after 0, 3, 6 and 10 days
WHC	Water holding capacity (%)
$\delta^{13}C$	Delta carbon 13 value; isotopic mass ratio of carbon 13 to carbon 12 ($^{13}C/^{12}C$) relative to the international standard Vienna Pee Dee Belemnite (VPDB)

1. Introduction

One of the eight major threats to soil is contamination. In Europe 340.000 contaminated sites have been identified and 2.5 million sites are potentially contaminated. Management of these sites was estimated to cost 6 billion Euros per year (Panagos et al., 2013). The most important causes for soil pollution were inappropriate industrial and municipal waste-treatment as well as the commercial and industrial sector. Main contaminants are petroleum based hydrocarbon compounds, organic solvents, halogenated organics (e.g. pesticides), heavy metals and metalloids (Chirakkara et al., 2016). The effects on the environment and human health depend on the contaminants, their properties, concentrations and behaviour in soil, the potential for dispersion, bioavailability and environmental conditions (Panagos et al., 2013).

Conventional remediation approaches like soil washing, solidification, dig and dump often require considerable amounts of chemicals and energy and cause external effects such as hazardous waste and CO_2 emissions. In economic terms conventional clean up approaches can cause substantial costs of several hundred thousand dollars per hectare (Witters et al. 2011). Additionally, these activities are often invasive and destroy soil functions and biological activity. But over the past decades various gentle remediation strategies have been studied intensively and are considered to be cost-effective and more sustainable as energy, water and material use can be minimized. Risks to human health and environment can be alleviated while soil functions, land and ecosystems can be preserved (Witters et al. 2011). The gentle remediation approaches include use of living organisms such as plants and associated microorganisms to restore contaminated sites and are then termed bio-remediation (Haritash and Kaushik, 2009). Whereas organic compounds can be degraded to less hazardous substances, heavy metals cannot undergo biodegradation and potentially accumulate in soils and stay there for a long time (Chirakkara et al., 2016). Often sites are contaminated with both organic as well as inorganic pollutants (Gerhardt et al., 2009). These sites are complex to remediate, as for example the degradation of organic compounds can be inhibited by metal(loid) toxicity (Sneath et al., 2013).

One remediation approach aims to stabilise heavy metals and metalloids by the use of soil amendments that can immobilise the contaminant and hence avoid translocation to other ecosystems and availability to organisms (Kumpiene et al., 2008). Also plants possess the ability to reduce mobility of heavy metals and metalloids, which is also termed phytostabilisation (Wenzel, 2009). The combined use of plant roots and associated microbes for the transformation of organic contaminants to less hazardous substances is called rhizodegradation (El Amrani et al., 2015). The processes behind plant-based remediation are often not well understood and need to be optimised for effective clean-up, but studies investigating the complex interactions between plants and associated microorganisms for bioremediation are still relatively rare (El Amrani et al., 2015). Furthermore, while numerous studies focused on the remediation of either only one pollutant

group or one remediation strategy, knowledge of a combination of the named remediation approaches is still limited (Chirakkara et al., 2016).

The present master thesis focused on the remediation of a shooting range soil co-contaminated with lead (Pb) and polycyclic aromatic hydrocarbons (PAHs). The aim was to investigate the complex interactions of Pb, soil amendments, nitrogen fixing plants (red clover) and degrader microorganisms, as knowledge of such a multifaceted system is still limited. The effects on PAH degradation were revealed by conducting a plant-based biotest (rhizotest) and microbial analysis combined with stable isotope probing. This experiment was a simplified approach that aimed to reveal short time dynamics and temporal changes in the rhizosphere in order to gain better knowledge about the complex interactions during PAH degradation in the rhizosphere.

The first section of the thesis provides an overview about the contaminants, phytoremediation and selected soil amendments. Then a short presentation of the framework project follows. In the next chapter the hypotheses and research questions were formulated. The following chapter contains material and methods and details of the experiment. Finally, the results will be presented and discussed.

2. Literature overview

In the following chapters the issue of mixed-contaminated sites, lead and polycyclic aromatic hydrocarbons will be treated, and then an overview of remediation strategies and the mechanisms of selected soil amendments used for metal stabilisation will be provided.

2.1. Soil pollution and remediation of mixed contaminated sites

Most heavy metals are omnipresent in soil in small amounts. Some of them are necessary for many biological processes, e.g. zinc and copper. But if critical concentrations are exceeded, they become of environmental and public concern due to toxicity (Kabata-Pendias, 2011). In contrast to inorganic contaminants, most of the organic pollutants in soil can be degraded. However, they have become of environmental concern as the production of synthetic organic chemicals has increased and large quantities have been released in the last few decades. Moreover, most of the organic contaminants can cause toxicity to organisms even at low concentrations, because the accumulation in tissues can lead to toxic levels (Chirakkara et al., 2016).

Remediation of soil contaminants is complex. The applicability of remediation approaches depends on the type of contaminant as well as of site-specific conditions like soil type, depth of the contamination, proximity to the groundwater, costs and the end use of the site. Remediation solutions for one site and contaminant cannot be just extrapolated to other sites or contaminants. As even the remediation of only one class of contaminants can be challenging, the remediation of sites polluted with several contaminants is even more complex. Unfortunately, hazardous sites co-contaminated with both inorganic and organic pollutants are very abundant. Some examples are sites irrigated with waste water or fertilised with sludge, industrial sites like asphalt production, landfills or sites where industrial waste like oil, chemicals were discharged, bus depots and shipyards or along roads (Chirakkara et al., 2016; Johnsen et al., 2002; Palmroth et al., 2007). As the contaminants have different physico-chemical properties, they will respond differently to remediation technologies. Moreover, interactions between the contaminants can limit the efficiency of the remediation. For example the efficiency of solidification/stabilisation or soil washing for immobilising or removing metals can be impaired by the presence of organic compounds that either act as surfactants or have a high viscosity. Furthermore, the toxicity arising from heavy metals can hamper the degradation of organic contaminants or inhibit plant growth, biomass production, metal uptake during phytoremediation (Chirakkara et al., 2016).

Shooting ranges are one example for mixed-contaminated sites. Worldwide there are over 100,000 shooting ranges and annually millions of kg Pb are deposited into the soil (Rodríguez-Seijo et al., 2016). Ammunition usually consist of 90-97% Pb, the other 3-10% are arsenic, antimony, cadmium, nickel, copper, zink (Rodríguez-Seijo et al., 2016). Nowadays, also Pb-free ammunition is available but Pb shots are still preferred because of the ballistic efficiency, malleability and high specific gravity (Rodríguez-Seijo et al., 2016).

On shooting ranges Pb concentrations in soil are very heterogeneous and can be up to 150,000 mg kg^{-1} in the backstop of the shooting galleries (Rodríguez-Seijo et al., 2016). Pb bullets in soil are subject to weathering and can be transformed from metallic Pb into dissolved and particulate species and hence pose a risk to the environment (Cao et al., 2003). Furthermore, on shooting ranges also organic contaminants like PAHs can be of environmental concern. Clay targets are mainly composed of limestone (70%) and an organic binding material (30%) like pitch, tar or bitumen. This binding material contains a mix of several PAHs, such as phenantrene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(a)pyrene, benzo(b)fluoranthene. Clay targets can have PAHs contents in the range of 3,000 to 40,000 mg kg^{-1} (Lobb, 2006). As the PAHs are strongly bound within the targets, they present little risk to organisms, but following degradation of targets in soil, PAHs might become mobile. As the PAH concentrations in soil can be far above toxicity thresholds proposed by the authorities this mobilisation can be of environmental concern (Lobb, 2006).

2.2. Phytoremediation

Bioremediation is a technology to remediate contaminated soils by using living organisms to eliminate, transform or attenuate pollutants biologically (Wenzel, 2009). The utilisation of higher plants for these purposes is defined as phytoremediation. Phytoremediation processes mainly take place in the rhizosphere, i.e. the soil around plant roots that is affected by root activities (Hinsinger et al., 2006). As there can be more than 400 million kilometres of roots per hectare, phytoremediation can have a great potential (Gerhardt et al., 2009). The term phytoremediation can be refined by distinguishing four major remediation processes:

- Phytostabilisation or – immobilisation aims to prevent pollutant transfer to the food chain or other ecosystem compartments. Often plants and soil amendments are used in combination to stabilise contaminated sites physically and chemically to avoid pollutant transfer by wind or water erosion, leaching or uptake into organisms and thus alleviate the risk arising from the contaminant (Chirakkara et al., 2016; Wenzel, 2009).
- Phytoextraction takes advantage of some plants that possess the ability to (hyper-) accumulate pollutants in their above-ground biomass (Wenzel, 2009). Contrary to phytostabilisation, pollutants are removed from soil by subsequent harvest of these plants. The biomass could be further used for the generation of energy (Witters et al., 2012) or as a raw material for industrial processes (e.g. phytomining) (Sheoran et al., 2009).
- Phytovolatilisation describes the removal of soil pollutants through transformation by the plant metabolism to volatile compounds that are released into the atmosphere (Wenzel, 2009).

- Phytodegradation or Rhizodegradation is a process that uses the metabolic capabilities of plants and associated microorganisms to degrade organic contaminants to less toxic compounds or to mineralise them totally to H_2O and CO_2 (Chirakkara et al., 2016). The microbes either degrade the pollutant or support the plant growth under stress conditions.

The advantages of the use of plants in remediation are the establishment of a vegetation cover that is aesthetically appealing, the preservation soil functions and the applicability for remediation of large sites with a low budget. However, the potential remobilisation of accumulated metal(loids) during degradation of dead roots should be considered, as well as the transfer into the food chain due to browsing by animals (Martínez-Alcalá et al., 2012). Phytoremediation has its limitations (Gerhardt et al., 2009): a lot of effort has been done to identify suitable plants that can tolerate the pollutants, nutrient poor and extreme pH conditions but some sites may be too toxic for plant growth. So the step from experiments in the laboratory and greenhouse to the field has proven challenging, as temperature, nutrient status, precipitation, drought, pathogens, herbivory by insects and animals and competition by better adapted species are often not encountered in laboratory and greenhouse trials. Furthermore, phytoremediation is limited to the depth of root growth, which is around 50 cm in the case of herbaceous plants and can be up to 3 m when trees are used. However, the longer time required for establishment and appropriate remediation compared to conventional approaches like soil excavation is a major drawback (Gerhardt et al., 2009).

2.3. Legumes and rhizobia – A symbiosis for nitrogen fixation and phytoremediation

Nitrogen (N) is necessary for the synthesis of enzymes, nucleic acids, proteins and chlorophyll and often N is the limiting macronutrient for plant growth (Gopalakrishnan et al., 2015). Although the atmosphere consists of 78% dinitrogen (N_2) and N is present in various organic forms, plants can mainly use N in its mineral forms, nitrate (NO_3^-) and ammonium (NH_4^+) (Angus et al., 2013). Legumes are known for their symbiosis with nitrogen (N) fixing nodule bacteria that can fix up to 600 kg N per hectare and year (Fuchs, 2007).

These nodule bacteria belong to genera like *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Azorhizobium*. They are Gram-negative bacteria and strictly aerobic. In legume roots they convert atmospheric N_2 to plant available ammonia (NH_3) or ammonium (NH_4^+) for the exchange of carbon and energy. The legume roots attract free living rhizobia with signal compounds (plant- and bacteria-specific flavonoids) and the bacteria move towards the roots of the host via chemotaxis. On the root hair the bacteria produce nod-factors that cause a curling of the root hair, an infection thread is built and the bacteria move further in the cell of the root hair and cause division of root cortex cells – a nodule forms. The bacteria differentiate to bacteroids that are able to fix N. The nodules can be red due to leghaemoglobin that is formed by the legume and reacts with oxygen in order to protect the enzyme nitrogenase that catalyses the reduction of N_2 to NH_3 or NH_4^+ (Fuchs, 2007).

There are many factors that affect this symbiosis between legumes and nodule bacteria such as low water availability, extreme temperatures, soil acidity, high salinity or the presence of toxic substances (Andrés et al. in Maheshwari, 2012). In metal contaminated soils the ability of nodule bacteria may be inhibited by toxicity (Zaidi et al., 2012). In experiments nodules often did not develop or the biological nitrogen fixation was ineffective (Gopalakrishnan et al., 2015; Houben and Sonnet, 2015; Martínez-Alcalá et al., 2010). Broos et al. (2004) found that the legume host was able to protect rhizobia from heavy metal stress. At the same time rhizobial species are able to support plants in contaminated soils by producing siderophores that can build stable complexes with heavy metals, by assisting the uptake of nutrients, e.g. iron or by releasing phytohormones that stimulate plant growth. Furthermore, rhizobia are also known for their biocontrol abilities against plant pathogens (Gopalakrishnan et al., 2015).

Legumes are considered good candidates for phytoremediation because of their potential for N-fixation, tolerance of a wide range of pH. The legume white lupin (*Lupinus albus*) has been suggested for phytoimmobilisation of heavy metal contaminated soils for its restricted transfer of metals from root to shoot and because it was able to release root exudates that changed the fractionation of metals in the rhizosphere and hence reduced uptake (Martínez-Alcalá et al., 2009).

2.4. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds that consist of fused benzene rings, which are arranged in linear, angular or clustered structures (Johnsen et al., 2005). They are omnipresent in the environment and cause concern due to their persistence, toxicity and potential carcinogenic and mutagenic properties (Haritash and Kaushik, 2009). They form during incomplete combustion of organic matter. Anthropogenic sources are burning of fossil fuels, home heating, waste incineration, especially burning of plastic materials, coal gasification and automobile exhausts (Bisht et al., 2015; Johnsen et al., 2005; Lu et al., 2011). PAHs are also components of asphalt, tar, bitumen, creosote and motor oil or develop during industrial production of PVC and other plastic materials, colours and pesticides (Freudenschuß et al., 2008).

According to the US Environmental Protection agency following 17 PAHs are considered to be harmful and should be remediated, 15 among them are carcinogenic (Zhang et al., 2006):

- Naphtanlene
- Naphtylamin
- Acenaphthene
- Acenaphtylene
- Fluorene
- Anthracene
- Phenanthrene
- Fluoranthene
- Chrysene
- Pyrene
- Benz[a]anthracene
- Benzo[b]fluoranthene
- Benzo[k]fluoranthene
- Benzo[a]pyrene
- Dibenz[a,h]anthracene
- Benzo[g,h,i]perylene
- Indeno[1,2,3-c,d]pyrene

Human are exposed to PAHs mainly via contaminated food, drinking water or respiration, especially inhalation of cigarette smoke (Freudenschuß et al., 2008). PAHs accumulate in soils and sediments via precipitation. Due to their high hydrophobicity and low water solubility they are very resistant to microbial degradation and so soil becomes the major sink (Zhang et al., 2006). On both sides of the ring structures of PAHs are dense clouds of π-electrons, which form strong bonds and make them resistant to nucleophilic attack (Johnsen et al., 2005). Generally, the more rings a PAH has, the less soluble it is, as the water solubility decreases logarithmically with increasing molecular weight (Johnsen et al., 2005). Additionally, PAH sorb strongly to organic matter and during aging they may also be sequestered in soil micropores, which increases their recalcitrance even more, because degrading bacteria cannot penetrate into pores smaller than 0.8-0.2µm (Johnsen et al., 2005).

Bacterial PAH degradation takes place inside the cells and requires multiple enzymes. Bacteria take up two atoms of an oxygen molecule and a PAH, then they oxidise the PAH to cis-dihydriols. These are further oxidised to aromatic dihydroxy compounds (catechols) and finally transformed via the ortho- or meta-cleavage pathway (Johnsen et al., 2005) to pyruvate that provides energy or can be used to form amino acids (Seo et al., 2009).

Lignin degrading fungi are capable of oxidising PAHs via unspecific exo-enzymes (Johnsen et al., 2005). In contaminated soils yeasts also play an important role in PAH degradation (Haritash and Kaushik, 2009).

There are three ways why PAHs are degraded by microorganisms (Johnsen et al., 2005):

- Assimilative biodegradation or mineralisation in order to use PAHs as a source of carbon and energy
- Intracellular detoxification: for the excretion of the PAH it has to be transformed into a water-soluble form
- Co-metabolism, the degradation of PAHs by enzymes due to structural similarities with other substrates, such as lignin or aromatic root exudates

Bacterial groups from contaminated sites have been isolated and some groups have been identified to use PAHs as a sole carbon and energy source, these were mainly attributed to Pseudomonas, Burkholderia, Sphingomonas, and Mycobacterium (El Amrani et al., 2015). However, PAH degradation in soil is mostly driven by microbial consortia rather than particular specialists and co-metabolism plays an important role (El Amrani et al., 2015; Zhang et al., 2006). Co-metabolism occurs when non-specific enzymes react with PAHs, because they are structurally similar to the primary substrate. The PAHs are then degraded but without the generation of energy or carbon for the organism that released the enzyme. However, the resulting metabolites can then be used as a substrate by other microorganisms (Johnsen et al., 2005; Zhang et al., 2006). In general individual species do not comprise the entire degradation pathway, but microbial consortia work closely together

(Gerhardt et al., 2009). Once hydroxylated, PAHs become increasingly soluble in water and hence can be excreted and further degraded by other microorganisms (Teng et al., 2010).

The main limitation for degradation is bioavailability of PAHs. A prerequisite for microbial PAH degradation is that the PAHs have dissolved in the water phase, but they have a low solubility in water (Johnsen et al., 2005). Furthermore, PAHs are not homogenously distributed in soil, they are often present as tar droplets having a low surface to volume ratio or they are trapped inside of organic particles. Bacteria depend on diffusion of PAHs to the cells, which is limited by the tortuosity of soil pores and retardation by soil particles (Johnsen et al., 2005). Plant roots and fungi play an important role in the distribution of both, PAHs and bacteria. Roots aerate and penetrate the soil and hence open up the contaminated site for the rather immobile bacteria. The mycosphere can be like the rhizosphere a beneficial habitat for microorganisms. The hyphae can act as a “fungal highway” to mobilise bacteria by chemotaxis but they can also be a “fungal pipeline” as PAH taken up by the fungi can be translocated via cytoplasmic streaming and hence make the contaminants accessible for bacteria (El Amrani et al., 2015). But microorganisms have also several strategies to solubilise PAHs for degradation, such as the production of bio-surfactants and extracellular polymeric substances or the formation of biofilms (Johnsen et al., 2005). Genes for PAH degradation are located on plasmids and can be spread rapidly among a bacterial community, even between phylogenetically different members, via horizontal gene transfer (Johnsen et al., 2005).

Factors influencing biodegradation of PAHs are temperature, oxygen and water regime, soil texture, redox potential, substrate concentration and the presence of co-substrates and nutrients. Alternative soluble carbon sources are said to be beneficial for PAH degradation (Adam et al., 2015; Teng et al., 2010). For complete degradation of PAHs supplemental nitrogen N may be needed (Zhang et al., 2006). Moreover the presence of heavy metals can inhibit PAH degradation because of their toxicity towards microorganisms (Chirakkara et al., 2016; Touceda-González et al., 2015).

Plant activities play an important part in PAH degradation. On the one hand plants can degrade PAHs directly and on the other hand plants indirectly stimulate the microbial degradation with root exudates (rhizosphere-effect). Plant secondary metabolites such as soluble phenols, flavonoids, lignin substances are analogous to PAH contaminants and can stimulate PAH degradation pathways. As a consequence the rhizosphere is often habitat of potential PAH degraders even in non-contaminated soils. Root exudates also serve as a carbon source for the initial ring hydroxylation. Furthermore, plants can take up PAHs actively via transporters or passively via diffusion and transform them by oxidation, reduction and hydrolysis to less toxic compounds, which are excreted afterwards. Besides transformation, plants possess further detoxification pathways like compartmentation in vacuoles, incorporation into the cell wall or conjugation to endogenous molecules (El Amrani et al., 2015). Plant roots also release enzymes like dehalogenase, nitroreductase, peroxidase, laccase that are able to transform organic contaminants by catalyzing chemical reactions in soil (Haritash and Kaushik, 2009). However, PAHs can still inhibit plant growth

due to oxidative stress (Gerhardt et al., 2009) and lead to leaf transformations, necrosis and transcriptional changes (El Amrani et al., 2015).

Figure 1 summarises the before mentioned processes behind rhizodegradation.

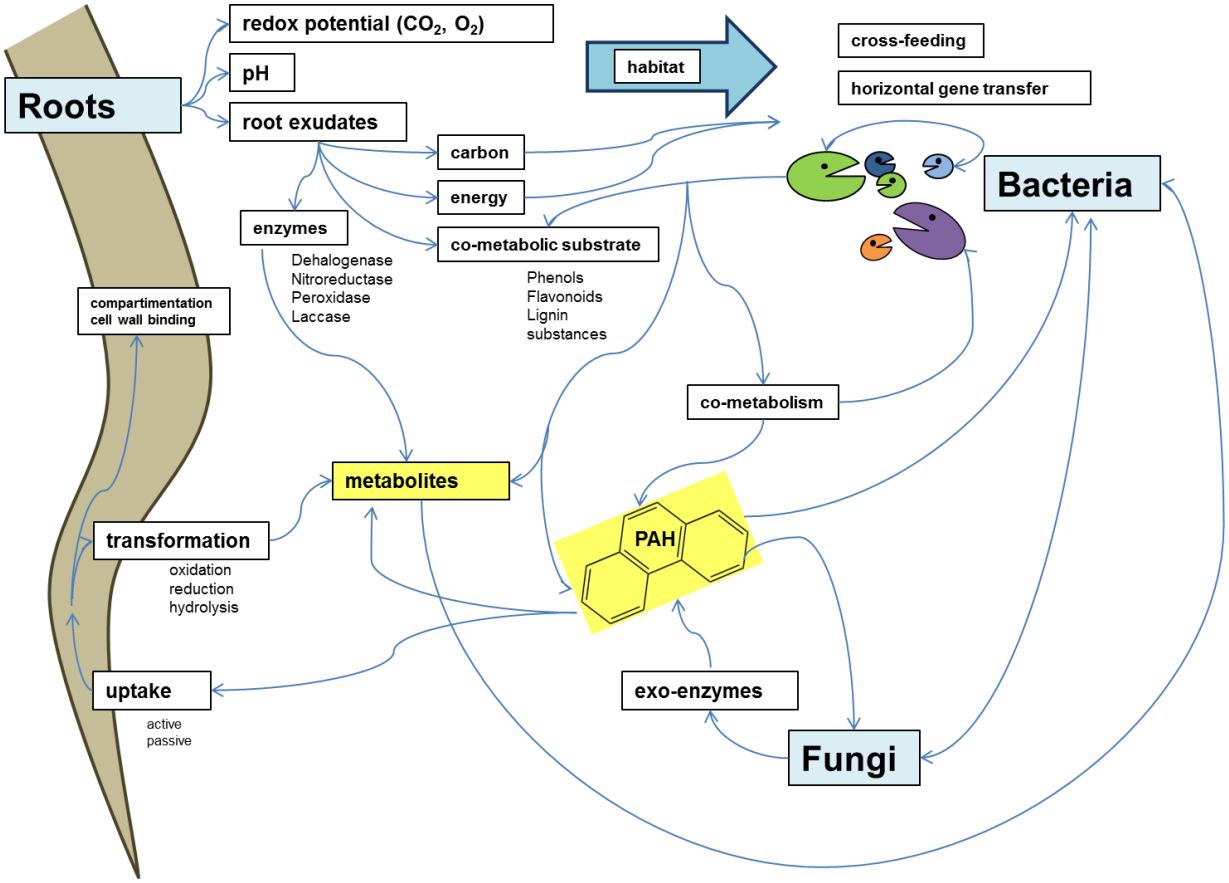

Figure 1. Rhizodegradation - complex interactions between plant roots, fungi and bacteria for the degradation of polycyclic aromatic hydrocarbons

2.5. Lead

Lead (Pb) contaminated soils are widespread and particularly top horizons are polluted (Kabata-Pendias, 2011). Pb is one of the top 20 Hazardous Substances of the Agency for Toxic Substances and Disease Registry (Khalid et al., 2016). Since the Roman ages, mining and smelting have been major causes for Pb contamination in soils. In more recent times, especially leaded gasoline, pigments in paint, sewage sludge, Pb ammunition and automobile batteries have also been important sources. Pb is volatile and atmospheric deposition has caused vast contaminated areas besides mining sites and heavily frequented roads, concentrations up to 7000 mg kg^{-1} have been observed (Steinnes, 2013).

Major risk pathways for human exposure are dust inhalation and soil ingestion (Kabata-Pendias, 2011). Another risk is contamination of the food chain, and also leaching of Pb into shallow groundwater bodies may occur (Cao et al., 2003). Pb can be harmful even at mild

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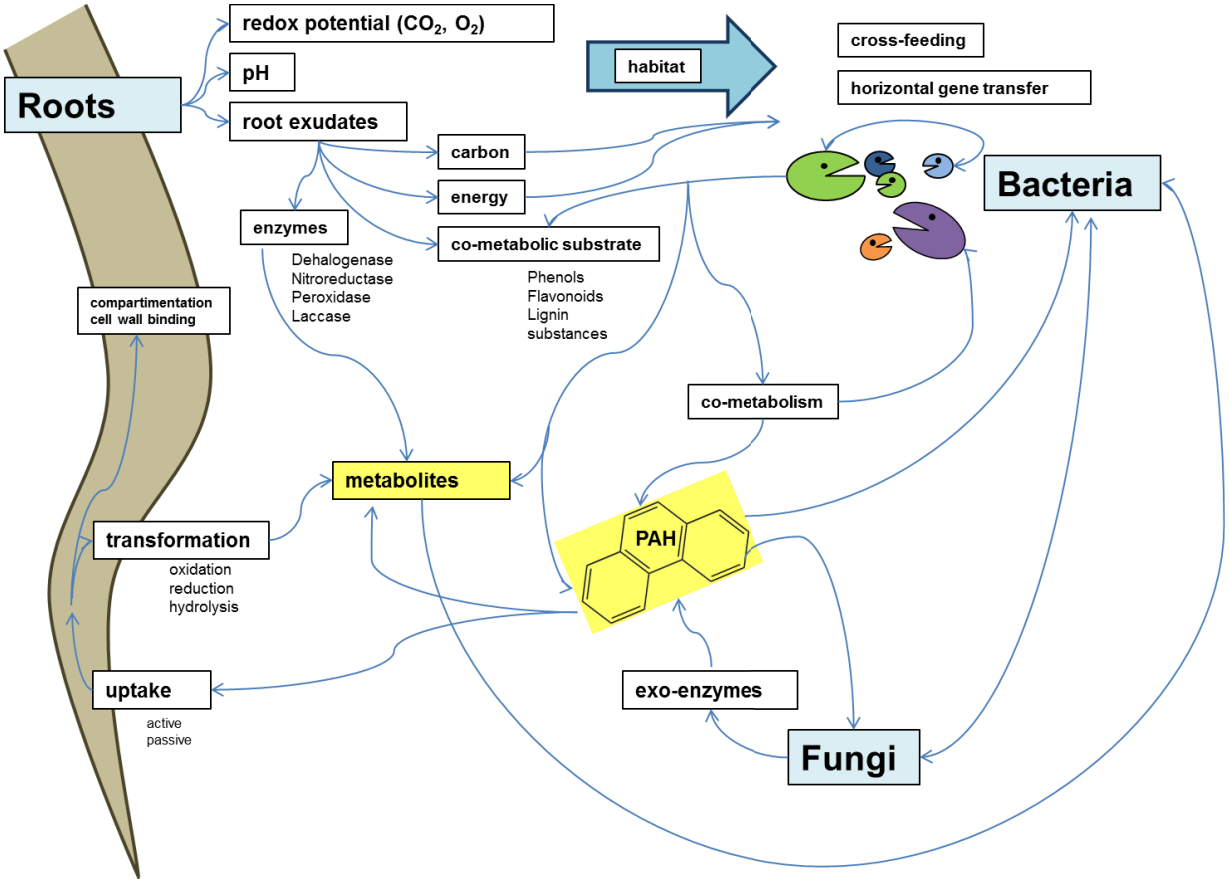

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exposures and negatively affect the human central nervous system, blood pressure, kidney and brain functions. It is also known to cause respiratory allergies, asthma and potentially cancer (Mahar et al., 2015).

Under oxidizing conditions the dominant Pb species in soil is Pb^{2+}, which is also the most bioavailable form. Its solubility decreases with increasing pH and generally Pb is rather immobile compared to other metals, because it has a strong affinity to clay, iron and manganese oxides and organic matter (Steinnes, 2013). Dissolved Pb builds organo-Pb complexes with OM. Soil organic matter may increase Pb bioavailability. But it is also an important sink, because Pb-soil organic matter sorption increases with increasing pH (Kabata-Pendias, 2011). However, also the potential for downwards migration of these complexes is elevated at high pH (Steinnes, 2013). Nevertheless, at alkaline pH Pb may also precipitate as hydroxides, phosphates, carbonates. Pb can also substitute divalent ions on sorption sites or in minerals (K^{2+} in silicate lattices or Ca^{2+} in carbonates). Pb from smelter emissions is present in mineral forms (PbS, PbO, $PbSO_4$, $PbO*PbSO_4$). Alkylated Pb-automobile exhausts form unstable halide salts in soil, that are converted into oxides, carbonates and sulfates or methylated into water soluble and thus bioavailable forms (Kabata-Pendias, 2011).

Although Pb is not essential for plants, tissues can contain between 1-20 mg kg^{-1} Pb and when growing on contaminated sites up to 1000 mg kg^{-1} Pb (Kabata-Pendias, 2011). Pb affects photosynthesis, mitosis, water adsorption and enzyme activity. As a consequence plant growth is reduced. There exist several plant tolerance mechanisms. One of them is accumulation of Pb in roots, or cell wall binding as insoluble, amorphous Pb precipitates (for example with phosphorus) (Martínez-Alcalá et al., 2010). Other tolerance mechanisms comprise modification of soil properties in the rhizosphere in order to immobilise metals, for example the exudation of water soluble organic substances that can complex free metal ions or change of the redox potential in root proximity can cause transformation of redox-sensible elements such as chromium (Martínez-Alcalá et al., 2009). Translocation of Pb from roots to shoots is usually low (Steinnes, 2013). Root membranes are likely disturbed by Pb, as Pb binding influences cell wall elasticity and causes wall rigidity. So uptake of co-present pollutants may be stimulated (Kabata-Pendias, 2011).

Bacteria living in the rhizosphere are able to promote plant growth and plant tolerance by influencing metal availability or uptake. These so-called plant growth-promoting bacteria (PGPB) can also influence soil fertility and availability of nutrients, release plant growth-promoting compounds such as phytohormones, antimicrobial compounds and induce plant stress defence mechanisms. PGPB can also cause complexation, precipitation, im/mobilisation of heavy metals (Touceda-González et al., 2015).

2.6. In-situ stabilisation by the use of soil amendments

Legislations for site assessment are often based on total contaminant concentrations, but they do not necessarily reflect how hazardous a site is (Lobb, 2006). The risk of soil pollutants depends on their availability to organisms and the susceptibility for translocation which depends on the pollutant's behaviour. This is determined by the pollutant properties but also by the soil type. Factors that control the bioavailability of soil contaminants are: pH, organic matter content, redox potential, cation exchange capacity, electrical conductivity and the presence of other compounds that can interact with the pollutant or degrade it in the case of organic pollutants. Inorganic contaminants cannot be degraded and remain in soil for a long time. One mechanism to alleviate potential risks is in-situ stabilisation. The aim of in-situ stabilisation techniques is to reduce contaminant exposure without having destructive effects on soil structure or biological activity. This is done with the help of metal immobilising agents (Khalid et al., 2016). The processes to render the contaminants immobile are sorption, precipitation, complexation and ion exchange. In-situ stabilisation allows revegetation of contaminated sites, prevents contaminant dispersion and generates no hazardous waste (Kumpiene et al., 2008).

2.6.1. Biochar

Biochar is a porous, carbon-rich organic material with a high specific surface area and a large amount of reactive surface groups like hydroxyl (-OH) and carboxyl (-COO) groups, hence a high exchange capacity. Biochar is widely used as a soil conditioner because of its ability to supply nutrients, enhance water holding capacity, improve soil fertility, soil structure and soil carbon content (Beesley et al., 2011; Frišták et al., 2014). It is also used for carbon sequestration as it is recalcitrant to microbial mineralisation (Watzinger et al., 2014). Moreover, biochar can be used for the remediation, re-vegetation and restoration of soils contaminated with organic and inorganic pollutants (Beesley et al., 2011). Biochar is produced from biological residues like greenwaste, wood poultry litter, crop residues by pyrolysis at high temperatures (200-900°C) without the addition of oxygen. The higher the temperature during the pyrolysis, the higher is the degree of carbonisation and hence the surface area. It has an alkaline pH in the range of 7-9 (Beesley et al., 2011). Biochar characteristics can vary depending on the source material, the charring conditions (Sneath et al., 2013). Biochar consists also of mineral components such as phosphates and carbonates, which can reduce the bioavailability of HM by precipitating with the metals to insoluble metal-phosphates (Beesley et al., 2011). But the major immobilisation mechanism is due to the liming effect as cationic metals are more retained by the soil particles. The pH increases because the biochar increases the amount of negative charges on variable-charge sites which reduces the competition of H^+ ions with metal cations for these exchange sites (Karer et al., 2015). Furthermore, the hydroxyl and mainly carboxyl functional groups of biochar play crucial roles in sorption processes of metal cations (Frišták et al., 2014). Which process dominates depends on the site conditions. In soils with a low metal retention

capacity (e.g. a sandy loam), the prevailing immobilisation mechanism deriving from the addition of biochar will be due to the higher cation exchange capacity. In an alkaline, clay-rich soil, which has a higher retention capacity, precipitation will be more important but also the co-mobilisation of metals complexed by dissolved organic matter should be considered as biochar could increase soluble carbon (Beesley et al., 2011). Biochar can also retain nutrients which can be on the one hand desirable because nutrient efficiency can be enhanced due to reduced leaching, but on the other hand in nutrient deficient conditions this might negatively affect plant growth. A combination with compost is thus recommended (Beesley et al., 2011). For the remediation of mixed-contaminated sites biochar can be used to immobilise metals but it also sorbs organic pollutants which inhibits their degradation and can be undesired (Beesley et al., 2011).

2.6.2. Gravel sludge

Gravel sludge is a waste product from the gravel industry. It consists of fine grains of dolomite, calcite, muscovite, illite, feldspar, chlorite and quartz and has a pH in the alkaline range (7-8) due to its calcium carbonate content (Krebs et al., 1999). The clay minerals, muscovite and illite, and the calcite in the gravel sludge provide a large surface for sorption of heavy metals. The specific surface can range from 6 to more than 10 $m^2 g^{-1}$ (Karer et al., 2015; Krebs et al., 1999). Despite the fact that gravel sludge also contains to some extent heavy metals such as Pb, Zn, Cu, Ni and traces of Cd it has been shown by Karer et al. (2015) that its application did not significantly contribute to the initial contamination but efficiently reduced mobile (NH_4NO_3-extractable) heavy metal fractions. The immobilisation of heavy metals caused by gravel sludge can be attributed to the large reactive surface and negatively charged clay minerals, but also to its alkaline pH which can increase soil pH (Krebs et al., 1999) and hence elevate the binding of metals on soil particles. As a waste product gravel sludge is cheap, available in large amounts and thus suitable for soil remediation on the large scale. However adverse effects on soil structure and nutrient supply are possible and re-mobilisation due to acidification in the long-term may occur (Krebs et al., 1999).

2.6.3. Oxides

Soil oxides are omnipresent in soils and formed during weathering. Although they usually occur only in small quantities (tens to thousands of mg kg^{-1}) they play a crucial role in soil chemistry (Komárek et al., 2013). Soil oxides and especially iron oxides have a high potential for stabilisation of metal(loids) due to specific and non-specific sorption as well as co-precipitation. Fixation of metal cations by oxides occurs via three mechanisms. Firstly, metals are covalently bond to the oxide via chemical (specific) sorption, i.e. formation of inner-sphere complexes where the hydration sphere between metal and oxide is lost. This process forms stable complexes and is rather irreversible (Komárek et al., 2013). Moreover,

also physical (non-specific) adsorption is an important process in metal stabilisation. The -OH groups of oxides can be negatively charged (deprotonated) at high pH and positively charged at low pH. These charges determine the overall charge and metal cations can be electrostatically bond to the oxides at alkaline to neutral pH while the hydration sphere is maintained. Furthermore, oxides can co-precipitate with the target metals (Komárek et al., 2013). Iron grit, a by-product from siderite mining, can be used for metal immobilisation because of its high pH (7-8) and the high content in oxides, especially iron oxides that make up around 40%. It contains also oxides from aluminium, manganese, magnesium, calcium and sodium (Karer et al. 2015). The high amounts of elemental iron (Fe(0)) in iron grit can be slowly oxidised to amorphous or poorly crystalline Fe oxides. This is associated with a net release in OH^- ions that increase in pH (Komárek et al., 2013). The sorption of metal(loids) depends on the chemical speciation of the metal(loids), pH, the surface properties of the oxides and the presence of organic and inorganic ligands such as phosphates and sulphates. Low molecular weight organic acids (citrate, malate, oxalate, tartarate) from roots and microorganisms, as well as humic and fulvic acids can form soluble complexes with As, Cu and Pb and increase their solubility at alkaline pH but they can also form surface-metal(loid) ligand complexes and hence promote adsorption. In conclusion, the addition of iron oxides has shown to effectively immobilise metal(loids) but it is suggested to combine the application with compost, because nutrients like P and Mg may also be sorbed and thus limit nutrient availability (Komárek et al., 2013) and soil structure can be negatively affected because oxides potentially cement particles together (Sneath et al., 2013).

2.7. Introduction into the ISOMON project

The aim of the project ISOMON ("Isotopenanwendungen für Sanierung, Nachsorge und Monitoring von kontaminierten Standorten") was to investigate and develop the application of isotope methods for the monitoring of in situ remediation of contaminated sites. Analyses using stable isotopes should reveal the behaviour of pollutants during the treatment of abandoned landfills and industrial sites using defined remediation methods. This innovative monitoring approach was based on changes of isotopic ratios and should be compared with conventional monitoring methods. Pollutants, soil and water have characteristic isotopic ratios which are changing during physical, chemical and biological processes. Observing these isotopic fractionations enables tracing pollutants, identifying pollutant sources, investigating the redox-state of landfills and revealing remediation processes and quantifying biological degradation.

The project was a cooperation of the Austrian Institute of Technology and the University of Natural Resources and Life Sciences, Vienna and it was financially supported by the province of Lower Austria.

ISOMON was organised in following four working packages:

1. *In situ aeration of abandoned waste disposal sites and aftercare of landfills*

Within this working package a method to monitor the state of landfill stabilisation should be developed and validated. Stable isotopes in the leachates and Fourier Transform Infrared spectra should be compared with conventional parameters for monitoring (ÖAV, 2014).

2. *Application of stable isotopes to provide evidence for natural attenuation*

The degradation rate of chlorinated hydrocarbons at a site was determined by investigating the isotope fractionation factor depending on the distance to the pollutant source and compared with a conventional method for modelling the degradation (ÖAV, 2014).

3. *Investigation of the influence of in situ remediation of heavy metal contaminated sites on the degradation of organic pollutants*

Microbial degradation is linked to an isotopic fractionation. In enzymatic reactions, substrates containing lighter isotopes are thermodynamically favoured over those containing heavier isotopes (^{13}C, ^{2}H, ^{37}Cl, ^{15}N, etc.). Thus, microbially mediated degradation reactions result in a relative enrichment of heaver isotopes in the substrate, whereas depletion of it occurs in organisms and metabolites. Abiotic processes like dispersion, volatilisation or sorption only contribute marginally to the fractionation. This working package aimed at analysing the decrease in the isotope ratio of carbon ($^{12}C/^{13}C$) during microbial degradation of organic pollutants in the presence of heavy metals in order to qualify and quantify the degradation. Moreover, the influence of heavy metals on the microbial community should be revealed by analysing the ^{13}C-Phospholipid fatty acid patterns of microbes. Heavy metals negatively influence microbial biomass and composition and hence degradation of organic pollutants might be reduced or incomplete. Shifts in microbial communities could be

identified by using ^{13}C labelled biomarkers. Soil additives to immobilise heavy metals and biological measures to improve soil fertility were tested in their potential to improve microbial degradation. Knowledge to accelerate and support the degradation should help to find and evaluate appropriate methods to remediate mixed-contaminated sites (ÖAV, 2014).

4. *Quantification of chemical in situ oxidation of organic pollutants using stable isotopes*

Here the aim was to develop methods to monitor in situ remediation using alternative electron acceptors such as nitrate and sulfate (ÖAV, 2014).

2.8. Research questions and objectives of the thesis

This master thesis was implemented within working package 3 of the ISOMON project. It aimed to investigate the influence of soil amendments and root activities on microbial degradation of polycyclic aromatic hydrocarbons PAHs in a soil co-contaminated with Pb and PAHs.

It was hypothesised that PAH degradation can be enhanced by reducing Pb toxicity to PAH-degrading microorganisms using metal-immobilising soil amendments. Furthermore, root exudates and nitrogen provided by a legume and its associated rhizobia may be beneficial for degrading microorganisms and hence PAH degradation may be further increased in the rhizosphere compared to bulk soil. Pb is also toxic for plants and associated nitrogen fixing bacteria and their impact on PAH degradation may be inhibited by the adverse effects of Pb. So the combination of soil amendments and plants may enhance PAH degradation even further.

Following research questions were formulated to test the hypotheses:

- How effectively do amendments immobilise Pb and can toxicity towards microorganisms and plants be reduced? More precisely: Can the combined application of biochar, gravel sludge and iron oxides reduce mobile Pb fractions and minimise toxic effects to plants and microorganisms?

- What are the specific effects of legumes and associated rhizobia on Pb bioavailability, in particular in the rhizosphere? Legumes are known for rhizosphere acidification (Houben and Sonnet, 2015) which may lead to mobilisation of Pb. Can amendments mitigate this potential effect? Contrary to this, legumes could immobilise Pb by changing rhizosphere conditions such as the redox potential, by releasing organic compounds that complex with Pb or by uptake and cell wall binding or accumulation in vacuoles (Martínez-Alcalá et al., 2012). Is Pb more mobile in the rhizosphere than in bulk soil?

- The rhizosphere is a beneficial environment for microorganisms. Is microbial PAH degradation in the rhizosphere enhanced due the provision of nutrients (C, N)? Furthermore, excess nitrogen produced during biological N-fixation could establish optimal conditions for PAH degradation. However, also a competition for nutrients between plants and microorganisms could inhibit PAH degradation.

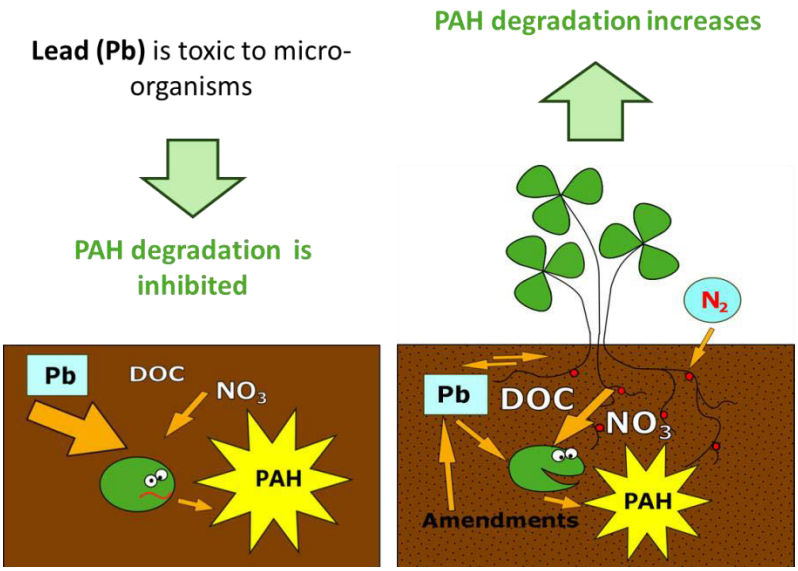

Figure 2. Hypotheses: Amendments reduce Pb toxicity, legumes provide nutrients (dissolved organic carbon [DOC] and nitrate [NO_3]) and thus enhance microbial PAH degradation

3. Materials and Methods

3.1. Soil characterisation

The experimental soil was taken from a clay pigeons shooting range in Austria. It was slightly acidic with a pH of 6.5 ($CaCl_2$) and contaminated with Pb and PAHs due to shot and clay pigeons debris. Clay pigeons consist of 70% limestone ($CaCO_3$) and 30% coal tar pitch, a binder material that contains PAHs (Lobb, 2006). PAH concentration in the sampled soil was 135±24 mg kg^{-1} and the initial Pb concentration ranged from 350 to 450 mg kg^{-1}. Shooting ranges are not homogenously polluted with Pb, common Pb concentrations can vary between 1500-10500 mg kg^{-1} (Cao et al., 2003) and often even higher concentrations could be observed (Sorvari et al., 2006). In order to see clear toxicity effects and to simulate a typical Pb level for a shooting range, we spiked the soil with Pb oxide up to 2000 mg kg^{-1}. Pb oxide seemed to be suitable to simulate weathered Pb shots, as metallic Pb bullets are oxidised to PbO (Hashimoto, 2013). Arsenic and antimony are also typical contaminants on shooting ranges, but in the experimental soil the concentrations were not extremely elvated and hence not discussed in this thesis.

Spiking: Per kg air dried soil 1200 mg Pb(II)oxide (puriss, Sigma Aldrich) were added and shaken in a bucket as can be seen in Figure 3. The spiked soil was incubated at 75% water holding capacity for two weeks. Then 1% (m/m) biochar, 1% (m/m) gravel sludge and 1% (m/m) iron oxides were added. The composition of gravel sludge and iron oxides is shown in Table 1. Biochar was produced from greenwaste residues at maximal 500°C and a residence time of 120 min using nitrogen to establish uniform and inert heating conditions (Frišták et al., 2014). It had a pH(H_2O) of 9, a CEC of 317 $mmol_c$ kg^{-1} and a surface area of 31.5 m^2 g^{-1}. It consisted mainly of carbon (80%), hydrogen (1.6%) and nitrogen (0.7%) (Frišták et al., 2014). Soil and amendments were mixed in a bucket and tap water was added to reach 80% water holding capacity (WHC). Soil with and without amendments (control) were allowed to equilibrate, i.e. they were regularly moistened with tap water to 65% WHC and incubated for 5 weeks at 22 °C.

Table 1. Amendment properties

Amendment	pH	SiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	C_{org}	Cu	Pb	Zn
		g kg^{-1}								mg kg^{-1}		
Iron oxides	7.7	123	43	394	21	34	94	1	282	56	11	78
Gravel sludge	7.5	405	121	69	2	68	125	12	165	85	54	116

References for gravel sludge and iron oxides: Karer et al., 2015; Touceda-González et al., 2015.

Figure 3. Soil spiking: Mixing soil with lead oxide (left figure), Addition of soil amendments: biochar, gravel sludge and iron oxides (from left to right) (right figure)

In order to determine total metal concentrations in the soil an *aqua regia* extraction according to ÖNORM L1085 was conducted by digesting 0.5 g oven-dried and milled soil with 4.5 mL 37% hydrochloric acid, 1.5mL 65% nitric acid (HNO_3) and one drop of Iso-octanol for 3 hours at 150°C in an automated heating block. Digests were then filled up with 50 mL deionised water (Millipore HQ) and filtered with folded filters (pore size 45 µm, Munktell). Metals in soil were measured in filtrates diluted with HQ water to 2% HNO_3 by inductively coupled plasma mass spectrometry ICP-MS (Elan 9000 DRCe, Perkin Elmer) using Indium as internal standard.

Plant available potassium (K) and phosphorus (P) were extracted with calcium-acetate-lactate (CAL, 77g L^{-1} calcium acetate, 39.5 g L^{-1} calcium lactate, 89.5 mL L^{-1} acetic acid, dilution with HQ to 1:5), according to ÖNORM L 1087. Soil was shaken for 2 hours in an overhead shaker at 20 revolutions per minute using a soil:solution ratio of 1:20 with the CAL working solution. After filtration with folded filters (pore size 45 µm, diameter 150 mm, Munktell), samples were stored at 4°C before measurement of K and P on inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 8300pv, Perkin Elmer).

Cation exchange capacity was determined in soil extracts with 0.1 M $BaCl_2$ in a soil:solution ratio of 1:20 according to a modified ÖNORM L 1086-89 method. Extractant and soil were left over night for equilibration, shaken the next day for 2 hours at 20 revolutions per minute and filtrated with folded filters (pore size 45 µm, diameter 150 mm, Munktell). Exchangeable cations (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}, Fe^{3+}, Al^{3+}) were measured on ICP-OES (Optima 8300pv, Perkin Elmer).

Total carbon and nitrogen were determined by dry combustion in an elemental analyser (vario MACRO cube, Elementar Analysensysteme GmbH) according to ÖNORM L 1080 using homogenised, fine-grained, dried soil.

Inorganic carbonates were measured volumetrically by Scheibler apparatus using 10 mL 10% HCl and 2 g oven dry soil (ÖNORM).

Data for texture analysis according to ÖNORM L1060 (2002) were provided by Wawra (2015, personal information).

All extractions and digestions were conducted using sieved soil (<2 mm). For quality assurance blanks and in-house reference material were included in all extraction and digestion procedures. Plastics and glassware for digestions and extractions were acid washed in 5% nitric acid (HNO_3) before use.

3.2. Experimental setup

In this study we used the rhizotest to observe the degradation of PAHs over 10 days. The rhizotest was developed by (Chaignon and Hinsinger, 2003) and is a standardised, plant-based test for assessing rhizosphere processes and bioavailability of trace elements, according to ISO 16198:2015. It facilitates harvest of roots and rhizosphere soil because roots and soil are separated by a mesh, which however enables rhizosphere processes. The Rhizotest approach consist of two phases: (I) a hydroponic pre-growth period where plants are grown in growth cylinders in nutrient solution (visualised in Figure 4a), when a dense root mat has developed (II) the soil-plant contact is enabled and the growth cylinders are put on a thin layer of soil (approximately 3 mm), which is supplied with water and macronutrients via filter wicks (visualised in Figure 4b).

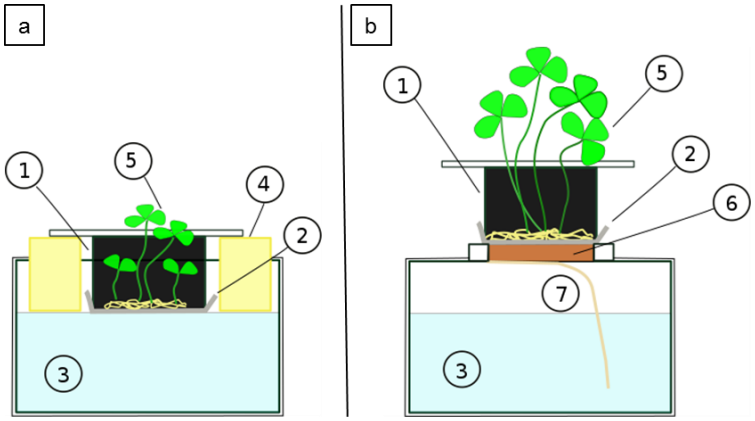

Figure 4. a: Rhizotest hydroponic pre-growth, b: Rhizotest soil-plant contact

Description: Figure 4.a: Rhizotest hydroponic pre-growth: growth cylinder (1) with membrane (2) floating on nutrient solution (3) with the help of foamed polystyrene (4). Plants (5) growing until a dense root mat has developed.
Figure 4.b: Rhizotest soil-plant contact: growth cylinder (1) put on a thin layer of soil (6 g dry weight, ~3 mm thickness), which is supplied with water and macronutrients via a filter wick (7) hanging in nutrient solution (3).

The experiment was conducted in three replicates (three rhizotest units) per treatment and sampling date: control (C) or amended soil (A), with or without plants representing rhizosphere (rhizo) and bulk (bulk) soil after three (T3), six (T6) and ten (T10) days. For T10 nine rhizotest units per treatment were prepared in order to combine three replicates (three rhizotest units à 6 g soil per unit) for having enough soil for the analyses. For T0 soil, either with or without amendments, was combined corresponding to three rhizotest units, to obtain three replicates for each treatment. An overview is provided in Table 2.

Table 2. Number of Rhizotest units per treatment and sampling day

Treatment	T0	T3	T6	T10
C bulk	9 → 3 repl.	3	3	9 → 3 repl.
A bulk	9 → 3 repl.	3	3	9 → 3 repl.
C rhizo		3	3	9 → 3 repl.
A rhizo		3	3	9 → 3 repl.

At the beginning and at the end of the experiment (T0 and T10) soils from three rhizotests were combined in order to have enough soil for analysis resulting in 3 replicates per treatment (*n=3*).

As experimental plant red clover (*Trifolium pratense)* was chosen because it is a legume and could naturally occur at the site. Seeds were sterilised with 6% hydrogen peroxide (H_2O_2) for 3-5 minutes as shown in Figure 5a, rinsed 6 times with deionised water and then inoculated for 90 minutes with Rhizobia using 10% Radicin Trifol (Jost GmbH, Germany). 35 seeds per rhizotest unit were then transferred into growth cylinders, which consisted of a cylinder and a 30 µm polyamide mesh (Klein und Wieler oHG). The growth cylinders were put in a floating assembly as shown in Figure 5 in aerated culture tanks filled with 5 L germination solution containing 0.6 mM $CaCl_2$ and 2 µM H_3BO_3. An aluminium foil covered the seeds during germination. After two days the foil was removed and germination solution was exchanged against a modified half strength N-free Hoagland nutrient solution containing no nitrogen N but higher amounts of K and P in order to provide good conditions for nodule development (Gopalakrishnan et al., 2015). The composition of the nutrient solution is shown in Table 3. The nutrient solution was exchanged every four days and seeds affected by fungal diseases were removed regularly. As plants showed symptoms of N deficiency and nodules did not develop, inoculation with rhizobia was repeated 3 times by pipetting 0.2, 2 and 2.5 mL of 10% Radicin Trifol (Jost GmbH, Germany) in 0.2% sucrose onto the seeds or roots in each growth cylinder. The plants were grown in a climate chamber with day/night temperatures of 22/14°C, a 14h day period and 70% humidity. After 5 weeks of hydroponic pre-growth a dense root mat had developed, but nodules were still not visible.

The soil for the experiment was incubated in the climate chamber. After 5 weeks of ageing it was supplied with nutrient solution up to 80% WHC, left for drying overnight, sieved the next day and moistened to 70% WHC.

Table 3. Composition of the modified half strength N-free Hoagland nutrient solution for hydroponic pre-growth of clover

Nutrients	Concentration mM L^{-1}	Compounds
Mg	2.05	$MgSO_4$ x 7 H_2O
K	4.36	K_2SO_4, K_2HPO_4, KH_2PO_4
SO_4	3.45	$MgSO_4$ x 7 H_2O, K_2SO_4, $ZnSO_4$ x 7 H_2O, $CuSO_4$ x 5 H_2O
PO_4	1.56	K_2HPO_4, KH_2PO_4
Fe	0.01	EDT-Fe
Ca	2.00	$CaCl_2$ x 2 H_2O
Cl	4.00	$CaCl_2$ x 2 H_2O, $MnCl_2$ x 2 H_2O
Micronutrients	µM L^{-1}	
Mn	5.00	$MnCl_2$ x 2 H_2O
B	25.00	H_3BO_3
Zn	0.40	$ZnSO_4$ x 7 H_2O
Cu	0.15	$CuSO_4$ x 5 H_2O
Mo	0.01	$NaMoO_4$ x 2 H_2O
Na	0.01	$NaMoO_4$ x 2 H_2O

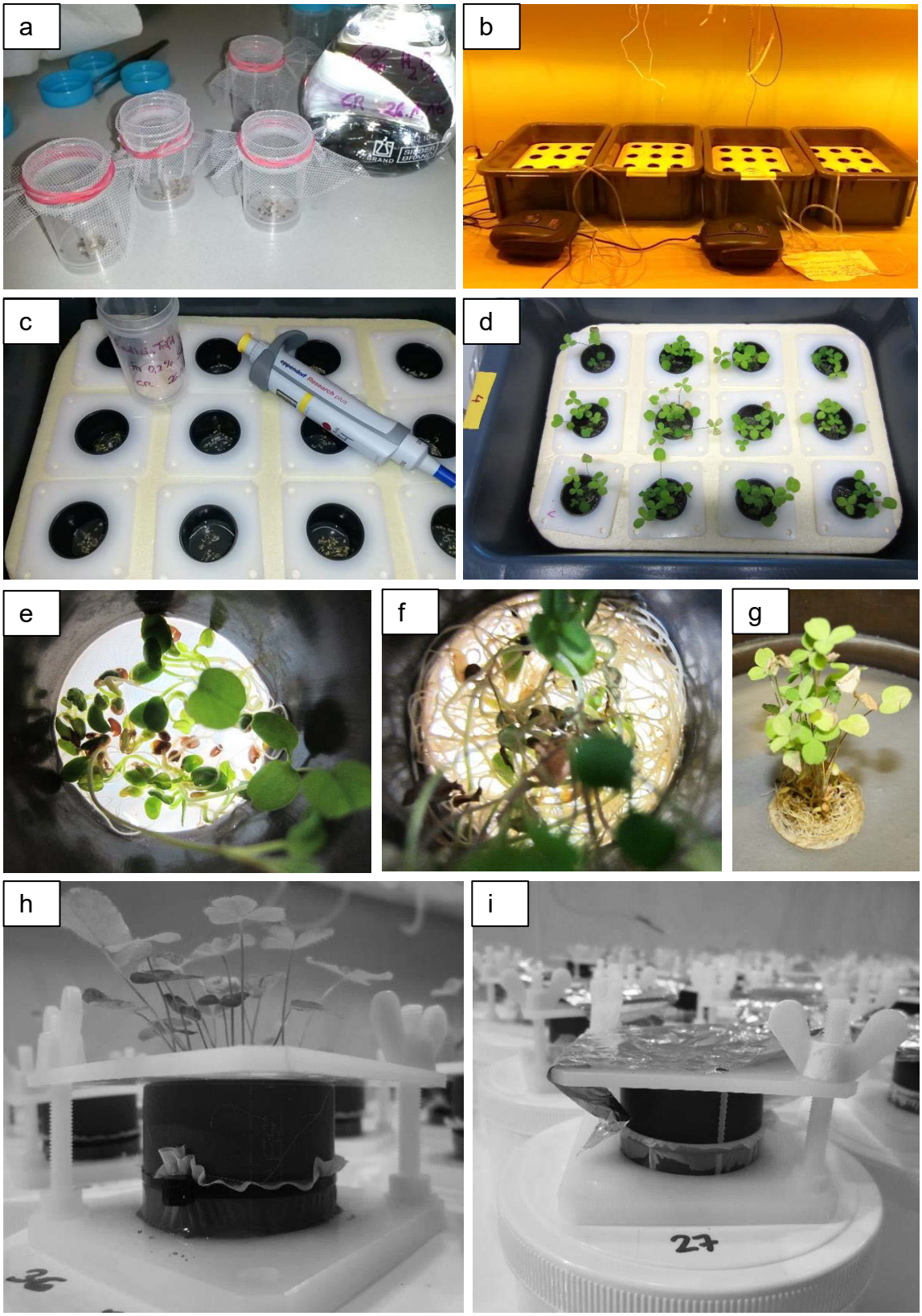

Figure 5. Rhizotest experiment

Description of Figure 5: a)Seed sterilisation , b)Hydroponic system for plant pre-growth in aerated nutrient solution, c) Inoculation with Rhizobia, d) Red clover in hydroponic set-up, 4 weeks old, e) some days after germination, f) root mat at T6, g) harvest of clover at T6, h) Rhizotest unit with plants (rhizosphere soil) and i) without plants (bulk soil)

3.3. Tracing PAH degradation using labelled phenanthrene

In order to evaluate treatment effects on microbial community a ^{13}C-labelled model PAH (phenanthrene, PHE) was added to the soil. For tracing its degradation over time and for identifying the main microbial degraders a phospholipid fatty acid (PLFA) extraction was conducted. Microbial membranes consist of phospholipids with a hydrophobic tail that is composed of two fatty acids. The length, saturation, ring structure and substituted groups of these PLFAs are specific for certain taxonomic groups and can be used as biomarkers. PLFA extraction combined with stable isotope probing helps to reveal soil microbial processes by tracing the labelled substrate temporally within microbial communities (Evershed et al., 2006; Watzinger, 2015). The amount of total PLFAs extracted from a soil sample can be used as a proxy for microbial biomass. Furthermore, analysing the PLFA pattern allows taxonomic interpretation of low resolution such as differentiation into Gram-positive and Gram-negative bacteria, actinomycetes and fungi. PLFAs represent only living organisms as they are rapidly degraded in soil. Thus, shifts in composition of microbial communities can be observed and hence treatment effects can be identified. By addition of the ^{13}C-labelled compound, the incorporation of the labelled material can be used to identify the actively degrading groups of microorganisms, their contribution to total degradation and treatment effects on the degradation. In a chemical reaction different isotopes of the same molecule participate and thus cause an isotope fractionation. During many biochemical processes the lighter isotope is preferred over the heavier one. So microbial degradation leads to relative depletion of the heavy isotope in the microbes and accumulation it in the substrate (Watzinger, 2015). The resulting isotope mass ratios ($^{12}C/^{13}C$) can be expressed as delta notation (δ) relative to an international standard material (Vienna Pee Dee Belemnite) in per mill (‰). Usually the $\delta^{13}C$ values are negative and there is a difference between $\delta^{13}C$ of the soil and of $\delta^{13}C$ of the fatty acids, which is relatively constant (Watzinger, 2015). If a labelled ^{13}C substrate is taken up into microbial membranes they become enriched in ^{13}C and the $\delta^{13}C$ value of a PLFA becomes less negative. Measuring these isotope ratios from the PLFAs by gas chromatography-combustion-isotope mass spectrometry (GC/c/IRMS) hence enables the identification of microbial groups that metabolised the model PAH. In this study PAH degradation was observed over a time period of 10 days to detect qualitative and quantitative differences in rhizosphere and bulk soil in amended and control soils.

As a labelled substrate $^{13}C_{14}$-Phenanthrene (^{13}C-PHE, Sigma Aldrich), a tricyclic hydrocarbon consisting of 14 carbon atoms with the relative atomic mass of 13 was used. The label was mixed with unlabelled phenanthrene in acetone (HPLC grade, Sigma Aldrich) to obtain 1g L^{-1} phenanthrene with a $\delta^{13}C$ value of 1003.6 ‰.

<u>For setting up the rhizotest the soil was prepared as follows:</u>

One day before the rhizotest setup for each rhizotest unit 0.24 g quartz sand (50-70 mesh particle size, Sigma Aldrich) were weighed into glass vials and 0.12 mL acetone containing the label were put on the sand. The acetone was then put under a gentle stream of nitrogen for evaporation. On the first day of the experiment (T0) 6 g incubated soil with or without

amendments was weighed into glass beakers and subsequently mixed with the labelled sand (as shown in Figure 7a) resulting in soil with a phenanthrene concentration of 20 mg kg^{-1} and 4% sand. The soil was then put on the rhizotest units and the growth cylinders containing the pre-grown clover plants or empty ones were put on top and fixed with screws, as can be seen in Figure 5h,i. Empty growth cylinders represented bulk soil and were covered with aluminium foil.

Harvest of soil and plants:

For T0 soil was mixed with PHE-sand following the same procedure as described above and frozen immediately at -20°C. For assessing plant-available Pb at the start of the experiment soil was put on DGT assemblies. Soil (C and A) was mixed with 4% sand, brought up to 90% WHC with water, incubated overnight and put on DGTs for 24h. Afterwards soils were frozen for further analysis. After 3 and 6 days (T3 and T6) the rhizotest soils were frozen for subsequent PLFA analysis. After 10 days soils of 3 rhizotest units were combined to one replicate. About 4 g soil were immediately put on DGTs for 24h and then frozen. The rest of the soil was frozen instantaneously. T0 and T10 were used for PLFA analysis, PAH extraction and $CaCl_2$ extraction. Plants were harvested at all sampling days (T0, T3, T6, T10). They were first washed with tap water to get rid of seed pods and then rinsed with deionised water. Plants were counted and examined for nodules; shoots and roots were separated and dried for 72h at 65°C in an oven.

Figure 6 shows an overview of the experiment from the preparations over the rhizotest setup and harvest to the analyses that were conducted.

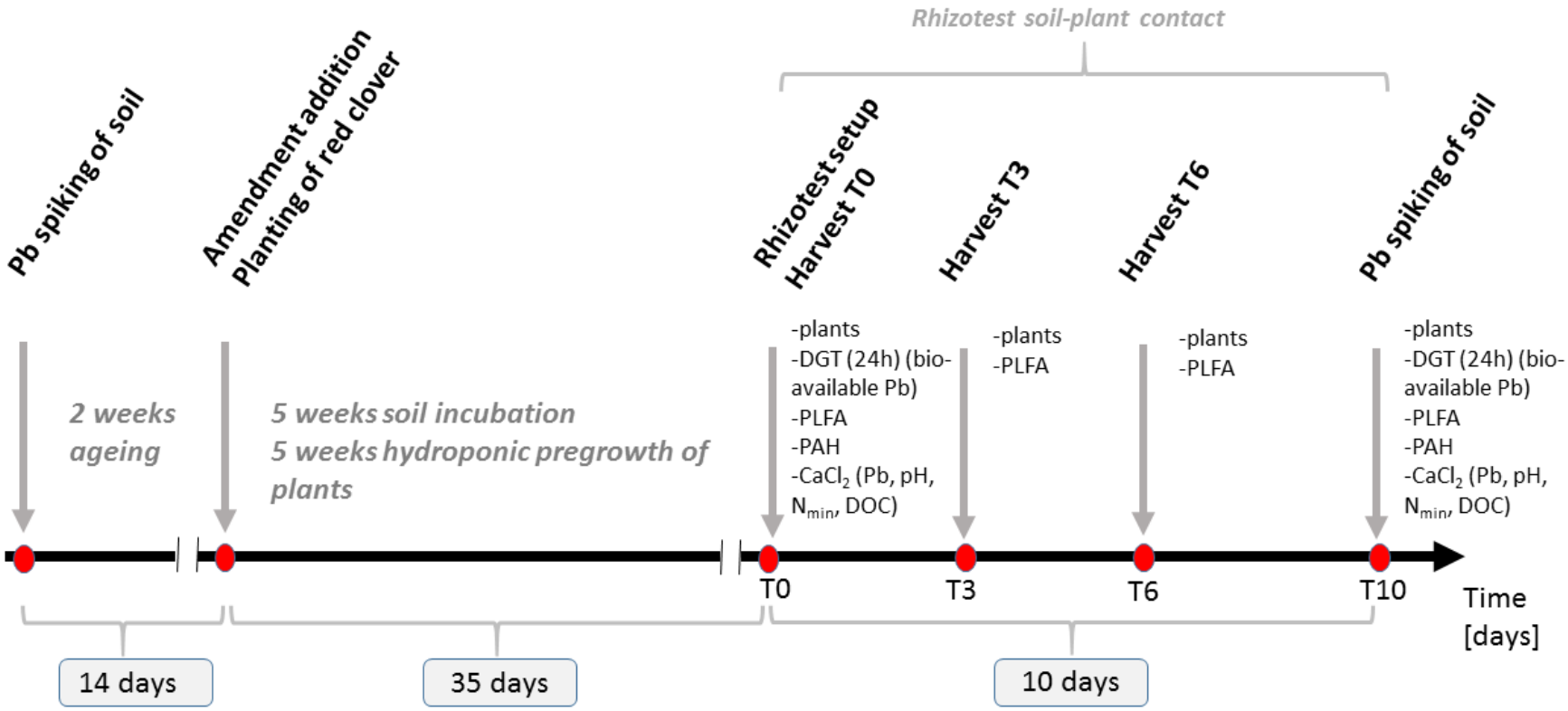

Figure 6. Experimental timeline

Figure 7. a) Addition of PHE-labelled sand, b) plant harvest at T10, c) nodules at T10

3.4. Soil and plant analysis

3.4.1. Plant-available Pb - DGT

Bioavailable Pb was assessed by diffusive gradients in thin films DGT according to Zhang et al. 2005. At the beginning (T0) and at the end (T10) of the experiment about 4 g fresh soil was put on DGT devices and incubated for 24 hours in a water-saturated atmosphere at 20 °C. The DGT devices consisted of a membrane (cellulose nitrate, 100 µm thickness, 0.45 µm pore size), a diffusive gel layer (0.8 mm thick polyacrylamide gel) and a polyacrylamide resin gel containing suspended Chelex 100 particles for binding cations. After 24 hours deployment, soils were removed from the DGT devices and frozen immediately for further analysis. Gels were rinsed 3 times in beakers with HQ-water and then eluted in 5 mL 1 M HNO_3 for several days. Metal concentrations in elution solution were measured by ICP-MS (Elan 9000 DRCe, Perkin Elmer).

The DGT assesses the flux of labile species from soil to the gel during deployment time. The diffusion into the gel simulates the diffusion-limited plant uptake of elements, thereby providing a better predictability of bioavailability than conventional equilibrium-based extraction approaches.

Concentrations in soil were calculated applying following calculations according to (Zhang, 2017)

Mass of metal accumulated in the resin gel layer M

$$M = \frac{C_e * (V_{HNO3} + V_{gel})}{fe\ [\mu g]}$$

Where C_e is the concentration in the elution solution [µg L-1], V_{HNO3} corresponds to the volume of HNO_3 (here 0.005 L 1M HNO_3) to eluate metals from the resin gel, V_{gel} is the volume of the resin gel (0.0015 L) and fe is the elution factor for a certain metal (here 0.8).

By dividing the mass in the gel M by the deployment time t (t = 24*60*60 s) and the gel surface A (3.14 cm^2), the flux F is calculated.

$$F = \frac{M}{t * A}\ [ng\ s^{-1}cm^{-2}]$$

Metal concentration measured by DGT at the interface of soil and the DGT device C_{DGT} is calculated using following equation:

$$C_{DGT} = \frac{F * \Delta g}{D}$$

Where ∆g is the sum of the thickness of the diffusive gel (0.08 cm) and the filter membrane (0.014 cm) and D is the diffusion coefficient of metal in the gel (D_{Pb} = 6.99x10^{-6} cm^2 s^{-1}).

3.4.2. Calcium chloride extraction (pH, Nmin, metal concentrations and DOC)

After DGT deployment experimental soils were frozen immediately and later extracted with 0.0125 M calcium chloride in order to determine mineral (inorganic) nitrogen N_{min} (modified procedure of ÖNORM L1091). As experimental soil per treatment was limited, the $CaCl_2$ extracts were also used to analyse pH, DOC and metals in soil solution. Briefly, 20 mL of 0.0125 M $CaCl_2$ were added to the soils of T0 and T10 in acid washed centrifuge vials and put into an end-over-end shaker for 30 min at 20 revolutions per minute. To obtain as much extraction solution as possible from the limited amount of available soil the vials were then centrifuged at room temperature for 5 min at 613 x g. Immediately after filtration, 1 mL of extract was acidified for metal analysis by ICP-MS (Elan 9000 DRCe, Perkin Elmer). The rest of the extracts was used for pH measurement and then frozen for determination of dissolved mineral N and dissolved organic carbon DOC by an elemental analyser (ELEMENTAR Vario TOC Cube, Hanau, Germany).

3.4.3. Microbial biomass and ^{13}C-PLFA analysis

Phospholipid fatty acids (PLFAs) were extracted from soil according to Watzinger (2015). Briefly, lipids were extracted overnight from 2 g fresh soil with chloroform, methanol and citrate buffer in a 1:2:0.8 ratio (v/v), at which the citrate buffer was adjusted to the soil water

content in order to correct it. The fatty acid methyl ester 19:0 PC 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabastar, USA) was added at the beginning of the extraction as an internal standard to quantify individual PLFAs. Lipids were separated in silica solid phase extraction columns (ISOLUTE SI, 500 mg Silica 3 mL, Biotage) using chloroform, acetone and methanol to eluate neutral lipids, glycolipids and phospholipids separately. Phospholipids were collected with methanol. For the conversion of PLFAs into fatty acid methyl esters (FAMEs), which allows measurement by gas chromatography GC, methylation using methanolic KOH was conducted for 20 minutes at 40°C and stopped with acetic acid. The FAME 13:0 PC 1,2-ditridecanoyl-sn-glycero-3-phosphocholine standard (Avanti Polar Lipids, Alabastar, USA) was added during the methylation step.

For determination of microbial biomass, the FAMEs were measured by gas chromatography equipped with a flame ionisation detector (GC-FID, Agilent 7890A, Santa Clara, USA) and an Agilent J&W HP-5ms GC Column (60 m, 0.25 mm, 0.25 µm). The temperature of the column was held at 70 °C for 2 minutes, then ramped from 70 to 160 °C at 15 °C min^{-1} and then to 280 °C at 2.5 °C min^{-1}. Helium was used as carrier gas and the injection volume was 2 µL.

To identify PHE degrading microbes, isotopic ratios in FAMEs were measured by gas chromatography-combustion-isotopic ratio mass spectrometry GC/c/IRMS (HP5890 Series II, Agilent, Santa Clara, USA) as described in Watzinger (2015). The GC/c/IRMS was connected to a Delta S via a Combustion II Interface (Finnigan, Bremen, Germany) and the column temperatures were like described above, but the injection volume was 4 µL. The temperature of the combustion oven was 940°C and the pressure of the helium gas flow was set to 1.5 bar. Due to the small peaks the samples were pre-concentrated by a gentle stream of nitrogen just before the measurement and injected manually because the autosampler could not be adjusted to the small sample volume remaining after pre-concentration.

The PLFA peaks in the chromatogram (shown in Figure 9) were identified according to their retention time compared to a standard mix of bacterial acid methyl esters. Due to co-elution, some PLFAs could not be separated without causing severe bias in the areas and the $\delta^{13}C$-values and hence combined area and $\delta^{13}C$-values were reported. This was the case for following PLFAs: 16:1ω7 and 16:1ω6, i17:1ω8 and 10Me16:0, 10Me18:0 and 12Me18:0, cy19:0 and 19:1.

The nomenclature of the PLFAs was based on Frostegård et al. 1991 (reviewed by Frostegård et al., 2011). The iso- and anteiso-branched saturated fatty acids (i14:0, i15:0, a15:0, i16:0, i17:0, a17:0) indicated Gram-positive bacteria. The cyclopropane (cy17:0, cy19:0) and the monounsaturated (16:1ω7, 16:1ω6, 16:1ω5, 18:1ω5c, 18:1ω7) fatty acids were used as biomarkers for Gram-negative bacteria. The methyl branched fatty acids (10Me16:0, 10Me17:0, 10Me18:0, 12Me18:0) represented actinomycetes. The fatty acids 18:2ω6,9 and 18:1ω9c were considered as typical fungal biomarkers. The PLFAs 16:0 and 18:0 are ubiquitous fatty acids and the fatty acids 14:0, 15:0, 17:0, i17:1ω8, 17:1ω8, 19:1 were not distributed to any of the mentioned taxonomic groups. The sum of all 27 PLFAs

was used to calculate the total microbial biomass. The mass of ^{13}C incorporated into the PLFAs and total PLFAs was calculated with the isotope mass balance equation based on the measured $\delta^{13}C$-values and the microbial biomass. The calculation is explained in the appendix (Stable isotope analysis).

Figure 8. Solid phase extraction of PLFAs with silica columns

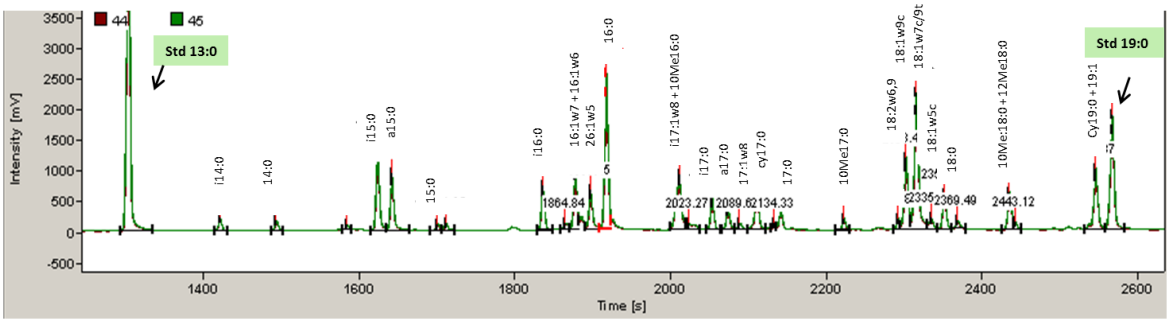

Figure 9. PLFA-chromatogramm from GC/c/IRMS measurement

3.4.4. Extraction of polycyclic aromatic hydrocarbons

For analysis PAHs a Soxhlet extraction was carried out using 10 g of frozen experimental soil from T0 and T10 and 130 mL of ethyl acetate (puriss, Sigma Aldrich) as an extraction solvent. 10 g soil was weighed into extraction thimbles and extracted via the reflux cycle within 5 hours at boiling phase to recover 100 mL of extract. 10 mL of the extracts were then concentrated to 1 mL under a gentle stream of nitrogen and stored in the fridge until measurement by high pressure liquid chromatography HPLC (Agilent 1100 series) equipped with a degasser, a quaternary pump and a diode array detector (DAD) and a fluorescence detector (FLD). Acetonitrile was used as an eluent. Column temperature was held at room

temperature and standards (Supelco EPA TCL PAH Mix) were used for the calibration. The wavelengths 310, 350, 420, 440 and 500 nm were used for determining following 16 PAHs:

Acenaphtylene, Naphthalene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Pyrene, Benz[a]anthracene, Chrysene, Benzo[a]pyrene, Dibenz[a,h[anthracene, Benzo[g,h,i]-perylene, Fluoranthene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Indeno[1,2,3-cd]pyrene

To calculate the PAHs concentrations in soil following equation was applied:

$$PAH\left[\frac{mg}{kg}\right] = cPAH\,[\frac{mg}{L}] * \frac{Vsolvent\,[mL]}{CF} * \frac{\frac{100}{dm\%}}{fw\,[g]}$$

Where cPAH [mg L^{-1}] is the concentration calculated from the peak area and the calibration curve, Vsolvent is the Volume of the extract, CF represents the concentration factor (10), dm% is the percentage of dry matter in the sample and fw represents the weighted sample.

3.4.5. Plant analysis

Shoots and roots were dried and weighed. Due to the small biomass total plant parts were digested in PFA-vials for 120 min at 160 °C using 65% HNO_3 and 30% H_2O_2 (5:1, v/v) in a plant/solution ratio of 0.04. Digests were diluted with HQ-water to receive an 8% HNO_3 solution. Filtration was conducted using acid 50 mL PE-syringes and nylon filters (0.45 µm, Rotilabo, Roth, Germany). Plant digests were analysed for Pb on ICP-MS (Elan 9000 DRCe, Perkin Elmer). For each digestion batch blanks and certified reference plant material (Tobacco leaves INCT-OBTL-5, Institute of nuclear chemistry & technology, Warsaw, Poland) were included.

3.5. Statistical analysis

The statistic software R (version 3.3.1, R-studio) was used for the statistical analysis. After screening the data for normality within the groups (Shapiro-Wilk test) and equality of variances between the groups (LeveneTest), two-sample t-tests and multi-variate ANOVAsd were conducted. If the assumptions for normality or homogeneity of variance could not be met, Kruskal-Wallis tests with the Bonferroni method were applied. The level of significance was 5%. Outliers were identified by Grubbs test and a 1% significance level.

Firstly, differences in soil properties (CEC, C_{org}, N_{total}, P_{CAL}, K_{CAL}, total Pb) and pH, $PAHs_{T0}$, mobile Pb ($CaCl_2$, DGT), NO_3 and DOC between control soil and amended soil at T0 were identified with t-tests.

For analysing changes in pH, mobile Pb ($CaCl_2$, DGT), NO_3, DOC, PAHs and PLFAs between T0 and T10, two-way ANOVAs with the factors “harvest date” (=T0, T10) and “treatment” (=bulk /rhizosphere & with/without amendments) were conducted. Then the influence of plants was analysed for T10 (and for PLFAs also T3 and T6) with a two-way

ANOVA with the factors “soil” (=control or amended soil) and “plant” (=bulk/rhizosphere). Pb concentrations in plants were analysed by two-way ANOVA with the factors “harvest date” and “soil” (hydroponic, control or amended soil).

Several principal component analyses (PCA) with the command “prcomp” were conducted to identify patterns in the PLFA biomass and label uptake. The data were scaled and the factor loading of the principal components were obtained by applying a “rotation command” on the PCA.

5. Results and discussion

5.1. Soil characteristics

Table 4 shows soil properties of control and soil with amendments at the beginning of the experiment. The addition of amendments increased organic carbon content from 22±0 to 30±0 g kg^{-1} (two-sample t-test, $p<0.05$), which resulted in a significantly higher C:N ratio of 15±0 in amended soils compared to control soils (11±0), total nitrogen remained constant (0.2 %). The biochar in the amendments significantly increased plant-available potassium (K_{CAL}) ($p<0.05$, log transformed data) from 7±1 mg kg^{-1} in control soil to 42±2 mg kg^{-1} in amended soils. However, there was no significant change in cation exchange capacity (CEC) due to the addition of amendments. Plant-available phosphorous, dissolved organic carbon (DOC) and NO_3^- were also unaffected by the addition of amendments. During the treatment of the soil with HCl in the Scheibler apparatus no CO_2 evolved and thus revealed that the soil did not contain any carbonate.

Table 4. Soil characteristics

Soil properties*	Control soil	Soil with amendments
Clay [%]	17.0	n.a.
Silt [%]	32.1	n.a.
Sand [%]	50.9	n.a.
pH_{T0} ($CaCl_2$)	6.5 ± 0.1[b]	6.9 ± 0.0[a]
CEC [mmolc kg^{-1}]	129 ± 1	133 ± 3
Base saturation [%]	99.8 ± 0.1	99.9 ± 0.1
Corg [g kg^{-1}]	22 ± 0	30 ± 0
Ntotal [g kg^{-1}]	2.0 ± 0.0	1.9 ± 0.0
C:N	10.9 ± 0.1[b]	15.3 ± 0.1[a]
P_{CAL} [mg kg^{-1}]	26 ± 1	27 ± 1
K_{CAL} [mg kg-1]	7 ± 1[b]	43 ± 16[a]

Values represent means ± standard deviation (n=3), letters indicate significant differences according to t-test ($p<0.05$).

5.2. Pb mobility, plant uptake and soil pH

The amendment addition could significantly increase the pH by 0.4 units ($p < 0.01$) from 6.5±0.1 to 6.9±0.0 at T0, as shown in Table 5. During the rhizotest soil contact phase, pH was stable in control soils but dropped by 0.1 units in soils with amendments. In the planted treatments of amended and control soils pH decreased by about 0.2 units, from 6.5±0.1 to 6.3±0.1 and from 6.8±0.0 to 6.6±0.0 in control and amended soils, respectively.

Table 5. Soil pH

pH	Harvest	Control soil		Soil with amendments	
Treatment		**bulk**	**planted**	**bulk**	**planted**
pH	Day 0	6.5 ± 0.1^{b}		6.9 ± 0.0^{a}	
	Day 10	6.5 ± 0.1^{bc}	6.3 ± 0.1^{c}	6.8 ± 0.0^{a}	6.6 ± 0.0^{ab}

Values represent means ± standard deviation (n=3), letters indicate significant differences according to Kruskal Wallis test and Bonferroni post-hoc test ($p<0.05$).

Means of mobile Pb are shown in Table 6. The total Pb concentrations in the soil (2150±230 mg kg^{-1}) exceeded the Austrian Standards S 2088-2 (2014) assessment value of 100 mg kg^{-1} for agricultural and horticultural soils. The NH_4NO_3-extractable Pb concentrations were 1.57±0.03 and 0.7±0.02 mg kg^{-1} in control and amended soil. Both were above the critical value of 0.3 mg kg^{-1} proposed by the Austrian Standards S 2088-2 (2014) and hence would pose a need for remediation. In control soils the amount of $CaCl_2$-extractable Pb was 0.21±0.02 mg kg^{-1}, which was nearly twice as high as in amended soils (0.12±0.01 mg kg^{-1}) and the difference was significant ($p<0.001$). Within the 10 days of the rhizotest experiment, the $CaCl_2$-extractable Pb slightly decreased in control soils and slightly increased in amended soils as can be seen in Table 6. At T10 no significant difference between control and amended soils could be observed and also rhizosphere soils were not significantly elevated despite the lower pH. Although the means of the Pb concentrations assessed by the DGT method at T0 were slightly lower in amended soils (56±12 and 39±2 ng cm^{-2} in C and A, respectively) the difference was not significant (T0, t-test). At the end of 10 days of soil-plant contact, the DGT analysis could not reveal any significant differences in Pb mobility in rhizosphere and bulk soils, which is similar to the results of the $CaCl_2$ extraction.

Table 6. Lead mobility

Lead fraction	Harvest	Control soil		Soil with amendments	
Treatment		**bulk**	**planted**	**bulk**	**planted**
Total [mg kg^{-1}]	Day 0	2150 ± 230		1820 ± 100	
NH_4NO_3 [mg kg^{-1}]	Day 0	1.57 ± 0.03^{a}		0.70 ± 0.02^{b}	
$CaCl_2$ [mg kg^{-1}]	Day 0	0.21 ± 0.02^{a}		0.12 ± 0.01^{b}	
	Day 10	0.18 ± 0.01	0.22 ± 0.06	0.19 ± 0.08	0.17 ± 0.04
C_{DGT} [ng cm^{-3}]	Day 0	56 ± 12		39 ± 2	
	Day 10	63 ± 15	52 ± 19	40 ± 7	39 ± 13

Values represent means ± standard deviation (n=3), the letters indicate significant differences according to t-test ($p<0.05$)

The plant biomass harvested after 6 days was higher than after 10 days. This could have been due to a bias in plant selection at the beginning of the experiment. Although the selection was randomised it was difficult to get similar plant biomasses in rhizotest units because a huge amount of plants died during the hydroponic pre-growth period so that from the 35 seeds only 4-12 plants survived the pre-growth period and had very differing biomasses. It is possible that plants competed for nutrients, especially N, as they showed significant N deficiency symptoms (light coloured leaves and beginning chlorosis) at the beginning of the experiment. The looked much more healthy after some days on soil and after 6 days also nodules had developed.

Within 10 days, Pb concentrations in roots (shown in Table 7) increased from 8±5 mg kg^{-1} up to 113±39 mg kg^{-1} and 73±12 mg kg^{-1} in control and amended soils, respectively. Within the first 6 days of soil exposure the Pb uptake into roots was not significantly different between plants on soils with or without amendments but after 10 days Pb concentrations in plants grown on control soil showed significantly ($p<0.05$) higher Pb concentrations than plants from previous days or amended soils (t-test with log-transformed data). Transfer from roots to shoots remained low in all treatments and Pb concentrations in shoots ranged between 2-12 mg kg^{-1}, whereas no significant differences between control and amended soil could be observed. Accumulation of Pb in roots while keeping translocation of metals from roots to shoots low could make red clover a good candidate for phytostabilisation (Martínez-Alcalá et al., 2012). However, the experimental setup does not allow drawing any conclusions for red clover towards natural and long-term immobilisation of Pb.

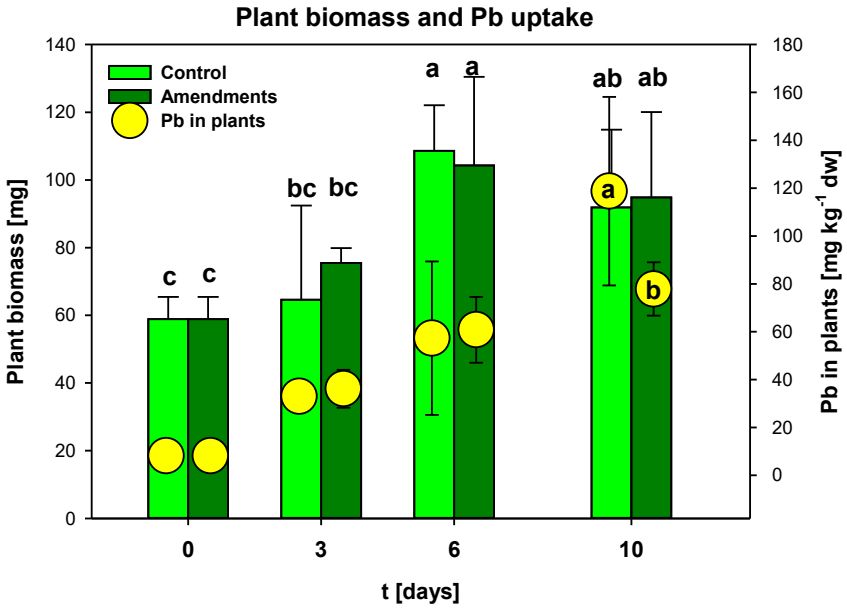

Figure 10. Biomass development of red clover and Pb concentrations in plants

Bars show mean dry weight of clover at each harvest date, circles show Pb concentrations in plant tissues (shoot + root), and error bars indicate standard deviation. Plant biomass was highest after 6 days, the letters show significant different groups assessed by ANOVA and post-hoc Tukey HSD test. After 10 days Pb concentrations were significantly ($p<0.01$) lower in clover grown on soils with amendments (t-test with log-transformed data).

Table 7. Plant biomass and Pb concentrations in plants

Plants		Control soil	Soil with amendments
Biomass [mg]	Day 0	59 ± 7	
	Day 10	92 ± 23	95± 25
Pb in shoots [mg kg^{-1}]	Day 10	5 ± 2	5 ± 2
Pb in roots [mg kg^{-1}]	Day 10	113 ± 39[a]	73 ± 12[b]
Pb in plant [mg kg^{-1}]	Day 10	119 ± 39[a]	78 ± 11[b]
Bioconcentration	$=c_{plant}/Pb_{total}$	0.04 ± 0.01	0.05 ± 0.01
Translocation	$=c_{shoot}/c_{root}$	0.05 ± 0.02	0.07 ± 0.05

Data are means ± standard deviation of 6 and 9 replicates for Day 0 and day 10, respectively. The letters indicate significant differences according to t-test ($p<0.05$)

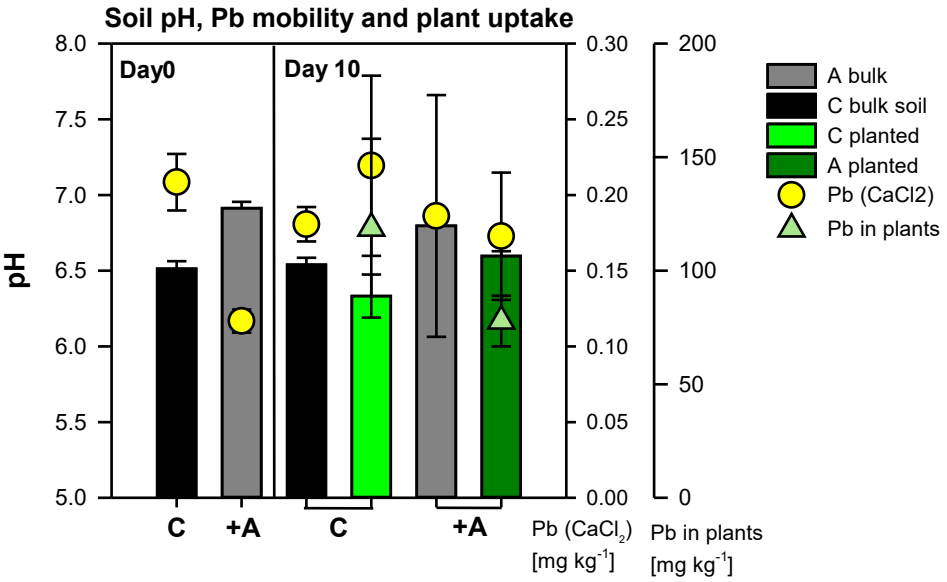

Figure 11. Soil pH, Pb mobility and plant uptake.

Amendments did not decrease extractable Pb but still reduced plant Pb concentrations. Bars show means of pH (n=3) with standard deviation, yellow circles show CaCl2-extractable Pb (n=3) and green triangles Pb plant concentrations (n=9)

The addition of 3% soil amendments (biochar, gravel sludge and iron grit) could reduce the NH_4NO_3 and $CaCl_2$-extractable fractions by 55% and 43%, respectively. The added biochar and the iron grit probably caused a redistribution of Pb from the exchangeable pool to the carbonate- and Fe oxide-bound fractions (Houben and Sonnet, 2015; Komárek et al., 2013) and hence reduced the fractions assessed by the extractions. Bioavailable Pb assessed by DGT did not show a significant difference at T0. This probably indicated that resupply of Pb from the solid phase following depletion was not strongly affected by the amendment addition (Zhang et al., 1998). This suggests that on the one hand the exchangeable Pb

fraction was decreased, but on the other hand resupply dynamics remained unchanged. Hence the pH effect caused lower concentrations of Pb in the $CaCl_2$- and NH_4NO_3-extracts but apparently no redistribution of the fractions, as pH is one of the major factors controlling the mobility of Pb (Friesl-Hanl et al., 2009; Kumpiene et al., 2008). After 10 days of the rhizotest experiment also the $CaCl_2$ extractable fractions were no more significantly different, this may be due to the slight drop in pH in amended soils. The presence of more H^+ ions could induce desorption of Pb from binding sites (Beesley et al., 2011). In rhizosphere soils the pH dropped by 0.2 units and even a slight but not significant increase of mobile Pb in control soil could be observed. The drop in pH might have been a result of rhizosphere acidification. One possible reason for rhizosphere acidification could be that the plants tried to mobilise nutrients such as Fe, Cu and Zn by releasing root exudates (Hinsinger et al., 2006). The plant concentrations of these nutrients decreased over the 10 days of soil contact compared to concentrations of plants harvested from nutrient solution at T0 (data not shown). This highlights the limited period of a rhizotest experiment. The nutrient solution used to keep the soil in the rhizotest units moist contained only macronutrients like Mg, K, P and Ca but no micronutrients such as B, Cu, Zn, Fe or Mn. Another cause for a pH decrease in the rhizosphere could be root and microbial respiration, since the emerging CO_2 can form carbonic acid (Hinsinger et al., 2006). Furthermore, plant roots release protons when they take up more cations than anions to maintain their charge balance, this can decrease the rhizosphere pH up to 2 units (Hinsinger et al., 2006).
The addition of amendments could not counteract the rhizosphere acidification, either because the amount of 3% amendments was not sufficient for increasing the soil buffer capacity to maintain the pH increase or because the plants grown on soil with amendments released more acidifying compounds than plants on control soil. Biochar is known to retain also plant nutrients (Beesley et al., 2011). In an experiment similar to a rhizotest conducted to assess the impact of biochar on metal dynamics in the rhizosphere, the authors Houben and Sonnet observed a two-fold higher net production of H^+ ions by white lupin in the presence of biochar (Houben and Sonnet, 2015). They explained this by the high nutrient supply from biochar which promoted uptake of cationic nutrients such as K and hence a release of protons to compensate for the charge imbalance. They found a redistribution of Zn, Cd and Pb from the exchangeable pool to the carbonate-bound fraction due to the application of 5% biochar, but for Pb the shift was not significantly different to soil without amendments.

Despite the insignificant differences in Pb mobility assessed by several extraction methods and DGT, at T10 the Pb concentrations in plant roots were significantly less in clover grown on soils with amendments. The Pb plant concentrations were not correlated to the Pb concentrations determined by $CaCl_2$ or DGT indicating that these methods were poor predictors for Pb plant uptake in this study. Probably several phytoimmobilisation processes that are not considered in chemical extractions reduced the uptake into roots grown on amended soils. Although DGT would have been the more suitable method for assessing plant-available Pb, the possible immobilisation effect by the amendments was not pronounced enough considering the measurement uncertainties (high standard deviation).

Furthermore, plants possess the ability to change soil properties in the rhizosphere like reduction of the redox potential or the presence of organic ligands that can form stable complexes with metals, especially with Pb (Martínez-Alcalá et al., 2009). Martínez-Alcalá et al. (2009) also found more highly oxidized conditions in the rhizosphere of white lupin grown in an acid soil, which helped maintaining iron as Fe^{3+}, which is able to retain HM on Fe oxides and hydroxides. They also reported that water-soluble organic carbon derived from mucilage, exudates, sloughed-off cells effectively immobilized Pb and Cu by formation of organo-metal complexes, which would be in the 0.1 M $CaCl_2$ extract but not necessarily taken up into the roots or the DGT sampler. It could be possible that the clover grown on soil with amendments released more organic compounds that complexed Pb and hence avoided root uptake. So the amendments did not directly but indirectly reduce Pb uptake. However, it has to be considered that the experiment lasted only 10 days and the dynamics might have changed if the exposure lasted longer as in the case of Houben and Sonnet (2015) who concluded that their 21 day biotest similar to this rhizotest overestimated the root effects.

5.3. Nitrate and dissolved organic carbon

Over 10 days the nitrate (NO_3^-) concentrations decreased in all treatments as shown in Table 8. Contrary to the formulated hypothesis that the rhizobia associated with the clover would fix nitrogen (N), NO_3^- concentrations in rhizosphere soils were significantly lower than in bulk soils. The overall decline in N within the 10 days could be attributed to high activity of soil organisms and plants. The fact that N depletion was more expressed in rhizosphere soils than in bulk soils could either result from an even higher microbial activity in the rhizosphere or from additional plant uptake, which is supported by the fact that the clover plants showed clear symptoms of N-deficiency at the start of the experiment and nodules were only found after 6 days of soil contact. Rhizobial N-fixation may have started only after some days on soil and the duration of the experiment was too short to see clear effects. Fåhraeus (1957) observed the earliest infection of root hairs of white clover already after 48 hours after inoculation. He reported the appearance of the nodules when the seedlings were 10-15 days old (Fåhraeus, 1957). Prior to the rhizotest soil contact phase the plants had been inoculated 3 times and had been growing for 5 weeks in nutrient solution. An investigation of a plant during this period under the microscope revealed that some root hairs showed a typical “sheperd’s crook” indicating infection by rhizobia (Fåhraeus, 1957). However, the observation of N-deficiency symptoms and missing nodules suggest no biological N-fixation during the hydroponic pre-growth. The reason for no obvious N-accumulation in rhizosphere soil despite the presence of nodules after 6 days of soil contact might either be due to a lag phase of rhizobial N-fixation or ineffective N-fixation, as heavy metals are known to inhibit N-fixation (Zaidi et al. 2010). If Pb toxicity hindered rhizobial N-fixation, it might have been less negatively affected in amended soils. Analysing the plants for N would have made clear, if rhizobial N-fixation was effective and if it was higher in soil with amendments. But in this study all plant biomass had to be digested in order to investigate Pb uptake. Unfortunately evidence for effective or ineffective biological

N-fixation is missing and so the hypothesis that N supply by legumes to promote PAH degradation could not be verified with this experiment.

Dissolved organic carbon (DOC) was expected to represent easy available carbon in the form of organic acids, carbohydrates and amino acids that could serve PAH-degrading MO as a co-substrate. But DOC concentrations in the $CaCl_2$-extracts showed no differences between bulk and rhizosphere soils or harvest dates. Measuring DOC in $CaCl_2$ extracts might not be a suitable measure to quantify root exudates (Oburger and Schmidt, 2016).

Table 8. Nitrate and dissolved organic carbon

Nitrate and DOC	Harvest	Control soil		Soil with amendments	
Treatment		bulk	planted	bulk	planted
NO_3^- [mg kg^{-1}]	Day 0	211 ± 13		190 ± 10	
	Day 10	119 ± 18^{a}	98 ± 10^{ab}	98 ± 10^{ab}	87 ± 7^{b}
DOC [mg kg^{-1}]	Day 0	31.5 ± 3.7		29.2 ± 2.0	
	Day 10	23.5± 2,6	24.4 ± 2.8	26.4 ±4.4	21.9 ± 3.4

Data are means ± standard deviation (n=3). The letters indicate significant differences according to t-test ($p<0.05$)

5.4. PAH degradation

Total concentrations of PAHs did not significantly change within the 10 days of the rhizotest experiment resulting in concentrations of 146±16 and 137±29 mg kg^{-1} PAHs compared to initial concentrations of 144±36 and 128±20 mg kg^{-1} in control and amended soil, respectively. The incubation of the soils with amendments for 5 weeks before the soil contact phase did not result in lower PAH concentrations at T0 either. Also the presence of plants during the 10-day experiment could not reduce the concentration of total PAH or single PAHs. Bioavailability and hence degradation of PAHs in the experimental soil was limited because the contaminants had already been aged for several years. This means that the most bioavailable fraction of PAHs was already degraded. The remaining PAHs were likely sequestered, i.e. strongly bound to soil particles, absorbed inside of organic matter or expandable clays and hence not bioavailable (Haritash and Kaushik, 2009). Soil structure is also crucial for PAH bioavailability. If PAHs are trapped in pores smaller than 0.2-0.8 µm they become inaccessible for bacteria (Johnsen et al., 2005). The variation in PAH concentrations could possibly be attributed to the heterogeneous distribution of clay pigeon particles. As bacteria are not very mobile in soil, microbial degradation depends on diffusive transport of PAHs to the cells which is impeded by the tortuosity and retardation of PAHs by the soil solid phase (Johnsen et al., 2005), hence PAHs recalcitrance is not only attributed to their chemical structure but also to soil properties. Being inaccessible to the solution due to sequestration, PAHs would not pose a threat to the environment and remediation would cause unnecessary costs while having minimal benefits for health and safety (Haritash and Kaushik, 2009). However, the soil was sieved prior to the experiment and hence particles

bigger than 2mm were removed. These particles could still pose a risk in case of dissolution of PAHs (Lobb, 2006).

Phenanthrene (PHE) concentration was significantly reduced after 10 days from 14.4±4 mg kg^{-1} to 8.9±1.4 mg kg^{-1}, indicating degradation of the easy available PHE that was added as a labelled substrate. However, treatment effects did not significantly affect the total PHE degradation as can be seen in Figure 12.

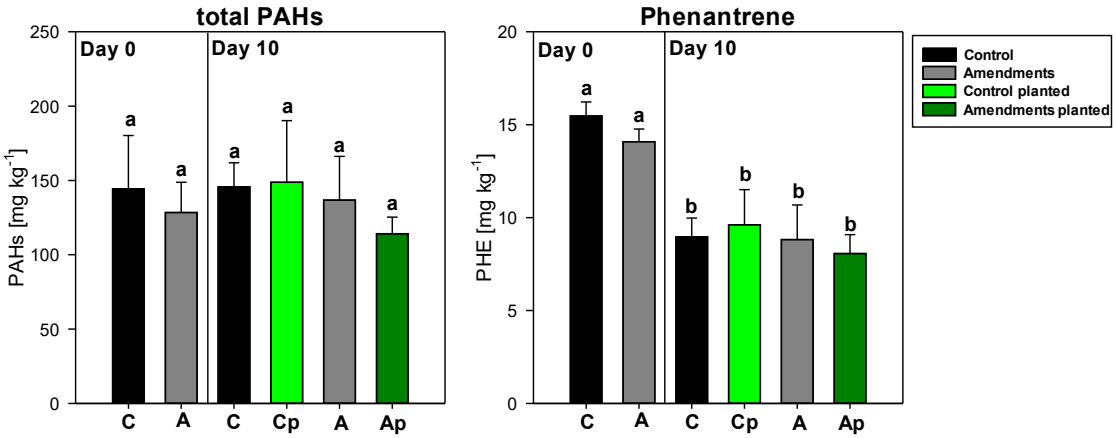

Figure 12 . Development of PAHs and PHE concentrations.

Bars show total PAH concentrations (left) expressed as means of the sum of 16 PAHs and means of PHE concentrations (right) on day 0 and 10, the error bars indicate standard deviation (n=3) and the letters show significantly different groups according to anova with pos-hoc Tukey HSD test.

5.5. PLFA analysis

5.5.1. Microbial biomass

At the beginning of the experiment, the total microbial biomass assessed by all 27 PLFAs (sumPLFA) was 166±18 and 182±19 nmol g^{-1} soil in control and amended soils, respectively. In bulk soils the biomass after 3 and 6 days was slightly lower than at day 0, but it then increased again at day 10 to 195±41 and 198±27 nmol g^{-1} soil in control and amended soils, as visualised in

Figure **13**. There was no significant difference between the soils and treatments except for day 6, where total microbial biomass was higher in rhizosphere than in bulk soils. At this harvest date also the plant biomass was largest (even more than after 10 days) which could have made plant effects more pronounced, as discussed later. Microbial biomass was very low compared to another study using the same soil for a pot experiment (unpublished data). But in that study the soil was not spiked with Pb which very likely had a detrimental effect on microbial biomass in the present experiment.

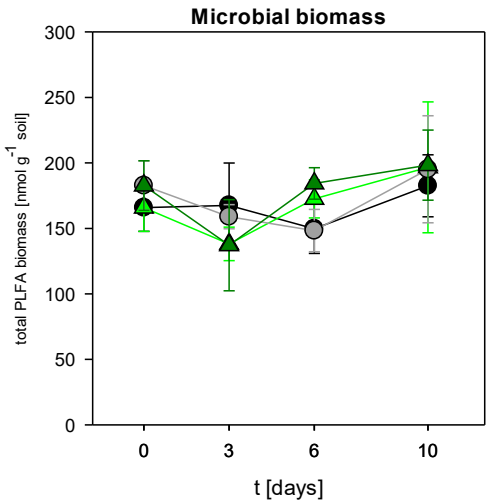

Figure 13. Development of microbial biomass over 10 days
Symbols show means (n=3) and error bars indicate standard deviation

Single PLFAs showed no significant differences between the treatments after 3 or 10 days but after 6 days microbial biomass of following PLFAs was significantly elevated in rhizosphere soils:

- 16:1ω6+7, 18:1ω7c, cy17:0 (Gram-negative bacteria)
- i15:0, a15:0, i16:0 (Gram-positive bacteria)
- 16:0, 18:0 (ubiquitous PLFA that is present in various taxonomic groups)
- 18:1ω9c (fungi)
- 16:1ω5 (either arbuscular mycorrhiza or gram-negative bacteria (Frostegård et al., 1996)

Regarding the microbial composition, the most abundant taxonomic group of microorganisms were Gram-negative bacteria, which made up 34.1±0.9 % of the total biomass. Second largest group were Gram-positive bacteria with 19.9±1.3 %, followed by fungi (11.2±0.8 %). The ubiquitous PLFAs (14:0, 15:0, 16:0 and 18:0) had an abundance of 17.9±0.4 %. These straight-chain PLFAs are found in several taxonomic groups, which renders interpretations difficult (Frostegård et al., 2011; Ruess and Chamberlain, 2010). Actinomycetes had a share of 6.8±0.5 % and other PLFAs that could not be distributed to the above mentioned groups made up 10.2±0.3 % of the microbial community. The amounts of single PLFAs are listed by taxonomic group in Table 9.

Figure 14 visualises the community composition. There were no significant differences in relative biomass between the treatments. Over the 10 days, there were some small changes in the composition at T3 and T6 in the range of 1-2%, but at T10 the relative microbial composition was not significantly different from T0.

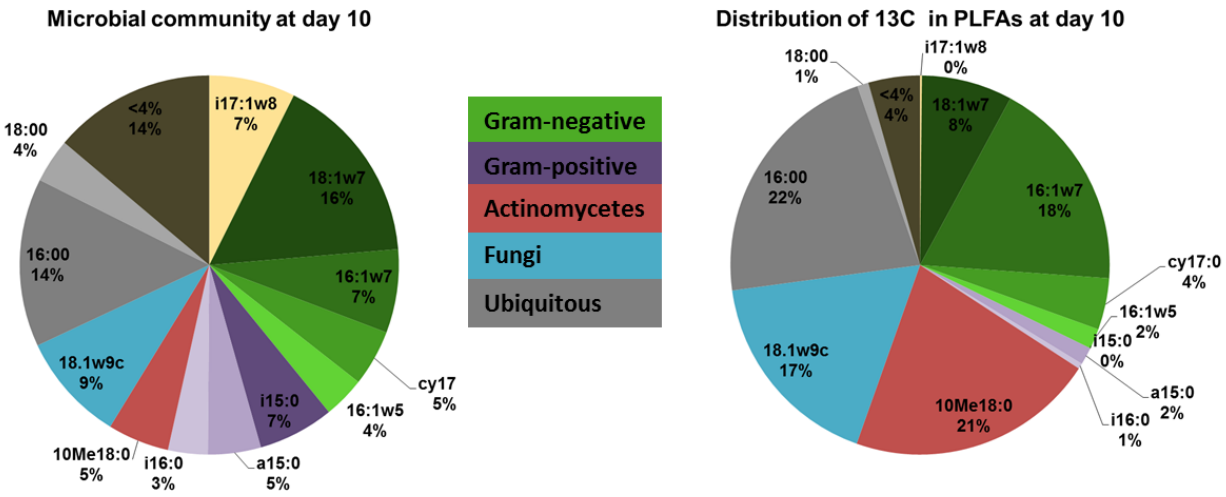

Figure 14. Composition of microbial community and distribution of ^{13}C in individual PLFAs at day 10

Table 9. Composition of microbial community and uptake of ^{13}C into most important PLFAs at day 10

Taxonomic group	% of total PLFA biomass	PLFA	Biomass [nmol g^{-1} soil]	% of total PLFA	^{13}C Control [nmol g^{-1} soil]		^{13}C Amendments [nmol g^{-1} soil]	
					bulk	planted	bulk	planted
total		**Sum 29 PLFAs**	193 ± 32		21.7 ± 1.8	27,1 ± 7.6	20.6 ± 5.5	24.8 ± 5.1
Gram-negative bacteria	34.1±0.9 %	**18:1ω7**	31.5 ± 4.2	16.4 %	1.8 ± 0.1^{ab}	2.7 ± 0.9^{a}	1.0 ± 0.8^{b}	1.7 ± 0.5^{ab}
		16:1ω7+6*	7.1 ± 0.2	7.1 %	4.2 ± 0.5	4.8 ± 1.8	3.6 ± 1.1	4.5 ± 1.0
		cy17:0	4.8 ± 0.2	4.8 %	0.9 ± 0.1	1.0 ± 0.4	1.0 ± 0.3	1.3 ± 0.1
		16:1ω5	3.5 ± 0.1	3.5 %	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Ubiquitous PLFA	17.9±0.4 %	**16:0**	27.8 ± 5.3	14.3 %	4.8 ± 0.6	5.5 ± 1.8	4. ± 1.2	5.3 ± 1.0
		18:0	7.3 ± 1.0	3.8 %	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
Fungi	11.2±0.8 %	**18:1ω9c**	17.9 ± 2.6	9.3 %	3.5 ± 0.3	4.9 ± 1.1	3.6 ± 1.1	4.4 ± 1.1
Actinomycetes	6.8±0.5 %	**10+12Me18:0***	10.1 ± 1.4	5.3 %	4.3 ± 0.2^{b}	5.8 ± 1.0^{a}	4.5 ± 0.8^{b}	5.5 ± 1.2^{a}
Gram-positive bacteria	19.9±1.3 %	**i15:0**	12.5 ± 2.9	6.4 %	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		a15:0	8.7 ± 2.0	5.4 %	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
		i16:0	6.5 ±1.2	3.4 %	0.2 ± 0.0^{a}	0.2 ± 0.1^{a}	0.1 ± 0.1^{b}	0.0 ± 0.0^{b}
Other groups/unidentified	10.2±0.3 %	**i17:1ω8***	14.2 ± 2.6	7.3 %	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0

*some PLFAs could not be separated properly by GC-C-IRMS and were thus analysed together: i17:1ω8 + 10Me16:0, 10Me18:0+12Me18:0, 16:1ω7+16:1ω6

Data are means ± standard deviation (n=3), different letters indicate significantly different groups according to anova and post-hoc Tukey HSD test

5.5.2. PLFA 13C-analysis

During degradation of the labelled hydrocarbon ^{13}C-PHE microorganisms incorporated ^{13}C into their cell membranes. As a consequence the δ^{13}C-values, indicating the ratio of ^{12}C/^{13}C relative to the international standard VPDB, became less negative over 10 days, as can be seen in Figure 15. According to the increasing δ^{13}C-values, the mass of ^{13}C incorporated into total PLFAs increased, as visualised in Figure 16. In planted soils the mass of ^{13}C was slightly higher than in bulk soils, which indicated more pronounced degradation of PHE in the rhizosphere. However, the difference was not statistically significant for total PLFAs but for individual PLFAs which will be discussed later on.

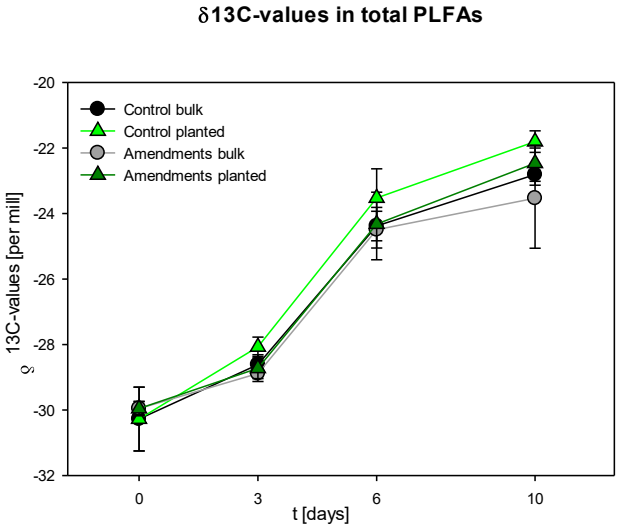

Figure 15. Delta ^{13}C-values in total PLFAs increased in all treatments over 10 days
Symbols show means (n=3) and error bars indicate standard deviation

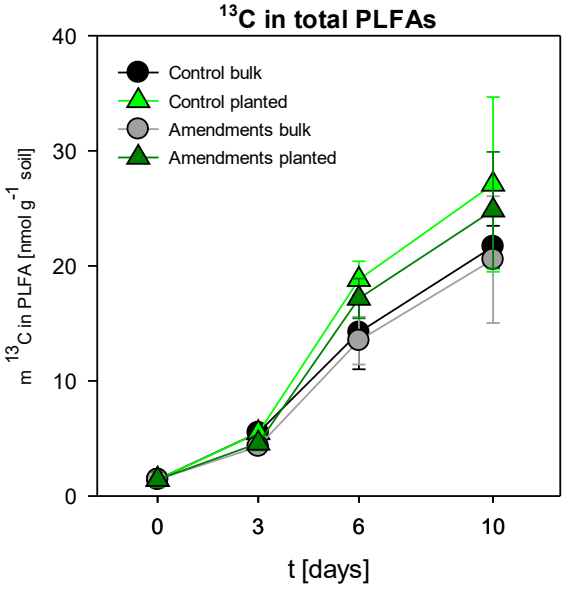

Figure 16. Mass of ^{13}C in total PLFA
Symbols show means (n=3) and error bars indicate standard deviation

Figure 17 shows the mass of ^{13}C in individual PLFAs per treatment. The symbols represent means of 3 replicates per harvest date and error bars show standard deviation. After 6 days (brown squares) the mass of ^{13}C in the PLFAs 16:1ω7, 16:0, 18:1ω9c, 18:1ω7c and 10+12Me18:0 was clearly elevated in all treatments. After 10 days (orange diamonds) also the PLFA cy17:0 was enriched in ^{13}C.

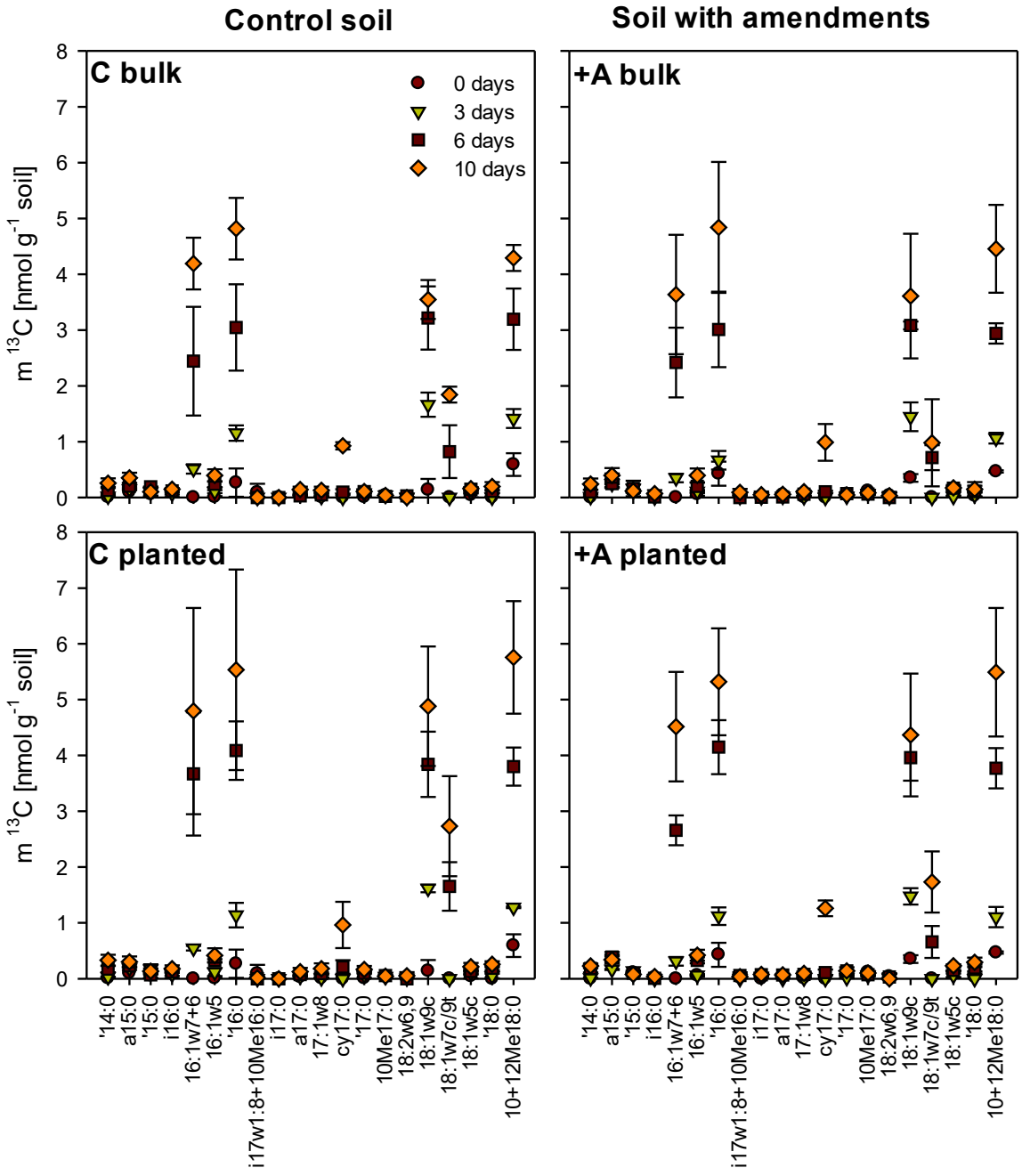

Figure 17. Mass of ^{13}C in individual PLFAs in each treatment and over 10 days

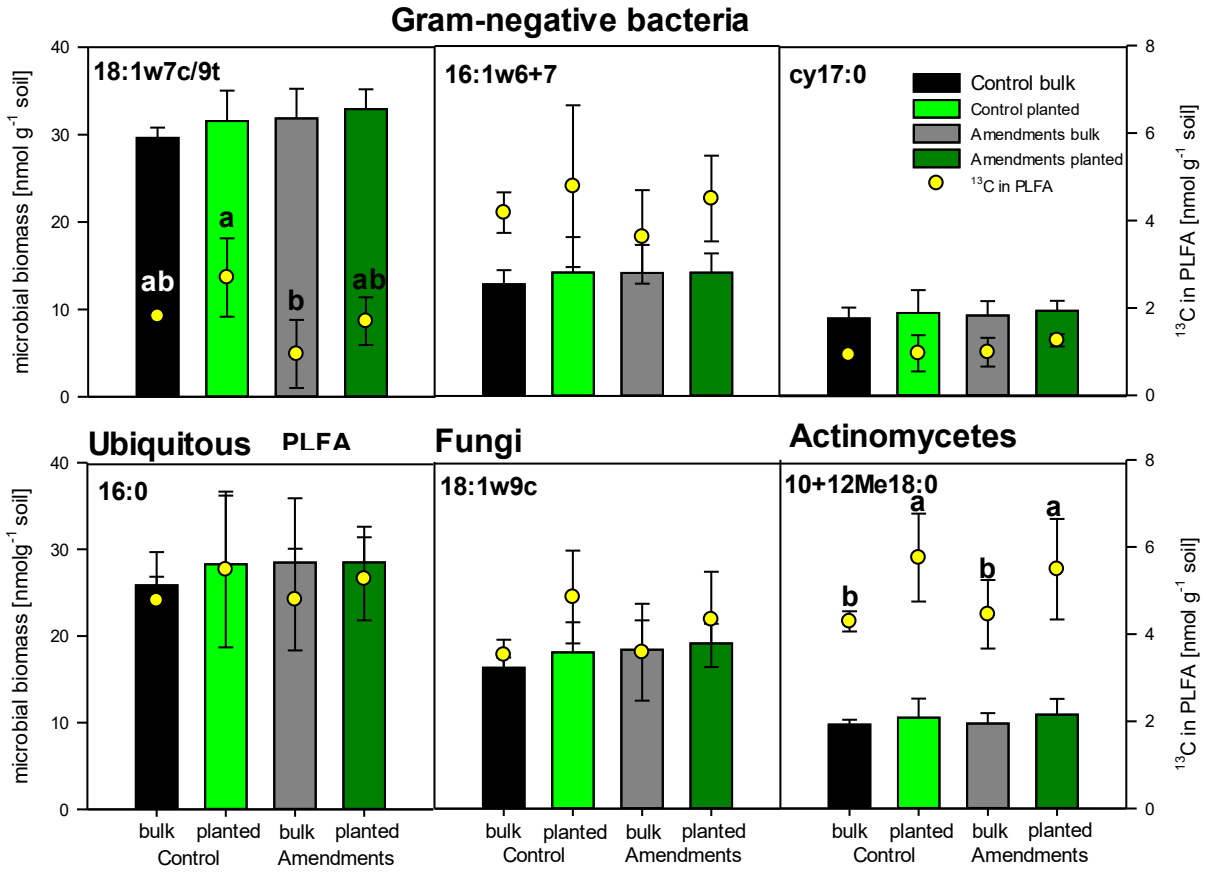

Figure 18. Microbial biomass (bars) and uptake of ^{13}C in main PHE degraders at day 10

The bars in Figure 18 represent means of microbial biomass (n=3) and yellow circles show means of ^{13}C incorporated into PLFAs (n=3) at day 10. Error bars show standard deviation, different letters indicate significant differences between the treatments assessed by HSDtest.

Interestingly, the PLFA 18:1ω7c was the most abundant group among the PHE-degraders but incorporated less ^{13}C than the other Gram-negative bacteria 16:1ω6+7. The Actinomycetes (PLFA 10+12Me18:0) only made up 5.3±0.3% of the total microbial biomass but they incorporated 21.4±1.9% of the label. The relation of abundance and label uptake is also visualised in Figure 14.

Actinomycetes have been previously identified as important pyrene degraders in an experiment with compost-amended and fertilised soil (Adam et al., 2015) and as dominant phenanthrene degraders in a diffusively polluted roadside soil, whereas they were of minor importance in other soils (Johnsen et al., 2002). Although there were no significant differences in microbial biomass between the treatments after 10 days, uptake of labelled ^{13}C into fatty acids of actinomycetes was more pronounced in rhizosphere soils, as shown by the yellow circles in Figure 18 at the right bottom.

Gram-negative bacteria with the PLFA 18:1ω7c incorporated higher amounts of ^{13}C in control soils than in soils with amendments despite the similar microbial biomass. As shown by the letters in the first plot Figure 18, again uptake of ^{13}C was more pronounced in rhizosphere soils. Biochar, clay minerals and metal oxides in the amendments could have

sorbed the PHE and made it hence less bioavailable and inhibited the degradation (Beesley et al., 2011; Cébron et al., 2011). But this seemed not to be the case because no other PLFA than 18:1ω7c showed less ^{13}C-uptake in amended soils. It has been widely reported that microbial biomass increased upon biochar application; often this was in line with changes in the community composition and enzyme activities (Lehmann et al., 2011). However, in the work presented here there were no differences in biomass of the PLFA 18:1ω7c while uptake of ^{13}C was lower in soils with amendments. This could possibly be explained by labile organic carbon provided by the biochar. The Gram-negative bacteria may have fed less on PHE but on leachable carbon fractions provided by the biochar that can be structurally similar because of aromatic structures (Lehmann et al., 2011). So while having the same biomass and activity as in controls they had an additional carbon source that possibly competed with PHE. Another possibility could be that bacteria with PLFA 18:1ω7c relied on enzymes for the degradation of PHE but these enzymes were maybe partly sorbed on biochar (Lehmann et al., 2011). So they had to use another substrate.

For fungi (18:1ω9c) and the Gram-negative bacterial fatty acids 16:1ω6+7 the differences in uptake of ^{13}C between rhizosphere and bulk soil or soils with or without amendments were not statistically different after 10 days. But after 6 days, where plant biomass was higher than at T10, uptake of ^{13}C in rhizosphere soils was higher than in bulk soils for the PLFAs 16:0 (ubiquitous PLFA), 18:1ω9c (fungi), 10+12Me18:0 (actinomycetes) and for total PLFAs, as visualised in Figure 19. The bars in Figure 19 show means of ^{13}C taken up into total PLFAs after 6 days, error bars show standard deviation (n=3), different letters (HSDtest) indicate the significant higher incorporation of ^{13}C into PLFAs in rhizosphere soils compared to bulk soils.

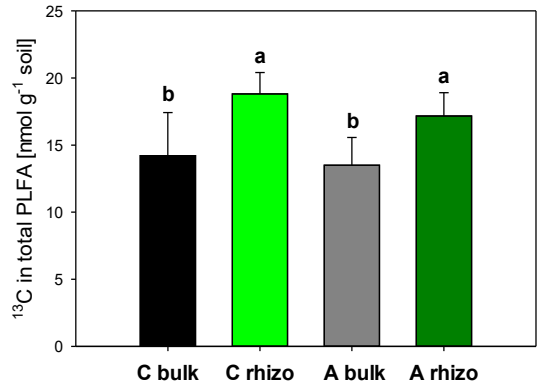

Figure 19. Uptake of 13C into total PLFAs after 6 days

There are several possibilities how clover could have enhanced microbial degradation of PHE. Firstly, it has been shown in several studies that the presence of plant roots increased the microbial biomass and thus enhanced PAH degradation (Bisht et al., 2015; Cébron et al., 2011; El Amrani et al., 2015; Haritash and Kaushik, 2009; Palmroth et al., 2007). So the higher uptake of ^{13}C into fungal PLFA 18:1ω9c is in line with the higher fungal biomass in the rhizosphere after 6 days. But in this experiment microbial biomass of actinomycetes was not affected, there were no differences between bulk and planted treatments. So there might

be other explanations for the higher mass of ^{13}C in PLFAs in planted treatments: It has been shown that the presence of easily available carbon sources stimulated microbial enzymatic activity by acting as a co-metabolic substrate (Teng et al., 2010). This means that the production of enzymes targeting root-released compounds could have been increased due to the availability of root exudates, but these enzymes were possibly simultaneously enabling the decomposition of PHE. At the same time the exudates were likely used as an energy-source for oxidation of PHE to a dihydriol, which is the most difficult step for bacteria and requires energy (Johnsen et al., 2005). Furthermore, enzymes released by roots like dehalogenase, nitro-reductase, peroxidase, laccase could have transformed PHE and catalysed its degradation (Haritash and Kaushik, 2009). Plants could also have taken up PHE, transformed it by oxidation, reduction or hydrolysis to less toxic compounds which were then excreted in the rhizosphere (El Amrani et al., 2015) could then have served in the form of root exudates as a carbon source for the microbes. However, the contribution of plant uptake to PAH degradation was likely of minor importance as it accounted only to 1% of PAH removal in a pot experiment over 75 days conducted by Meng et al. (2011).

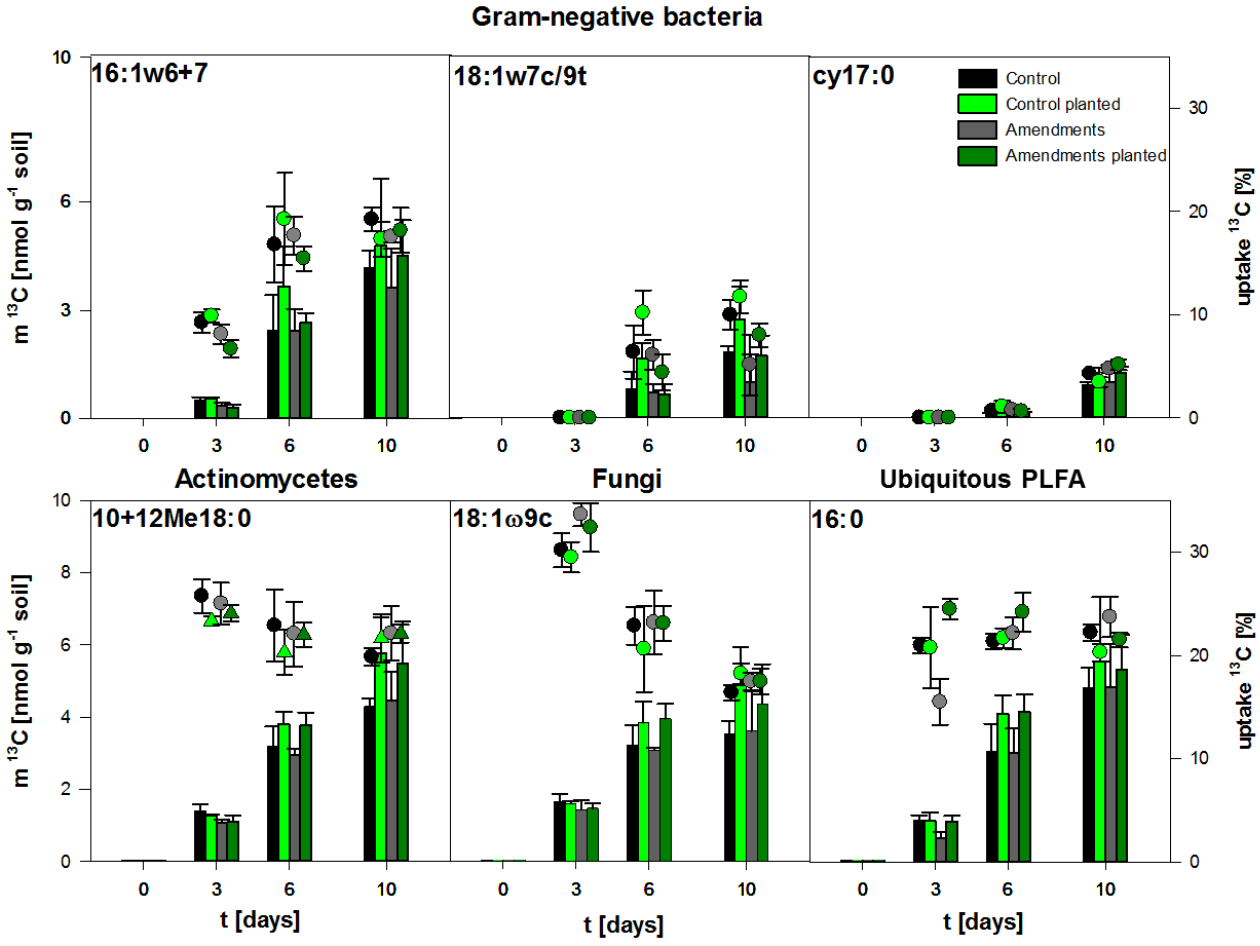

Figure 20. Uptake dynamics of main PHE-degraders over 10 days

The bars in Figure 20 show that the mass of ^{13}C (in nmol g^{-1} soil) increased in all microbial groups involved in PAH degradation over time. But their relative contribution to PAH degradation changed over the 10 days, as can be seen by the coloured circles.

On day 3 the fungi and actinomycetes were the most important PHE degraders, they incorporated 31±2 % and 25±1 % of the total amount of ^{13}C assimilated, respectively. The relative share of ^{13}C incorporation into fungal PLFA (18:1ω9c) decreased from then on as the incorporation of ^{13}C into other PLFAs became more important. Fungi are known to release exo-enzymes like lignin peroxidase, manganese peroxidase and laccases that are non-specific and oxidise various organic compounds and hence also PAHs (Johnsen et al., 2005). Once hydroxylated, PAHs become more soluble and thus easier to be taken up by bacteria that can degrade PAHs only inside their cells. The generation of PAHs metabolites by exo-enzymes may not be a benefit for the organism releasing the enzyme, but it paves the way for subsequent attack by other organisms. This phenomenon is called co-metabolism (Johnsen et al., 2005). However, incorporation of ^{13}C into fungal PLFAs indicated productive PHE degradation, i.e. use of the PHE by fungi as a source for carbon.

In this sense one must consider that the PHE-derived carbon was very likely also used to build up other substances than cell membranes (PLFAs). The turn-over of for example labelled pyrene into proteins was observed to be faster than turn-over into PLFAs (Adam et al., 2015). As a consequence, the contribution of fungi to PAH degradation was possibly even more important. Adam et al. (2015) observed that ^{13}C in amino acids was 2-3 times higher than in PLFAs. They stated that PLFA analysis underestimated the actual incorporation of PAH-derived carbon into microbial biomass.

Gram-negative bacteria characterized by the fatty acid cy17:0 were found to incorporate ^{13}C only after 6 days. A possible explanation for the delayed uptake could be that this PLFA did not actively degrade PHE but fed on degradation metabolites for example produced by fungi. This would reinforce the theory of co-metabolism. But these bacteria could also have fed on degraded dead microorganisms that had incorporated ^{13}C, because PLFAs are quickly degraded after cell death (Frostegård et al., 2011) and hence ^{13}C is provided to the microbial community is not limited to PHE degradation. (Adam et al., 2015) found that despite the dominance of Gram-negative bacteria in degradation of labelled pyrene, cross-feeding of bio-processed ^{13}C could be a reason for the distribution of ^{13}C within the whole microbial community with proceeding incubation time. In their long-term experiment over 3 to 5 months, they observed a maximum incorporation of ^{13}C with a subsequent decrease, which reflected the fast turn-over of PLFAs. Another explanation for a delayed start of ^{13}C incorporation could be transfer of genes necessary for PAH degradation from bacteria that already possessed the ability to degrade PAHs. This phenomenon is known as horizontal-gene transfer and could be possible as genes for PAH degradation are localised on plasmids that can be transferred rapidly within the bacterial community, even between different taxonomic groups (El Amrani et al., 2015).

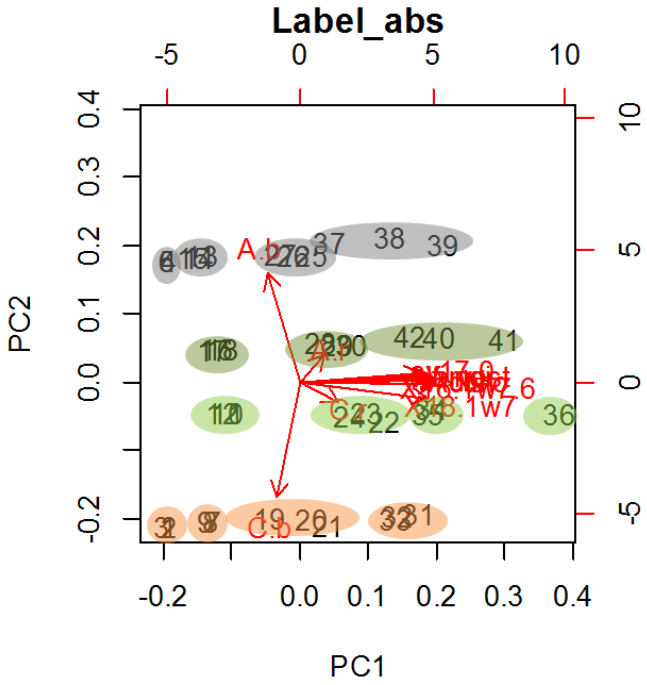

Figure 21. Biplot of principal component analysis with mass of ^{13}C incorporated into main degraders

The two principal components in Figure 21 explain 72% of the variance in the data of ^{13}C in degrader PLFAs. The factors “harvest date” and the single PLFAs contributed each by 12-13 % to PC1. PC2 was mainly determined by the factor treatments “control bulk” and “amendments bulk” (36 % and 35 % respectively). Data points of soil with amendments are labelled grey and dark green for bulk and rhizosphere soil respectively. Orange and lighter green indicate bulk soil and rhizosphere of controls. The principal component analysis thus shows how the uptake of ^{13}C (in nmol g^{-1} soil) into main degrader PLFAs can be distinguished according to harvest date, described by the change in the values of the single PLFAs (PC1) and the treatment (PC2). The proximity of both rhizosphere soils in the plot corresponds well with the results from statistical analysis, which did not identify significant differences between the rhizosphere soils of controls and soils with amendments.

The higher uptake of labelled Phenanthrene in planted treatments could indicate the beneficial impact of plants on microbes (rhizosphere effect, i.e. higher microbial degrading activity). Although the DOC measurement did not show enrichment in dissolved organic carbon in rhizosphere soils, the pH decrease indicates rhizosphere acidification, which might have been caused by organic acids released by roots. These organic acids can be used by microbes as a carbon source, thus enabling co-metabolism of PAHs.

6. Conclusion

The combined application of biochar, gravel sludge and iron grit increased the soil pH and hence reduced NH_4NO_3-extractable Pb but the immobilisation was not effective enough for rendering Pb less bioavailable within the rhizotest experiment, as assessed by DGT and $CaCl_2$ extraction. However, plants grown on soils with amendments accumulated less Pb suggesting more pronounced phytostabilisation, probably as a consequence of more pronounced root exudation in soils with amendments. Root exudates released in order to mobilise nutrients and might have immobilised Pb by formation of organo-Pb complexes. Despite the presence of nodules after 6 days it remained unclear if rhizobia could fix nitrogen and support microbial degradation. Nevertheless, uptake of ^{13}C from labelled PHE into microbial PLFAs was more pronounced in rhizosphere soils. This can be partly attributed to higher microbial biomass in the rhizosphere, especially fungal biomass (PLFA 18:1ω9c). But also stimulation of microbial degradation by root exudates played an important role as ^{13}C incorporated by actinomycetes (PLFA 10+12Me18:0) in the rhizosphere was more pronounced than in bulk soil while biomass was similar. Root exudates likely served as a carbon and energy source for ring cleavage and enzymes targeting exudates could also have degraded PHE due to structural similarities. The main PHE degraders in this experiment were actinomycetes (PLFA 10+12Me18:0), fungi (PLFA 18:1ω9c) and Gram-negative bacteria (PLFAs 16:1ω6+7, 18:1ω7, cy17:0). Whereas actinomycetes and fungi started ^{13}C incorporation immediately after PHE addition, Gram-negative bacteria (18:1ω7, cy17:0) had a delay of 6 days. This indicates that actinomycetes and fungi were likely primary degraders and Gram-negative bacteria probably fed on metabolites produced by actinomycetes and fungi.

This experiment demonstrated the complex interactions of plants, microbes, soil and soil amendments in a co-contaminated soil and revealed the short time dynamics of PAH degradation by microbial consortia. Interpretation of the results from this laboratory test in respect to natural conditions is somehow limited due to the artificial setup, i.e. hydroponic pre-growth of plants and abrupt soil contact, limited nutrients in the thin soil layer, physical restrictions for root growth in the growth cylinders, soil spiking with Pb-oxide and easily available phenanthrene. Moreover, labelled PHE was likely not only incorporated into microbial membranes (PLFAs) but also remained in metabolites or was mineralized to CO_2 and H_2O which were not analysed in this experiment but could have helped revealing PHE degradation more profoundly. Other drawbacks of the experiment were that the PLFA analysis has a low taxonomic resolution and that the experiment was too short to see clear effects of biological nitrogen fixation. However, despite these limitations the experiment was a first attempt to complement long-term studies focusing on PAH degradation by tracing the incorporation of PHE within the first few days. As case study for mixed-contaminated soils and the application of amendments the results may serve as one of many parts for revealing the big puzzle of PAH degradation and phytoremediation. To conclude, the rhizosphere effect on PHE degradation was confirmed in this experiment and hence phytoremediation is proposed as a promising remediation approach for mixed-contaminated sites.

7. Appendix

7.1. Corrections for water content in soil samples

All calculations were based on soil dry weight (dw). Therefore water content (WC) was assessed by weighing and drying about 1 g fresh soil over night at 105°C. The weight difference between fresh weight (fw) and dw corresponded to the WC, which was expressed in percent of dw.

$$WC\ [\%] = \frac{fw - dw\ [g]}{dw\ [g]} * 100 \qquad \text{(eq. 1)}$$

For transformation of weighted samples to a dry weight basis following calculation was applied:

$$dw\ [g] = \frac{fw\ [g]}{1 + \frac{WC\ [\%]}{100}} \qquad \text{(eq. 2)}$$

7.2. Calculations PLFA

7.2.1. PLFA biomass analysis

The concentrations of PLFA in soil ($conc_{FA}$) were used as a proxy for microbial biomass for the taxonomic groups. The calculation was as follows:

$$conc_{FA}\ [\mu g\ g^{-1} soil] = Area * \frac{conc_{19:0}}{Area_{19:0}} * \frac{Input_{CHCl_3}}{Output_{CHCl_3}} * \frac{1}{soil\ [g]} \qquad \text{(eq. 3)}$$

$$conc_{FA}\ [nmol\ \ g^{-1} soil] = \frac{conc_{FA}\ [\mu g\ g^{-1} soil]}{molar\ weight_{FA}\ [g\ mol^{-1}]} * 1000 \qquad \text{(eq. 4)}$$

The areas were taken from the GC-FID measurement because the FID better separated peaks than the GC-C-IRMS. The areas were expressed relative to the internal standard (19:0) that was added at the beginning of the extraction resulting in a concentration of 12 µg per sample. Chloroform ($CHCl_3$) input and output were also considered.

Outliers in PLFA-data were identified by Grubbs outlier test at a significance level of 99% and then replaced by the mean of replicates.

7.2.2. Stable isotope analysis

Methanol correction: The PLFAs had to be transformed to fatty acid methyl esters (FAMEs), which could be measured by GC-FID and GC-C-IRMS. In order to obtain $\delta^{13}C$ values for PLFAs the measured $\delta^{13}C$ values from FAMEs were corrected with the following equation:

$$\delta^{13}C_{PLFA}[‰] = \frac{f_{FA}}{f_{FAME}} * \frac{\delta^{13}C_{measured}}{1000} + \frac{f_{MeOH}}{f_{FAME}} * \delta^{13}C_{MeOH} \qquad \text{(eq. 5)}$$

Where f_{FA}, f_{FAME} and f_{MeOH} represented the number of carbon atoms in the fatty acid (FA), FAME and in methanol, respectively. $C_{measured}$ was the ratio of $^{13}C/^{12}C$ in relation to the international standard VPDB (Vienna Pee Dee Belemnite) in per mill.

Isotope mass balance equation:

The isotope mass balance equation was used to quantify the incorporation of the ^{13}C-labelled PHE into single and total PLFAs, as described by Watzinger (2015).

$$m_{label+soil\ FA}F_{label+soil\ FA} = m_{label\ FA}F_{label\ FA} + m_{soil\ FA}F_{soil\ FA} \qquad \text{(eq. 6)}$$

Where $m_{label+soil\ FA}$ represented the mass of carbon (m) [mol] and $F_{label+soil\ FA}$ the fractional abundance of FA in the soil after addition of the labelled substrate. It was calculated by using following equation:

$$^{13}F = \frac{^{13}R_{sample}}{(^{13}R_{sample}+1)} \qquad \text{(eq. 7)}$$

Where $^{13}R_{sample}$ was the isotope ratio between the isotopes ^{13}C and ^{12}C in the sample and calculated by using the methanol corrected $\delta^{13}C_{PLFA}$ values and following equation, which was obtained by transforming $\delta^{13}C_{PLFA}$ (that is expressed in per mill and relative to the international Standard VPDB):

$$^{13}R_{sample} = \left(\frac{\delta^{13}C_{PLFA}}{1000} + 1\right) * {}^{13}R_{VPDB} \qquad \text{(eq. 8)}$$

In the mass balance $m_{soil\ FA}$ and $F_{soil\ FA}$ represent the mass and fractional abundance of FA using only native soil organic matter. $F_{soil\ FA}$ was calculated by subtracting the concentration of ^{13}C in total FA ($\delta^{13}C_{total\ FA}$) from the concentration of ^{13}C in soil organic matter ($\delta^{13}C_{C-org(soil)}$) (see eq. 9). Then the equations (eq. 7) and (eq. 8) for calculating the ratio and the fractionation were applied.

$$\delta^{13}C_{soil} = \delta^{13}C_{C-org(soil)} - \delta^{13}C_{total\,FA} \qquad \text{(eq. 9)}$$

Where $\delta^{13}C_{C-org(soil)}$ was obtained from EA-IRMS Analysis. $\delta^{13}C_{total\,FA}$ was obtained from soil with and without amendments frozen at T0 where no labelled PHE but unlabelled PHE was added. It was calculated as follows:

$$\delta^{13}C_{total\,FA\,T0} = \frac{\sum(conc_{FA\,T0}\,[nmol\ g^{-1}soil] * \delta^{13}C_{PLFA\,T0}[‰])}{\sum conc_{FA\,T0}\,[nmol\ g^{-1}soil]} \qquad \text{(eq. 10)}$$

$m_{label\,FA}$ and $F_{label\,FA}$ in the mass balance represented the mass of carbon and the fractional abundance of the FA using only the labelled material. $F_{label\,FA}$ was obtained by first subtracting $\delta^{13}C_{soil}$ from $\delta^{13}C_{PHE-label}$ (=1004 ‰) (eq. 11) and then applying the equations for the ratio and fractionation calculation (eq. 7 and eq. 8).

$$\delta^{13}C_{label} = \delta^{13}C_{PHE-label} - \delta^{13}C_{soil} \qquad \text{(eq. 11)}$$

Finally, the mass of labelled FA ($m_{label\,FA}$) was calculated by rearranging the mass balance equation:

$$m_{label\,FA} = m_{label+soil\,FA} * \frac{(F_{label+soil\,FA} - F_{soil\,FA})}{F_{labelFA} - F_{soil\,FA}} \qquad \text{(eq. 12)}$$

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9. List of figures

10. List of tables

- The rhizosphere is a beneficial environment for microorganisms. Is microbial PAH degradation in the rhizosphere enhanced due the provision of nutrients (C, N)? Furthermore, excess nitrogen produced during biological N-fixation could establish optimal conditions for PAH degradation. However, also a competition for nutrients between plants and microorganisms could inhibit PAH degradation.

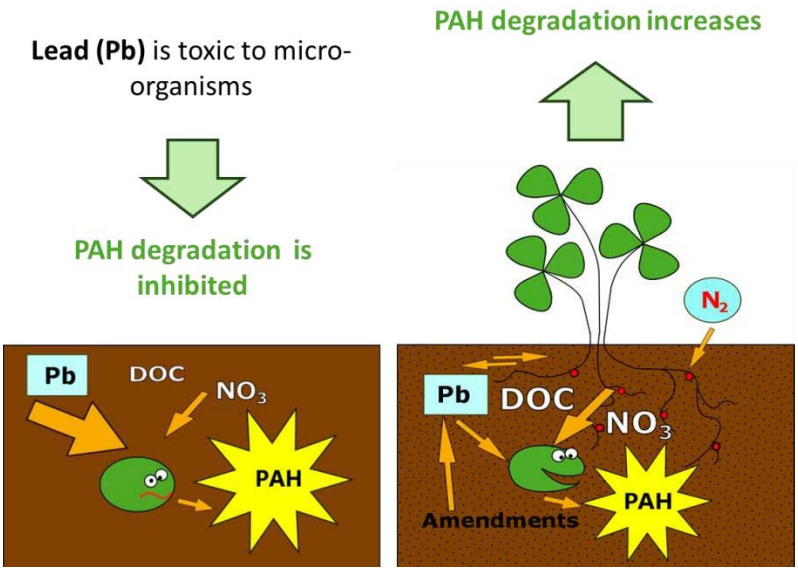

Figure 2. Hypotheses: Amendments reduce Pb toxicity, legumes provide nutrients (dissolved organic carbon [DOC] and nitrate [NO_3]) and thus enhance microbial PAH degradation

Total carbon and nitrogen were determined by dry combustion in an elemental analyser (vario MACRO cube, Elementar Analysensysteme GmbH) according to ÖNORM L 1080 using homogenised, fine-grained, dried soil.

Inorganic carbonates were measured volumetrically by Scheibler apparatus using 10 mL 10% HCl and 2 g oven dry soil (ÖNORM).

Data for texture analysis according to ÖNORM L1060 (2002) were provided by Wawra (2015, personal information).

All extractions and digestions were conducted using sieved soil (<2 mm). For quality assurance blanks and in-house reference material were included in all extraction and digestion procedures. Plastics and glassware for digestions and extractions were acid washed in 5% nitric acid (HNO_3) before use.

3.2. Experimental setup

In this study we used the rhizotest to observe the degradation of PAHs over 10 days. The rhizotest was developed by (Chaignon and Hinsinger, 2003) and is a standardised, plant-based test for assessing rhizosphere processes and bioavailability of trace elements, according to ISO 16198:2015. It facilitates harvest of roots and rhizosphere soil because roots and soil are separated by a mesh, which however enables rhizosphere processes. The Rhizotest approach consist of two phases: (I) a hydroponic pre-growth period where plants are grown in growth cylinders in nutrient solution (visualised in Figure 4a), when a dense root mat has developed (II) the soil-plant contact is enabled and the growth cylinders are put on a thin layer of soil (approximately 3 mm), which is supplied with water and macronutrients via filter wicks (visualised in Figure 4b).

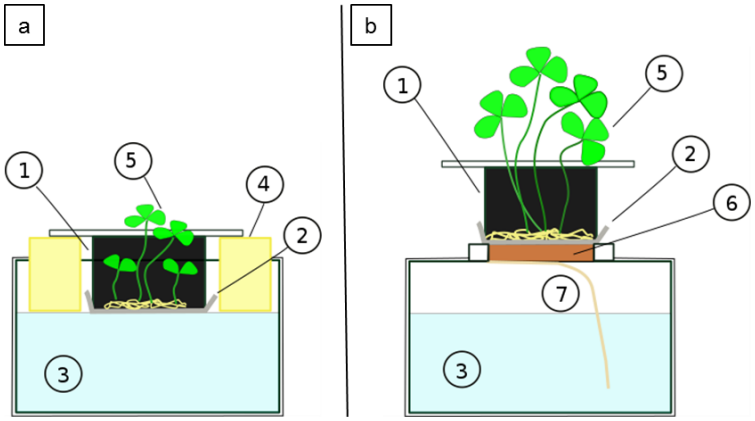

Figure 4. a: Rhizotest hydroponic pre-growth, b: Rhizotest soil-plant contact

Description: Figure 4.a: Rhizotest hydroponic pre-growth: growth cylinder (1) with membrane (2) floating on nutrient solution (3) with the help of foamed polystyrene (4). Plants (5) growing until a dense root mat has developed.
Figure 4.b: Rhizotest soil-plant contact: growth cylinder (1) put on a thin layer of soil (6 g dry weight, ~3 mm thickness), which is supplied with water and macronutrients via a filter wick (7) hanging in nutrient solution (3).

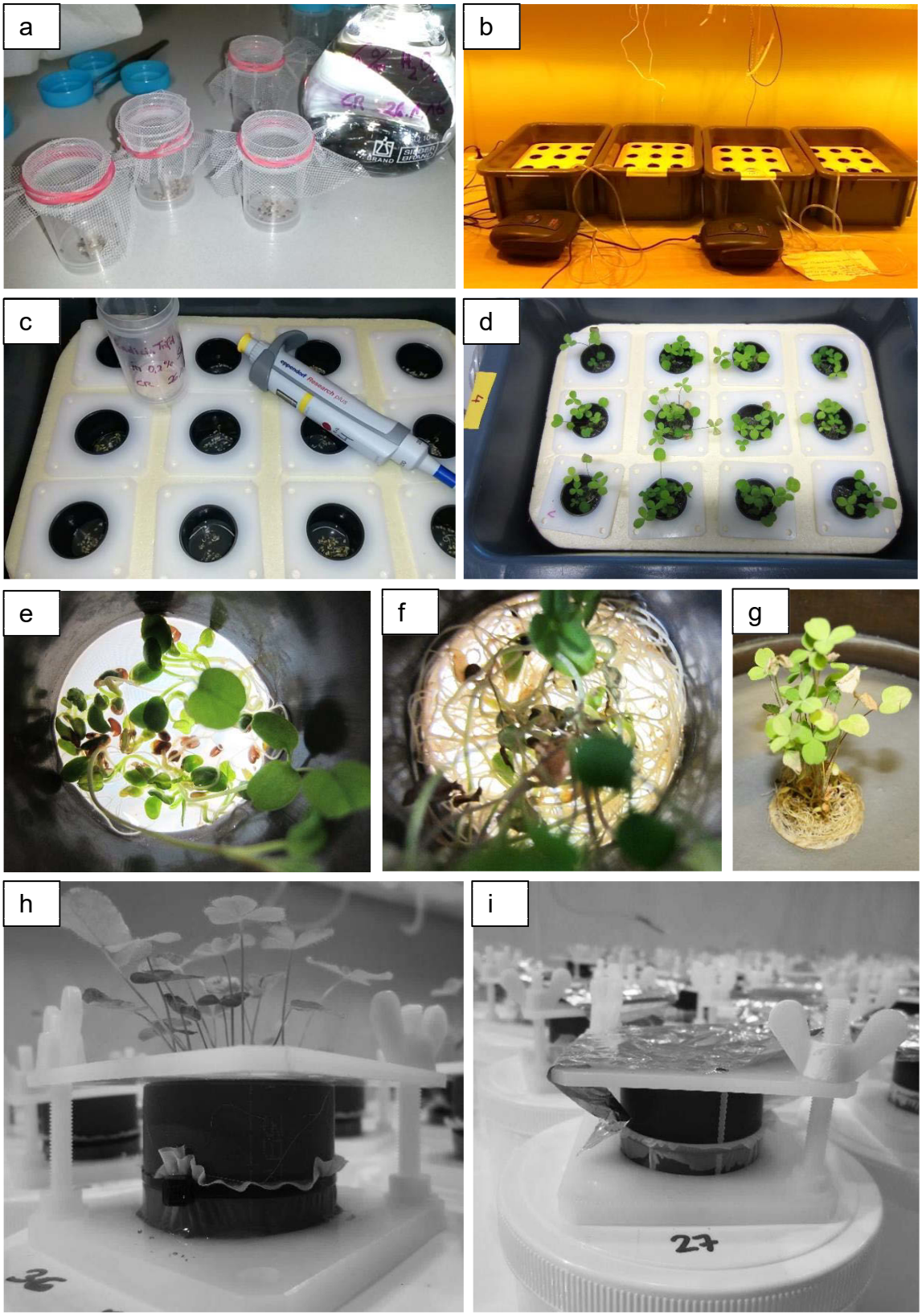

Figure 5. Rhizotest experiment

Description of Figure 5: a)Seed sterilisation , b)Hydroponic system for plant pre-growth in aerated nutrient solution, c) Inoculation with Rhizobia, d) Red clover in hydroponic set-up, 4 weeks old, e) some days after germination, f) root mat at T6, g) harvest of clover at T6, h) Rhizotest unit with plants (rhizosphere soil) and i) without plants (bulk soil)

Figure 7. a) Addition of PHE-labelled sand, b) plant harvest at T10, c) nodules at T10

3.4. Soil and plant analysis

3.4.1. Plant-available Pb - DGT

Bioavailable Pb was assessed by diffusive gradients in thin films DGT according to Zhang et al. 2005. At the beginning (T0) and at the end (T10) of the experiment about 4 g fresh soil was put on DGT devices and incubated for 24 hours in a water-saturated atmosphere at 20 °C. The DGT devices consisted of a membrane (cellulose nitrate, 100 µm thickness, 0.45 µm pore size), a diffusive gel layer (0.8 mm thick polyacrylamide gel) and a polyacrylamide resin gel containing suspended Chelex 100 particles for binding cations. After 24 hours deployment, soils were removed from the DGT devices and frozen immediately for further analysis. Gels were rinsed 3 times in beakers with HQ-water and then eluted in 5 mL 1 M HNO_3 for several days. Metal concentrations in elution solution were measured by ICP-MS (Elan 9000 DRCe, Perkin Elmer).

The DGT assesses the flux of labile species from soil to the gel during deployment time. The diffusion into the gel simulates the diffusion-limited plant uptake of elements, thereby providing a better predictability of bioavailability than conventional equilibrium-based extraction approaches.

was used to calculate the total microbial biomass. The mass of ^{13}C incorporated into the PLFAs and total PLFAs was calculated with the isotope mass balance equation based on the measured $\delta^{13}C$-values and the microbial biomass. The calculation is explained in the appendix (Stable isotope analysis).

Figure 8. Solid phase extraction of PLFAs with silica columns

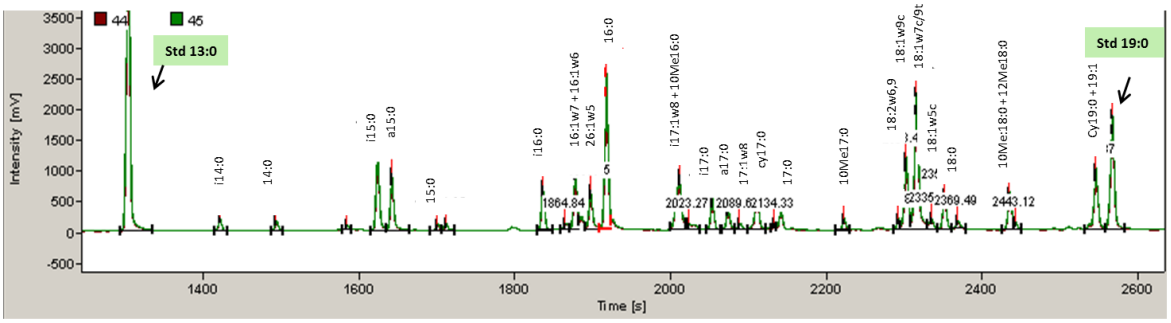

Figure 9. PLFA-chromatogramm from GC/c/IRMS measurement

3.4.4. Extraction of polycyclic aromatic hydrocarbons

For analysis PAHs a Soxhlet extraction was carried out using 10 g of frozen experimental soil from T0 and T10 and 130 mL of ethyl acetate (puriss, Sigma Aldrich) as an extraction solvent. 10 g soil was weighed into extraction thimbles and extracted via the reflux cycle within 5 hours at boiling phase to recover 100 mL of extract. 10 mL of the extracts were then concentrated to 1 mL under a gentle stream of nitrogen and stored in the fridge until measurement by high pressure liquid chromatography HPLC (Agilent 1100 series) equipped with a degasser, a quaternary pump and a diode array detector (DAD) and a fluorescence detector (FLD). Acetonitrile was used as an eluent. Column temperature was held at room

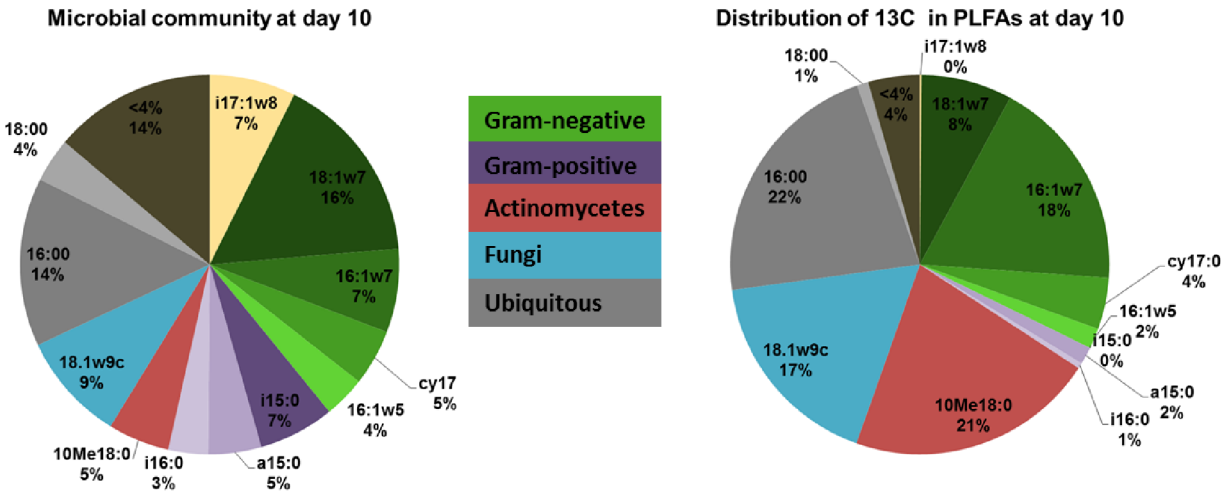

Figure 14. Composition of microbial community and distribution of ^{13}C in individual PLFAs at day 10

5.5.2. PLFA 13C-analysis

During degradation of the labelled hydrocarbon ^{13}C-PHE microorganisms incorporated ^{13}C into their cell membranes. As a consequence the δ^{13}C-values, indicating the ratio of ^{12}C/^{13}C relative to the international standard VPDB, became less negative over 10 days, as can be seen in Figure 15. According to the increasing δ^{13}C-values, the mass of ^{13}C incorporated into total PLFAs increased, as visualised in Figure 16. In planted soils the mass of ^{13}C was slightly higher than in bulk soils, which indicated more pronounced degradation of PHE in the rhizosphere. However, the difference was not statistically significant for total PLFAs but for individual PLFAs which will be discussed later on.

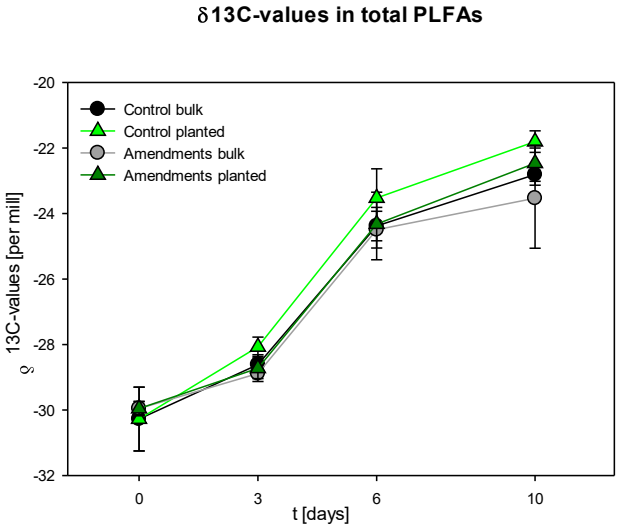

Figure 15. Delta ^{13}C-values in total PLFAs increased in all treatments over 10 days
Symbols show means (n=3) and error bars indicate standard deviation

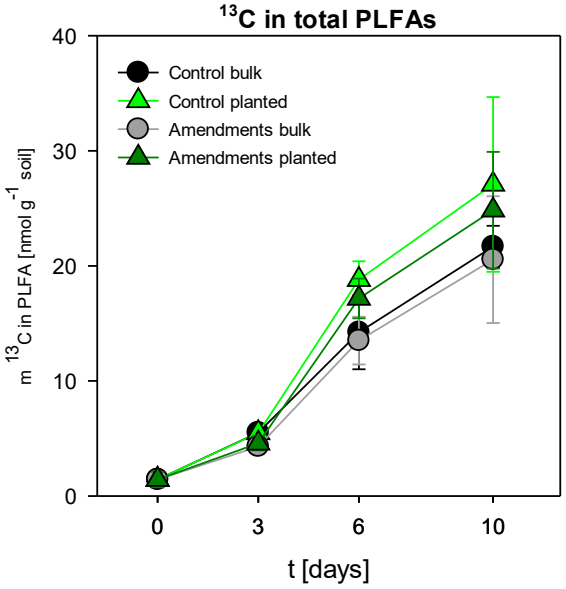

Figure 16. Mass of ^{13}C in total PLFA
Symbols show means (n=3) and error bars indicate standard deviation